Natural Polysulfides- Reactive Sulfur Species from *Allium* with Applications in Medicine and Agriculture

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“Read in the name of your Lord who created, created man from clot of blood. Read ! your Lord is the most Bounteous, Who has taught the use of the pen, has taught man what he did not know”. *[Quran 96.1-5]*

Dedicated to my loving Parents

The creativity, determination and sense of joy with which they respond to life's challenges has led me to seek this in others.

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While writing this thesis I jumped back 28 years in my thoughts to my first day in school in my village Bai, an old building with no chairs, I learnt ABC and enjoyed the berries during recess time. That school does not exist anymore but my heartiest gratitude to those teachers who taught me ABC and today I stand here writing my PhD thesis.

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This thesis is a small tribute to anyone who can benefit out of this- from a student still anxious to learn.

Awais

Abstract

Natural sulfur compounds from plants, bacteria, fungi and animals frequently exhibit interesting biological activities, such as antioxidant, antimicrobial and anticancer activity. Considering the recent developments in medicine (*e.g.* oxidative stress in ageing, antibiotic resistant bacteria, and selective anticancer agents) and Agriculture (*e.g.* 'green' pesticides), several of these compounds have become the focus of interdisciplinary research. Among the various sulfur agents isolated to date, polysulfides, such as diallyltrisulfide, diallyltetrasulfide (from garlic) and dipropyltrisulfide, dipropyltetrasulfide (from onion), are of particular interest, since they combine an unusual chemistry and biochemical mode(s) of action with a distinct biological activity, which includes antimicrobial activity and cytotoxicity against certain cancer cells. As part of this PhD thesis, the activity of diallyltrisulfide and diallyltetrasulfide against the fairly 'robust' Caco-2 colon cancer cell line and induction of apoptosis and cell cycle arrest in U937 cells have been confirmed. Accordingly, diallyltetrasulfide triggered the programmed cancer cell death- both *via* the extrinsic and intrinsic pathway. Similarly, these polysulfides showed a good activity in nematode toxicity assays considering them as potential green nematicides. Controls with the long chain carbon analogue 1,9-decadiene eliminate the possibility of solely lipophilic effects of diallyltetrasulfide and, together with the 'ranking' of activity, point toward a special sulfur redox chemistry which emerges when shifting from the di- to the trisulfide. The electrochemical studies and thiol oxidation assays, however, count against the notion of diallyltrisulfide and diallyltetrasulfide as effective oxidants. On the contrary, the rather negative oxidation and reduction potentials associated with these agents point toward a reducing chemistry, which is confirmed in the Nitrotetrazolium Blue assay. It is therefore likely that diallyltrisulfide and diallyltetrasulfide are reduced inside the cancer cells to perthiols and hydropolysulfides, which in turn trigger a lethal oxidative burst via superoxide radical anion formation. Further interdisciplinary studies are required to investigate in more detail the rather complicated chemical and biochemical processes which ultimately may explain the biological activity which is clearly associated with many natural polysulfides.

Kurzfassung

Natürliche Schwefelverbindungen von Pflanzen, Bakterien, Pilzen und Tieren zeigen oft interessante biologische Aktivitäten, wie antioxidative, antimikrobiale und Antikrebs-Wirkungen. Angesichts der derzeitigen Entwicklung in Medizin (z.B. oxidativer Stress beim Alterungsprozess, gegen Antibiotika resistente Bakterien und selektive Antikrebs-Mittel) und Landwirtschaft (z.B. „grüne“ Pestizide) haben verschiedene dieser Verbindungen ein besondeses Augenmerk in interdisziplinärer Forschung erhalten. Unter diversen bislang isolierten Schwefel-Agenzien sind Polysulfide, wie Diallyltrisulfid, Diallyltetrasulfid (von Knoblauch), so wie Dipropyltrisulfid und Dipropyltetrasulfid (von Zwiebeln) von besonderem Interesse, und diese Verbindungen vereinen eine ungewöhnliche Chemie und biochemische Wirkungsweise mit einer ausgeprägten biologischen Aktivität, welche antimikrobiale Aktivität and Zytotoxizität gegenüber bestimmten Krebszellen umfasst, Als Teil dieser Dissertation wurde die Aktivität von Diallyltrisulfid und Diallyltetrasulfid gegen die „robuste“ Caco-2 Darmkrebs-Zellinie, so wie die Induktion von Apoptose und Zellzyklus-Arrest in U937-Zellen bestätigt. Dementsprechend löst Diallyltetrasulfid programmierten Zelltod über den extrinsischen als auch den intrinsischen Weg aus. Gleichermaßen zeigten diese Polysulfide eine gute Aktivität im Nematoden-Toxizitäts-Assay, welches sie als potenzielle grüne Nematizide im Betracht ziehen lässt. Durch Kontrollen mit dem langkettigen Kohlenstoff-Analogen 1,9-Decadien lässt sich die Möglichkeit der alleinigen lipophilen Effekte von Diallytetrasulfid ausschließen, was zusammen mit dem „Ranking“ der Aktivitäten auf eine besondere Schwefel-Redoxchemie hindeutet, wie sie beim Übergang vom Di- zum Trisulfid auftritt. Die elektrochemischen Studien und Thiol-Oxidations-Assays sprechen gegen Diallyltrisulfid und Diallyltetrasulfid als effektive Oxidantien. Im Gegensatz, die eher negativen Oxidations- und Reduktionspotentiale deuten auf eine Reduktionschemie hin, welches durch das Nitrotetrazoliumblau-Assay bestätigt wurde. Deshalb ist es wahrscheinlich, dass Diallyltrisulfid und Diallyltetrasulfid in Krebszellen zu Perthiolen und Hydropolysulfiden reduziert werden, die wiederum einen letalen oxidativen Burst durch Superoxid-Radikalanionen-Bildung auslösen.

Abbreviations

AAT	Aminotransferase
AGE	Aged Garlic Extract
AM	Allylmercaptan
ARE	Antioxidant Responsive Element
Bn-SSH	Benzyl Hydrodisulfide
Bn-SH	Benzyl Mercaptan
BSA	Bovine Serum Albumin
Caco-2	Human Caucasian colon adenocarcinoma
CBS	Cystathionine-*β*-synthase
CSE	Cystathionine-γ-lyase
DATTS	Diallylteterasulfide
DPS	Dipropylsulfide
DPDS	Dipropyldisulfide
DPTS	Dipropyltrisulfide
DPTTS	Dipropyltetrasulfide
***E*pa**	Electrode potential (anodic)
***E*pc**	Electrode potential (cathodic)
FSC	Forward scatter
GPx	Glutathione Peroxidase
GSH	Glutathione (Reduced)
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
HDAC	Histone Deacetylase

MDR	Multidrug Resistance
MO	Mustard oil
MP	Mitochondrial Membrane Potential
MPST	3-mercaptopyruvate sulfur transferase
4-MP	4-mercaptopyridine
MT	Metallothioneins
NBT	Nitrotetrazolium Blue
NIK	NF-κB-inducing Kinase
OS	Oxidative stress
PARP	Poly-(ADP-Ribose)-Polymerase
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PrSH	Protein Thiols
ROS	Reactive oxygen species
RSS	Reactive sulfur species
SAC	*S*-allylcysteine
SMAC	*S*-allylmercaptocysteine
SSC	Side scatter
SOD	Superoxide Dismutase
U937	Human leukemic monocyte lymphoma cell line

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Chapter 1

Garlic & Onion – History & Chemistry

Garlic, onions and related plants of the genus *Allium* have been part of Folk Medicine for many Centuries and all over the world [1, 2]. Although the precise health benefits of such vegetables and their products are still a matter of debate, recent research has provided a number of new insights which deepen our understanding of *Allium* plants and derived products, such as garlic and onion oils and powders [3]. The name 'allium' itself is derived from the Celtic word "all", which means pungent, refering to the characteristic odor of these plants. Numerous biologically active ingredients, including allicin and polysulfides, have been identified and their interactions with biomolecules, cells and organisms described. In many cases, there seems to be a broad-range antimicrobial activity associated with sulfur-containing compounds derived from *Allium* species, which manifests itself in diverse antibacterial, antifungal and 'pesticidal' activities [4]. These activities seem to be the result of an intrinsic cytotoxicity of various (sulfur) compounds present in these plants.

1.1 Nature's Ancient Superfood

For the last 5,000 years of human history, Garlic (*Allium sativum*) and Onion (*Allium cepa*), has been both cherished and reviled, both sought for its healing powers and shunned for its pungent after effects. From miracle drug to vampire repellent to offering for the gods, this unassuming plant has had an undeniably important place in many aspects of human history, and today enjoys a renewed surge in popularity as modern medicine unearths the wonders of this ancient superfood.

Unlike that mysterious Tupperware lurking at the back of fridge, garlic has been employed in a variety of functions for millennia. Although the place where garlic grew

initially is not known, its cultivation is said to date back about 4,000 years ago. Generally accepted history is that garlic originated from Central Asia, then spread either to the west, the Tigris-Euphrates area, and Egypt, or to the east, China and then to Korea and later to Japan [5]. Modern phytochemical analysis and gene technologies performed on garlic by several researchers also support its Asian origin. According to Tsuneyoshi *et al.* [6], and Maas *et al*. [7], *Allium longicuspis*, an old garlic species having fertility (flowering and seed forming abilities), could be found only in the west area of the Tien Shan Mountains of China. Archeologists have discovered clay sculptures of garlic bulbs and paintings of garlic dating about 3200 B.C. in Egyptian tombs in El-Mahasna.

In ancient Greece and Rome, garlic enjoyed a variety of uses, from repelling scorpions to treating dog bites and bladder infections to curing leprosy and asthma. It was even left out as an offering to the Greek goddess Hectate. Early Greek military leaders fed garlic to their troops before battles to give them courage and promise victory (and perhaps in an attempt to fell the opposing army with one good whiff). Even Greek Olympic athletes counted on garlic to stimulate performance. Ancient Transylvania, home of the vampire legend, found garlic to be an effective mosquito repellent as well as a way to ward off toothsome visitors. In the Middle Ages, garlic was thought to combat the plague and was hung in braided strands across the entrances of houses to prevent evil spirits from entering.

Similarly, traces of onion remains were found in Palestinian Bronze Age settlements alongside fig and date stones dating back to 5,000 BC. Onion is native to South Asia, and is widely used in Indian cuisine. It is not clear however, if these were cultivated onions. Archaeological and literary evidence such as the Book of Numbers 11:5 suggests cultivation probably took place around two thousand years later in ancient Egypt, at the same time that leeks and garlic were cultivated. The Ancient Egyptians worshipped it, believing that its spherical shape and concentric rings symbolized eternal life. Onions were even used in Egyptian burials as evidenced by onion traces found in the eye sockets of Ramesses IV. They believed that, if buried with the dead, the strong scent of onions would bring breath back to the dead.

In ancient Greece, athletes ate large quantities of onion because it was believed that it would lighten the balance of blood. Roman gladiators were rubbed down with onion to firm up their muscles. Doctors were known to prescribe onions to facilitate bowel movements and also to relieve headaches, cough, snakebite and hair loss. The onion was

introduced to North America by Christopher Columbus on his 1492 expedition. Onions were also prescribed by doctors in the early 1500s to help with infertility in women, and even to treat dogs and cattle and many other household pets. Recent evidence has shown that dogs, cats, and other animals should not be given onions in any form, due to toxicity during digestion. On the botanical side, onions have many varieties; *Allium cepa* (common onion), *Allium aggregatum* (potato onion), *Allium ascalonicum* (shallot), *Allium proliferum* (tree or Egyptian onion), and *Allium viviparum*.

1.2 General composition and Sulfur compounds derived from Garlic and Onion

As with most other vegetables, both garlic and onion contain nutrients, carbohydrates, proteins, lipids as well as vitamins (**Table 1.1** and **1.2**). The contents of major nutrients in onions are quite low, however, when compared with the one of garlic, and the only component comparable to garlic is vitamin C. However, considering the higher onion consumption about 5 to 10-fold compared to garlic, these lesser amounts of general components in onion should not be neglected. The general composition is, of course, important to the nourishment of either garlic or onion eaters, nonetheless, with respect to the food function, the extraordinary high content of sulfur compounds in these vegetables should be much more important. These compounds are present as a group of sulfur-containing amino acids in their intact tissues, especially in the cloves of garlic bulb or in an onion bulb. (**Table 1.3**)

In an intact bulb, there are two major sulfur containing amino acid derivatives, namely γ-glutamylcysteines, and S-alkylcysteine sulfoxides. According to Lawson *et al* ., [8] and Ceci *et al*. [9], γ-glutamylcysteine is more abundant in mature bulbs than in immature young bulbs, and rapidly decreases at sprouting, which in turn is accompanied by increases of alliin, and related amino acids (methiin and isoalliin) [10]. Transformation of γ-glutamylcysteine to alliin, methiin, or isoalliin is accomplished enzymatically by the enzymes, γ-glutamyltranspeptidase (EC 2.3.2.2) and γ-glutamylpeptidase [10].

Table 1.1 General Composition of Fresh Garlic and Onion Bulbs. Adopted from Standard tables of Food Composition of Japan. 5th Revised Edition.

Component	Garlic [a]	Onion [a]	
		White	Red
Energy (kcal)	134	3	38
Water (g)	65.1	89.7	89.6
Protein (g)	6.0	1.0	0.9
Lipids (g)	1	0.1	0.1
Carbohydrates (g)	26.3	8.8	9.0
Minerals (mg)			
Sodium	9	2	2
Potassium	530	150	150
Calcium	14	21	19
Magnesium	25	9	9
Phosphorus	150	33	34
Iron	0.8	0.2	0.3
Zinc	0.7	0.2	0.2
Copper	0.18	0.05	0.04
Manganese	0.27	0.15	0.14

[a] Average value/100g flesh weight.

Table 1.2 Vitamins, Fatty Acids and Dietary Fibres in Fresh Garlic Cloves and Onion Bulbs. Adopted from Standard tables of Food Composition of Japan. 5th Revised Edition.

Compound	Garlic [b]	Onion [b]	
		White	Red
Vitamins (mg)			
A	0	0	0
D	0	0	0
E	0.5	0.1	0.1
K	Tr	Tr	Tr
B1	0.19	0.03	0.03
B2	0.07	0.01	0.02
Niacin	0.7	0.1	0.1
B6	1.5	0.16	0.13
B12	0	0	0
Folic acid	0.0	0.02	0.02
Pantothenic acid	0.55	0.19	0.15
C	10	8	7
Fatty acids (mg)			
Saturated	0.18	0.01	0
Monounsaturated	0.04	Tr	0
Polyunsaturated	0.41	0.03	0.03
	5.7	1.6	1.7
Dietary fibres (g)			
Water soluble	3.7	0.6	0.6
Water insoluble	2.0	1.0	1.1

[b] Average value/100 g flesh weight.

Table 1.3 Sulfur and non-sulfur compounds that characterize fresh Garlic cloves. Values obtained for dry weight of garlic by Ueda *et. al*. (1991) [11] are represented after multiplying with 0.35 for the flesh weight (65% moisture).

Compound	Amount g/100 g [a]
Total sulfur compounds	2.3
Sulfur	0.48
Cysteine sulfoxides	
S-Allylcysteine sulfoxide (alliin)	1.7
S-Methylcysteine sulfoxide (methiin)	0.23
S-trans-1-Propenylcysteine sulfoxide (isoalliin)	0.02-0.12
Cycloalliin	0.25
γ-Glutamylcysteines	
γ -Glutamyl-S-trans-1-propenylcysteine	0.3-0.9
γ -Glutamyl-S-cis-1-propenylcysteine	0.006-0.015
γ -Glutamyl-S-allylcysteine	0.2-0.6
γ -Glutamyl-S-methylcysteine	0.01-0.04
Nonsulfur compounds	
Saponins, mostly in β-sitosterol based	0.035-0.042
Sapogenin, as β –sitosterol	0.019

1.2.1 Enzymatic generation of reactive sulfur species (RSS) as part of binary plant defence systems

A range of Reactive sulfur species (RSS), such as allicin **(3)** and dialk(en)ylpolysulfides are not simply synthesised and stored in the plant. In garlic, allicin is synthesised in the clove 'on demand', *i.e.* when the clove is physically injured (*e.g.* chopping, crushing) or attacked by microbes [2]. The chemistry behind allicin synthesis is rather intriguing. The cysteine derivative γ-glutamyl-*S*-alk(en)yl-*L*-cysteine is hydrolysed and oxidised to yield alliin **(1)** (**Figure 1.1**). Alliin, which is present in the cytosol, is a comparably chemically unreactive sulfoxide. Alliin is converted to the highly reactive thiosulfinate allicin by the C-S-lyase enzyme alliinase (EC 4.4.1.4), which is normally stored separately from alliin in vacuoles and only encounters its substrate when the garlic clove is damaged. The conversion of alliin to allicin is proceeds via an intermediate sulfenic acid **(2)**.

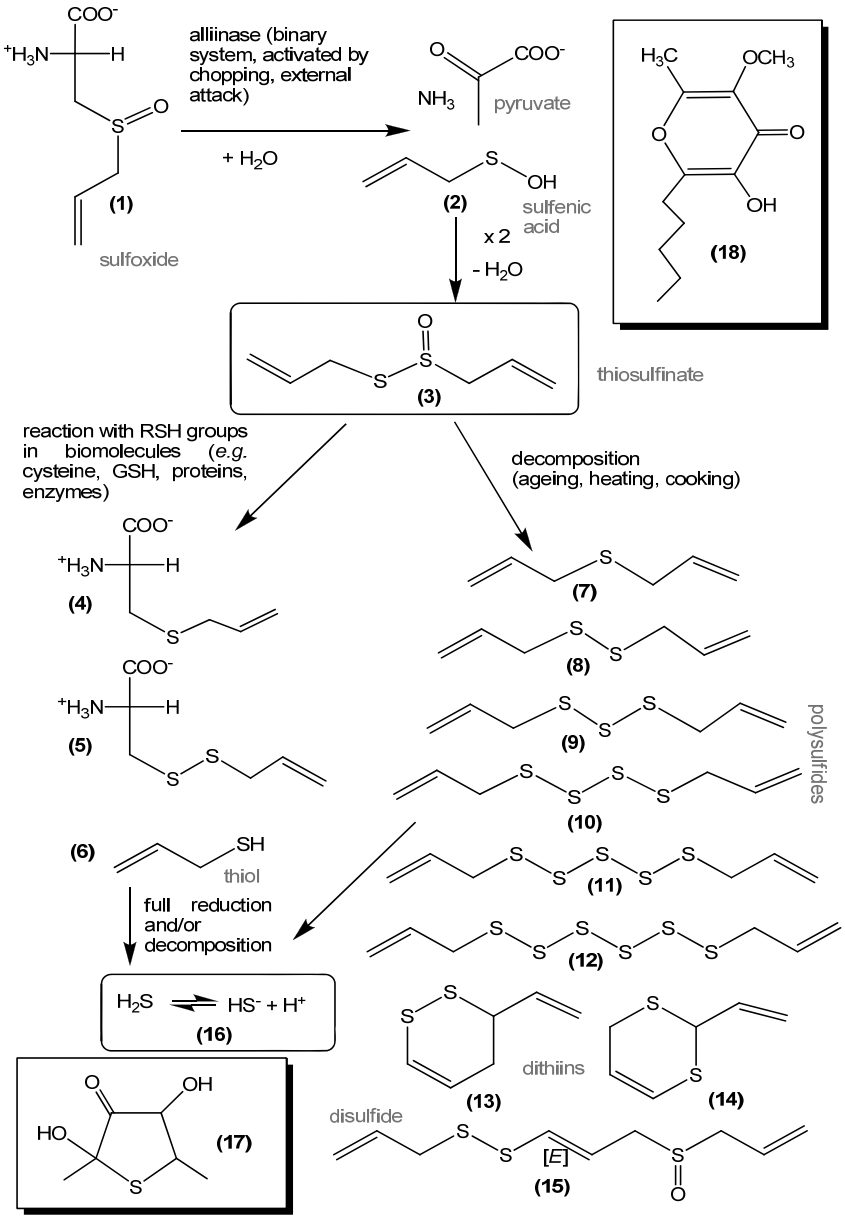


Figure 1.1 A selection of the sulfur chemistry found in garlic adopted from Jacob & Anwar *et.al.* [12]. Alliin **(1)**, allyl sulfenic acid **(2),** allicin **(3)**, *S*-allyl cysteine (SAC **(4)**), *S*-allylmercaptocysteine (SAMC **(5)**), allylmercaptane (AM **(6)**), diallylsulfide (DAS **(7)**), diallyldisulfide (DADS **(8)**), diallyltrisulfide (DATS **(9)**), diallyltetrasulfide (DATTS **(10)**), diallylpentasulfide **(11)**, diallylhexasulfide **(12)**, 3-vinyl-3,4-dihydro-1,2-dithiin **(13)**, 2-vinyl-2,4-dihydro-1,3-dithiin **(14)**, E-ajoene **(15)**, thiacremonone **(17)**, allixin **(18)**.

A similar 'binary' substrate/enzyme system able to generate an aggressive sulfur species on demand also exists in onions (**Figure 1.2**). Here, most of the chemistry of biologically active species is associated with the formation and subsequent reaction pathways of the lachrymatory factor (LF) **(22)**, although a garlic-like thiosulfinate chemistry also exists. The LF is formed from isoalliin (*trans*-1-propenyl-*L*-cysteine sulfoxide, PRENCSO) **(19)** via 1-propenyl sulfenic acid **(20)**, in the presence of the (onion) enzymes alliinase and lachrymatory-factor synthase (LFS) [13]. The LF is chemically unstable and highly reactive and endows onions with an extensive and complicated sulfur chemistry, which is quite distinct from the one of garlic, yet equally interesting from a biological perspective. The dimer of the LF, *trans*-3,4-diethyl-1,2-dithietane-1,1,-dioxide **(23)**, differs dramatically from the dimer of **(20)** or **(2)** and is given as just one example to illustrate the rather unusual chemistry based on the LF.

Most of the agents formed in this context, such as LF and allicin are either too unstable chemically or too reactive to survive for long in the presence of other biomolecules, especially of the ones containing thiol groups. Allicin, for instance, is sensitive to elevated temperatures and will be 'destroyed' by cooking or decompose after a couple of days at room temperature (**Figure 1.1**). Alternatively, it may react with thiols present in small molecules, peptides, proteins or enzymes, to form a range of follow-on products, such as *S*-allylmercaptocysteine (SAMC **(5)**) [14]. As a consequence, a human diet rich in garlic and onions will not automatically also be rich in allicin, LF or similarly aggressive sulfur agents. A recent review by Amagase entitled "Clarifying the Real Bioactive Constituents of Garlic" has underlined the importance of avoiding any such misunderstandings, for instance when dealing with aged garlic extracts (AGE) [15].

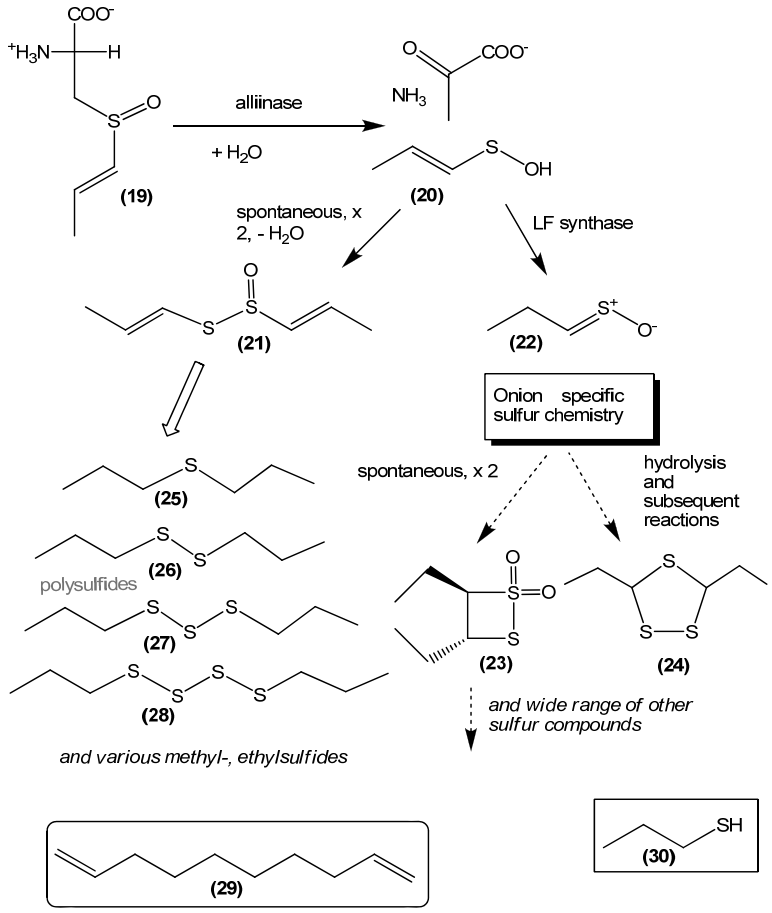


Figure 1.2 Aspects of sulfur chemistry found in onions. Figure adopted from Jacob & Anwar *et.al.* [12]. Isoalliin (*trans*-1-propenyl-*L*-cysteine sulfoxide, PRENCSO) **(19)**, 1-propenyl sulfenic acid **(20)**, lachrymatory factor (LF) **(22),** *trans*-3,4-diethyl-1,2-dithietane-1,1,-dioxide **(23)**, 1,2,4-trithiolanes **(24)**, dipropylsulfide (DPS **(25)**), dipropyldisulfide (DPDS **(26)**), dipropyltrisulfide (DPTS **(27)**), dipropyltetrasulfide (DPTTS **(28)**). The carbon analogue of tetrasulfide, 1,9-decadiene **(29)** and propylmercaptane **(30)** are not part of onion chemistry but mentioned here as they are part of this study.

1.2.2 Follow-on products formed by degradation of reactions with biomolecules

Allicin **(3)**, once formed , reacts readily with a wide range of thiol-based targets, such as cysteine residues in peptides, proteins and enzymes [16], forming a range of 'follow-on' products, such as *S*-allylcysteine (SAC **(4)**), *S*-allylmercaptocysteine (SAMC **(5)**) and, quite frequently, allylmercaptane (AM **(6)**). Interestingly in human body, allicin is also likely to be sequestered by reduced glutathione (GSH), leading to the formation of *S*-allylmercaptoglutathione.

Apart from such reactions, allicin also decomposes upon 'ageing' or heating. These decomposition processes are chemically far from trivial and result in a wide range of sulfur compounds, including diallylsulfide (DAS **(7)**), diallyldisulfide (DADS **(8)**), diallyltrisulfide (DATS **(9)**), diallyltetrasulfide (DATTS **(10)**), as well as a range of 'higher' sulfides, such as diallylpentasulfide **(11)**, diallylhexasulfide **(12)** and diallylheptasulfide**.** In addition, 3-vinyl-3,4-dihydro-1,2-dithiin **(13)**, 2-vinyl-2,4-dihydro-1,3-dithiin **(14)** and ajone **(15)** are also formed as a part of – chemically rather complicated – allicin decomposition pathways.

Full reduction of sulfur, for instance *via* successive exchange reactions or thermal degradation results in the liberation of the cell signalling molecule hydrogensulfide (H_2S), which at pH 7.4 is in equilibrium with the HS^- anion **(16)**. Recently, thiacremonone **(17)** has been found in garlic as yet another sulfur agent, and with a hitherto unknown formation pathway. Importantly, garlic is also rich in a number of compounds, which are biologically active yet do not contain sulfur, such as allixin **(18)**. Please note that this selection of compounds shown in **Figure1.1** provides only a fraction of the sulfur compounds present in garlic.

In onion, major follow-on products of the LF reaction pathway are *trans*-3,4-diethyl-1,2-dithietane-1,1,-dioxide **(23)**, 1,2,4-trithiolanes **(24)** , as well as propylsulfides, such as dipropylsulfide **(25)**, dipropyldisulfide **(26)**, dipropyltrisulfide **(27)**, dipropyltetrasulfide **(28)**, and various methyl-, and ethylsulfides (**Figure1.2**) [17]. The precise composition of such preparations varies widely and depends critically on the manufacturing process.

1.2.3 Chemical recycling of allicin

The loss of allicin from garlic due to decomposition or chemical reactions has frequently raised the question if aged garlic extracts (AGE), powders or oils are indeed still biologically active and beneficial to human health. The presence of various biologically active follow-on agents other than allicin may point towards such a longer-term activity of cooked or processed garlic. In addition, it has been reported that allicin is in part being 'recycled' from its own decomposition product DADS **(8)** by cytochrome P-450 and

flavin-containing monooxygenase enzymes which are present in many human cells - and especially in certain cancer cells [18].

Is it therefore possible that certain cancer cells convert DADS to allicin. After all, 'oxidative activation' of reactive (sulfur) species, such as thiosulfinates, has been discussed before in the context of oxidative stress (OS), and allicin is synthesised chemically by oxidation of DADS in the presence of H_2O_2 [2]. The notion of allicin formation *in situ* is particularly intriguing since it is supported by two possible reaction pathways, both of which are relevant in the context of human cells. Firstly, enzymatic conversion of DADS **(8)** to allicin by oxidase enzymes may preferably occur in cancer cells, which often exhibit higher levels of such enzymes when compared to healthy cells. To a certain degree, such oxidative 'activation' would therefore be cancer-cell specific and may allow DADS (and other active ingredients of *Allium* vegetables) to act as 'selective' agents. Secondly, there is some evidence of 'redox interplay' between DADS on the one side, and DATS **(9)** and DATTS **(10)** on the other. Recent reports by Munday and colleagues have pointed towards the ability of DATS and DATTS to (catalytically) generate superoxide ($O_2^{\bullet-}$) - and therefore also H_2O_2 - in cells in the presence of dioxygen and GSH [19]. Since DADS, DATS and DATTS are usually jointly present in garlic products and consumed together, H_2O_2 generation by DATS and DATTS may result in allicin formation (from DADS) and therefore trigger the chemopreventive, *i.e.* cytotoxic effects associated with this thiosulfinate. Such hypotheses and their implications for chemoprevention obviously still need to be explored further.

1.3 Commercial products of Garlic

Garlic products are sold worldwide in markets or drug stores, and recently, as over-the-counter (OTC) products which place first rank in the selling records of Germany. There are many methods for preparing garlic products, and hence, components involved are different from one product to another. In contrast, all the various garlic-derived preparations have name "garlic" on their labels. This must be a problem for people using the product for their health promotion. Production of different garlic products is described next.

1.3.1 Aged Garlic Extracts (AGE)

AGE have been produced by prolonged (about 6 months) soaking of chopped garlic into 20% alcohol [20]. During the aging, γ-glutamyl-S-allylcysteine, 2 and γ-glutamyl-S-1-propenylcysteine, are hydrolyzed into S-allylcysteine **(4)**, 8 and S-1-propenylcysteine, respectively [21]. Therefore, preparation of AGE is useful for releasing functional S-alkenylcysteines from their parent peptides without further production of allicin **(3)** and sulfides. In addition, the aging affords novel compounds, such as S-allylmercaptocysteine **(5)** and cystine, which are absent in garlic, although the content of total sulfur compounds decreases by about 50%. On the other hand, the longtime storage produces 'caramelized' compounds, because of which the extracts are usually colored green. Aging may also cause deterioration of products. The discoloration will be mentioned in the section of onion products (onion powder).

1.3.2 Garlic Oil

Garlic oil is prepared by steam distillation of chopped garlic, and is used for production of some sauces, pizza, cakes, ham and sausages. The major compounds of garlic oil are diallyldisulfide **(8)**, diallyltrisulfide **(9)**, and allyl methyl trisulfide. These compounds do not change chemically during long time storage, especially at 4 °C.

1.3.3 Garlic Powder

There are many types of garlic powder products produced in different ways. Surprisingly, most powdered products have no detectable amounts of alliin and sulfides. Producing such powders, manufacturers would usually take the easiest way: *e.g.*, chopping garlic into fine pieces, followed by dehydration and pulverization. Although the ingredients of the powder should be controlled to meet the formulation for a desired product, such as spiced sausages and sauces, the loss of sulfur compounds should be minimized. The only sulfur compound detected in such powders was γ-glutamyl-S-allylcysteine. In contrast, some powders known as 'allicin potential' contain both alliin and alliinase, in addition to γ-glutamylcysteines. When preparing such a powder, the alliinase activity is likely to be inhibited under freezing conditions during the production. If such powder with 'allicin potential' is ingested as acid-resistant capsules to avoid acid

inactivation in the stomach, the allicin, which must be generated in the intestine, may work somehow, although the true activity *in vivo* remains to be clarified.

1.4 Commercial products of Onion

1.4.1 Dehydrated Onion Pieces

Cured or dried onion has 4 to 5% moisture to allow good storage and acceptable quality. The product is processed to make powder, granules, flakes or slices, then used for the formulation of sausages, meat products, many kinds of soups and sauces as well as dressings.

1.4.2 Onion Powder

Onion powder is prepared either from dehydrated onion pieces or from puree. A stronger flavored product is obtained by spray drying. The powder is a uniform product of which 95% passes a sieve of 0.25 mm aperture size. This is the finest among onion products including grifts, flakes, slices and rings, and used for soups, relishes, sauces, and products that do not require onion appearance and texture. Discoloration develops during the processing of onion. Many publications have described its cause, since it is of particular interest to manufacturers producing high quality onion products. Lukes *et al*. [22] demonstrated that in garlic puree, the content of *S*-1-propenylcysteine sulfoxide, was significantly correlated to the development of a green pigment. This group also demonstrated that storing the puree at 23 to 28 °C could prevent color development for as long as 32 days. At lower temperatures, 12 °C or 3 °C, the purree colored green upon 18 days of storage, and dark to blue-green upon 32 days of storage. These evidences clearly suggest that quick conversion of *S*-1-propenylcysteine sulfoxide to its metabolites (sulfinyl compounds and sulfides) by the enzymatic action of alliinase is a positive factor in preventing discoloration of garlic. The same mechanism may be adapted to onions.

1.4.3 Onion Oil

As garlic oil, onion oil is obtained by distillation of minced onion. Most onion oil components are generated enzymatically from their precursors such as S-1-propenylcysteine sulfoxide, S-1-propylcysteine sulfoxide and S-methylcysteine sulfoxide. Therefore, essential oil is an incorrect term to be used for oil from onion or garlic. The minced onion is allowed to stand at ambient temperature for a few hours prior to distillation to complete the enzymatic and successive chemical reactions. The onion oil can be obtained in 0.002 to 0.03% yields as a brown-amber liquid, and collected from the bottom of a vessel placed under a steam condenser.

The chemical composition of onion oil is confined to a series of sulfides, namely dimethyl sulfide, dipropenyl disulfide, dipropyl disulfide, and dipropenyl trisulfide. According to Fenwick and Hanley [23], onion oil possesses (on a weight basis) 800 to 1,000 times the odor strength of fresh onion, but its commercial value may be many thousands times that of the onion. Actually, the product smells extraordinarly strong, and its availability expands to many food productions because of its solubility, lack of color, and strong aroma. From a functional food view point, onion oil is now comparable to garlic oil.

1.4.4 Onion Salt

Onion salt is a mixture of onion powder and salt for use at the table or in cooking. It can be used whenever salt is required. The product is prepared with an anticaking agent (calcium stearate, 1 to 2%) and hydrogenated vegetable oil.

1.4.5 Pickled Onion

Small onions such as a button onion or a silver skin onion may be preserved in vinegar as pickled products. A translucent product with a desired texture is preferable. Usually, onion is soaked in 10% saline solution for 24 h, is transferred to a bottle, and spiced vinegar is added. This is best eaten after 2 weeks, and it may be used within 6 months. As mentioned in the section of garlic, pickling onion in the acid solution fully retains cysteine derivatives, which may exhibit hypoglycemic and hypolipidemic activities.

1.5 Other Sources Of Polysulfides[1]

Over the years, there have been sporadic reports of thiosulfonates and thiosulfinates isolated from plants and found to possess (weak) antibiotic activity (**Figure 1.3**). 2,4,5,7,-tetrathiaoctane 4,4,-dioxide **(31)**, together with several other S-oxides (such as **(32)**) occurs in the fruit of *Scorodocarpus borneensis*, also known 'wood garlic', which belongs to the *Olaceae* family. This plant grows naturally on the island of Borneo and the Malay Peninsula and whose fruit is locally used as a seasoning [24]. This thiosulfonate was active against a range of bacteria and fungi, such as *Bacillus subtilis*, *Microbacterium smegmatis*, *Escherichia coli*, *Candida albicans*, *Saccharomyces cervisiae*, *Mucor racemosus* and *Aspergillus niger*, with minimum inhibitory concentrations around 50 µg/ml [24]. Interestingly this thiosulfinate is less active, yet more widely applicable than penicillin (against bacteria) and nystatin (against fungi). The three thiosulfonates 2,3,5,7-tetrathiaoctane-3,3-dioxide **(33)**, methyl methanethiosulfonate **(34)** and 2,3,5-trithiahexane-3,3-dioxide **(35)** have recently been isolated from the bark of *Scorodophloeus zenkeri*, a small tree which is found in Central Africa, which has a distinct smell of garlic (its seeds, bark and wood are locally used as spices) [25, 26]. 2,3,5-trithiahexane-3,3-dioxide **(35)** is weakly active against bacteria such as *Escherichia coli* and *Bacillus subtilis*, where it significantly reduces the cellular ATP content[26].

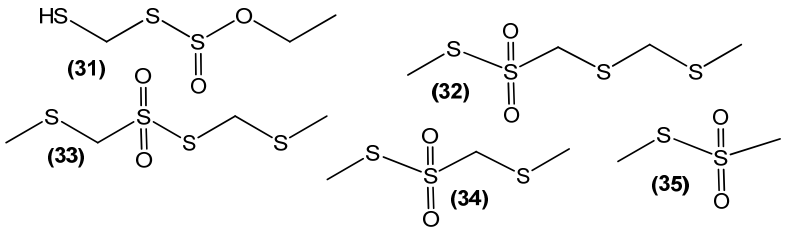


Figure 1.3 Additional Thiosulfonates and thiosulfinates found in *Scorodocarpus borneensis* **(31)** and **(32)** ,*Scorodophloeus zenkeri* **(33)-(35)**.

[1] The term polysulfide here is denoted to organic and inorganic sulfur species which contain sulfur-sulfur chains with sulfur chain length of three or more sulfur atoms. This is due to the fact that most natural, organic compounds discussed here are commonly known as 'sulfides', such as diallyltrisulfide. Please note, however, that strictly speaking organic molecules containing such groups (RS_xR, $x \geq 3$, $R \neq H$) should be referred to as 'polysulfanes', while inorganic species of the type S_x^{2-} ($x \geq 3$) should be called 'polysulfides'.

Polysulfides are found in many plants and lower organisms in addition to *Allium* species. Linear polysulfides, such as DATS **(9)**, DATTS **(10)** and chemically related methyl-, ethyl- and propyl-based tri- and tetrasulfides also occur in trees, such as in barks of *Scorodophleus zenkeri* Harms from which a range of different di- and trisulfides with antibacterial and antifungal activity have been isolated **(36)-(38)**) [25, 26]. (**Figure 1.4**)

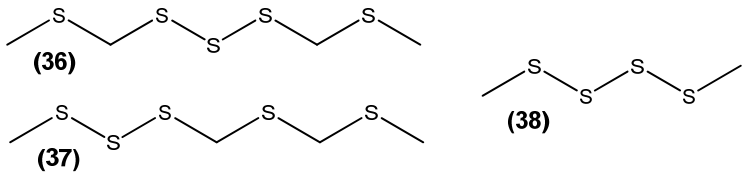


Figure 1.4 Linear polysulfides isolated from the bark of *Scorodophleus zenkeri* (**(36)-(38)**)

Various edible species also contain cyclic polysulfides, such as Shiitake mushrooms (lenthionine **(39)** and 1,2,3,4,5,6-thiepane **(40),** 1,2,4,6-tetrathiepane **(41)**, 1,2,4-trithiolane **(42)**) [27-29], asparagus (1,2,3-trithiane-5-carboxylic acid **(43)**, a plant growth inhibitor also suspected to act as contact allergen in dermatitis) [30] and the Mimosaceae *Parkia sp*. (lenthionine and a range of di- and polysulfides, such as **(44)**) [31]. Seeds of the latter, when treated with water, emit a distinct onion-like odour, are a favourite food ingredient in Indonesia and have been employed in folk medicine as antibacterial agents. Cyclic polysulfides have also been found in the New Zealand ascidian *Aplidium sp.* (1,2,3-trithiane derivatives **(45)**) [32, 33], the alga *Chara Globularis* (1,2,3-trithiane derivatives **(46)**) [34] and the South American tree *Cassipourea guianensis* (*N*-(2-hydroxyethyl)-5-hydroxy-1,2,3-trithiane-4-carboxamide **(47)**) (**Figure 1.5**) [35].

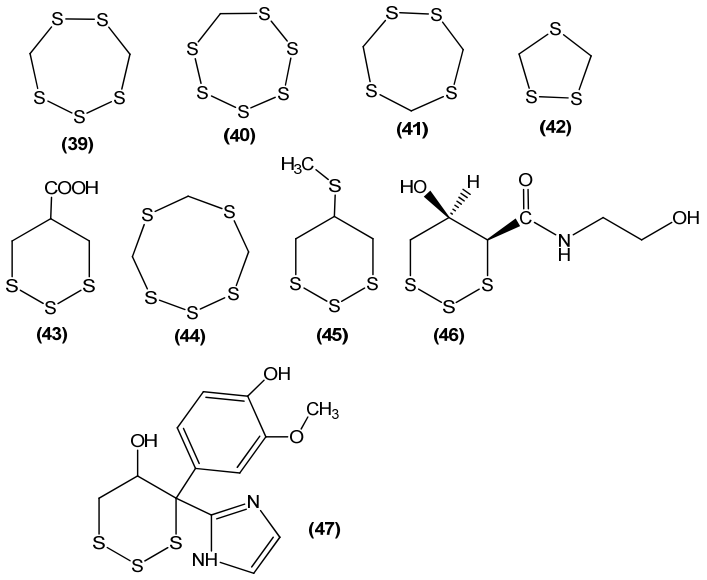


Figure 1.5 Cyclic polysulfides found in shiitake mushroom (**(39)-(42)**), Asparagus **(43)**, Mimosaceae *Parkia sp.* **(44),** ascidian *Aplidium sp.* **(45),** algae *Chara Globularis* **(46),** and *Cassipourea guianensis* **(47)**

Cyclic polysulfides with significant therapeutic potential have been isolated from the far-eastern ascidian genus *Lissoclinum* (varacin **(48)**[36], lissoclinotoxin A **(49)**[37]) and *Polycitor sp* (varacin A-C **(50)**, **(51)**, **(52)**[38]).(**Figure 1.6**)

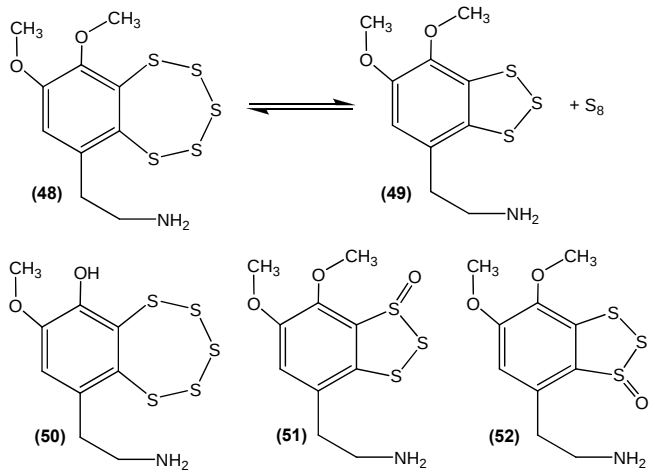


Figure 1.6 A range of chemically closely related polysulfides and polysulfide-S-oxides have been isolated from ascidians, including varacin **(48)** and formation of varacin A **(49)** with release of sulfur molecule, lissoclinotoxin **(50),** varacin B **(51)** and varacin C **(52)**.

Interestingly, the thiolhistidines ergothioneine (ESH **(53)**) are catalytically active. Ergothioneine, 2-thiol-L-histidine, isolated first from ergot in 1909, is produced by certain fungi and the *Actinomycetales* bacteria (*e.g. M. tuberculosis*) [39]. It is taken up and enriched by many plants, fungi, bacteria and animals, including humans [40]. For example, human red blood cells contain up to 3.7 mg ESH per 100 ml of blood and up to 115 mg ESH per 100 g lens [39, 40]. ESH uptake in human cells is not random, as the recent discovery of the first ESH transporter in erythrocytes and monocytes has illustrated [41]. (**Figure 1.7**)

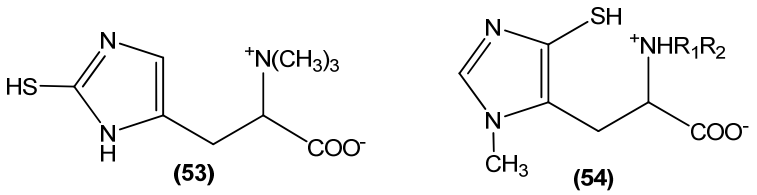


Figure 1.7 Ergothionine **(53)** isolated from ergot and fungi and Ovothiol **(54)** isolated from the eggs of sea urchin.

Ovothiols **(54)** form a group of three chemically closely related 1-methyl-4-thiol-histidine derivates and have been identified in echinoderms, *i.e.* in the eggs of sea urchins, in 1985 [42]. **(Figure 1.7)**. The intracellular concentration of OvSH in these eggs is estimated at 6.8 mM, and they seem to fulfil an antioxidant role during cell division. Within this context, ovothiols are known to facilitate catalytic removal of H_2O_2 [43].

Chapter 2

Biological activities associated with Polysulfides

2.1 Selective activity against cancer cells

The possible therapeutic significance of diallylsulfides and related natural polysulfides has been boosted by recent studies on anticancer effects of DATS **(9)**. Although these studies must be considered as preliminary, they have provided insight into biological activities and rather unexpected regulatory cellular events associated with polysulfides. Such activities will be explained here as part of this PhD thesis.

In order to briefly emphasize the importance of these developments from a pharmacological point of view, it is worth comparing current efforts to employ allicin **(3)** and DATS **(9)** as anti-cancer agents. Unlike bacteria, fungi and microbes, cancer cells are difficult to reach. The cytotoxic effects associated with allicin and diallylsulfides in cell culture may therefore not translate into proper anticancer activity in animals. As a consequence, allicin, being chemically unstable and highly reactive, has recently played a part in an elegant, yet highly complex system of antibody-directed enzyme prodrug therapy (ADEPT), developed during the last couple of years by researchers at the Weizmann institute [44, 45]. This approach employs a cancer cell selective antibody-alliinase hybrid and alliin as selective 'allicin generating' system, and has shown some promise in a nude mouse model.

In contrast, several independent studies published during the last two to three years have found that DATS **(9)** may be stable enough to reach the tumour site without delivery system. In addition, it may also selectively attack cancer cells without the need of

complicated delivery or recognition and by a biochemical mechanism quite different from the one known for allicin.

In 2005, Yuan and colleagues reported an important link between DATS **(9)** and cancer cell death [46]. They found that cells of the human gastric cancer cell lines MGC803 and SGC7901 were killed by the trisulfide with an IC_{50} value of around 7 μg/ml. Cell death bore certain hallmarks of necrosis and was associated with significant increases of cell numbers in the G_2-M phase and decreases in the G_0-G_1 phase, as well as an increased expression of p21.

These findings were mirrored in a study by Seki and colleagues published the same year, who noticed that proliferation of cells of the human colon cell lines HCT-15 and DLD-1 was inhibited by DATS **(9)** with an IC_{50} value of 11.5 and 13.3 μM, respectively [47]. This effect was investigated further and found to be the result of DATS **(9)**-induced G_2-M cell cycle arrest. Apoptosis of the cells seemed to be associated with oxidative modification of β-tubulin: The trisulfide at 10 μM was found to selectively thiolate β-tubulin cysteine residues Cys-12 and Cys-354 to form S-allylmercaptocysteine modifications and inhibit tubulin polymerisation and microtubuli formation in an *in vitro* cell free system. In contrast, 100 μM concentrations of the corresponding mono- and disulfide had no effect on microtubuli formation. Antitumour activity of DATS **(9)** was also confirmed in a HCT-15 xenograft mouse model, where the compound significantly reduced tumour volume (apparently by necrosis) without any apparent side effects to the animals [47].

The biochemical basis of the cytotoxic behaviour of DATS **(9)**, which stands in stark contrast to its mono- and disulfide analogues, was also investigated by Singh and colleagues [48-51]. Research of this group demonstrated the ability of DATS **(9)** (20-40 μM), but not diallylsulfide or DADS **(8)** (at the same concentrations), to induce G_2-M phase cell cycle arrest in cultured PC-3 human prostate cancer cells. This event seemed to be related to a DATS **(9)**-induced increase in intracellular levels of oxidative stress. Amazingly, cultured normal PrEC prostate ephithelial cells were not affected by DATS **(9)**, even at 40 to 80 μM concentrations [48]. Quite surprisingly, these findings point towards a selective toxicity of DATS **(9)** in cancer cells, but not in the corresponding normal cells.

These studies indicate that DATS **(9)** may surpass allicin **(3)** as far as chemical stability, toxicity and maybe even selective targeting are concerned. It is therefore important to study the biological activity of DATS **(9)**, DATTS **(10)** and possibily higher polysulfides such as diallylpentasulfide **(11)** and diallylhexasulfide **(12)**. From a chemist's point of view, the biochemical findings need to be related to chemical properties of polysulfides, which seem to be surprisingly well suited for the various biochemical tasks at hand.

2.2 Cell cycle arrest and induction of apoptosis

Sulfur compounds are known to interfere with a range of cellular pathways such as inhibition of phase I metabolic enzymes [3], induction of phase II enzymes [52], inhibition of histone deacetylase (HDAC) [53] and cell cycle arrest and induction of apoptosis [48]. Perhaps even more interesting, however, are events which not only slow cancer cell proliferation, but kill cancer cells. In this context, DATS **(9)** and DATTS **(10)** have received considerable attention. A series of independent studies published during the last five years have found activity against MGC803 and SGC7901 human gastric cancer cell lines (Yuan and colleagues) [54], HCT-15 and DLD-1 colon cancer cell lines (Seki and colleagues) [47], Caco-2 and HT-29 colon carcinoma cancer cells (Jakubikova and Sedlak) [55] and PC-3 and DU145 prostate cancer cell lines (Singh and colleagues) [48], to name just a few. Singh and colleagues also noted a cancer-cell selective activity of DATS against PC-3 cells, whilst normal prostate cells (Pr-EC) were largely unaffected. The possibility that a chemically simple molecule such as DATS **(9)** may distinguish between cancer cells and normal cells is intriguing, and has led to an intensive search for the biochemical causes of this effect [50].

Among them, oxidative modification of cysteine residues in proteins, such as β-tubulin, *via* a thiol/trisulfide exchange mechanism involving DATS **(9)** is a possibility, although DATS is not a particularly strong oxidant [47]. Interestingly, it appears that polysulfides such as DATS **(9)** are also able to (catalytically) generate $O_2^{\bullet-}$ in the presence of dioxygen and GSH and hence raise intracellular levels of OS, which may push cancer cells already high in ROS over a critical ROS threshold [16, 19]. In this context, Singh and

colleagues have suggested that DATS **(9)** may actually trigger the degradation of ferritin, which is associated with iron ion release, subsequent ROS generation, G2/M cycle arrest and apoptosis, a chain of events which may in part be under the control of the c-Jun NH_2-terminal kinase (JNK) signalling axis [53].

Apart from DATS **(9)**, a mitotic arrest has also been observed for DAS **(7)**, DADS **(8)**, SAMC **(5)** and ajoene **(15)**, although the underlying (bio)chemistry is less apparent (yet may still be associated with ROS formation). Interestingly, cell cycle arrests are not limited to the G2/M phase: DADS **(8)** may also cause arrest in the S-phase, while DATS **(9)** can cause arrest in the G1 phase *via* a decrease in cyclin D1 and an increase in p27 in human gastric cancer cell line BGC823 [53].

The ability of sulfur compounds to kill cancer cells is not solely linked to mitotic arrest *via* ROS formation. Induction of apoptosis by changing the Bcl-2 to Bax ratio provides another entry point which leads to apoptosis *via* the caspase pathway [53]. In this context, DATS **(9)**, ajoene **(15)**, and, to a lesser extent, DADS **(8)** and DAS **(7)**, have been associated with a decrease in Bcl-2 levels, which triggers the intrinsic pathway of cell death. The biochemistry surrounding apoptosis in cancer cells is, of course, quite complex, and other pathways, such as the Akt-Bad pathway (in the case of DATS) and calcium-dependent pathways leading to increased levels of H_2O_2 and caspase-3 activity (in the case of DADS) have also been implicated. The role of p53 in sulfur compound-induced cell death is still not fully understood.[2] Finally, the possibility of H_2S release from polysulfides, which has been postulated for DATS and DATTS (and also DADS), may associate these agents with a host of different cell signalling pathways [57]. If, and to which extent, this may also include signalling pathways associated with cancer promotion or progression is still unclear and needs to be investigated further.

[2] For more details on sulfur compounds from garlic and their role in apoptosis of cancer cells see Herman-Antosiewicz *et al.* [53]. Two recent reviews, one by Mersch-Sundermann and colleagues [56], the other by Shukla and Kalra [3] provide further, more general details of the rather complicated relationship between sulfur compounds from *Allium* vegetables, cancer chemoprevention in different cancer cells (cell lines) and the possible modes of action.

2.3 Nematicidal effects

Farmers worldwide face the dilemma of controlling pests and increasing food production in a manner that is economical and protective of the environment at the same time. Given the estimated 12 billion dollar worldwide annual market for insect control, and driven by the economic and human costs of crop losses, as well as the persistence of pesticides in the environment and their adverse biological impact, there is a clear need for "green" alternatives [58]. A biomimetic approach, based on naturally evolved mechanisms for plant protection, is an appealing option. The extensively studied sulfur compounds derived from genus *Allium* plants such as garlic and onions [14, 59] represent excellent candidates for detailed studies as green nematicides, particularly in view of recent work suggesting that such pungent natural compounds transmit a pain signal through activation of the Transient Receptor Potential (TRP) family of ion channels, *e.g*. TRPA1, and that this family of ion channels is conserved in species ranging from nematodes to humans. It is further suggested that these natural compounds activate TRPA1 ion channels through covalent modification of cysteines, consistent with work reported as early as 1945 that the garlic compound allicin readily reacts with cysteine.

There has in fact been considerable interest in the development of pesticides based on organosulfur compounds derived from garlic, onions and other *Allium* species since such food-based compounds should be environmentally benign, and potentially widely available and low in cost. It is known that *Allium* species are resistant to insects and fungi and that extracts and distillates of these plants can function as nematicides [60] and repellents against a range of pests [61]. The repellent effect of garlic toward the nematode *C. elegens* has been specifically noted [62]. Onion oil, DPDS **(26)** and DPTS **(27)** were tested for their nematicidal activity against the pine wood nematode, *Bursaphelenchus xylophilus*. The LC_{50} values of onion oil against male, female and juvenile pine wood nematodes were 17.6, 13.8 and 12.1 mug/ml, respectively [63], [64]. Similarly, allicin **(3)** was tested against a tomato root knot nematode, *Meloidogyne incognita*.

Not surprisingly, *Allium* species are used in inter-cropping to protect crops such as carrots from pests [65]. In addition, certain of the compounds from *Allium* species have been found to function as growth stimulators. These green chemicals are not harmful for

environment and their potential application as individual component as well distilled oils of garlic and onion were extensively studied as part of this PhD thesis.

2.4 Other Effects

Suppression of platelet aggregation is the most prominent effect of garlic intake. Bordia and Bansal (1973) [66] described the suppressive effect of garlic or garlic oil against platelet aggregation in human studies. Agriga *et al*. (1981) [67] isolated a potent inhibitory compound from garlic oil, and identified it as methyl allyltrisulfide. Ajoene **(15)** was discovered as the most potent platelet inhibitor from an oil-macerated crushed garlic by Apitz-Castro *et al.* (1983)[68]. Sulfur compounds from garlic were considered to protect insulin from its inactivation with cysteine, glutathione, and albumin by blocking their SH groups [69]. Since, alliin (200 mg/kg) is known to reduce the plasma glucose level in the alloxan-induced diabetic rats, and regenerate their pancreas [70]. Eating boiled garlic, which has no annoying odour, may render the antidiabetic effect.

Garlic and its sulfur compounds have many other beneficial effects *e.g*., blood pressure-lowering, diuretic, anti-inflammatory, immunomodulatory, and hormone secretory effects, as well as enhancing effects, on vitamin B1 absorption. The recent findings by Oi *et al*. [71] are noticeable. They found that garlic compounds, especially allyl-containing structures, stimulate both noradrenaline secretion from nervous system and testosterone from testis. These hormones may act to modulate lipid and protein metabolisms (enhancement of lipid catabolism and protein anabolism [72]).

Chapter 3

Physiochemical properties of Polysulfides

Apart from stimulating a therapeutic interest in diallylpolysulfides, these findings also raise several important chemical and biochemical questions related to the mode of action of trisulfides and tetrasulfides in biological systems. Why, for instance, are polysulfides RS_xR' ($x \geq 3$) toxic against bacteria, fungi and certain types of human cells? How do they interact with other cellular components, such as glutathione (GSH), peptides, proteins, DNA and membranes? Is there an optimal sulfur-chain length *x* for maximum biological activity? How may simple molecules such as diallyltrisulfide distinguish between normal and cancer cells?

DATS **(9)** and DATTS **(10)** seem to rely on thiols as intracellular reaction partners to trigger a highly complicated biological (redox) chemistry which has hardly been explored to date – yet may explain a lot of the biological findings currently associated with polysulfides. As a part of this PhD thesis, different biochemical events associated with polysulfides from a chemical point of view have been explored which are discussed in this chapter.

3.1 Sulfur as Redox Chameleon

The chemical element sulfur is highly abundant in most organisms. In the average 70-kg human adult, it is present in amount of about 140 g, which is similar to the occurrence of potassium. Apart from its high abundance, the distinct redox, catalytic and metal-binding properties of sulfur ensure that it plays a central role in biology [73].

The hallmarks of this sulfur chemistry, which set the element apart from most other members of the periodic table, can be summarized as follows: sulfur is able to occur in

around 10 different oxidation states *in vivo*, which range from -2 in thiols (RSH) to +6 in sulfate (SO_4^{2-}) anions and include several fractional formal oxidation states, such as -0.5 in the disulfide radical anion ($RSSR^{\bullet-}$). These sulfur oxidation states are found in numerous sulfur chemo-types, *i.e*. sulfur-containing compounds with distinct sulfur functional groups, such as thiols, thiyl radicals, disulfides, sulfinic and sulfonic acids, thiosulfinates, thiosulfonates and polysulfides (**Table 3.1**). Considering that each of these chemo-types represents many individual sulfur compounds, the diversity of sulfur species in biology becomes apparent [4].

The occurrence of sulfur-containing agents *in vivo* is reflected by the chemical properties and (bio-) chemical reaction behaviour associated with them. Redox-active sulfur agents are able to participate in one- and two-electron transfer, hydride transfer, hydrogen atom transfer, radical processes and exchange reactions. Many sulfur compounds, such as thiols, also blind to metal ions and give rise to a range of bioinorganic (transition) metal complexes. The latter are found, for instance, in iron/sulfur, zinc/sulfur and copper/sulfur proteins. Ultimately, all embracing, yet distinct, *in vivo* redox networks of sulfur are emerging for various organisms, such as plants, bacteria, fungi, yeasts, insects, nematodes and also for humans, which incorporate chemical reactivity at the level of molecules into intracellular events and physiological processes affecting the whole organism [4].

As far as the redox-activity of sulfur is concerned, all species depicted in **Table 3.1** are redox-active and can be derived in principle from the thiol oxidation state by nucleophilic substitution and/or oxidation. Oxidation processes play a major role inside living cells. Thiols can act as ‘sacrificial’ and catalytic reducing agents able to counteract a range of dangerous oxidative stressors (*e.g.* peroxides, hydroxyl radicals, peroxynitrite). Indeed, thiol and sulfide oxidation during oxidative stress (OS) results in a cocktail of reactive sulfur species which contains most of the chemotypes shown in **Table 3.1**.

The underlying sulfur redox-mechanisms differ widely and include one- and two-electron transfer reactions, hydride and oxygen transfers, thiol/disulfide exchanges, nucleophilic exchanges and radical reactions. In some instances, these reactions also

Table 3.1 Summary of RSS, their general formula, oxidation state of sulfur, occurance and reactivity, adopted from Jacob 2006 [4].

Reactive species	Formula	Sulfur oxidation state	Occurrence (examples only)	Reactivity
Thiol	RSH	-2	GSH and proteins	Reducing agent
Disulfide radical anion	RSSR	-1.5 (overall)	Intermediate of RS radical reaction	Strongly reducing and may generate O_2
Disulfide radical cation	R2S SR2	-1.5 (overall)	Intermediate of methionine oxidation	Unclear and one –electron oxidant
Thiyl radical	RS	-1	One-electron oxidation of RSH, e.g. in enzymes	Reducing or oxidizing and dimerises to RSSR
Perthiol	RSSH	-1	Formed by reduction of polysulfides	Strongly reducing and may generate O_2
Perthiyl radical	RSS	-1, 0	Intermediate in perthiol reactions	Dimerises to polysulfide
Disulfide	RSSR	-1	GSSG and numerous proteins	Mild oxidant (reduced to RSH) andcan be oxidised
Trisulfide	RSSSR	-1,0,-1	GSSG, diallyl polysulfides and leinamycin products	Mild oxidant and interesting follow-on chemistry
Tetrasulfide	RSSSSR	-1,0,0,-1	DATTS in garlic	Mild oxidant with interesting follow-on chemistry
Pentasulfide	RSSSSSR	-1,0,0,0,-1	Varacin	Mild oxidant with complex follow-on chemistry
Hexasulfide	RSSSSSSR	-1,0,0,0,0,-1	Unclear, 1,2,3,4,5,6-hexathiepae in Shiitake mushrooms	Probably very weak oxidant and chemistry mostly unknown
Sulfenic acid	RSOH	0	Prx and NADH oxidase	Oxidising or reducing and demerises to RS(O)SR
Sulfinic acid	RS(O)OH	+2	Prx and proteins oxidised during OS	Dead-end product of thiol oxidation and in Prx reversed by sulfiredoxin
Sulfonic acid	RS(O)2OH	+4	Taurine and overoxidised proteins	Irreversible oxidation product of RSH oxidation
Sulfate	SO4	+6	Glucosinolates and heparin sulfate	Sulfatation of S and O atoms and cleavage of such esters can trigger follow-on reaction
Thiosulfinate	RS(O)SR	+1, -1	Allicin and Prx	Highly reactive thiolation agent and oxidising
Thiosulfonate	RS(O)2SR	+3,-1	Unclear	Highly reactive thiolation agent and oxidising
Isothiocyanate	RSCN	-2	Products of myrosinase	Strongly electrophilic and modifies thiols and amines

enable sulfur-based redox-catalysis, superoxide radical generation, and various decomposition reactions [74].

Thiols, disulfides, sulfenic and sulfinic acids are also able to coordinate to metal ions, with each modification exhibiting its own preference for copper, zinc, cadmium, iron and cobalt ions. As a result, thiol agents play a major role in metal trafficking and detoxification. The interplay of redox-activity and metal-binding forms the basis for several biochemical control mechanisms, such as redox-control of metal binding in the metallothioneins (MTs) [75] and metal-based control of cysteine enzyme activity, which has been reviewed recently [73], [76].

3.1.1 Thiosulfinates, thiosulfonates and sulfones

Among the natural thiosulfinates, allicin **(3)** has been extensively studied as an active ingredient of garlic. The redox behaviour of thiosulfinates is characterized by a high reactivity and selectivity towards thiols which allows one thiosulfinate molecule to consume up to four thiol equivalents [77-79]. The initial reaction of thiosulfinate RS(O)SR with a protein or low molecular weight thiol R'SH results in a mixed disulfide RSSR' and a sulfenic acid RSOH (**Figure 3.1**). The latter reacts with a second thiol equivalent R'SH to form a mixed disulfide RSSR' and water. Depending on the relative redox-potentials and concentrations of RSSR' and RSH, the two RSSR' formed in this process might react further with two R'SH to form two R'SSR' and two RSH. As an example, one allicin molecule can oxidise four GSH molecules to generate two GSSG and two allylmercaptan **(6)** molecules. Allylmercaptan has its own biochemical activity, which might not always be beneficial; by acting as an undesired ligand for certain metal ions, it might inhibit metalloenzymes, as has been observed for various thiols [73].

The oxidising power of thiosulfinates is limited to thiols, they do not interact with other common cellular reducing agents, such as NAD(P)H or vitamin C [80]. Nonetheless, the attack of reactive thiosulfinates towards thiols is 'ruthless', *i.e.* they oxidise virtually any cysteine protein they encounter. While mammalian cells are protected against such an assault by millimolar concentrations of GSH, many microbes, bacteria and fungi seem to be readily killed by thiosulfinates such as allicin - and stand little chance of developing a resistance against such an unspecific cysteine protein oxidant.

This extraordinary oxidising behaviour has significant implications for the therapeutic use of thiosulfinates. Allicin exhibits antimicrobial, antibacterial and antifungal properties, although many of these effects may not be significant in a clinical setting (for an overview of biological activities of garlic ingredients see Agarwal [81] and Davis[82]).

The spectrum of natural thiosulfinates is not limited to allicin and its derivatives, and includes two closely related cyclic thiosulfinate analogues of the disulfide gerrardine, which have recently been isolated from *Cassipourea guianensis* [83]. Little is known about their formation pathway or biological activity (**Figure 3.1**, **(56)**).

Similarly, thiosulfonates ($RS(O)_2SR'$) have only sporadically been studied to date. Theoretically, either oxidation of a disulfide or thiosulfinate are possible thiosulfinate formation pathways, although the reaction of certain C-S-lyase cleavage products might also result in this chemotype. Like thiosulfinates, thiosulfonates react readily with thiols, yet are less potent as oxidants: The sulfinic acid formed in place of the sulfenic acid is normally unreactive towards thiols - and one thiosulfonate therefore oxidises two thiol equivalents instead of four. Indeed, pseudoallicin **(55)**, the thiosulfonate analogue of allicin, was found to possess only about half the antibacterial activity of allicin (**Figure 3.1**) [84]. Apart from reactivity and reaction stoichiometry, parameters such as chemical stability, membrane permeability and kinetic effects need to be considered in this context, too.

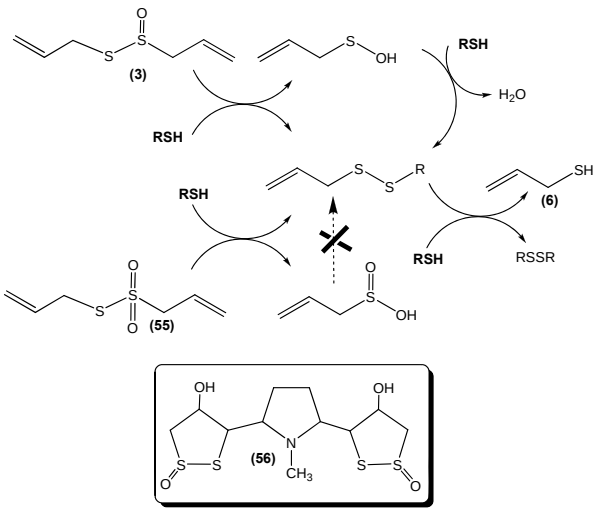


Figure 3.1 The natural thiosulfinate allicin **(3)** is strong yet thiol specific oxidizing agent which can consume 4 RSH equivalents to generate RSSR and allylmercaptan **(6)**, pseudoallicin **(55)**. There has also been claim of a naturally occurring thiosulfinate in *Cassipourea guianensis* **(56)**.

Within this context, it should be mentioned that a range of chemically closely related sulfones, some which have also been isolated from *Scorodocarpus borneensis* (**(57)** and **(58)**) and Shiitake mushrooms **(59)**, exhibit a similar, or even better antimicrobial activity when compared to thiosulfonates [24]. This raises the question, if sulfones possess a particular biochemical activity profile or if an enzymatically formed thiosulfinate or thiosulfonate, rather than the sulfone, is responsible for the observed activity, in analogy with the sulfoxide/thiosulfinate conversion. There is little doubt that the formation pathway(s) of thiosulfinates and thiosulfonates need to be evaluated further. Thiosulfonates, which possess a similar reactivity, yet are more stable than thiosulfinates, might also provide new leads for the development of antibiotic agents.

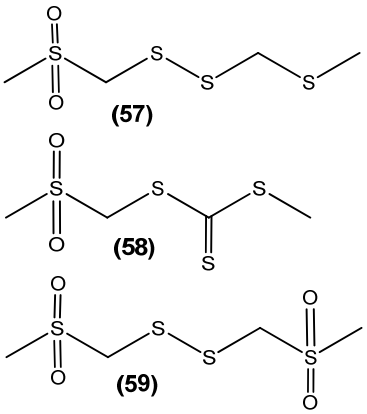


Figure 3.2 Structures of various sulfones, isolated from *Scorodocarpus borneensis* (**(57)** and **(58)**) and Shiitake mushroom **(59)**.

3.2 Polysulfides as multifaceted toxins

In analogy with allicin **(3)**, disulfides and polysulfides are often considered as oxidants able to modify protein thiols to mixed disulfides, with concomitant disturbance of protein function and subsequent cellular responses, including cell death [47]. The important role such thiolation reactions play as part of various cellular signalling processes is currently becoming apparent. For instance, a very recent study by Julius and colleagues has linked the covalent modification of cysteine residues present in the nonselective cation channel TRPA1 of sensory nerve endings by diallyldisulfide to acute pain [85, 86]. Disulfides react with thiols, including cysteine residues in proteins, *via* thiol/disulfide

exchange reactions. The latter may also be seen as thiolation or thiol oxidation reactions, since they result in a mixed disulfide at the protein site.

If aspects of this disulfide chemistry are projected to tri- and tetrasulfides, those compounds may undergo a similar kind of thiol/polysulfide exchange reaction, which would result in a mixed disulfide, *i.e.* thiolated protein, and a reduced species, such as RSH or RSSH (**Figure 3.3**). For instance, diallyltrisulfide is thought to thiolate β-tubulin and hence disturb the protein's biochemical function [47]. Similar thiolation reactions have also been associated with calicheamine, which reacts with GSH [87].

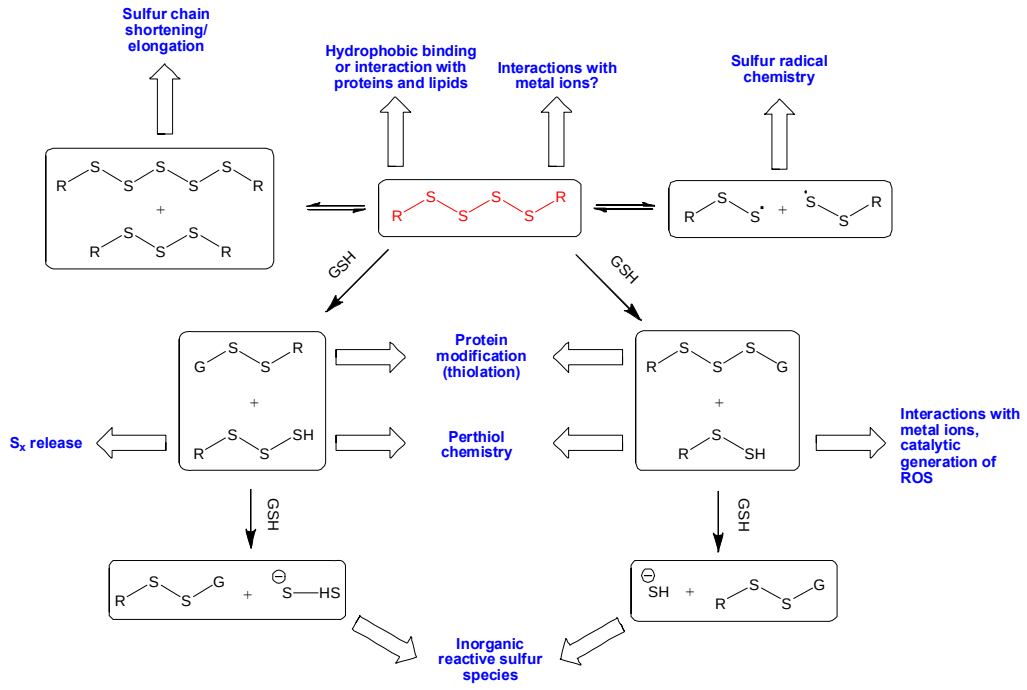


Figure 3.3 Schematic overview of chemical reactions and biochemical actions associated with a polysulfide such as diallyltetrasulfide. Figure adopted from Muenchberg and Anwar *et.al.* [16]. Some biochemical effects, such as binding to hydrophobic parts in proteins and membranes or to metal ions, may be associated with the polysulfide itself. Other biological activities may be the result of polysulfide reaction products, such as thiols, perthiols and inorganic sulfur species. Please note that almost all biochemical effects are in one way or another detrimental to the living cells.

This rather straightforward view ignores, however, three major aspects of polysulfide chemistry. Firstly, thiolation reactions, such as the modification of β-tubulin, should be reversible in cells with sufficient levels of reduced glutathione (GSH). In the absence of oxidative stress, such cells should overcome the effects of micromolar concentrations of polysulfides rather easily, even if 'complete' reduction of a tri- or tetrasulfide may require several equivalents of GSH.

Secondly, while trisulfides and tetrasulfides may be more reactive towards protein thiols than disulfides, their rates of reaction are unlikely to be of the same order as the ones of allicin **(3)**, which reacts with most thiols within seconds to minutes [88, 89]. Nonetheless, diallyltetrasulfide **(10)** and allicin **(3)** have comparable biological activities.

Thirdly, the chemistry polysulfides are able to conduct in a biological setting is considerably more complex than simple thiol/polysulfide exchanges and, based on *in vitro* evidence, may also include various types of oxidation, radical generation, protein modification and enzyme inhibition reactions. These 'additional' polysulfide reactions also need to be taken into account before a likely mode of action is proposed. Just focusing on thiolation alone would be an insult to the chemical diversity of tri- and tetrasulfides.

Initially, it is therefore worth taking an open, unbiased and all-embracing view when considering the different physical and chemical properties of polysulfides – and only ruling out individual possibilities once firm experimental counter-evidence has been found. The latter may, for instance, come from biological activity rankings, such as the relationship between the number of sulfur atoms and activity.

Opening the chemist's treasure chest, we find various oxidation, radical generation and decomposition reactions as well as enzyme inhibition and hydrophobic interactions associated with polysulfides and their diverse follow-on products, such as thiols, perthiols, thiyl radicals, perthiyl radicals, S_x and inorganic (poly-)sulfide anions. In order to explain aspects of the biological activity of polysulfides, such as toxicity against bacteria, fungi and cancer cells, one may therefore construct a provisional, necessarily incomplete network of chemical reactions and biochemical responses (**Figure 3.3**).

The network emerging hints at an interplay of several different chemical reactions, some of which may trigger others, and as a whole may interfere with response and signalling pathways in cells. It should be noted upfront that the following discussion of these processes is speculative at times. Aspects of this network will be studied as part of this PhD thesis.

3.2.1 Thiolation reactions

As mentioned above, one common explanation for the biological activity of tri- and tetrasulfides is their ability to react with (protein) thiols. In many ways, this reaction is most familiar to us, and well known from the thiolation behaviour of disulfides such as glutathione disulfide (GSSG). Some known examples of suspected thiolation reactions by diallyltrisulfide have already been discussed. Although aspects of thiol/polysulfide exchange reactions involving proteins, such as thermodynamic and kinetic parameters, are often unknown, a reaction mechanism similar to the one of the thiol/disulfide exchange reaction is assumed. The biochemical importance of the reduced product of this exchange is sometimes ignored. In the case of trisulfides (RSSSR), the reaction with a thiol (R'SH) results in a mixed disulfide (RSSR') and a persulfide (RSSH). Some researchers consider the latter as the actual biologically active form of the polysulfide [19] [87] [90].

The case of diallyltetrasulfide **(10)** is even more complicated since in theory, this molecule contains two possible positions for nucleophilic attack, *i.e.* at one of the two 'terminal' S-S-bonds and at the central S-S-bond. Apparently, attack at the central bond with formation of a trisulfide (RSSSR') and a hydropersulfide (RSSH) are preferred [19]. Nonetheless, the alternative, *i.e.* formation of a disulfide (RSSR') and a hydrogen trisulfide (RSSSH) from tetrasulfide should not be completely ruled out at this point [91]. RSSSH would, of course, open up an additional set of chemical reactions, such as S_2 and reductive S_2^{2-} release.

3.2.2 Homolytic S-S-bond cleavage

The central S-S-bond in polysulfides (RS_xR, $x \geq 4$) not only forms a position for nucleophilic attack, it is also weaker and slightly longer than the terminal ones, with S-S-bond dissociation energies of alkyltetrasulfides around 146 kJ/mol, compared to 184

kJ/mol and 293 kJ/mol for the corresponding tri- and disulfides, respectively [92, 93]. The weakness of this bond is mostly due to the Lewis character of divalent sulfur which exerts a bond weakening influence on adjacent bonds. In this case, the perthiyl radical product ($RSS^{\bullet}$) is stabilised by partial double or π-bond formation, an effect absent in thiyl radicals ($RS^{\bullet}$) [94]. In essence, this implies that polysulfides may undergo homolytic S-S-bond cleavage, resulting in perthiyl radicals ($RS_x^{\bullet}$, $x \geq 2$). In the case of dimethyltrisulfide and dimethyltetrasulfide, this type of reaction has been known for several decades [93]. Nonetheless, its biological importance may only now become apparent, especially since $RS_x^{\bullet}$ radicals can also be formed by one-electron oxidation of perthiols.

3.2.3 S_x transfer reactions

Model studies have shown that various polysulfides, such as benzotrithiepane can formally transfer S_2 and S_3 units to molecules containing one or two conjugated double bonds, such as norborn(adi)enes [95]. Recent studies by Greer and colleagues indicate that this kind of sulfur-transfer reaction may also be mirrored in Biology. S_3-transfer has been associated with the biological activity of the pentasulfide varacin from *Lissoclinum vareau* [96]. To date, it is still unclear if this transfer occurs as a concerted action, or if a highly electrophilic, ozone-like S_3 species is released from the pentasulfide first.

Both pathways are debateable. Neutral S_x species (x = 2, 3) are known to form from elemental sulfur and sulfur compounds at high temperature [94, 97-99], yet their occurrence in aqueous solution at room temperature is questionable. Alternative mechanisms for the transfer of sulfur atoms or S_x units may involve the initial formation of a thiosulfoxide (**Figure 3.4**) [91, 100]. Then again, conversion of a polysulfide to a thiosulfoxide requires considerable energy which may not be available *in vivo* or exceed the S-S-bond dissociation energy [101].

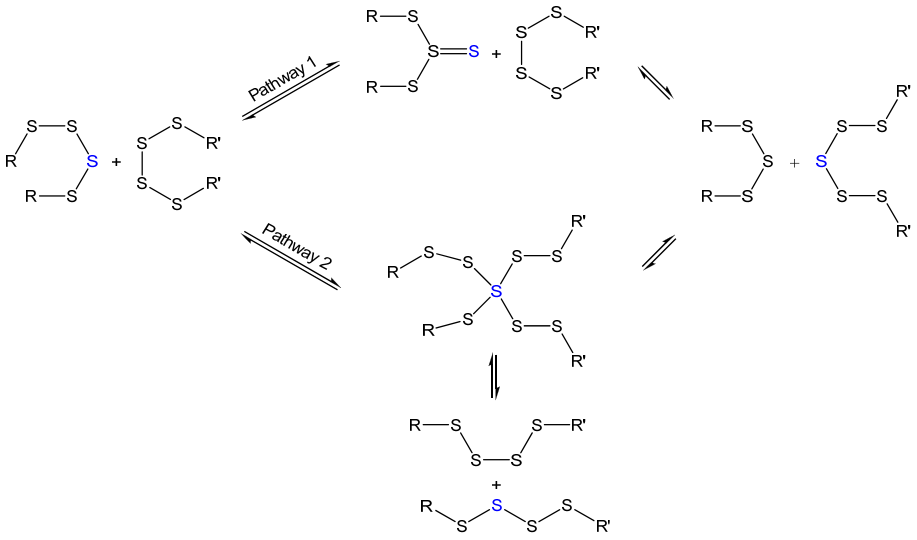


Figure 3.4 'Sulfur transfer' between polysulfides resulting in simultaneous chain shortening/elongation, a process commonly observed for diallylpolysulfides. Pathway 1 proceeds *via* a thiosulfoxide intermediate, Pathway 2 *via* a tetra-coordinated sulfur species [16].

3.2.4 Hydrophobic interactions

Not all explanations concerning the biological activity of polysulfides do have to be that complicated. One property of longer-chain polysulfides, which is often ignored, is their similarity with (toxic) organic solvents, such as nonane and decane, the saturated carbon analogues of diallyltrisulfide and diallyltetrasulfide, respectively. Although such a comparison is speculative at this time, some of the toxicity of longer chain polysulfides, especially when applied in higher concentrations, may well result from hydrophobic interactions, such as disruption of cellular membranes, dissolution of (nematode) skin, or binding to hydrophobic pockets of proteins with subsequent unfolding of the protein structure.

Within this context, it is of interest that the structure of diallylpolysulfides may not be linear. Extensive studies and *ab initio* calculations have shown that S-S-S torsion angles in the $-S_x-$ units may result in 'folded' or even helical arrangements, using + and - 'motifs' as basic structural units [91]. To date, it is unclear how such polysulfides behave once they encounter cellular membranes, cytosolic components, DNA or metal ions.

3.2.5 Metal binding

The interaction of polysulfides with metal ions represents another important, yet rather surprising aspect of their chemistry, which is often ignored. While thiolates are excellent ligands for a range of (transition) metal ions, most disulfides hardly coordinate to metal ions. Polysulfides, on the other hand, seem to form metal complexes, possibly due to their ability to coordinate with several sulfur atoms at a time and therefore, act as multi-dentate ligands.

Within this context, it is interesting to note that dimethyldisulfide, another natural product from *Allium* plant species, exerts its insecticidal toxicity by inhibiting cytochrome *c* oxidase [102]. Since this effect is comparable to the one of cyanide, one wonders if adventitious binding of dimethyldisulfide, or rather its reduced form, methanethiol (CH_3SH), to the active site iron atom of cytochrome *c* oxidase is the reason for toxicity. Such inhibitory reactions are known to occur with various organic thiols and also HS^-, and may be more widespread in biological sulfur chemistry than commonly thought. Then again, dimethyldisulfide may simply follow 'classic' disulfide chemistry and thiolate cysteine residues essential for enzymatic activity in cytochrome *c* oxidase.

To be frank, there is no direct evidence available to date to indicate direct binding of diallyltri- or tetrasulfide to either membranes, hydrophobic pockets in proteins or metal ions. Occasional comparisons of the activity of diallylsulfides with their sulfur-free carbon-analogues in biological assays indicate, however, that the sulfur-containing agents are considerably more active. As a consequence, hydrophobicity alone may not be enough to explain toxicity of these agents. On the other hand, those comparisons do not account for three dimensional structural aspects and metal binding associated with polysulfides. They also provide only limited information as far as the follow-on products of polysulfides are concerned.

3.3 Role of Perthiol Chemistry

Several recent biochemical studies conducted on polysulfides have concluded that hydropersulfides (RSSH, also known as perthiols) and hydropolysulfides (RS_xH, $x > 2$, also known as polysulfanes), should be considered the actual active form of polysulfides *in vivo* [19, 90]. Indeed, RS_xH species exhibit an extensive chemistry on their own. Similar to the broad range of chemical reactions associated with thiols, RS_xH may participate in redox-reactions, radical chemistry, catalysis and metal binding. In addition, RS_xH species are also able to act as oxidants and release inorganic S_x^{2-} species, something thiols are unable to do. It is therefore, worthwhile to consider briefly the properties and possible *in vivo* formation and reaction pathways of these compounds.

3.3.1 Generation of Reactive Oxygen Species

This matter becomes particularly important once redox-reactions at the 'terminal' sulfur atom are concerned. Compared to RSH, certain RSSH are strong reducing agents which react rapidly with oxidants, such as dioxygen and oxyhemoglobin, to form Reactive Oxygen Species (ROS), such as the superoxide radical anion ($O_2^{\bullet-}$) and hydrogen peroxide [19, 90]. This reaction also generates a perthiyl radical ($RS_x^{\bullet}$, $x \geq 2$). The latter may dimerise to form a polysulfide. Alternatively, and in analogy to the thiyl radical ($RS^{\bullet}$), it may react with GSH to form a radical anion $RS_xSG^{\bullet-}$, itself a good reducing agent which may reduce a further molecule of dioxgen to $O_2^{\bullet-}$ whilst forming a polysulfide RS_xSG. Since a polysulfide is 'regenerated', one may consider this as a (pseudo-)catalytic redox cycle which relies on the polysulfide-perthiol-perthiyl radical combination to generate ROS from O_2 whilst converting RSH to RSSR (**Figure 3.5**).

Consumption of thiols and generation of ROS are, of course, both processes which can severely damage cells by creating oxidative stress. $O_2^{\bullet-}$ and H_2O_2 may damage membranes, peptides and proteins. In the presence of copper or iron ions, they are also converted to hydroxyl radicals ($HO^{\bullet}$), a highly aggressive species which indiscriminately attacks DNA, proteins and membranes. Since the ROS generating catalytic cycle simultaneously lowers the content of (antioxidant) thiols, it is particularly vicious and may

explain the toxicity of perthiol-generating tri- and tetrasulfides, such as the ones found in garlic.

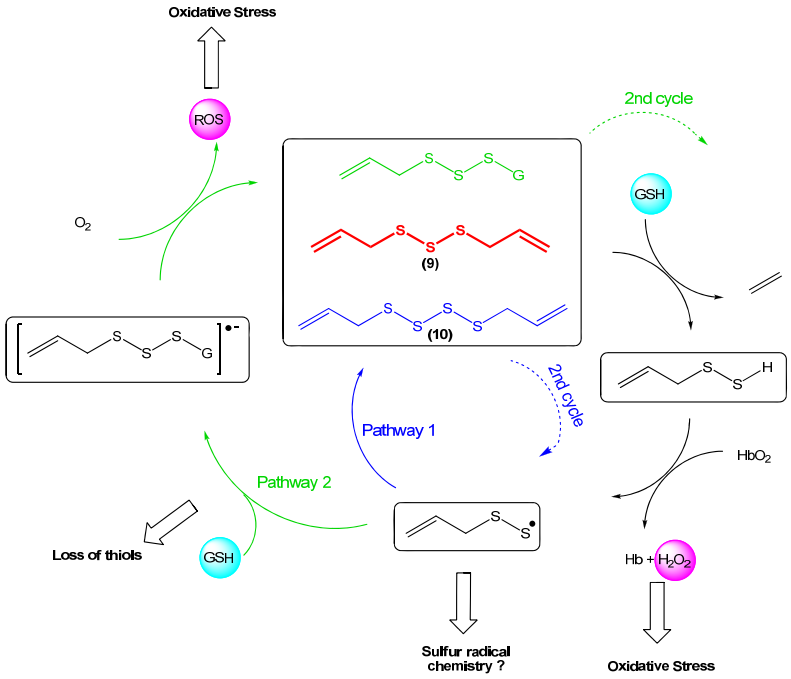


Figure 3.5 The reaction of polysulfides in the presence of intracellular components GSH and O_2 as exemplified for diallyltrisulfide **(9)** [16].

ROS generation by diallyltrisulfide may also explain the findings of elevated levels of oxidative stress in cancer cells killed by this compound [48]. Within this context, Gates and colleagues noticed that the DNA-damage caused by the pentasulfide varacin is most likely to be the result of $O_2^{\bullet-}$ generation and not protein thiolation [90, 103].

Similarly, Munday and colleagues have studied the redox behaviour of diallylsulfides in the presence of GSH and cellular oxidants, such as oxyhaemoglobin and methaemoglobin [19]. Using dioxygen consumption as a measure and superoxide dismutase and catalase as 'interceptors', they found that a mixture of GSH and oxy-/methaemoglobin (catalytic amounts) in the presence of diallyltrisulfide and diallyltetrasulfide (catalytic amounts) converted dioxygen to H_2O_2. In essence, the chain of reduction reactions starts with GSH, which reduces the polysulfide, and proceeds *via* the perthiol and haemoglobin to O_2, which is reduced to H_2O_2. Interestingly, dioxgen on its own was not strong enough as oxidant, *i.e.* it did not seem to oxidise allylperthiol directly

and hence no $O_2^{\bullet-}$ was formed in the absence of haemoglobin. As expected, the diallylmono- and disulfides were virtually inactive. Furthermore, the dipropyl-analogues of the diallyltri- and tetrasulfide were also active, albeit their activity was generally somewhat lower.

Munday's team was also able to demonstrate GSH depletion caused by polysulfides, in line with the proposed catalytic mechanism and/or the subsequent reaction of H_2O_2 with GSH. Importantly, this study also demonstrated that diallyltrisulfide and diallyltetrasulfide were able to increase the activity of Phase 2 enzymes quinone reductase and glutathione-S-transferase in various rat organs.

3.3.2 Metal binding by perthiols

Like thiols, perthiols should be good ligands for (transition) metal ions such as zinc, copper and iron [104]. The lack of an appropriate biological perthiol coordination chemistry is most unfortunate, since RSSH and related compounds are likely to bind strongly to a range of free and protein bound metal ions due to their low pK_a values. In turn, this would make them excellent inhibitors for various copper, zinc and iron enzymes, such as members of the mitochondrial respiratory chain, dehydrogenases and hydrolases: Perthiols may act either as 'adventitious ligands' or by depleting the cytosolic pool of 'free' metal ions.

Overall, the area of enzyme inhibition by thiols and perthiols derived from natural polysulfides such as the diallyl- and dipropyldi-, tri- and tetrasulfides is a promising area for further studies. For instance, there is little evidence yet of enzyme inhibition by allylmercaptan, a compound almost certainly formed reductively from diallyldi-, tri- and tetrasulfide inside the cell. The vast area of biological perthiol chemistry also remains virtually unexplored. Do these perthiols bind strongly to active site metal ions with subsequent inhibition of the enzyme? Can perthiols replace thiols as ligands in metalloproteins? Do perthiols act as reducing agents, for instance, can they break disulfide bonds in proteins? The answers to these and similar questions are crucial for our understanding of (natural) polysulfide chemistry inside the living cell.

3.4 Garlic and 'Rotten Egg Connection' (Inorganic Sulfide Anions)

One of the most interesting aspects of RS_xH chemistry resides within the often ignored fact that such compounds are oxidants as well as reductants. Hydroper- and polysulfides may react with GSH to form quite a range of partially protonated S_x^{2-} species [105]. This area of inorganic sulfur species has been reviewed by Toohey in 1989 [100].

Unlike the neutral S_x species, these anions are rather stable and in biochemical terms considerably less aggressive. If and to which extent S_x^{2-} formation occurs *in vivo* will critically depend on the redox potentials of RS_xH and thiol species involved, as well as their relative concentrations. Considering that GSH occurs in millimolar concentrations in mammalian cells, release of inorganic sulfur species such as S^{2-} and S_2^{2-} from diallyltrisulfide and diallyltetrasulfide, respectively, becomes a real possibility.

One particular reaction, which has caught the attention of chemists, biochemists and cell biologists during the last couple of years, is the release of hydrogen sulfide from several sulfur-containing garlic compounds. The release of 'inorganic sulfide ions' from polysulfides is one possible reaction pathway open to polysulfides. Extensive experimental evidence for hydrogen sulfide release from DADS **(8)** and DATS **(9)** - and vasoactivity associated with this process – has been provided by David Kraus and colleagues, which deals with H_2S release from DADS, DATS and, implicitly, also with higher polysulfides (such as DATTS **(10)**). Similarly, Zhu and colleagues have been able to link hydrogen sulfide and cardioprotective effects to another ingredient of garlic, *i.e.* SAC **(4)** [106]. These findings have provided the basis for a range of exciting new ideas in the area of natural sulfur products, *Allium* research, hydrogen sulfide biochemistry and possible practical applications of sulfur agents in Medicine and Agriculture.

Within this context, the chemistry and biochemistry of mono-, di-, tri- and tetrasulfides, as well as related perthiols, hydropolysulfides and inorganic sulfides (such as hydrogen sulfide or, more generally, H_2S_x ($x \geq 1$) species) is now taking central stage. A brief overview of their ability to release hydrogen sulfide is given in **Figure 3.6**. Needless to say, these listings are necessarily incomplete and the true chemistry and biochemical impact of these chemical species is highly complicated and is still only poorly understood. Here, we will therefore focus on the issues surrounding the inorganic side of organic sulfur

compounds, *i.e.* hydrogen sulfide release, bearing in mind that there is also a huge (biological) chemistry of mono-, di-, tri- and tetrasulfides transcending H_2S_x release.

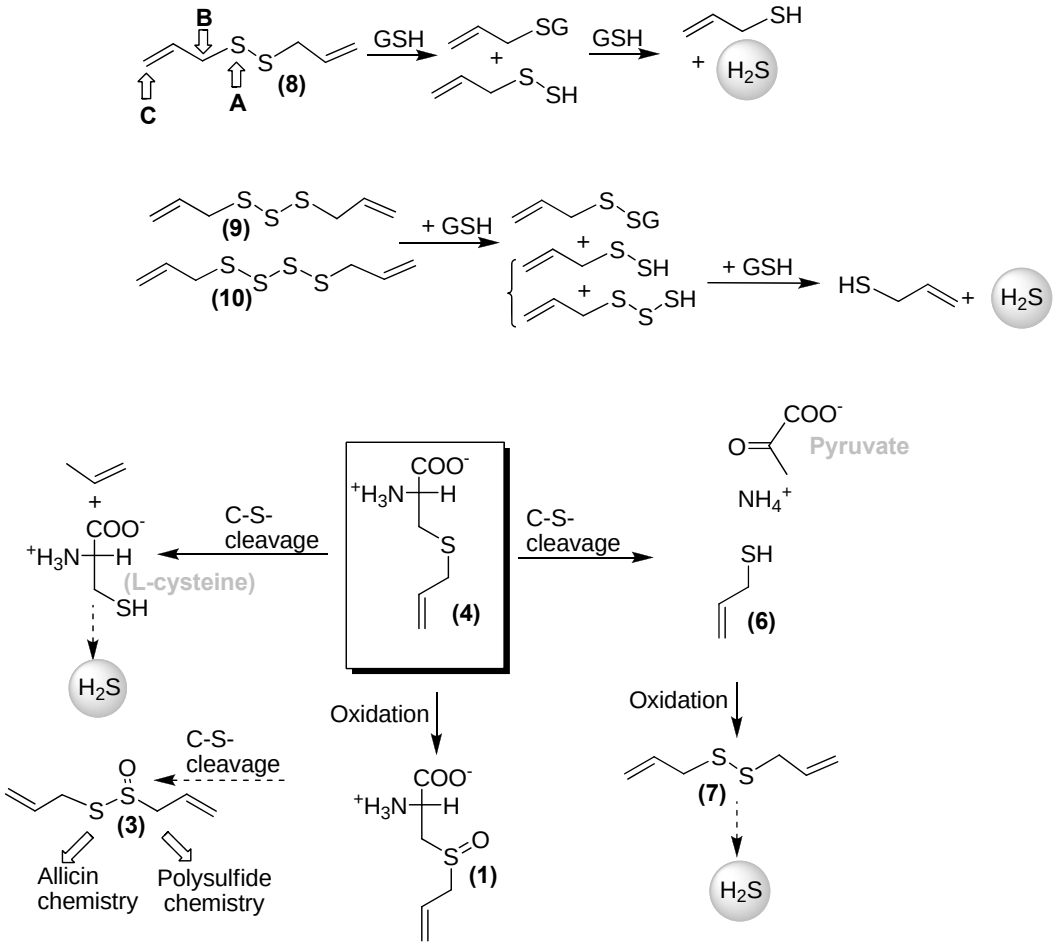


Figure 3.6 A selection of important chemical transformations which result in the formation of hydrogen sulfide. Figure adopted from Jacob & Anwar *et.al.* [107]. Please note that only reactions with a (suspected) relevance *in vivo* are shown. Cleavage of a carbon-sulfur bond may either result in the liberation of L-cysteine which may then feed into the Cystathionine β-synthase/ Cystathionine β-lyase catalyzed hydrogen sulfide generation process. Alternatively, ammonia, pyruvate and allyl mercaptan **(6)** may be formed. The latter may result in hydrogen sulfide release *via* the DADS **(7)** route. In addition, *S*-allylcysteine **(4)** may be oxidized to alliin **(1)**, which could then be converted to allicin **(5)** by an hitherto unidentified C-S-lyase. Although speculative at this time, allicin **(3)** formation would open the door to a distinct redox chemistry which may explain some of the biological activities associated with *S*-allylcysteine **(4)**.

3.4.1 Biochemical reactions of inorganic anions

If such inorganic sulfur species (HS) are formed inside a living cell, what happens next? Once formed, these inorganic anions seem to affect a range of biochemical processes in the cell (**Figure 3.7**) [108]. The underlying chemical reactions and biochemical mechanisms explaining such actions are mostly unknown, although interactions of HS^- with metal ions, either free or protein-bound, may, in some instances, provide a plausible explanation. Indeed, one of major chemical reactions associated with sulfide anions are complex formations with metal ions such as zinc, iron and copper. In this respect, the biochemical mode of action of inorganic sulfide species H_2S_x may resemble the one of RS_xH.

In line with these findings, HS^- has been known for many years to inhibit metalloenzymes, such as carbonic anhydrase, by acting as adventitious ligand to the metal ion (in this case zinc). Such studies go back to the 1960s [109] [110]. Recent work by Supuran and colleagues has shown that carbonic anhydrase enzymes of the α-class and the γ-class are inhibited by HS^- at concentrations between 0.6 μM (hCA I enzyme) and 50 μM (Zn-Cam enzyme) [111].

Other reactions of S_x^{2-} anions under physiological conditions may include the reaction with dioxygen to form $O_2^{\bullet-}$, H_2O_2 and sulfur centred-radicals ($S_x^{\bullet}$). More remote possibilities of S_x^{2-} anion ($x > 1$) interactions include the reaction of these anions with the thiol function of cysteine residues in proteins. Since S_x^{2-} anions can still be reduced further, thiols can, in principle, attack S-S-bonds in S_x^{2-}. If such a reaction takes place, a sulfur atom is formally transferred from S_x^{2-} to the thiol, resulting in a perthiol and S_{x-1}^{2-} [100]. Such cysteine modifications may inhibit enzymes, yet may also convey activity, as is the case in bovine liver rhodanese [112].

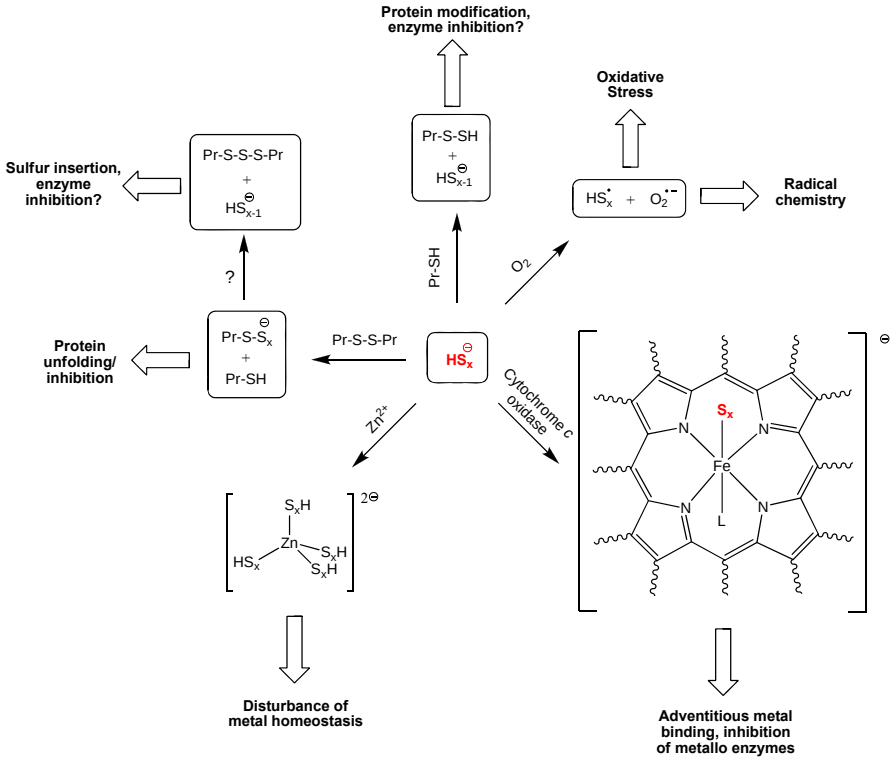


Figure 3.7 Inorganic sulfide S_x^{2-} anions as biochemical signalling molecules. Figure adopted from Jacob and Anwar *et.al.* [107]. Although little is known about the chemistry of such anions inside the living cell, *in vitro* data point towards a range of possible interactions.

In essence, this area of inorganic, reactive sulfur species in biology is still in its infancy. It provides ample opportunities for comprehensive bioinorganic research which may include mechanistic studies at the molecular level, but also biochemical investigations considering protein modifications, signalling pathways and toxicity in cell culture and animals.

3.5 The Objectives and Strategy of this Project

The last decade has witnessed a renewed interest in natural products and their possible applications in Medicine and Agriculture. In the field of Medicine, several developments have come together to challenge conventional drugs and highlight the requirement for new agents. Ageing Societies, for instance, have an increased need for disease preventive drugs, such as antioxidants able to fight oxidative stress (OS), which occurs naturally in ageing organisms and has been associated with range of diseases, including inflammatory diseases and possibly cancer [113]. Furthermore, several bacterial strains resistant to classical antibiotics have developed and pose a serious problem, often as part of hospital acquired infections [114]. Although there is an apparent demand for new (types) of antibiotics, pharmaceutical research so far has been unable to come up with effective solutions. At the same time, the demand for effective, yet selective anti-cancer agents is rapidly increasing.

The search for new lead compounds has led to a (re-)evaluation of a range of naturally occurring agents from various plants, fungi, bacteria and animals. This is hardly surprising, since Nature provides a treasure chest of natural products which 'by design' possess one or more biological activities, which in turn may be of considerable interest in medical and agricultural research.[3] Within this context, recent research has shown that naturally occurring sulfur compounds in particular provide a range of interesting leads for new antimicrobial and anticancer agents and may play a role as cancer cell selective cytotoxins and 'green' pesticides [4]. This area of research is as diverse as the different sulfur compounds found in Nature.

A similar picture is emerging in the agricultural arena. Conventional pesticides are associated with environmental damage and a long-term negative impact on the human food chain. As in Medicine, the hunt is on for innovative new agents, *i.e.* 'green pesticides',

[3] The label 'natural' in Medicine and Pesticide development therefore provides not (only) a social or political advantage. It also reflects the fact that natural products are often complicated chemicals well suited to fulfill a certain biological task. In many instances, they are even superior to 'artificial' drugs. Later on, compounds will be discussed with have formed part of effective natural host defense systems for millions of years.

which exhibit antimicrobial activity, yet pose virtually no damage to the eco-system and human health.

In the view of above the project was divided into four main areas: a) Synthesis of high purity polysulfides and characterization. b) Basic screening in cancer cells to highlight some agents with anticancer properties. c) First steps towards the development of green pesticides for use in agriculture. d) Mechanistic *in vitro* studies to evaluate the biochemical mode(s) of action of these molecules.

For this both ^{1}H NMR and HPLC methods were employed in the analysis of the polysulfide samples tested for biological activity to circumvent problems associated with their instability and reactivity. A new HPLC method was developed for enhanced purity of the samples. Straightforward synthetic methods were developed for higher polysulfides, *e.g*. diallylpenta- **(11)** and hexasulfide **(12)**.

The anticancer properties of polysulfides were evaluated by screening these agents in Caco-2 and U937 cells. In addition, the mechanism of induction of cell death in U937 cells by DATTS **(10)**, a compound that so far has hardly been examined in this context, has been studied.

Guided by *in vivo* assays using common species of crop-damaging nematodes, polysulfides with three or more sulfur atoms *i.e.* DATS **(9)**, DPTS **(27)**, DATTS **(10)**, DPTTS **(28)**, diallylpentasulfide **(11)**, diallylhexasulfide **(12)**, garlic oil, and onion oil were identified as compounds of greatest biological activity. The choice of higher polysulfides is based on evidence that tri- and tetrasulfides are generally biologically more active than the disulfides or monosulfide [115]. This is consistent with the progressive weakening of the S–S bond, and an anticipated increased reactivity towards cysteine in the homologous series of polysulfides. The diminished volatility and odour of the higher polysulfides is an additional bonus in the context of possible agricultural applications.

In summary, the objective of the current study is to investigate the potential of chemically simple organosulfur compounds of genus *Allium* for the development of anti cancer agents and green pesticides as well their possible mode of action. A combination of chemical and biochemical techniques are employed to evaluate the biological activities of polysulfides. These studies are multi disciplinary in nature and involve the use of

electrochemical methods, *in vitro* bioassays and cell culture techniques. The ultimate aim of the project is to identify promising lead candidates for the development of anticancer drugs and green pesticides.

Chapter 4

Materials and Methods

Diallylsulfide, dipropylsulfide, diallyldisulfide, dipropyldisulfide, sulfur monochloride, sodium thiosulfate, sodium sulfide, hydrogenperoxide, L-cysteine, ammonium hydroxide, pyridine, sodium sulfate, magnesium sulfate, potassium pentasulfide, sodium methanethiosulfonate, bis-tetrabutyl ammonium hexasulfide, allyl chloride, propyl chloride, allyl bromide, propyl bromide, allylisothiocyanate (mustard oil), allylmercaptan, propylmercaptan, glutathione (GSH), thiophenol (PhSH), 4-mercaptopyridine (4-MP), Nitrotetrazolium Blue (NBT), superoxide dismutase (Cu, Zn-SOD), haemoglobin, sodium hydrogen sulfide, potassium dihydrogen phosphate, potassium hydrogen phosphate, 1,9-decadiene and EDTA were purchased from Sigma-Aldrich, Munich (Germany). Garlic oil and Onion oil were purchased from R.C. Treatt Ltd. (UK). Diallylsulfide, Diallyldisulfide, Dipropyldisulfide and allylmercaptan were distilled at 1 mbar under vacuum before use. Deionised water (Millipore, 18.2 $M\Omega cm^{-1}$) was used unless stated otherwise.

4.1 Synthesis

As part of this project a total of 12 sulfur containing compounds were synthesized, mostly according to literature procedures with improvement to increase either yield or purity. A high purity is required for biological testing.

4.1.1 Synthesis of *S*-allylcysteine (4)

S-allyl cysteine (SAC) was synthesized according to literature procedure of Freeman *et.al.* [116] with some modifications.

To the ice cooled solution of L-cysteine [(R)-2-amino-3-mercaptopropanoic acid, 10.0 g, 82.6 mmol)] in NH_4OH (2 M, 240 mL) was added 3-bromopropene (15.0 g, 124

mmol) was vigorous stirring. The mixture was stirred at 0 °C for 40 min and filtered, and the filtrate was concentrated *in vacuo* (40 °C) to a small volume, and filtered. The solid was washed repeatedly with ethanol, dried *in vacuo*, and recrystallized from H_2O/EtOH (2:3) to yield white needles of SAC **(4)**.

4.1.2 Synthesis of Alliin (1)

10 g of **(4)** was dissolved in 250 ml of H_2O and 25 ml of 30% H_2O_2 added. The solution was stirred at room temperature for 5 hr, and crude racemic alliin was recovered by rotary evaporation followed by precipitation with ice-cold ethanol.

Crude racemic alliin (20 g) was purified. It was dissolved in H_2O (50 mL) at 50 °C and filtered. The filtrate was added to 250 mL of aqueous acetone (30 mL of H_2O). The suspension was kept at 4 °C for 24 hours and filtered. The crystals (4.2 g) were dissolved in H_2O (10 mL) at 50 °C and the solution was added to 40 mL of aqueous acetone (10 mL of H_2O). The suspension was kept at 0 °C for 24 h and filtered, and the solid was dried to afford pure alliin **(1)**.

4.1.3 Synthesis of Allicin (3)

Allicin was obtained by oxidation of diallyldisulfide with H_2O_2 according to the method of Lawson and Wang [117] which was improved to increase purity and yield.

2 g (13 mmol) of freshly distilled diallyldisulfide (about 99% pure) was dissolved in 5 mL of cold (4 °C) glacial acetic acid, to which 3 mL of cold 30% hydrogen peroxide was added slowly. After 30 min, the temperature was allowed to increase to room temperature and stirring continued for 2 h. The reaction was stopped with addition of 25 mL of water, and was extracted with 30 mL of dichloromethane. Acetic acid was removed by washing the extract several times with 5% $NaHCO_3$ and then washing with water to pH 6-7. Solvent was evaporated *in vaccuo* and the yellow oil obtained was re-dissolved in 200 mL water. Unreacted DADS was removed by double extraction with 0.1 vol of hexane and allicin was extracted with dichloromethane again, dried over $MgSO_4$ and concentrated *in vacuo*. It was purified further using silica gel chromatography petrol ether (40 ^oC-65 ^oC) :ethyl acetate (95:5).

4.1.4 Synthesis of diallyltrisulfide (9)

Diallyltrisulfide (DATS) was synthesized from allylchloride, sodium thiosulfate and sodium sulfide according to the method of Milligan and Swan [118] which was improved to increase purity.

A mixture of sodium thiosulfate pentahydrate (0.25 mole) and the appropriate alkyl or alkenyl halide (0.2 mole) in 60% v/v ethanol-water (100 ml.) was heated under reflux until the mixture was homogeneous, indicating substantially complete formation of the intermediate thiolsulfate. The mixture was then distilled at reduced pressure to remove most of the ethanol, cooled, extracted with light petroleum (b. p. 30-40 ^{o}C).

The aqueous phase was then treated with sodium sulfide (0.09 mole) in water (200 ml). The crude trisulfide, deposited as oil, was then extracted with light petroleum, washed with water, and dried. Removal of solvent gave a residue further purified by vacuum distillation at 1 mbar.

4.1.5 Synthesis of diallyltetrasulfide (10)

Diallyltetrasulfide (DATTS) was synthesized from allylmercaptan and sulfur chloride (S_2Cl_2) as described by Derbesy and Harpp [119].

A solution of allylmercaptane (10 mmol) and pyridine was taken in 25 mL ether. S_2Cl_2 in 50 mL of ether was cooled down to –78°C with stirring. To this S_2Cl_2 solution was added dropwise the first thiol solution over a period of 0.5 hr. The stirring continued for further 0.5 h after the addition of thiol keeping the temperature at –78°C. The second equivalent of thiol (Allyl mercaptane) and pyridine (10 mmol) in 25 mL of ether was added dropwise within 0.5 h (–78°C) and the reaction mixture was stirred for another hour.

After an hour of cooling, the reaction was stopped and the mixture stirred until room temperature was reached. The mixture was washed with 3 × 25mL water followed by 30 mL of 0.5 M NaOH until neutral to pH paper. The organic phase was dried with $MgSO_4$, filtered and evaporated to yield a yellow oil. The respective compound was purified by column chromatography using petrol ether: chloroform (97:3) as eluent.

4.1.6 Synthesis of diallylpentasulfide (11) and diallylhexasulfide (12)

Diallylpenta- and hexasulfide was synthesized and purified in collaboration of EcoSpray Ltd., UK.

4.1.6.1 Methanesulfonothioic acid, *S*-2-propenyl ester ($MeSO_2SAll$)

To a solution of sodium methanethiosulfonate [120] (1.34 g, 10.0 mmol) in water (10 mL) was added dropwise a solution of 2-propenyl bromide (1.21 g, 10.0 mmol) in CH_3OH (10 mL) at room temperature. The mixture was stirred for 2 h, and extracted with CH_2Cl_2 (3 × 50 mL), washed with brine, dried over Na_2SO_4, and concentrated *in vacuo* yielding the known title compound (1.4 g) as a light yellow oil.

4.1.6.2 Diallyl Polysulfides from Methanesulfonothioic acid, S-2-Propenyl Ester

Method 1. A solution of $MeSO_2SAll$ (304 mg, 2.0 mmol) in EtOH (5 mL) was added dropwise with vigorous stirring over 10 min at room temperature to a solution of potassium pentasulfide (K_2S_5; 238 mg, 1.0 mmol) in EtOH (15 mL). The mixture was stirred for 2 h, the solvent was removed *in vacuo* and the residue was extracted with hexane (3 × 40 mL). The solvent was removed affording a pale yellow oil identified by ^1H NMR and HPLC as a mixture of diallyltri- (11%), tetra- (26%), penta- and hexasulfide (50% total) (160 mg; yield ca. 70% based on average MW of polysulfides).

Method 2. A solution of bis(tetrabutylammonium) hexasulfide, $(Bu_4N)_2S_6$, (TBAS; 676 mg, 1.0 mmol) in acetone (15 mL) was cooled below -90 ^oC, and a solution of $MeSO_2SAll$ (304 mg, 2.0 mmol) in acetone (5 mL) was added dropwise over 10 min. The mixture was kept for 3 h at -90 ^oC, then warmed up to room temperature. The now pale yellow solution was stirred for 1 h at room temperature, concentrated *in vacuo*, and the residue extracted with hexane (3 × 40 mL). The solvent was removed affording a mixture of allyl polysulfides as a pale yellow oil (140 mg, yield ca. 60% assuming average composition DAS5). Analysis by ^1H NMR indicated a 0.3:3.5:2:1 tri:tetra:penta:hexasulfide molar ratio. Analysis by RP HPLC gave peak areas for these same compounds of 0.1:2:1.7:1. The individual compounds, diallylpentasulfide **(11)** and diallylhexasulfide **(12)**

were separated from mixture using RP HPLC.

4.1.6.3 Diallyl Polysulfides from 2-Propenesulfinothioic acid, S-2-Propenyl Ester (Allicin)

A solution of TBAS (676 mg, 1.0 mmol) in acetone (15 mL) was cooled below -90 ^{o}C, and a solution of allicin (324 mg, 2.0 mmol), prepared by oxidation of DAS2, in acetone (5 mL) was added dropwise over 10 min. The mixture was kept for 3 h at -80 ^{o}C and warmed to room temperature. The now pale yellow solution was stirred for 1 h at room temperature, concentrated *in vacuo* and the residue was extracted with hexane (3 × 40 mL). The solvent was removed affording a light yellow oil which from ^{1}H NMR analysis consisted of a ca. 3:2:1 tetra: penta: hexasulfide mixture (155 mg; ca. 74%).

The individual compounds, diallylpentasulfide **(11)** and diallylhexasulfide **(12)** were purified by preparative C18 HPLC with 80:20 MeOH: H_2O as eluent. The purity of all compounds obtained by this method was ≥ 93%. The separated fractions were extracted with hexane, dried with $MgSO_4$ and concentrated under vacuum.

4.1.7 Synthesis of dipropyltrisulfide (27)

Dipropyltrisulfide (DPTS) was synthesized from propyl bromide, sodium thiosulfate and sodium sulfide according to the method of Milligan and Swan [118].

Mixture of sodium thiosulfate pentahydrate (0.25 mol) and the propylbromide (0.2 mol) in 50% v/v ethanol-water (100 ml) was heated under reflux until the mixture was homogeneous, indicating substantially complete formation of the intermediate thiolsulfate. The mixture was then distilled at reduced pressure to remove most of the ethanol, cooled, extracted with light petroleum (b. p. 30-40 ^{o}C).

The aqueous phase was then treated with sodium sulfide (0.09 mol) in water (200 ml). The crude trisulfide, deposited as oil, was then extracted with light petroleum, washed with water, and dried. Removal of solvent gave a residue (pale yellow with onion smell) further purified by vacuum distillation at 1 mbar.

4.1.8 Synthesis of dipropyltetrasulfide (28)

Dipropyltetrasulfide (DPTTS) was synthesized from propylmercaptan and sulfur chloride (S_2Cl_2) as described by Derbesy and Harpp [119].

A solution of propylmercaptane (10 mmol) and pyridine was taken in 25 mL ether. S_2Cl_2 in 50 mL of ether was cooled down to −78°C with stirring. To this S_2Cl_2 solution was added dropwise the first thiol solution over a period of 0.5 h. The stirring continued for further 0.5 h after the addition of thiol keeping the temperature at −78°C. The second equivalent of thiol (propylmercaptane) and pyridine (10 mmol) in 25 mL of ether was added dropwise within 0.5 h (−78°C) and the reaction mixture was stirred for another hour.

After an hour of cooling, the reaction was stopped and the mixture stirred until room temperature was reached. The mixture was washed with 3 × 25mL water followed by 30 mL of 0.5 M NaOH until neutral to pH paper. The organic phase was dried with $MgSO_4$, filtered and evaporated to yield a yellow oil with strong onion smell. The respective compound was purified by column chromatography using petrol ether: chloroform (95:3) as eluent.

4.1.9 Synthesis of Lenthionine (39)

A solution of $Na_2S_{2.5}$ was prepared from $Na_2S{\cdot}9H_2O$ (300 g) and sulfur (60 g) in 1000 ml of water, to which was bubbled a stream of hydrogen sulfide gas to adjust the pH of the solution at 8. The solution at this pH was covered with methylene chloride (1000 ml) and the mixture was vigorously stirred at room temperature for several hours. The organic layer was separated, washed with water, dried over sodium sulfate and the solvent evaporated under reduced pressure to yield an oily material. On standing at room temperature, this oily material gradually went into a crystalline mass, which was separated from the liquid by centrifugation. Recrystallization from dioxane yielded 98.2% pure colorless prisms of lenthionine. Yield 2.1 g (40.7%).

4.1.10 Synthesis of 1, 2, 4-Trithiolane (42) and 1, 2, 4, 6- Tetrathiepane (41)

A solution of $Na_2S_{2.5}$ (pH 12.7), which was prepared from $Na_2S{\cdot}9H_2O$ (300 g) and sulfur (60 g) in 1000 ml of water, was covered with methylene chloride (1000 ml) and the mixture was vigorously stirred at room temperature for seven hours. During this period the pH of the solution shifted to 11.9. The organic layer was separated, washed with water, dried over sodium sulfate and the solvent was evaporated under reduced pressure to yield an oily material (24.9 g). Upon distillation under reduced pressure (1 mbar) a pale yellow liquid of 1, 2, 4-trithiolane **(42)** obtained with purity of 97%. Yield 3.4g (25.5%)

The residue in the flask was left at room temperature for 2 days which solidified to a crystalline mass. This was recrystallized from dioxane to yield white crystals of 98% pure 1, 2, 4, 6- Tetrathiepane **(41)**. Yield 1.65 g (30.4%)

4.2 Characterization of Polysulfides

4.2.1 NMR Spectroscopy

NMR spectra were recorded on Bruker (Rheinstetten) type DRX 500 and Avance 500. Measuring frequency was 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR. Measurements were carried out at room temperature (298K). Solvents used were $CDCl_3$ and CD_3OD. Chemical shifts are given in ppm. $CDCl_3$ reference signals are 7.26 ppm for ^{1}H-NMR and 77.16 ppm for ^{13}C-NMR. For spectral analysis XWinNMR and TopSpin (Bruker) were used.

4.2.2 HPLC for purification and identification of Polysulfides

Equipment used in the weighing and analysis of these samples is calibrated every 3 months. For all HPLC runs a commercial ‘Gold’ standard was used (first sample, a central sample and the last sample) to confirm the system is operating optimally *i.e.* retention time, peak shape and peak area are reproducible. Also three standards *i.e.* diallyldisulfide, dipropyldisulfide (Sigma, Germany) and diallyltrisulfide (Oxford chemicals UK) were used before each run.

An Agilent HP1050 series system with a degasser, column oven, thermostated autosampler, quaternary pump system and diode array detector was used. A Zorbax C_{18} (150 x 4.6 mm, 5µm) column was used.

4.2.2.1 Sample Preparation

Three replicates of each sample (3 x 5mg for each sample) were transferred into 2 ml screw-top micro-tubes using a 20 µl Gilson pipette; variance in sample weight was less than 0.1 mg. To each sample 1 mL methanol was added, the tubes were sealed and the samples were extracted for 20 min. During extraction samples were vortex mixed every 5 min to increase the efficiency of extraction. Each sample was then diluted (1:5) with 80% MeOH containing 20% H_2O (i.e. the solvent system for analysis). Each sample was taken up in separate 1 ml disposable sterile syringes, and any insoluble matter was removed by filtration through separate 0.2 µm Target® PTFE solvent filters (Chromos Express, Macclesfield, UK) prior to HPLC analysis.

4.2.2.2 HPLC Analysis procedure

HPLC analysis was performed using an Agilent HP1050 series HPLC system with diode array detection in combination with a Zorbax C_{18} column (150 x 4.6 mm, 5 µm) with a `Securityguard` C_{18} (2) pre-column. Auto-sampler temperature was 4^{o}C and the column temperature was 37 ^{o}C and cut-off pressure was 280 bar. Data was collected at 240 nm (with total data collected between 200-600 nm). The optimal injection volume for each sample was 10 µl. Please note that the solvent system used for separation was based on a literature method [121] with the following modifications:– A Zorbax C_{18} column and an isocratic gradient of 80% MeOH and 20% ultra-pure water. Method time was 40 min. A DATS standard (Oxford Chemicals Ltd., UK) was also used to create a calibration curve. The pattern of peaks in the Gold standard was identical to the chromatogram presented by Lawson *et. al.* 1991, except that separation of allylsulfides **(7) - (12)** was greatly improved by the method.

4.2.3 GC-MS

Mass spectrometry data were collected on a Hewlett-Packard GC/MS 5890 series. The GC column was an HP5-MS (5% phenyl, methyl silicone; reference 190915-433) bonded phase fused silica capillary column (30 m × 0.25 mm id, 0.25 μm film thickness) (Hewlett-Packard). N60 helium was chosen as the carrier gas and was used at a constant pressure of 85 kPa. Injections were made in the splitless mode, the injector temperature was 300 °C. The oven temperature, initially maintained at 70 °C for 1 min, was programmed to rise to 325 °C at 11.5 °C min, then kept at 325 °C for 5 min. Method time was 30 min.

4.3 Cell culture

The cell culture assays provide an estimate of (cancer cell selective) toxicity and, most importantly, a "ranking" of activity within the various sulfur compounds under investigation.

4.3.1 Caco-2 cell culture

Caco-2 cell culture studies were carried out in cooperation with the group of Prof. Claus-Michael Lehr, Institute of Biopharmaceutics and Pharmaceutical Technology, University of Saarland under the supervision of Dr. Nicole Daum.

Cell viability of cultured Caco-2 cells was measured using the standard MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [122]. Cells were seeded in 96-well plates at a seeding density of 10^5 cells/ml (*i.e.* 10^6 cells in 10 ml volume distributed in 80 cm^2) 24 hr before performing the viability test. As part of the test, the confluent, adherent cells were incubated with the sulfur compounds and controls in culture medium (DMEM containing 1% of MEM-NEA, 10% of FCS (heat inactivated for cytotoxicity tests, in the absence of antibiotics)) for up to 48 hours at different concentrations (150 nM to 1.4 mM final concentration, whereby 700 μM was found to be the most suitable concentration for comparison of effects and to avoid solubility problems). MTT staining was carried out according to the standard protocol with a background control of 0.1% DMSO. The conversion of MTT was taken as a measure of cell viability. It was

quantified after cell lysis and resolubilization of the intracellularly formed dye by absorbance measurements at 540 nm. All experiments were performed in triplicate, and average values and standard deviations were calculated accordingly.

4.3.2 U937 cell culture

U937 cell culture studies were carried out in cooperation with the group of Dr. Marc Diederich at Laboratoire de Biologie Moleculaire et Cellulaire du Cancer (LBMCC), Hôpital Luxembourg. These experiments were performed by our group postdoc Dr. Christiane Scherer.

U937 cells (human histiocytic lymphoma, DSMZ) were cultured in RPMI 1640 medium (Bio-Whittaker, Verviers, Belgium) containing 10% [v/v] of fetal calf serum (FCS) (Lonza, Verviers, Belgium), 1% [v/v] of antibiotic-antimycotic (Lonza, Verviers, Belgium) and 2 mM of L-glutamine (Lonza, Verviers, Belgium) at 37 °C, 5% CO_2. All experiments were performed in culture medium containing 0.1% of FCS. Peripheral blood mononuclear cells (PBMCs) were isolated from the non-coagulated peripheral blood of healthy adult human donors using the standard Ficoll-Hypaque (GE Healthcare, Roosendaal, The Netherlands) density separation method. After isolation, PBMCs were washed, counted, resuspended at a cell density of 1 × 106 cells/ml in RPMI 1640 supplemented with 0.1% of FCS, 2 mM L-glutamine and 1% of antibiotic-antimycotic and kept in a controlled atmosphere in an incubator for up to 48 hours (at 37 °C, 5% CO_2).

4.3.2.1 Assessment of metabolic activity (ATP-quantification) as a measure of cell viability

The percentage of cell death after incubation with the test compounds was determined using the Cell Titer Glo® Luminescent Cell Viability Assay Kit (Promega, Leiden, Netherlands). Assays were performed according to the manufacturer's instructions. Extent of luminescence was quantified as a measure of viable (ATP-producing) cells using an Orion Microplate Luminometer (Berthold, Pforzheim, Germany).

4.3.2.2 Elucidation of apoptosis by nuclear morphology (Hoechst) or by cytofluorimetric analysis (annexinV-FITC/propidium iodide-staining)

Percentage of apoptotic cells was quantified as the fraction of apoptotic nuclei (different stages of nuclear fragmentation) assessed by fluorescence microscopy (Leica-DM IRB microscope, Lecuit, Luxemburg) upon staining with the DNA-specific dye Hoechst 33342 (Sigma, Bornem, Belgium), as described by Ghibelli *et. al*. [123]. The fraction of cells with nuclear apoptotic morphology was counted (at least 300 cells in at least three independent fields) and the images were analysed using the Image J software online from Imperial College London.

In order to assess apoptosis by FACS (FACSCalibur, Becton Dickinson, San José, California) the AnnexinV–FITC Apoptosis Detection Kit I® (Becton Dickinson Biosciences, Erembodegem, Belgium) was used according to the manufacturer`s instructions. Data was recorded using the CellQuest software from BD Biosciences USA.

4.4 Nematode toxicity Assay

Nematode assays described here provide a robust, straightforward, easy to use and highly reliable *in vivo* assay to estimate (selective) toxicity of compounds used.

Nematodes, *Steinernema feltiae* and *Phasmarhabditis hermaphrodita*, were purchased as a nematode product for use in gardening (Just Green Ltd, UK or Schnecken Profi Hennstedt Germany). *Panagrellus redivivus* was supplied from the John Innes

Research Centre, Norwich, UK., having been grown on culture media using published methods [124].

Experiments were undertaken with *S. feltiae* and *P. hermaphrodita* using protocol 1 while protocol 2 was used for *P. redivivus* [63].

4.4.1 Protocol 1

The nematode products purchased have the texture of friable soft cake, when added to water, these product release thousands of very active *S. feltiae* nematodes into solution. Nematode populations were adjusted with water to produce ca. 300-400 in a 100 μL aliquot; the viability of the nematodes was checked prior to each set of experiments. Test solutions of polysulfides were prepared by dissolving the substance in EtOH to produce stock solutions. At the point of use, the stock solutions were diluted with water to leave 1% v/v of residual EtOH in addition to investigation at defined concentration of 400, 200, 100 and 50μM. Aliquots (900 μL) of the test solutions were then added to each of four 2 mL eppendorf tubes followed by addition to each tube of 100 μL of *S. feltiae* suspension (300-400 nematodes). The caps were closed and the combined solutions (nematodes and test substance) were gently mixed for a few seconds and allowed to settle at room temperature in air. At pre-determined time intervals, typically 2, 4, 6 and 24 h, the Eppendorf tubes (test cells) were subjected to gentle mixing and 100 μL of solution estimated to contain 30-40 nematodes was withdrawn from each of the test cells and carefully deposited discretely on the surface of a plastic petri dish such that the drops would not re-combine. The individual groups of four replicate droplets were then examined at 40 times magnification through a Meiji zoom binocular microscope (Meiji Techno USA). Nematodes were grouped as either alive or dead on the basis of movement. Percentage mortality in each droplet was then calculated. The experiment was repeated again after 3 days. Percentage mortality for the substance under investigation was determined as the mean from the four replicate test cells used. All experimental runs were referenced against a 1% v/v EtOH/H_2O (blank) with each set of experiments.

4.4.2 Protocol 2

The diallyl sulfides used in the protocol 2 studies were taken directly off the HPLC in 80:20 v/v methanol: water. The polysulfides were obtained by removing the methanol to

leave an aqueous suspension which was then re-introduced to the HPLC to confirm the precise concentration. Adjustments to the concentration were then made by addition of water to produce the test solutions. All test solutions were run against a 1% methanol blank.

To account for the different numbers of nematodes available from the different culture media as supplied by Ian Bedford, John Innes Research Institute UK, volumes of test solution and nematode containing aqueous aliquots were 800 µl and 200 µl respectively, which after combining produced estimated total populations of nematodes at 300-400/test cell. The caps were closed and the combined solutions (nematodes and test substance) were gently mixed for a few seconds and allowed to settle. At pre-determined time intervals, typically 1, 3, and 24 h, the Eppendorf tubes (test cells) were subjected to gentle mixing and 100 µL of solution estimated to contain 30-40 nematodes were withdrawn from each of the test cells and carefully deposited discretely on the surface of a plastic petri dish such that the drops would not re-combine. The individual groups of triplicate droplets were then examined at 40 times magnification through a Meiji zoom binocular microscope (Meiji Techno USA). Nematodes were grouped as either alive or dead on the basis of movement. Percentage mortality in each droplet was then calculated. Percentage mortality for the substance under test was determined as the mean from the three replicate test cells used.

Statistical analysis

Statistical analysis for the experiments was performed using Student's t-test and P values < 0.05 were considered significant. Data are presented as mean ± SEM.

4.5 *In vitro* studies

4.5.1 Cyclic Voltammetry

Cyclic voltammetry was used to define the general redox properties of different sulfur compounds. It is also a very useful method to estimate the oxidation and reduction potential of sulfur containing molecules.

Cyclic Voltammograms were recorded on a BAS CV-50W workstation linked to a controlled growth mercury electrode. The mercury electrode (drop size 16) served as working electrode, with a standard Ag/AgCl reference and a platinum wire counter electrode. The reference electrode was calibrated against ferrocene. All experiments were performed at room temperature and in triplicate. Cyclic Voltammograms were recorded between -900 and 0 mV *vs.* Ag/AgCl, with 3 full cycles *i.e.* 6 segments per experiment. Unless stated otherwise, potentials and currents were obtained from the 3rd cycle. As part of the pH study, voltammograms were recorded at pH 5.0, 6.0, 7.0, and 8.0. The scan rate study was performed at 50, 100, 200 and 500 mVs^{-1}. For a detailed and physiologically relevant comparison, voltammograms were obtained at pH 7.4 at a scan rate of 200 mVs^{-1}.

4.5.2 Thiophenol and 4-Mercaptopyridine Redox assays

All spectrophotometric assays were performed using a Cary50 *Bio* from Varian Inc. The thiophenol (PhSH) assay measures the oxidation of PhSH to PhSSPh [125]. It is frequently used to monitor thiol-specific oxidation reactions and catalytic oxidation events, for instance in form of a simple glutathione peroxidase (GPx) assay. As a part of this assay PhSH was dissolved in methanol to a final concentration of 0.5 mM. The assay was initiated by addition of sulfur compound (0.5 mM final concentration) and the formation of PhSSPh was monitored at 305 nm for 1 h at room temperature. Measurements were taken once every minute. All measurements were performed in triplicate. Zero order rates were calculated between 0 and 30 min.

The 4-mercaptopyridine (4-MP) assay represents a thiol-oxidation assay which is very similar to the PhSH assay. The major difference lies in the fact that the 4-MP assay can be performed in aqueous solutions [126]. It is therefore more representative of physiological conditions, yet is often marred by the inherent low water-solubility of (drug) compounds to be studied. For this reason, the assay solution frequently contains a certain percentage of methanol. 4-MP was dissolved in 50 mM sodium phosphate buffer (pH 7.4) containing 30% methanol and 2 mM EDTA to complex adventitious metal ions [126]. The final concentration of 4-MP in the assay was 1mM. Sulfur compounds were added to a final concentration of 1 mM. The reaction was monitored at 324 nm for 1 h at room

temperature. Measurements were taken every minute. All measurements were performed in triplicate. Zero order rates were calculated between 0 and 30 min.

4.5.3 Nitrotetrazolium Blue (NBT) assay

The Nitrotetrazolium Blue (NBT) assay is frequently used to measure SOD activity. It is based on the reduction of NBT to the corresponding formazan by $O_2^{\bullet-}$. Under certain conditions, this assay can also be used to monitor the formation of $O_2^{\bullet-}$. Since it is not specific for $O_2^{\bullet-}$ and NBT reduction may be caused by other reductants, a SOD control must be used.

NBT (final concentration 0.5 mM) was dissolved in 50 mM potassium phosphate buffer (pH 7.4), containing 15 mM EDTA to sequester adventitious metal ions (an 'EDTA free' control was used to see if trace amounts of redox active metal ions may indeed have an effect). In the first set of experiments, sulfur compounds, including GSH, were added (0.5 mM final concentration). The reduction of NBT to formazan was monitored at 570 nm for 30 min at room temperature, with measurements being taken every 30 s. In the second set of experiments, the sulfur compounds were incubated as before but 'activated' at the beginning of each measurement by addition of GSH (0.5 mM final concentration). A series of controls, such as NBT in combination with GSH or sulfur compounds in combination with GSH were performed to rule out eventual interferences. In order to investigate a possible involvement of $O_2^{\bullet-}$ radicals, the second series of experiments was repeated in the presence of Cu,Zn-SOD (final SOD activity was approx. 1 unit). All experiments were performed in triplicate. Zero order rates were calculated between 0 and 30 min.

4.5.4 Metal interaction studies

Interactions between haemoglobin and polysulfides were monitored by UV/Vis spectrophotometry using Cary50 *Bio* from Varian Inc.

In brief, haemoglobin was solubilized in phosphate buffer (pH 7.4, 20% DMSO). The final concentration of haemoglobin was estimated by UV/Vis using an extinction coefficient of $\varepsilon_{280} = 118872\ cm^{-1}/M$, it was 5.35×10^{-6} M. Polysulfide stock solutions of 10 mM in DMSO were used.

980 µL of hemoglobin stock was taken to a Eppendorf tube and 20 µL polysulfide solution was added (final concentration of polysulfide 200 µM). The samples were votrex mixed after every 15 minutes. After an hour the absorption was recorded at 405 nm as blank. The samples were incubated at room temperature and measurements were taken after 24 hrs. All experiments were performed in triplicate and mean values were taken for results. Zero reading was recorded with compound in DMSO as well.

4.5.5 Chemical stability study of Polysulfides

In order to determine the chemical stability of polysulfides, a range of experiments were carried out to investigate the influence of temperature and light.

Diallylsulfides were purified using the HPLC method described in section 4.2.2. Each methanolic fraction of diallylpolysulfide (99% pure) was kept in ice during separation and re-injected to confirm purity and used as blank reading. Each separated fraction under investigation was divided further into four categories.

a) Stability at -20 ^{o}C
b) Stability at -4 ^{o}C
c) Stability at Room temperature in dark
d) Stability at Room temperature in light

The fractions kept in dark were stored in amber vials and further protected with aluminium sheet. A standard was run before and after each run to confirm the peak identities. Fractions were analysed each day for two weeks.

Chapter 5

Results

5.1 Synthetic Considerations

The demand for natural and synthetic polysulfides, either for research or for product development, has stimulated a renewed interest in the production of chemically pure polysulfides and thiosulfinates, notably by chemical synthesis. Unfortunately, the wider issues associated with the synthetic chemistry of such sulfur compounds are sometimes ignored.

This is particularly true for the chemical purity of agents employed in biological assays. Many commercially available sulfur agents are only of a 'technical grade' and carry sufficient amounts of impurities to prevent any meaningful biochemical or biological investigation. Such chemicals should not be used without prior purification

For the purposes of this study, pure samples of polysulfides were required. As every serious chemist knows, polysulfide synthesis is a tricky and rather difficult area of synthetic chemistry often marred by separation and solubility problems and decomposition of products. Nonetheless, there are a few well-established procedures for the synthesis and purification of various biologically active, natural sulfur compounds. For example, thiols such as allyl mercaptan **(6)** and propyl mercaptan **(30)** are available commercially. To use them in biological test systems, they need to be purified, which can be achieved by distillation under vacuum. The same applies to monosulfides (**(7)**, **(25)**) and disulfides (**(8)**, **(26)**), which are chemically reasonably stable and can be distilled under reduced pressure at mildly elevated temperatures of around 40 °C. Distillation of trisulfides (**(9)**, **(27), (44)**) and tetrasulfides (**(10)**, **(28)**) from crude mixtures has also been reported, yet is

complicated by the fact that such compounds tend to decompose or polymerize at elevated temperatures and/or reduced pressure. The same applies to allicin, which decomposes under normal vacuum distillation conditions.

A variety of published routes to individual saturated polysulfides with a specific number of sulfur atoms cannot be used due to the presence of the double bonds which react with the electrophilic reagents used in the majority of these syntheses. Furthermore the ease of 2,3-sigmatropic rearrangements and associated sulfur extrusion from allylic thiosulfoxides renders allylic polysulfides considerably less stable than the corresponding saturated polysulfides. To achieve reasonable amounts of sulfur compounds for tests and to avoid unnecessary problems with purification, synthetic efforts were focused on methods which do not result in too many side products *i.e.* impurities.

Higher polysulfides, such as diallylpenta- **(11)** and hexasulfide **(12)**, which have been identified at very low concentration in garlic, have not been reported as far as their chemical synthesis is concerned. Based on the work of Baechler, Hummel and Mislow [127] who reported that diallyl sulfide is partially converted to diallyl disulfide by heating with elemental sulfur in DMSO, while "prolonged reaction leads to formation and steady accumulation of tri-, tetra- and higher polysulfides", the reaction of diallyldisulfide and excess sulfur *in the absence of solvent*. Indeed, addition of an equal weight of diallyl disulfide to well stirred, nicely fluid molten sulfur at 120 °C gives a heterogeneous mixture which quickly becomes clear and homogeneous. After a few minutes, cooling this liquid, separation from precipitated sulfur and analysis by both ^{1}H NMR spectroscopy and RP-HPLC indicated the presence of > 70% All_2S_n, $n \geq 5$, with minor amounts of **(7)**, **(8)** and **(9)**.

Our synthetic strategy focused on utilizing compounds derived from diallyl disulfide as the source of one or both of the allylthio groups, CH_2=$CHCH_2S$, comprising the polysulfide product, CH_2=$CHCH_2SS_nSCH_2CH$=CH_2, where $n \geq 2$. The first set of approaches, shown in eq 1, used derivatives of type CH_2=$CHCH_2SX$, where X is a leaving group which would render the adjacent sulfur electrophilic.

$$2CH_2{=}CHCH_2SX + S_n^{-2} \rightarrow 2CH_2{=}CHCH_2S_{n+2}CH_2CH{=}CH_2 \qquad \text{(Eq 1)}$$

5.2 Characterization

5.2.1 ^{1}H & ^{13}C NMR Spectroscopy

NMR is a particularly useful tool for the study of polysulfides. Since polysulfides are often labile, so that quantitative conclusions based on classical separation methods for molecules are often suspect [128]. The ^{1}H NMR spectrum of allyl polysulfides consists of a series of well separated doublets (J = 7.1) in the area of 3.1 to 3.7 ppm for the thioallylic protons (CH2=CHC***H***$_2$S), along with signals at 5-6 ppm for olefinic multiplets. There is virtually no absorption in the region of 0-1.8 ppm nor in the 2.7-3.1 and 3.7-5.0 ppm. The ^{1}H NMR spectra show a monotonic increase in shielding for the methylene shifts of CH2=CHC***H***$_2$S*n*- with increase in sulfur atom number *i.e.*, δ 3.11, 3.36, 3.52, 3.60, 3.63, 3.67 for n = 1-6, respectively.

Similar trends in shifts were found for dipropyl polysulfides (**(25) - (28)**). These spectra typically consist of a well separated series of triplets ranging from 2.6 to 2.9 for CH$_2$C***H***$_2$S$_n$ along with signals between 0.80 and 1.73 ppm for the aliphatic multiplets.

As the number of sulfur atoms (n) increases, the triplet associated with the RC**H**$_2$S$_n$ protons undergoes progressive deshielding in case of diallyl polysulfides (**(7) - (12)**) but less evidently in case of dipropyl polysulfides (**(25) - (28)**) and the other families of polysulfides [128], [61].

The ^{13}C NMR spectrum is equally simple and informative. Allylic methylene peaks are located at 33 to 42-43 ppm (CH2=CH***C***H2S-), and allyl group olefinic peaks are found at 116-119 ppm (***C***H)) and 132-134 ppm (***C***H$_2$)). With respect to the sulfur chain length the corresponding ^{13}C NMR shifts change in a more erratic manner *i.e.* for R***C***H$_2$Sn-, δ 33.3, 42.3, 41.6, 42.0 ppm for n = 1-4, respectively. Similarly, for dipropyl polysulfides ^{13}C NMR signals were observed at 40.22 to 41.72 ppm (CH3CH$_2$***C***H2S$_n$-) and aliphatic group 21.69 to 21.90 ppm for CH3***C***H$_2$CH2S$_n$- for n = 1-4.

The trend in ^{13}C NMR shifts is less predictable, with deshielding and shielding alternating as the number of sulfur atoms progressively increases. The relative peak areas determined by ^{1}H NMR can be compared *qualitatively* to the intensities of the various ^{13}C peaks which are well separated in *both*, the olefinic and aliphatic regions for each compound. The carbon shifts appear not to be significantly affected by the nature of the R group. The ^{1}H and ^{13}C NMR assignments are summarized in **Table 5.1**.

Table 5.1 ^{1}H and ^{13}C NMR analysis of polysulfides. The experimental trends observed here are consistent with published NMR data [119, 129].

Compounds	**δ** H1/C1 (**–CH_2S_n**)	**δ** H2/C2 (**–CHCH_2S_n**)	**δ** H2/C2 (**–$CH_2CH_2S_n$**)
Alliin **(1)**	3.13/ 55.42	5.71/ 125.6	
Allicin **(3)**	3.72/ 34.80	5.83/118.91	
SAC **(4)**	2.95/ 34.49	6.02/ 133.22	
AM **(6)**	3.23/ 28.29	6.02/ 133.1	
DAS **(7)**	3.12/ 32.92	5.01/ 116.5	
DADS **(8)**	3.35/ 42.21	5.11/ 118.7	
DATS **(9)**	3.51/ 41.65	5.19/ 120.14	
DATTS **(10)**	3.50/ 42.05	5.22/ 121.70	
Diallylpentasulfide **(11)**	3.63/ 42.5	5.26/ 122.03	
Diallylhexasulfide **(12)**	3.67/ -	-	
DPS **(25)**	2.61/ 40.22		1.63/ 21.69
DPDS **(26)**	2.78/ 41.17		1.65/ 21.78
DPTS **(27)**	2.91/ 41.51		1.69/ 21.83
DPTTS **(28)**	3.05/ 41.72		1.73/ 21.90
PM **(30)**	2.60/ 26.86		1.38/ 27.22
Lenthionine **(39)**	4.51/ 41.23	-	-
1,2,4-trithiolane **(42)**	4.20/ 42.86	-	-
1,2,4,6-tetrathiepane **(41)**	4.21/ 42.09	3.93/ 39.92	3.93/ 39.92

5.2.2 HPLC Purification

HPLC was used to estimate the purity of polysulfides, and in some cases, in order to identify polysulfides on the bases of their specific retention times. Also, higher polysulfides with six or more sulfur atoms **(11)** and **(12)** were synthesized as mixtures so separation of these molecules was achieved by using HPLC. For developing a proper HPLC method for the identification of these polysulfides, different mobile phases were tried on the basis of literature methods. In many cases, a good separation was not achieved or resulted in decomposition of higher polysulfides on the column. This was specially the case with DATTS **(10)**, diallylpentasulfide **(11)** and diallylhexasulfide **(12)**. After repeated HPLC analysis, a method employing MeOH:H_2O (80:20) was established , which provided the best separation for allyl-, methyl- and propyl polysulfides without decomposition on the column.

Three standards confirm the peak identity the alk(en)yl polysulfides and further support the identification of the other sulfides based on the elution pattern shown by Lawson & Wang [10]. The retention times of different dialk(en)yl polysulfide are provided in **Table 5.2** and **Figure 5.1**. It is apparent from the similar responses of the two diallyl standards used that diallyltrisulfide (DATS) equivalents can be employed to calculate concentrations of the alk(en)yl polysulfides in the sample.

This is clear from the fact that UV/Vis absorbance is clearly related to the allyl groups, rather than the number of sulfur atoms, which have a negligible affect on the response. Therefore, the concentration calculations for each dialk(en)yl polysulfide is not affected by sulfur chain length. Since the dialk(en)yl polysulfides constitute the major percentage of the sulfides within the product, the re-calculation of the other compounds (based on the other standards) would not significantly affect the overall sulfide concentration nor make a big difference to the total of the (poly)sulfide.

One additional point to note is that many of the commercial sulfide products, specifically those containing sulfides with more than one sulfur atom, are often not as pure as stated. This is without doubt due to the chemical instability of these molecules and the exchange of sulfur atoms between the various sulfides over time. This was observed

mostly in case of higher diallylpolysulfides and also in some commercial products such as DADS **(8)** and diallylmercaptan **(6)** (available as 80% and 60% technical grade, respectively). It was also noticed that pure compounds are more stable than technical grade products, DADS (technical grade 80%) for instance, changes its composition quite significantly during 6 months on the shelf, while the distilled DADS (99%) is stable enough for more than one year at 4 °C.

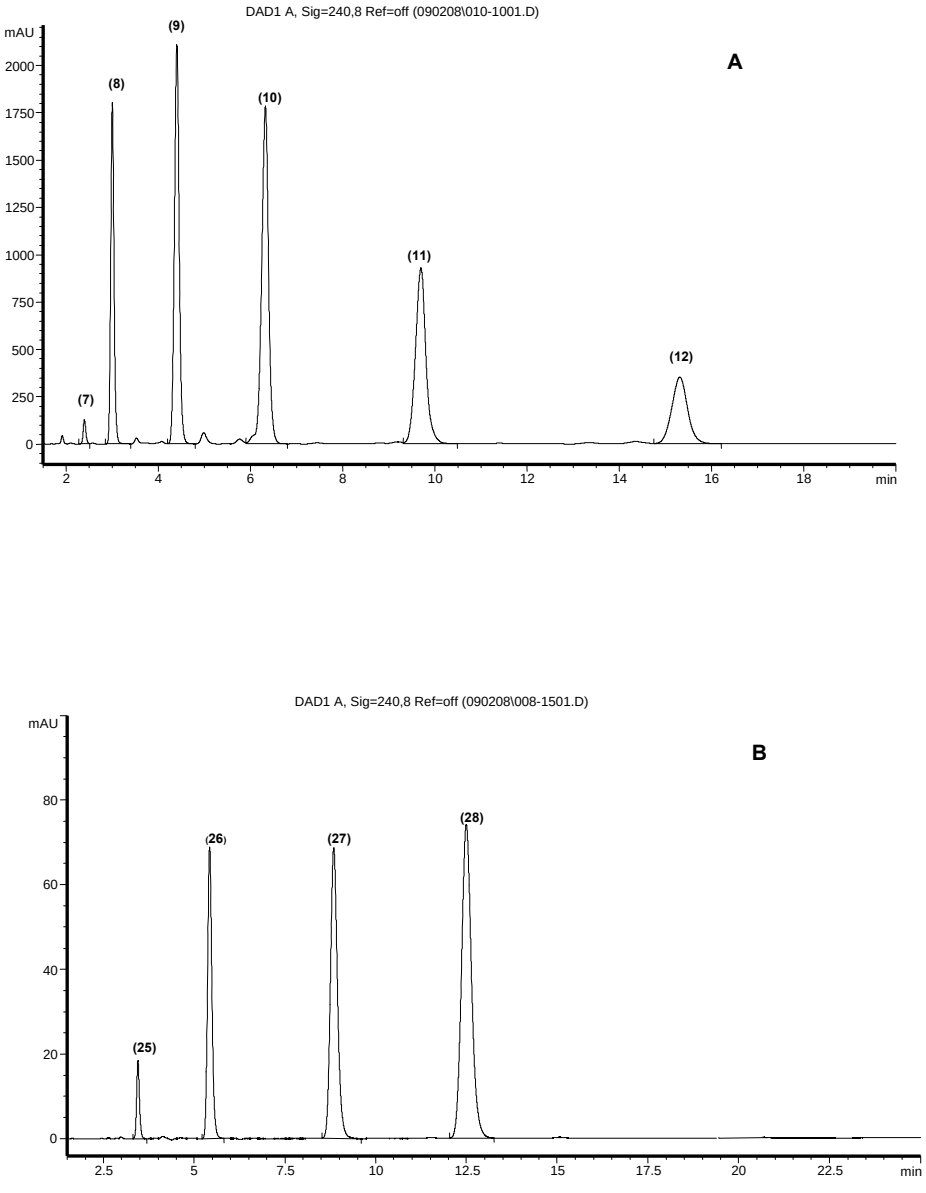


Figure 5.1 HPLC spectra of diallyl- **(A)** and dipropylsulfides **(B)**. The elution times of polysulfides using MeOH:H_2O (80:20). Diallylsulfide **(7)**, diallyldisulfide **(8)**, diallyltrisulfide **(9)**, diallyltetrasulfide **(10)**, diallylpentasulfide **(11)**, diallylhexasulfide **(12)**, dipropylsulfide **(25)**, dipropyldisulfide **(26)**, dipropyltrisulfide **(27)**, dipropyltetrasulfide **(28)**.

Table 5.2 Retention times and purity of different dialk(en)yl polysulfide with MeOH: H_2O (80:20) as eluent.

Compounds	Retention time (min)	Purity (%)
Allicin **(3)**	1.005	91.66
AM **(6)**	1.543	97.36
DAS **(7)**	2.399	98.56
DADS **(8)**	2.994	99.21
DATS **(9)**	4.385	97.31
DATTS **(10)**	6.287	96.02
Diallylpentasulfide **(11)**	9.648	93.87
Diallylhexasulfide **(12)**	15.258	91.42
DPS **(25)**	3.479	97.56
DPDS **(26)**	5.490	99.35
DPTS **(27)**	9.349	93.55
DPTTS **(28)**	12.689	96.04
PM **(30)**	1.810	96.23

5.2.3 GC- MS

The molecular masses of sulfides were confirmed by GC-MS. All compounds tested exhibited the expected m/z ratio. For compounds DAS **(7)** to DATTS **(10)** and DPS **(25)** to DPTTS **(28)** a simple GC-MS method as mentioned in "Materials and Methods" was used while for diallyl- penta **(11)** and hexasulfide **(12)**, a more sophisticated method was required due to instability of these molecules at higher temperature. Therefore, an Ag^+/ sulfide ion method was used and expected m/z ratio was obtained.

5.3 Cell Culture

5.3.1 Cytotoxic activity in the Caco-2 cell culture model

The Caco-2 cell culture model is frequently used as a model system to measure cytotoxic effects of compounds against a (cancer) cell line. Caco-2 cells are colon cancer cells. They are fairly resistant towards toxic agents, which makes them ideal for rapid screening and comparison of toxic and less toxic agents [55]. Unfortunately, rather high, close to millimolar concentrations of agents are often required in this particular cell line to produce a significant effect.

The results obtained in these studies are shown in Figures **5.2 A** and **B**. While various concentrations of agents between 15 nM and 1.4 mM were studied, a distinction of activity between the individual compounds was best possible at 700 μM. Compounds employed at lower concentrations had little activity even at 100 μM (80 to 90 % cell survival), whist experiments at higher concentrations were marred by solubility problems of these fairly lipophilic compounds. While at 700 μM, DAS **(7)** is virtually non-toxic against the Caco-2 cells (90 % cell survival), there is a measurable toxic effect of DADS when employed at the same concentration (80 % cell survival). Nonetheless, this effect is small compared to the effects caused by DATS **(9)** and DATTS **(10)** (57 % and 36 % cell survival at 700 μM polysulfide, respectively). In order to compare these results to previous studies, allicin **(3)** and mustard oil **(62)** were used as benchmarks. Both compounds are known to be fairly cytotoxic due to their high reactivity with thiol (and amine) groups. Interestingly, the activity of DATTS **(10)** (36 %) was comparable to the ones of allicin **(3)** (34 %) and allylisothiocyanate **(62)** (35 %).[4]

Similarly, DPS **(25)** and DPDS **(26)** has only marginal effects on the cell survival (86 and 81%) and a considerably more significant reduction was observed for DPTS **(27)** and DPTTS **(28)**, with 68% and 31% cell survival, respectively.

[4] An attempt to estimate IC_{50} values for these compounds in Caco-2 cell culture was made. The IC_{50} values of DAS **(7)** and DADS **(8)** are well above 1 mM. The IC_{50} value of DATS **(9)** appears to be close to 750 μM, while the IC_{50} values of DATTS **(10)**, allicin **(3)** and allylisothiocyanate **(62)** appear to fall within a similar range of between 500 and 700 μM.

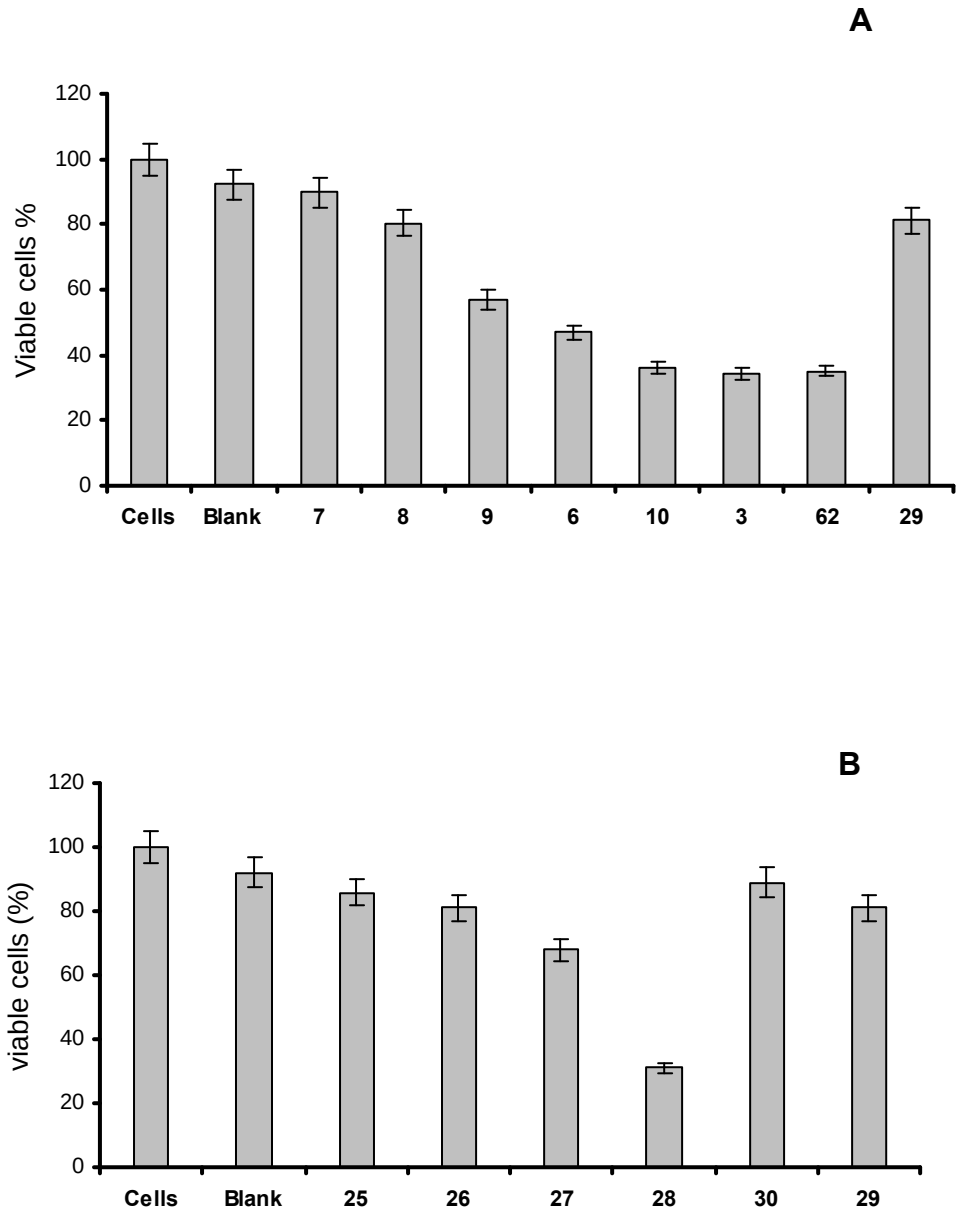


Figure 5.2 Viability of cultured Caco-2 colon cancer cells in the presence of various natural sulfur compounds and controls (given at 700 μM for comparison). While diallylsulfide **(7)** has only marginal effects on the cell survival, diallyldisulfide **(8)** reduces cell viability by approximately 20%. A considerably more significant reduction is observed for diallyltrisulfide **(9)** and diallyltetrasulfide **(10)**, with 43% and 64% reduction of cell survival, respectively. Diallyltetrasulfide exhibits a cytotoxicity comparable to the benchmark compounds allicin **(3)** and mustard oil (allylisothiocyanate **(62)**) while allylmercaptan **(6)** reduced the cell viability by 47% **[Panel A]**. Similarly the propyl polysulfides also showed considerable activity specially dipropyltri- **(27)** and tetrasulfide **(28)** with 32% and 69% reduction in cell survival, respectively. Propylsulfide **(25)**, propyldisulfide **(26)** and propylmercaptan **(30)** showed no activity. In contrast, the carbon analogue of diallyltetrasulfide, *i.e.* 1,9-decadiene **(29)**, shows only a very weak effect, counting against solely lipophilic interactions as reason behind polysulfide activity and pointing toward a special sulfur redox (bio-)chemistry as likely explanation. **[Panel B]**. All experiments were performed in triplicate

DATS **(9)**, DPTS **(27)**, DPTTS **(28)** and DATTS **(10)** are fairly lipophilic compounds. Their biological activity may therefore, at least in principle, arise from hydrophobic interactions - for instance with cellular membranes and/or proteins and enzymes - and not necessarily from the chemical reactivity associated with sulfur-sulfur bonds. After all, DATS **(9)** and DATTS **(10)** are analogues of 1,8-nonadiene and 1,9-decadiene, respectively. In order to investigate, if the polysulfide cytotoxicity observed is due to 'organic solvent'-like properties of the long-chain polysulfides, 1,9-decadiene was also tested in the Caco-2 cell culture model. Interestingly, this compound exhibited little toxicity at 700 μM (81 % cell survival), counting against any major hydrophobic or solvent-like toxic effects. Sulfur (redox) chemistry therefore seems to be the major cause of the biological activity observed in the case of DATS **(9)** and DATTS **(10)**. This was investigated further using the electrochemical method of Cyclic Voltammetry.

5.3.2 Cytotoxic activity & induction of apoptosis in U937 cells

These studies were carried out by our collaborators at Hôpital Kirchberg Luxembourg in the group of Dr. Marc Diederich.

Breifly, U937 cells were incubated with the panel of alk(en)yl polysulfides and their impact on cell viability was analysed by quantifying after 24 h the number of ATP-producing cells (**Figure 5.3**). When exposed to the alk(en)yl polysulfides at a concentration of 10μM DATS **(9)** and DATTS **(10)**, cell survival was reduced to 47% and 37%, respectively. Similarly, DPTS **(27)** and DPTTS **(28)** lead to levels of cell death between 60 and 70%.

In order to understand whether the reduction of cell viability was due to apoptosis, the appearance of typical apoptotic nuclear morphology in U937 cells after 24 h of incubation with polysulfides at 10 μM was investigated by Hoechst staining.

DATS **(9)**, DATTS **(10),** DPTS **(27)** and DPTTS **(28)** were very potent at inducing apoptosis in the range between 50 and 70% (**Figure 5.4**), whereas disulfides were almost inactive in this respect. Nuclei of all treated cells showed classical features of proceeding apoptotic events characterised by shrinkage or cleavage of nuclei (**Fig. 5.5**, white arrows). DATTS **(10)** is choosen here as a model representative compound [130, 131].

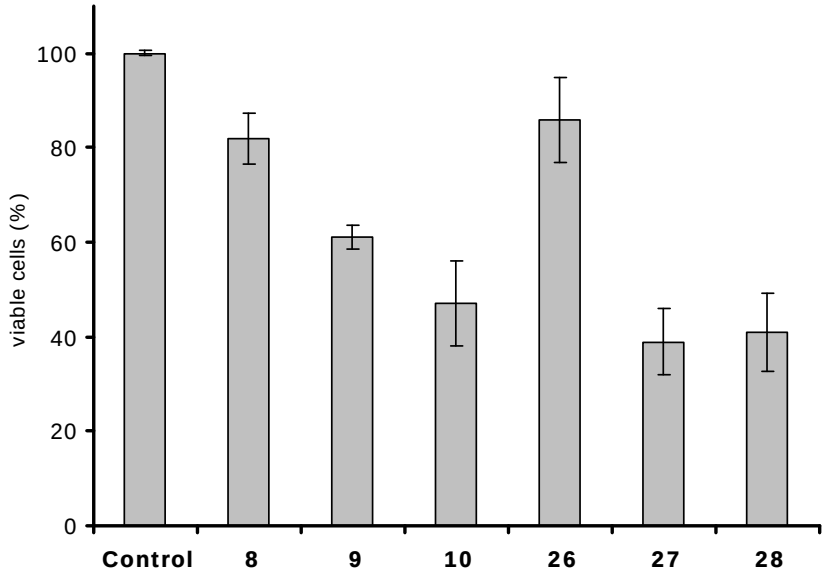


Figure 5.3 Luciferase-assay to assess effects of polysulfides on viability of U937 cells at 10 µM for 24 h. Afterwards, the number of viable cells was determined as the number of ATP-producing cells. DATS **(9)**, and DATTS **(10)** reduced the cell survival by 61% and 47% knocking out the DADS **(8)** which was virtually inactive with 82% cell survival. Interestingly DPTS **(27**) and DPTTS **(28)** showed similar toxicity to their respective allyl analogues (39% and 41 % cell survival respectively). Monosulfides (**(8)** and **(26)** were inactive). Data are depicted as mean ± SD. (n=3). Treated samples significantly different to the control were assessed by the t-test ($P < 0.05$). These studies were carried out by the group of Dr. Marc Diederich at Hôpital Kirchberg Luxembourg.

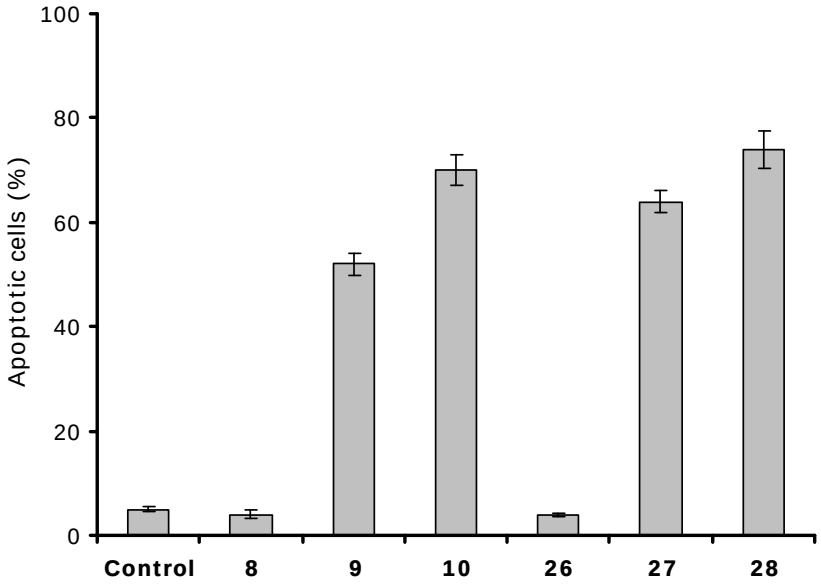


Figure 5.4 Percentage of apoptotic cells assessed by the morphology of cellular nuclei after 24 h of treatment with different polysulfides and subsequent Hoechst-staining. In the presence of DATTS **(10)** and DPTTS **(28)** apoptic cells were effectively increased (64% and 74%). DATS **(9)** and DPTS **(27)** also increased the apoptotic cells (52% and 60 %) whereas DADS **(8)** and DPDS **(26)** were inactive in this respect. Data are depicted as mean ± SD. (n=3). These studies were carried out by the group of Dr. Marc Diederich at Hôpital Kirchberg Luxembourg.

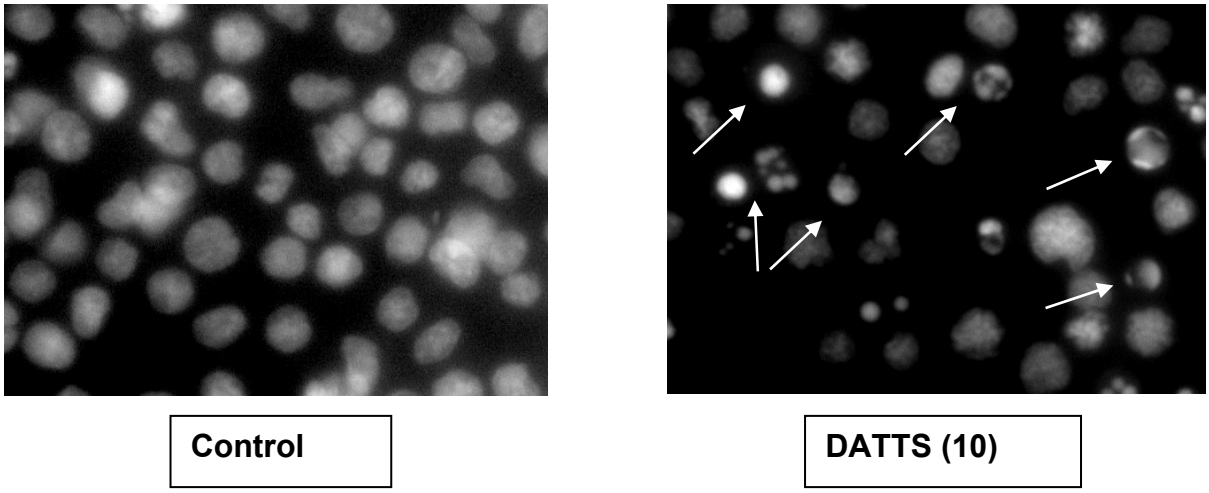


Figure 5.5 Representative images: DATTS **(10)** after 24 h induces typical apoptotic nuclear morphology: shrinkage and cleavage (white arrows) in comparison with a non-treated control. These studies were carried out by the group of Dr. Marc Diederich at Hôpital Kirchberg Luxembourg.

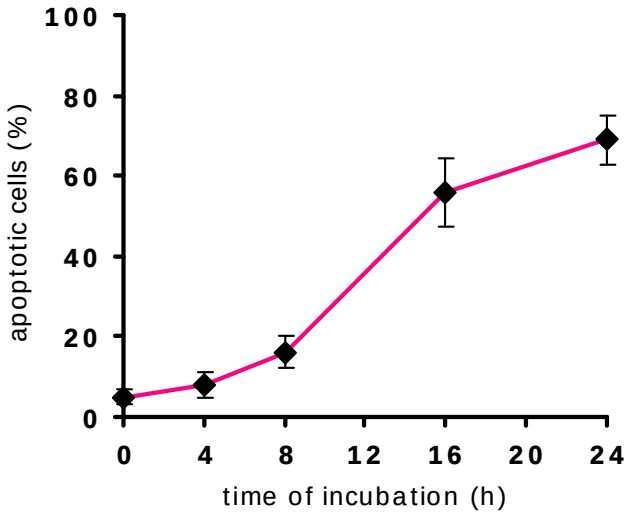


Figure 5.6 Kinetic analysis of apoptosis induced by DATTS **(10)**, evaluated as fraction of cells with apoptotic nuclei, stained with Hoechst. The number of apoptotic cells increased from 16% in 8 h to 69% after 24 h incubation. The data are the mean of n=3 independent experiments ± SD. These studies were carried out by the group of Dr. Marc Diederich at Hôpital Kirchberg Luxembourg.

Bearing in mind the above results, it was decided to choose DATTS **(10)** for ongoing mechanistic studies, since this is the most novel, promising and rarely investigated candidate in leukemia research [51, 132, 133]. The kinetic analysis of apoptosis induction was monitored after DATTS **(10)** treatment, as estimated by counting of cells stained with Hoechst (**Figure 5.6**). Further confirmation that cell death occurs *via* apoptosis came from FACS analysis after annexinV-FITC/propidium iodide staining, which gave similar results.

5.3.2.1 Summary of further mechanistic studies with DATTS (10)

Data for this ongoing research is not shown as this study was carried out by our collaborators at Hôpital Kirchberg Luxembourg by the group of Dr. Marc Diederich.

Before extending mechanistic studies on induction of cell death by DATTS **(10)** in U937 cells, the cancer selective activity of the compound comparing its effect on healthy analogues of the cancer model was investigated using primary monocytes from human blood as "healthy" controls [134]. The impact on cell viability of peripheral blood mononuclear cells (PBMCs) was assessed, testing their metabolic activity (ATP quantification). At a concentration of 10 μM, DATTS **(10)** does not exert any effect on cell viability of PBMCs. Furthermore, monocytes and lymphocytes were distinguished, by analysing their distribution by FACS and to see if the impact of compounds lead to alterations in size of blood cell population [135], using the biparametric analysis forward scatter (FSC) *vs* side scatter (SSC). Cells exposure to DATTS **(10)** at this concentration (10μM) did not result in any differences to non-treated samples that were assessed up to 48 h. This healthy state of the treated cells was additionally confirmed by analysis of the mitochondrial membrane potential, as a marker of cell metabolism and viability. In essence, the control "healthy" cells, *i.e.* PBMCs, were not significantly affected by DATTS at the concentration employed.

In contrast, caspase-3, 8 and 9 activation in U937 cells was confirmed when treated at 10 μM concentration of DATTS [136, 137]. Moreover, PARP was cleaved/degraded under the same conditions confirming caspase-3 activation, since the protein is one of the main substrates of the enzyme [137, 138].

The simultaneous activation of the caspase-8 and -9 was ascertained by the truncation of Bid [137] employing Western Blot analysis. It was found that Bid is not truncated after DATTS **(10)** treatment when compared to the control, *i.e.* by treating cells with Etoposide (VP16).

Aiming at the elucidation of the immediate target of DATTS **(10)** while triggering the intrinsic apoptotic pathway in U937 cells, mitochondrial injury was investigated [139] by analysing the mitochondrial membrane potential by FACS. The results confirmed the reduction of the mitochondrial membrane potential by time with release of cytochrome *c* into the cytosol which is often coupled with a loss of the mitochondrial potential.

As these above mentioned events are generally apoptotic signs occurring downstream to the activation of the pro-apoptotic proteins Bak and/or Bax [140-145], our experimental findings pointed towards the reduction of Bak and Bax when incubated with DATTS **(10).**

The levels of Bcl-2 and Bcl-xL of U937 cells upon treatment with DATTS **(10)** were also monitored. Inactivation of Bcl-2 [140, 141] after longer incubation times point towards the fact that protein was not an early target during the activation of this apoptotic pathway. Western Blot analysis of total cellular protein contents was performed to measure the levels of phospho-Bad. The results confirmed that the levels of phospho-Bad protein are strongly reduced by DATTS **(10)**, which is consistent with the notion that it could bind the anti-apoptotic proteins Bcl-2 and Bcl-xL [140] preventing them from counter acting Bax and Bak, thus favouring apoptosis [141].

As early as 4 h after incubation with DATTS **(10)**, a typical nuclear morphology of U937 cells was investigated. The nuclei, indeed, assumed a prophase-like pattern, suggesting an accumulation of cells in G2/M phase of the cell cycle. To ascertain whether the cell cycle was actually affected, a cell cycle analysis by FACS on propidium iodide stained cells was performed. An accumulation of cells in G2/M was observed from 4 h of treatment onwards, reaching a maximum at 8 h; then, apoptotic cells appear, with a progressive accumulation in sub-G1 phase, accompanied by a proportional disappearance of cells in G2/M phase.

The analysis of the presence of the phosphorylated form of histone H3 (H3P), which accumulates during early steps of mitotic chromatin condensation [146, 147] by FACS analysis, confirmed the accumation of H3P-positive cells after 4 h of treatment, with a further increase after 8 h, when apoptosis is still hardly detectable.

The accumulation of H3P-positive cells was correlated to a parallel change in the level of cyclin B1, which accumulated in G2/M phase, reaching the maximum expression in M phase [148]. Further studies by employing FACS and Western Blot analysis clearly indicated an accumulation of cyclin B1 level.

5.4 Nematode Toxicity Studies

Applying Protocol 2 (see Materials and Methods), the relative toxicity of the homologous series of diallylpolysulfides towards *Panagrellus redivivus* was obtained (**Table 5.3**). The data points towards an increase in toxicity once the sulfur chain length reaches 3 sulfur atoms. Diallylpentasulfide **(11)** was the most toxic molecule of the polysulfide series studied, with evidence of a toxic effect after just 1 h incubation. The toxicity of diallylhexasulfide **(12)** was no different to the one observed for blank.

Table 5.3 Mean percentage viability for the *Panagrellus redivivus*. Toxicity depends on the time of incubation. A concentration of 600μM concentrations of diallylpolysulfides was used in this study. The standard deviation is ≤ 5% (relative).

Compounds	**1 h**	**3 h**	**24 h**
Blank	99.1	96.8	77.4
DAS **(7)**	97.9	97.5	75.5
DADS **(8)**	96.2	95	73.6
DATS **(9)**	95.7	88.8	26.9
DATTS **(10)**	96.6	80.1	21.6
Diallylpentasulfide **(11)**	74.3	61.1	5.2
Diallylhexasulfide **(12)**	98.6	96.2	78.6

To investigate whether there are any physical effects of these molecules on *Panagrellus redivivus,* a detailed microscopic examination ("autopsy") was carried out. DATTS **(10)** was applied at a concentration of 200 µM. Initial examination revealed that as soon this compound was applied at higher concentrations, the nematodes became agitated and movement was very fast. After 6 h images were recorded at higher magnification (400X). (**Figure 5.7**)

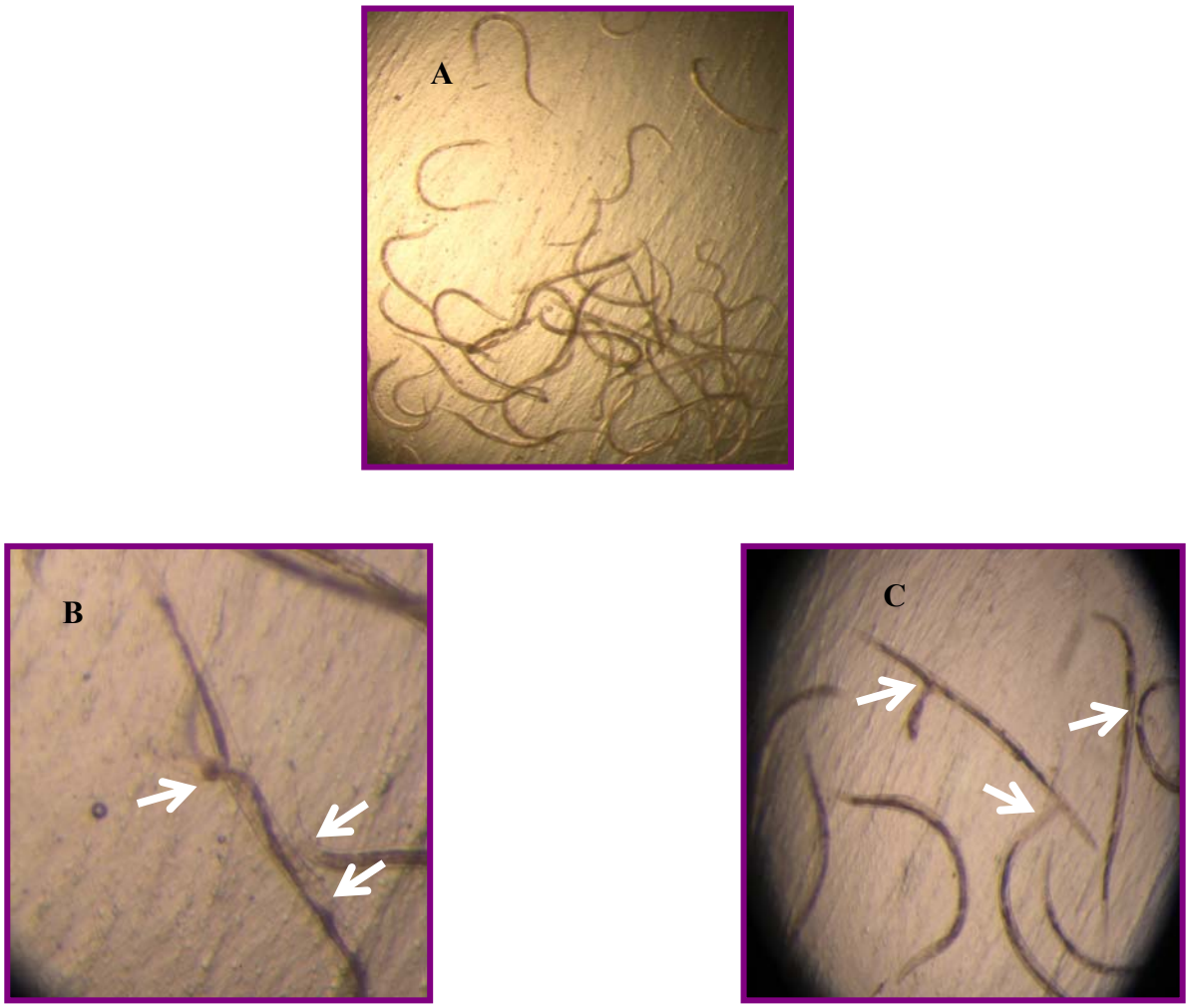


Figure 5.7 Effect of DATTS **(10)** on *Panagrellus redivivus*. Under normal conditions, the nematodes are very active (blank sample **(A)**); After 24 h treatment, it is clearly evident that the compound effectively has disrupted the epidermal layer (400X magnification) **(B & C)**. White arrows indicate the specific location of the disruption.

It was very interesting to notice that in most of cases the epidermal layer of the nematodes was completely disrupted and body fluids were leaking out. The surface damage was very evident and also in some cases a shrunk gut was observed.

This may point towards the mode of action of these molecules at least when applied at higher concentrations. As speculated by our own group in 2007 [16], the lipophillicity of such polysulfides may cause damage to membranes and could lead to (cell) death by dissolving or disrupting cellular membrane or even skin. Alternatively, observations may also be explained in terms of reaction of these molecules with body fluids, the generation of free radicals and by disturbing the metal homeostasis within the organism and /or cell. Although it is too early to speculate about the precise mode(s) of death and possible causes thereof- these initial results may provide a wide scope for detailed studies of the effects polysulfides have on these nematode species.

Applying Protocol 1 (see Materials and Methods), a very detailed study of polysulfide activity at different concentrations and time intervals and with two different species (*P. hermaphrodita* and *S. feltiae*) has been possible. The results obtained indicate a clear increase in toxicity once sulfur chain length reaches 3 sulfur atoms. Diallylpolysulfides (**(9), (10), (11), (12)**) and dipropylpolysulfides (**(27), (28)**) had an impact after 2 h, with diallylpentasulfide **(11)** being the most toxic at this time. After 24 h contact to *S. feltiae*, the diallytetrasulfide **(10)** had exerted a toxic effect which was similar to one of garlic and onion oil. The carbon chain analogue to the polysulfides, 1,9-decadiene was not toxic at any of the time intervals. (**Table 5.4**)

Interestingly, diallylhexasulfide **(12)** was not active within the first 4 h at a concentration of 200 µM or even 400 µM. Nonethless, it proved to be more toxic over a course of 24 h when compared to DATS **(9)** and DATTS **(10)**. As it was observed during the chemical stability study of diallylhexasulfide **(12)**, this molecule decomposes 30% to diallylpentasulfide **(11)** and 10% to diallyltetrasulfide **(10)**. The activity after 24 h may therefore be the result of actually a combined effect of the decomposition products rather than the hexasulfide itself. **(Figure 5.8)**

Table 5.4 Viability of nematodes after application of 200 µM of polysulfides. Readings were taken after 4 h. The data is presented as mean ± standard error of mean (SEM), n=4.

Compounds	Viability %age ± SEM	
	P. hermaphrodita	***S. feltiae***
Alliin **(1)**	90.37 ± 2.1	80.44 ± 1.5
Allicin **(3)**	67.6 ± 2	51.7 ± 1.1
AM **(6)**	70.9 ± 1.2	50.8± 1.9
DAS **(7)**	87.6 ± 0.5	81.5 ± 1.9
DADS **(8)**	80.3 ± 0.3	79.4 ± 1.4
DATS **(9)**	53.8 ± 1.7	43.7 ± 1.7
DATTS **(10)**	53.7 ± 1.3	39.4± 0.7
Diallylpentasulfide **(11)**	n.d	29.77 ± 2.1
Diallylhexasulfide **(12)**	n.d	67.7 ± 2.6
DPS **(25)**	88.7 ± 0.2	86.7 ± 1.6
DPDS **(26)**	86.3 ± 1.8	83.7 ± 2.1
DPTS **(27)**	67.8 ± 1.2	54.9 ± 1.1
DPTTS **(28)**	55.4 ± 1.3	49.2 ± 1.9
PM **(30)**	59.9 ± 2	45.1 ± 2.2
MO **(62)**	47.8± 2.1	42.4 ± 2.1
Lenthionine **(39)**	71.5 ± 2.2	64.41 ± 1.3
1,2,4-trithiolane **(42)**	81.58 ± 0.9	75.32 ± 2.1
1,2,4,6-tetrathiepane **(41)**	74.38 ± 1.7	68.98 ± 1.4
Ergothionine **(53)**	79.65 ± 1.6	70.05 ± 1.6
NaSH	71.72 ± 3.2	65.11 ± 1.4
Garlic Oil	51.75 ± 2.1	40.21 ± 3.2
Onion Oil	67.39 ± 1.8	57.33 ± 2.1
1,9-decadiene **(29)**	90.43 ± 1.7	86.22 ± 1.8
Blank	97.5 ± 1.8	96.5± 1.1

n.d .= not determined

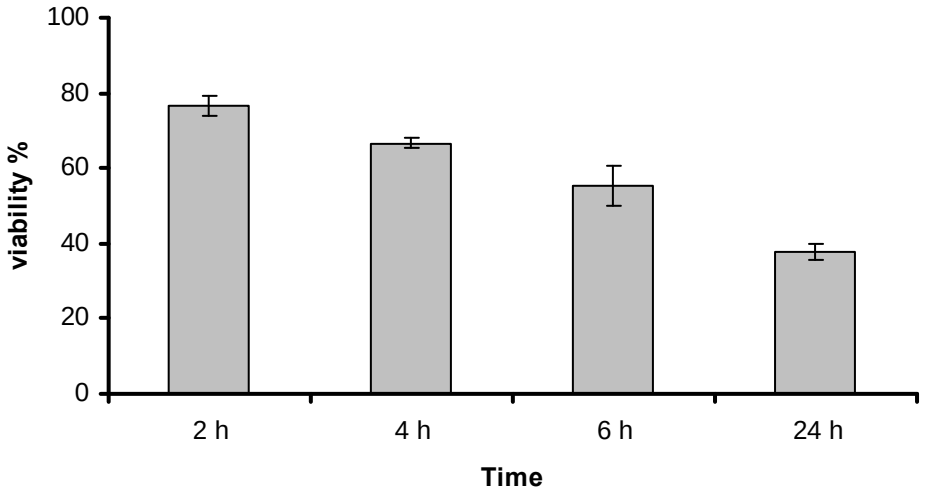


Figure 5.8 Viability of *S. feltiae* at 200µM of diallylhexasulfide **(12)** after 2, 4, 6 and 24 h. Please note that activity may result from diallylhexasulfide decomposition products rather than hexasulfide itself.

Also both nematode species demonstrated sensitivity to the polysulfides in a dose related manner. *S. feltiae* demonstrated sensitivity to both DADS (**(8)** 30% viability) and DPDS (**(26)** 17% viability) once 400 µM concentrations was reached. This was less evident with *P. hermaphrodita*. The dipropylpolysulfides demonstrated similar levels of toxicity to the diallylpolysulfides, but the effect was always numerically less in comparison. (**Table 5.4**)

Figure 5.9 gives a glimpse of dose response behaviour of *P. hermaphrodita* against active compounds such as DATTS **(10)** and DPTTS **(28)**. The results from these studies point towards a difference in activity between allyl and propyl compounds. Although the allyl compounds generally exhibited more activity when compared to propyl analogues, these differences were only marginal. With the passage of incubation time the activity was reduced.

Reduced forms of polysulfides such as allyl- **(6)** and propylmercaptane **(30)** also exhibited interesting activities, with 50% and 45% viability at a concentration of 200 µM, respectively, in *S. feltiae*. The toxicity of the mercaptane **(6)** is therefore similar (52%) to the one observed for allicin **(3)**. Mustard Oil **(62)** which posseses a totally different chemical structure and reactivity, was also active in both species (42% in *S. feltiae* and 47 % viability in *P. hermaphrodita*).

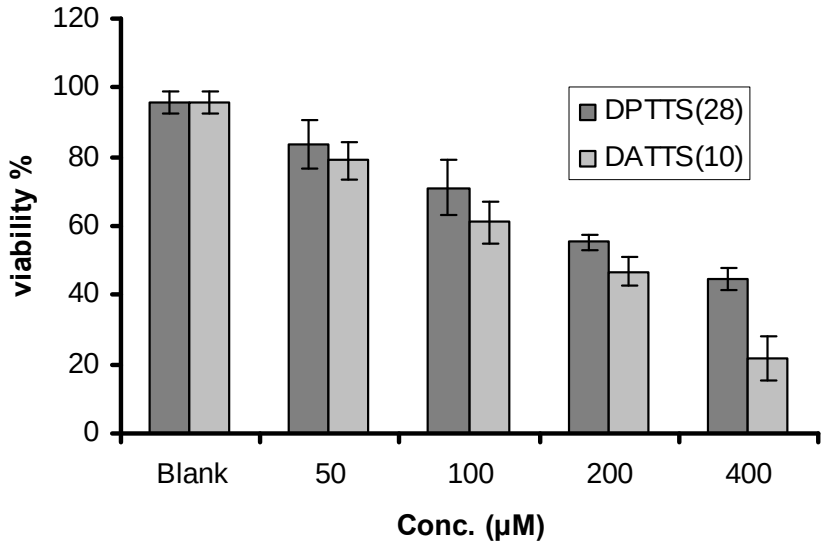


Figure 5.9 Effect of different doses of DATTS **(10)** and DPTTS **(28)** on *P. hermaphrodita* measured after 4 h at concentration at 400 µM. DATTS **(10)** exhibited a higher activity (21% viability) when compared with DPTTS **(28)** (44%).

Cyclic polysulfides such as lenthionine (a trisulfide **(39)**), tetrathiepane (a disulfide **(41)**), trithiolane (a disulfide **(42)**), ergothionine **(53)** also exhibited various activities in both species, albeit less when compared with the linear polysulfides. Lenthionine, a cyclic trisulfide, was found to be most active (29% reduction in survival in *P. hermaphrodita* at 200 µM). The activity of this cyclic trisulfide is found to about half of that associated with the linear trisulfide (DATS **(9)** and DPTS **(27)**). Cyclic disulfides were virtually in-active when compared with linear disulfides.

Garlic and onion oils were also active. Garlic oil reduced viability of *P. hermaphrodita* and *S. feltiae* to 51% and 40%, respectively. This activity is almost similar to the one observed for DATS **(9)**. One should bear in mind that garlic oil is mixture of DADS **(8)**, DATS **(9)** (45-50%) and some methyl-allyl sulfides.

Onion oil, which contains dipropylsulfides and various methyl-ethylsulfides, exhibited an activity which was less than the one of garlic oil. (**Table 5.4**)

5.5 *In vitro* Studies

5.5.1 Electrochemical properties of diallyldi-, tri- and tetrasulfide

Diallyldi- **(8)**, tri **(9)**- and tetrasulfide **(10)** contain one, two and three sulfur-sulfur bonds, respectively, which can be reduced and oxidized electrochemically using a dropping mercury electrode. Although the use of such an electrode is limited by a range of factors, such as strong adsorption of sulfur compounds on mercury, it provides an initial insight into the redox behavior of these compounds.

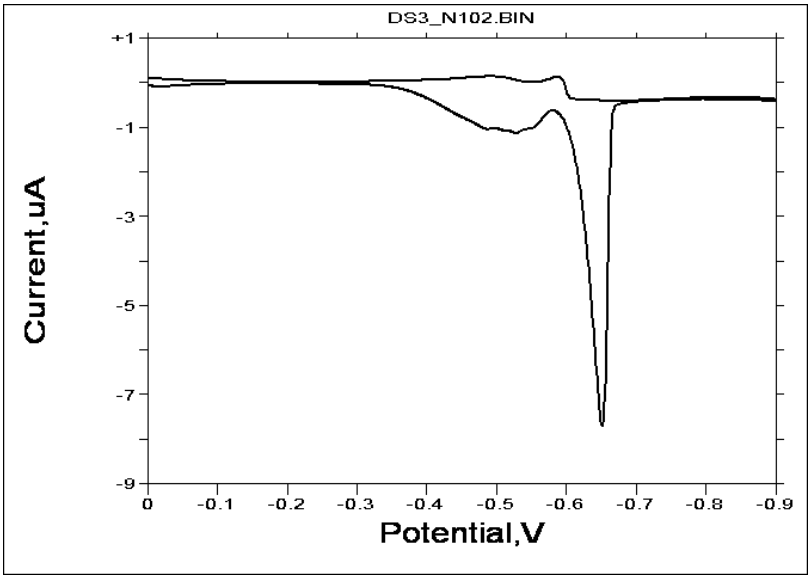


Figure 5.10 A typical voltagram of DATS **(9)** at pH 7.4

The oxidation and reduction peaks obtained are listed in **Table 5.5**. As expected, the monosulfide showed no oxidation or reduction signal in the potential range between -900 and 0 mV *vs*. the Ag/AgCl reference electrode. DADS **(8)**, DATS **(9)** and DATTS **(10)**, on the other hand, exhibited at least two reduction and oxidation signals, of which one reduction signal (*E*pc) and one oxidation signal (*E*pa) were dominant (**Table 5.5** and

Figure 5.11).[5] Both of these major signals seem to result from a thermodynamically quasi-reversible electron transfer process of electrochemically active species adsorbed on the mercury electrode (this was confirmed by a scan rate study, see below). The smaller signals are probably due to various other adsorption effects which complicate the voltammograms and allow only a very limited interpretation of the signals and their parameters. Nonetheless, a few interesting trends can be observed.

First of all, the reduction signal *E*pc generally becomes more negative with increasing sulfur chain length, *i.e.* the disulfide is more oxidizing compared to the trisulfide, which, in turn, is slightly more oxidizing than the tetrasulfide. This trend is clearly visible at a physiological pH of 7.4, where DADS **(8)**, DATS **(9)** and DATTS **(10)** exhibit *E*pc values of -590 mV, -657 mV and -680 mV, respectively (**Table 5.5**). These *E*pc values for the main reduction signals are fairly negative, compared, for instance, to the measured value for the reduction of GSSG (*E*pc = -463 mV). Based on this initial electrochemical estimate of reduction potentials, it is therefore unlikely that the DATS **(9)** or DATTS **(10)** *on their own* behave as strong oxidants, as may have been suggested by some cell culture studies [47].

Interestingly, the oxidation potential *E*pa observed for the di-, tri- and tetrasulfide also becomes more negative with increasing sulfur chain length, from -465 mV for the disulfide to -591 mV for the trisulfide and -603 mV for the tetrasulfide (at pH 7.4, 200 mV/s). In comparison, the *E*pa value for GSH under the same experimental conditions is -442 mV. In essence, this implies that the reduced forms of the tri- and tetrasulfide, *i.e.* most likely RSSH or RSSSH, are considerably more reducing than GSH and the reduced form of DADS, *i.e.* allylmercaptan (RSH) – and may well reduce O_2 to $O_2^{\bullet-}$. This finding is

[5] To avoid any confusion which may result from the use of Cyclic Voltammetry and the *E*pa/*E*pc redox pair notation: *E*pc arises from the reduction of the di-, tri- and tetrasulfide to a thiol, perthiol, hydropolysulfide or another species, while *E*pa reflects the oxidation of such a reduced species to the di-, tri- tetrasulfide (or any other product yet to be identified). In the cases discussed here, the redox processes seem to be reversible, *i.e.* the sulfides appear to be mostly re-cycled during the electrochemical sweep.

Table 5.5 Summary of oxidation and reduction signals of diallylsulfides. Standard deviation is ≤ 1% (absolute) [1].

Compound	*E*pc (mV)	*E*pa (mV)
DAS **(7)**	-	-
DADS **(8)**	-590	-463
Diallyltrisulfide **(9)**	-657	-591
Diallyltetrasulfide **(10)**	-680	-603

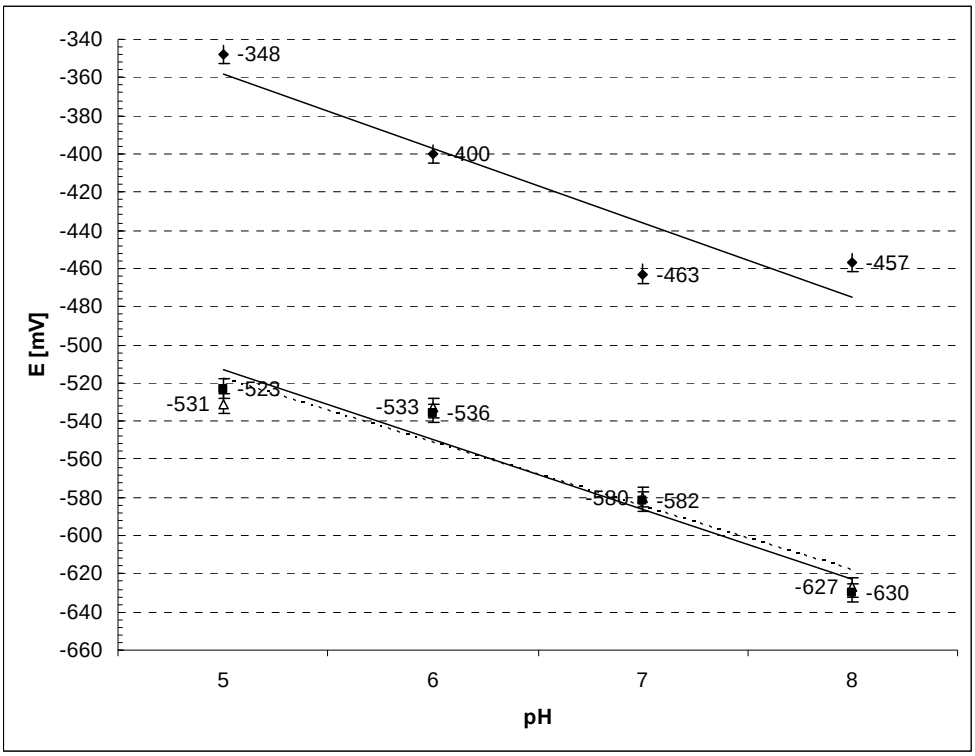


Figure 5.11 The influence of sulfur-chain length and pH on the oxidation potential *E*pa of the polysulfides under investigation. While DAS **(7)** shows no reduction or oxidation signal, DADS **(8)** (•), diallyltrisulfide **(9)** (▪) and diallyltetrasulfide **(10)** (▵) exhibit two major, distinct, pH dependent oxidation and reduction currents (*E*pa and *E*pc, respectively). The considerably more positive potentials measured for GSH and DADS **(8)** compared to the tri- and tetrasulfide indicate that the latter are rather poor oxidants, yet their reduced forms, with *E*pa values around -600 mV *vs.* Ag/AgCl, are strongly reducing and may be able to convert O_2 to $O_2^{\bullet-}$ (possibly requiring the presence of trace metal ions as catalysts). The observed decreases in potential with increasing pH values imply that this reducing behavior is particularly pronounced at physiological and alkaline pH [1].

in agreement with literature reports reporting lower p*K*a values for RSSH compared to RSH and an associated higher reactivity of RSSH as reducing agents (see below) [19].

The electrochemical studies also confirm a strong pH dependence of the oxidation and reduction potentials for the di-, tri- and tetrasulfide, which generally shift toward more negative potentials with increasing pH. The oxidation potentials, *E*pa, for instance, shift around 100 mV to more negative values between pH 5.0 and 8.0, which results in a Δ*E*/pH value of approximately 33 mV. The pH dependence observed in these studies is hardly surprising, considering that the redox processes involved heavily rely on protonation and deprotonation, *i.e.* the electrochemical potentials should theoretically be pH dependent. Interestingly, these findings also imply that the sulfides studied become less oxidizing at neutral and slightly alkaline pH, while their reduced forms become more reducing at these pH values. These findings agree with the biochemical notion that the deprotonated forms of thiols, *i.e.* thiolates, are more reducing than thiols, and also point towards a certain control of the polysulfide redox behavior by pH. (**Figure 5.11**)

In order to investigate the electrochemical properties of the sulfides on the mercury electrode further, scan rate studies were conducted for the oxidation and reduction signals. As expected, there is an apparent correlation between oxidation and reduction peak current (*I*pa and *I*pc, respectively) and scan rate, indicative of electrochemically active species adsorbed on the electrode surface. This adsorption phenomenon is observed for the reduction as well as oxidation signals, *i.e.* it seems that the di-, tri- and tetrasulfides, as well as their respective reduced forms, are adsorbed on the mercury electrode as seen in **Figure 5.12**

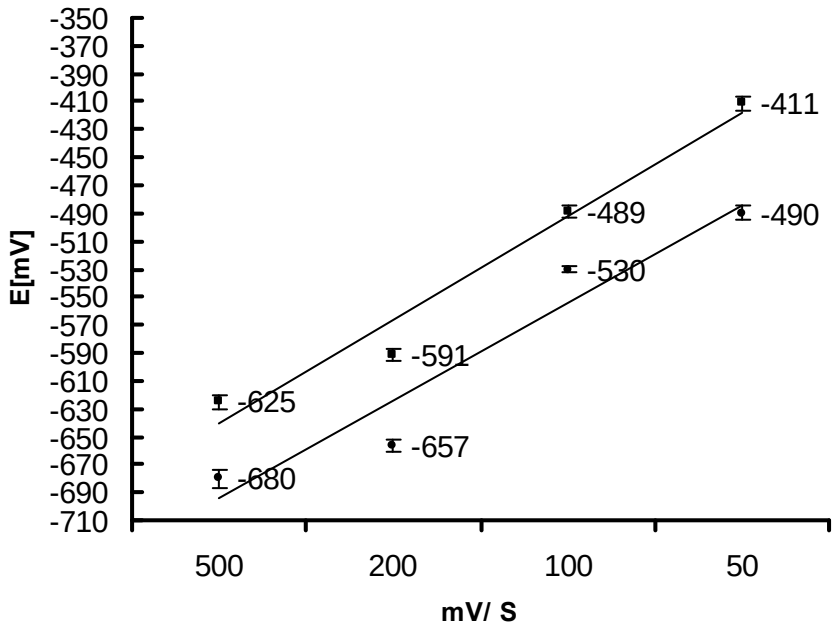


Figure 5.12 Influence of scan rate on *E*pa (•) and *E*pc (▪) values observed for DATS **(9)**. The linear correlation between *E*pa/ *E*pc and scan rate points towards the presence of an electrochemically active species adsorbed on the mercury electrode surface.

5.5.2 Thiophenol and 4-Mercaptopyridine assays

The electrochemical studies may only provide a preliminary insight into the apparent redox behavior of compounds, partially due to the adsorption phenomena on the mercury electrode. The redox properties of the sulfides were therefore investigated further using two well established spectrophotometric assays indicative of thiol oxidation [125]. The thiophenol (PhSH) assay measures the oxidation of PhSH to the disulfide PhSSPh in methanolic solution, while the corresponding 4-mercaptopyrdine (4-MP) assay operates in buffered aqueous media [126]. The former is ideally suited to study the redox behavior of water-insoluble compounds, while the 4-mercaptopyridine assay reflects physiological conditions more accurately (*e.g.* aqueous media, pH 7.4).

The results of these assays are summarized in **Table 5.6**. Bearing in mind that thiol/disulfide reactions are slow, the two assays were both run for 30 min or 1 h. During

this time, only the control compounds, *i.e.* allicin, mustard oil and H_2O_2, were able to form measurable amounts of PhSSPh and dipyridinedisulfide. In contrast, neither the DADS **(8)**, nor DATS **(9)** or DATTS **(10)** showed any significant PhSSPh or dipyridinedisulfide formation (**Figure 5.13**). These results support the findings of the electrochemical studies. Neither DATS **(9)** nor DATTS **(10)** are strong oxidants *on their own*; they do not readily interact with thiols, not even fairly reducing thiols such as PhSH. The known thiol oxidant allicin, on the other hand, readily oxidized PhSH and 4-MP to the corresponding (mixed) disulfides, therefore validating the assays and also emphasizing the inherent difference(s) in chemical reactivity and biochemical activity between this thiosulfinate on the one hand and the polysulfides on the other. It was therefore decided to employ the NBT assay.

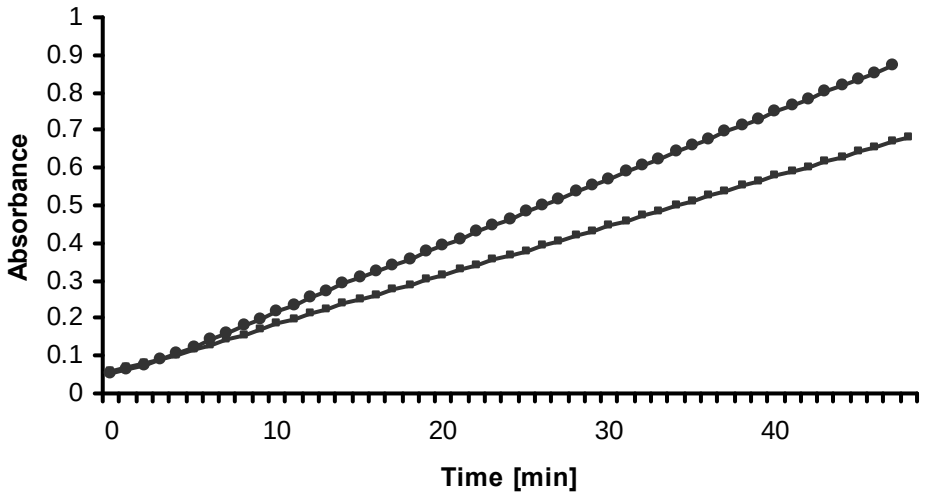


Figure 5.13 The thiophenol assay representing the oxidation of PhSH to disulfide (PhSSPh) by H_2O_2(▪) and allicin ((•) **(3)**). The reaction rates are summarised in Table 5.6.

5.5.3 Nitrotetrazolium Blue (NBT) assay

This spectrophotometric assay is commonly employed as an $O_2^{\bullet-}$ assay, for instance to estimate the activity of the enzyme superoxide dismutase (SOD). It monitors the one-electron reduction of NBT to a formazan by suitable reducing agents, such as $O_2^{\bullet-}$. In the context of the sulfur agents, it is employed to measure the formation of $O_2^{\bullet-}$ in the absence and presence of a reducing agent (GSH).

The results obtained in this assay are shown in **Figure 5.14** and **5.15,** while the reaction rates are summarized in **Table 5.6**. Neither DADS **(8)**, DPDS **(26),** nor DATS **(9)**, DPTS **(27),** DATTS **(10)**, or DPTTS **(28)** react with NBT in the absence of GSH. GSH, which itself does not react with NBT either, seems to 'trigger' the reduction of NBT in the presence of DATS **(9)**, DPTS **(27)** and DATTS **(10)**, DPTTS **(28)**, but not in the presence of DAS **(7)** or DADS **(8)**.

These results are highly revealing. First of all, there is clear difference in 'chemistry' between the disulfide on the one hand, and the tri- and tetrasulfide on the other. Secondly, the tetrasulfide is clearly the most 'active' of the compounds. Thirdly, the reaction needs a trigger, such as GSH, which is also reflected by an initial 'lag' phase frequently observed in the reaction of the tetrasulfide. This observation accounts for the formation of a reactive intermediate, such as RSSH or RSSSH. Therefore, the reduced form of DADS, *i.e.* allylmercapatan **(6)** (RSH), was also investigated and found to be inactive in this assay, either in the absence or presence of GSH (the reduced form of DATS and DATTS *i.e.* perthiols, are chemically unstable transient species and cannot be used). And fourthly, the reaction observed for DATS **(9)** and DATTS **(10)** is associated with a reduction process (either directly or indirectly), and not an oxidation event.

In order to investigate the possible involvement of $O_2^{\bullet-}$ radicals in NBT reduction, SOD was added to the reaction mixtures at the beginning of each assay (**Figure 5.14** and **5.15**; **Table 5.6**). This enzyme competes with NBT for $O_2^{\bullet-}$, which is converted by the enzyme to O_2 and H_2O_2. The significant suppression of the GSH triggered reaction of DATS **(9)** and DATTS **(10)** with NBT by SOD points indeed towards a chemical process which may involve $O_2^{\bullet-}$ radicals. It should be pointed out, however, that at this stage, the NBT assay used here has become fairly complex and alternative reactions, such as a direct reduction of NBT by (a highly reducing) perthiol, may not be ruled out completely at this point.

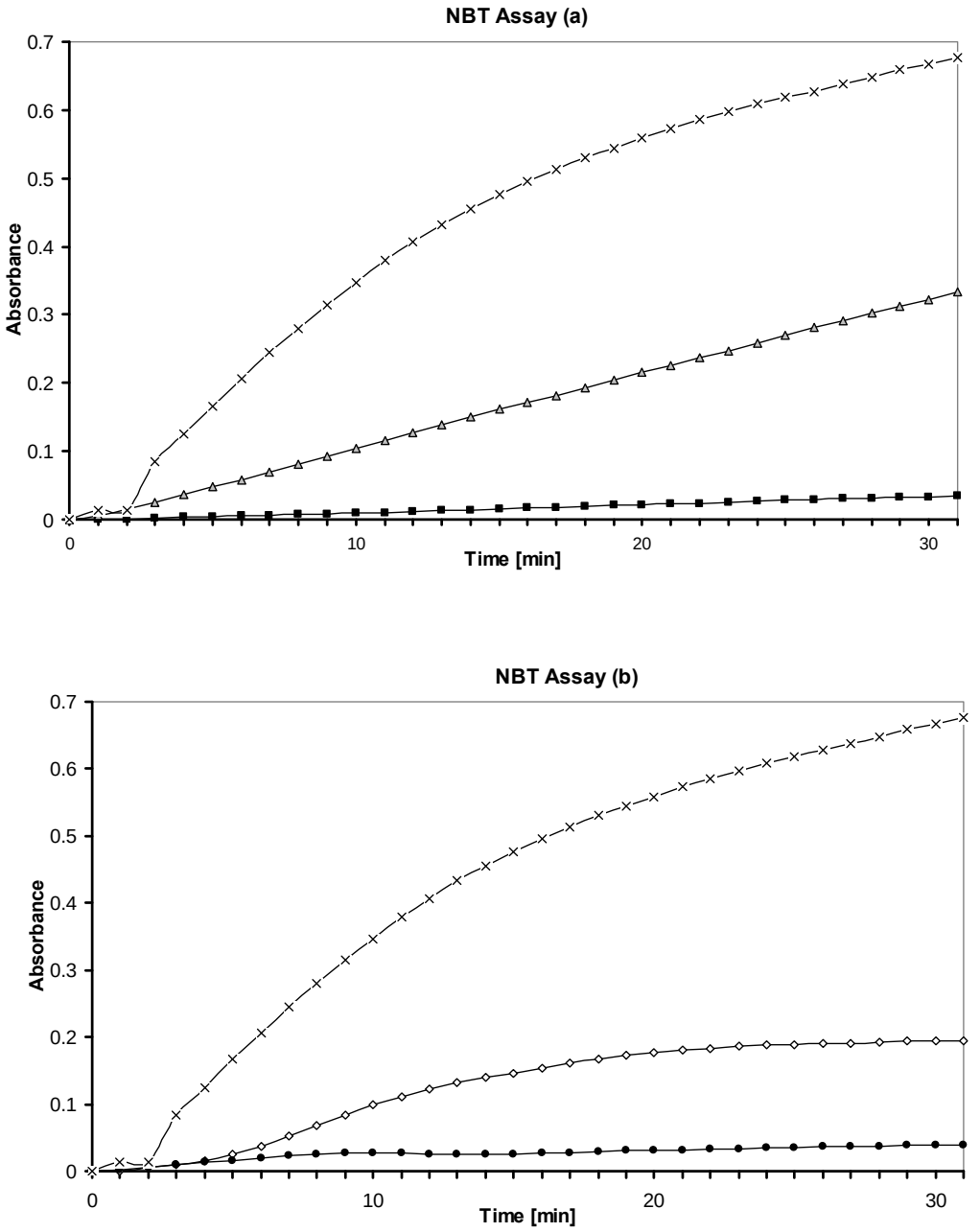


Figure 5.14 The Nitrotetrazolium Blue (NBT) assay is indicative of $O_2^{\bullet-}$ formation. In this assay, none of the four sulfides studied shows any significant activity on its own. In the presence of GSH, however, diallyltrisulfide **(9)** (-▲-) and diallyltetrasulfide **(10)** (-×-) cause the reduction of the NBT dye, while DADS **(8)** (-▪-) (DAS **(7)**, not shown) remain inactive [**Panel (a)**]. The activity of the diallyltrisulfide and diallyltetrasulfide depends critically on the presence of the reducing agent GSH, and is affected by SOD [**Panel (b)**]. While diallyltetrasulfide **(10)** and NBT do not react directly (-●-), the significant reaction in the presence of GSH (-×-) is partially inhibited by SOD (-◊-) [1].

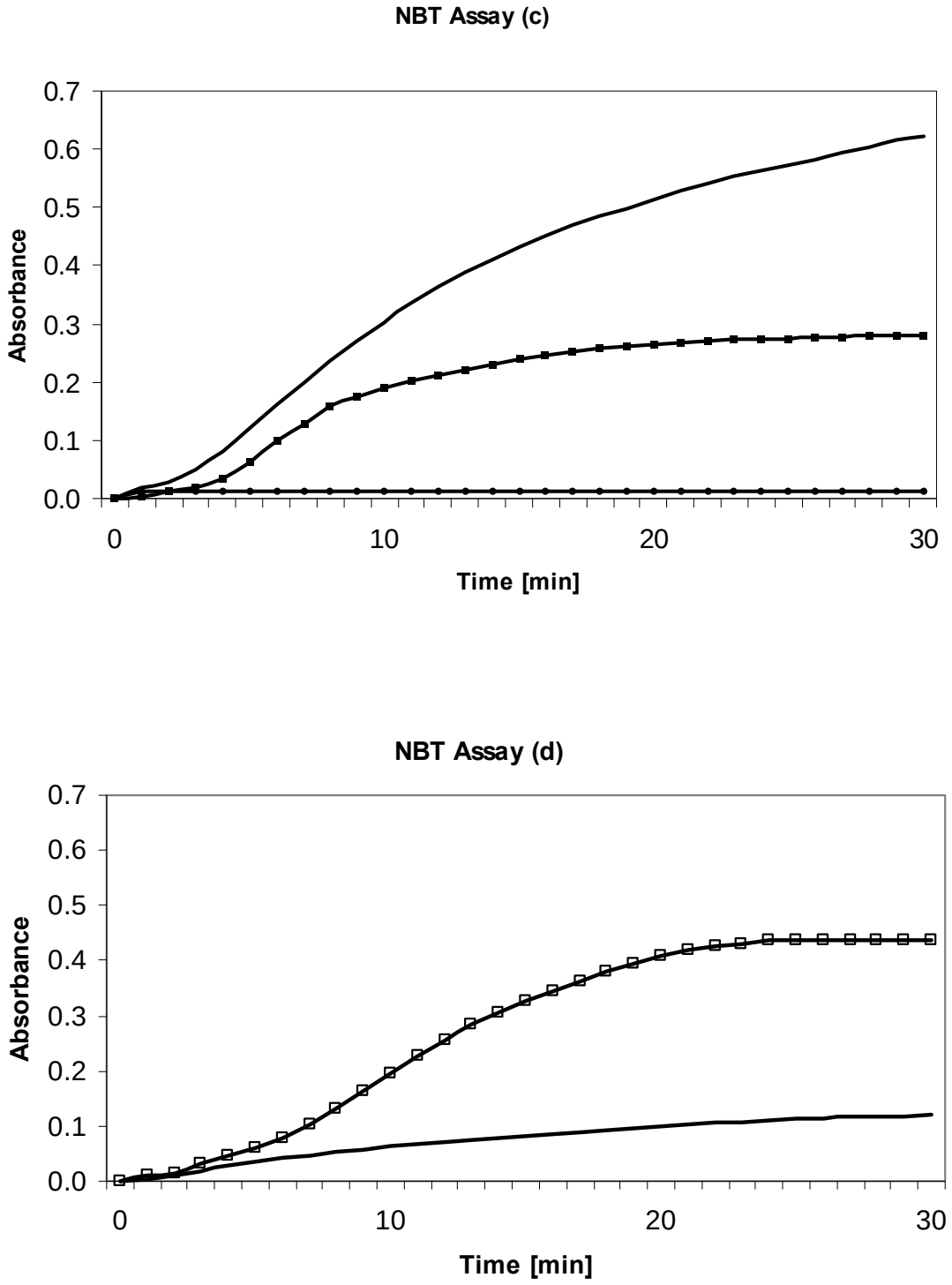


Figure 5.15 The Nitrotetrazolium Blue (NBT) assay for dipropylsulfides. While diropyltetrasulfide **(28)** and NBT do not react directly (•), DPTTS **(28)** (—) reacts in the presence of GSH. The activity was reduced when SOD was added (▪). DPTS **(27)** showed less activity for the one observed for diallyltrisulfide **(9),** while dipropyldisulfide **(26)** remained inactive (Panel (c)). The activity of the allicin **(3)** in the presence of GSH (▫) was greatly affected by SOD (—) (Panel (d)).

Table 5.6 Summary of reaction rates of different polysulfides observed in PhSH, 4-MP and NBT assays (in the presence of GSH). The standard deviation is ≤ 3% (absolute).

Compound	PhSH (10^{-9} Ms^{-1})	4-MP (10^{-9} Ms^{-1})	NBT (10^{-9} Ms^{-1})	
			Without SOD	With SOD
Allicin **(3)**	10.58	17.40	16.1	1.72
AM **(6)**	5.3	0	0.92	0
DAS **(7)**	0	0	0.37	0
DADS **(8)**	0	0	1.75	0
DATS **(9)**	0	0.84	19.74	8.62
DATTS **(10)**	0	2.81	39.77	11.32
DPS **(25)**	0	0	0.08	0
DPDS **(26)**	0	0	0.81	0
DPTS **(27)**	0	0.51	5.21	0
DPTTS **(28)**	0	2.19	19.54	4.03
PM **(30)**	3.3	0	0.80	0
Mustard oil **(62)**	3.68	5.61	6.8	3.56
Lenthionine **(39)**	2.2	0	3.4	0
Ergothionine **(53)**	0	0	0.64	0
NaSH	0	0	9.92	5.02
1,9-decadiene **(29)**	0	0	0	0

There have been reports in literature pointing towards the involvement of trace amounts of redox active metal ions (copper) in the $O_2^{\bullet -}$ formation process based on polysulfides [19]. Interestingly, NBT reduction by DATS **(9)** and DATTS **(10)** in the presence of GSH was identical in the absence and presence of EDTA (15 mM). This may point against a metal-catalyzed process, although one cannot dismiss the involvement of trace amounts of redox metals outright, since the metal-EDTA complexes may also be catalytically active.

Since DATS **(9)** and DATTS **(10)** are known to release hydrogen sulfide in the presence of reducing agents, the hydrogen sulfide donor NaHS was employed to examine a possible interaction of HS^- (or H_2S) with NBT. Under the reaction conditions chosen, there was no significant interaction of NaHS (1.5 mM) with NBT, either in the absence or presence of GSH (or indeed GSSG). Although these findings do not rule out the involvement of hydrogen sulfide in the reduction of NBT completely, they seem to point more toward reduction processes which are driven by RSSH or RSSSH species (and their respective anions).

The formation of $O_2^{\bullet -}$ radicals obviously requires the presence of O_2. While it is not possible to culture Caco-2 cells under anaerobic conditions, the NBT assay was performed in N_2 purged, *i.e.* low oxygen solution. This had only a marginal effect on the reaction, although the solutions used cannot be deemed completely O_2 free, and O_2 is also generated/'recycled' when $O_2^{\bullet -}$ reacts with and reduces NBT.

5.5.4 Metal interaction studies

The results obtained as a part of these experiment enhance further our understanding of polysulfides as weak oxidants. Interestingly, after 24 h of incubation, DATS **(9)** and DATTS **(10)** showed very little effect on heamoglobin. In contrast, the reduced forms, such as allyl- **(6)** and propylmercaptane **(30)**, completely changed the UV/Vis spectrum of heamoglobin. Monosulfides, disulfides and cyclic polysulfides did not show any effect. The results are shown in **Figure 5. 16**.

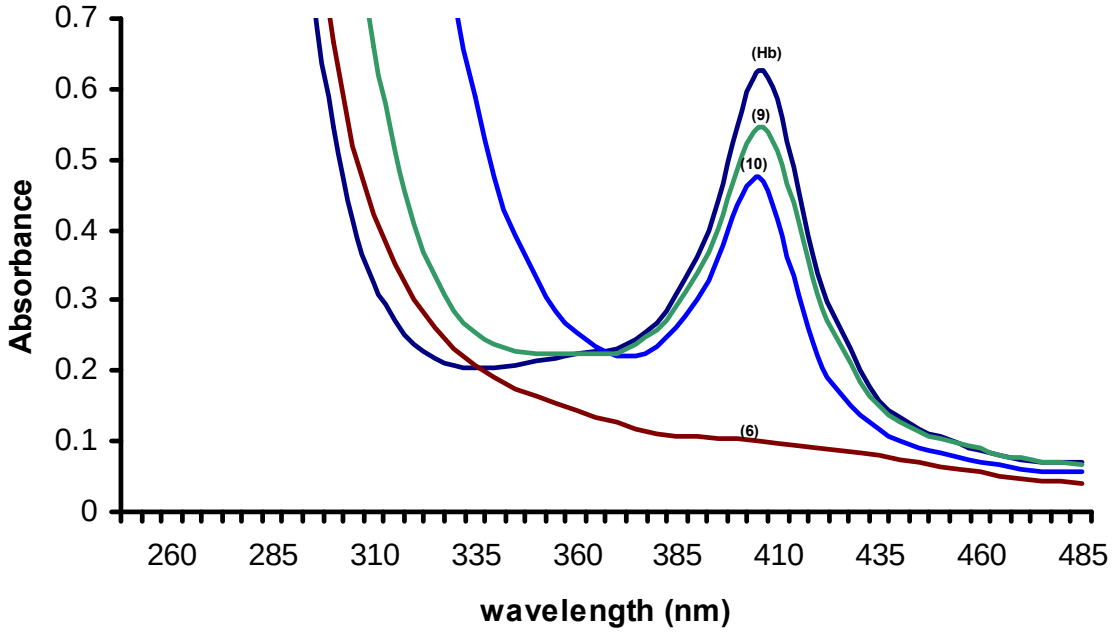
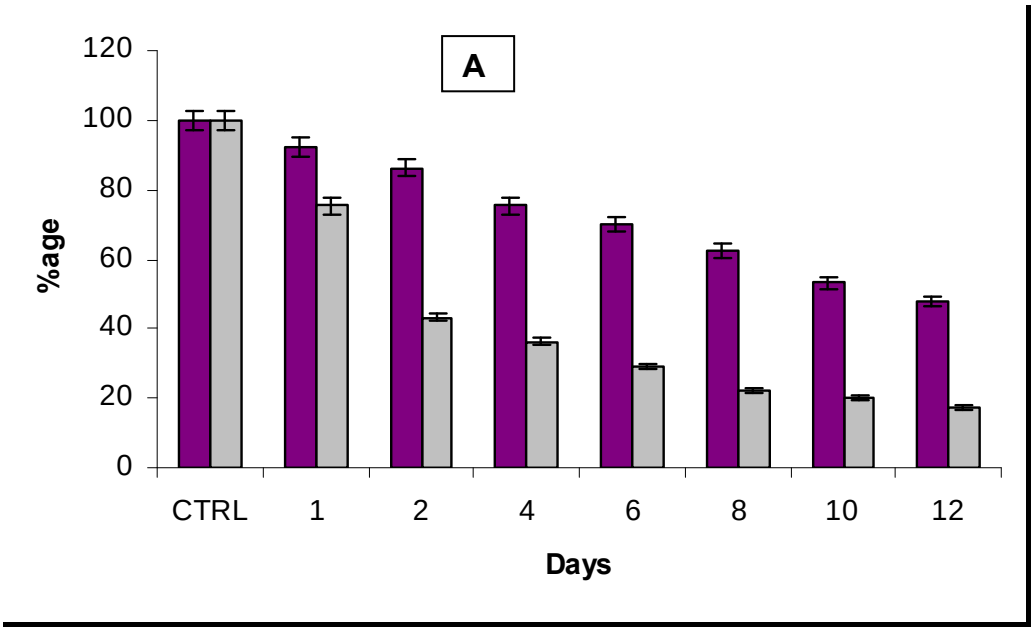


Figure 5.16 Interaction of polysulfides with haemoglobin (hb) after 24 h incubation. Final concentration of polysulfides was 200 µM while the concentration of haemoglobin was 5.35 X 10^{-6} M. DATS **(9)** showed a little effect as compared to DATTS **(10)** . The reduced form allylmercaptan **(6)** completely flatten the haemoglobin peak. The propyl analogues (data not shown) followed the same pattern but the effects were not observed as dramatic as in the case of the corresponding allyl analogues.

5.5.5 Chemical stability study of Polysulfides

The methanolic solutions of polysulfides DADS **(8)**, DATS **(9)** and DATTS **(10)** showed complete stability at 4 °C. Minor decomposition was observed when DATTS **(10)** was placed in light at room temperature. Diallylpentasulfide **(11)** and diallylhexasulfide **(12)** showed complete stability at cold temperatures but both decomposed at room temperature within 48 h and this effect was enhanced in the presence of light (**Figure 5.17**).

The suspected "exchange" of sulfur atoms between various sulfur species was also confirmed as part of the stability study. In the case of samples placed in light it was observed the diallylpenta- **(11)** and hexasulfide **(12)** initially decompose to



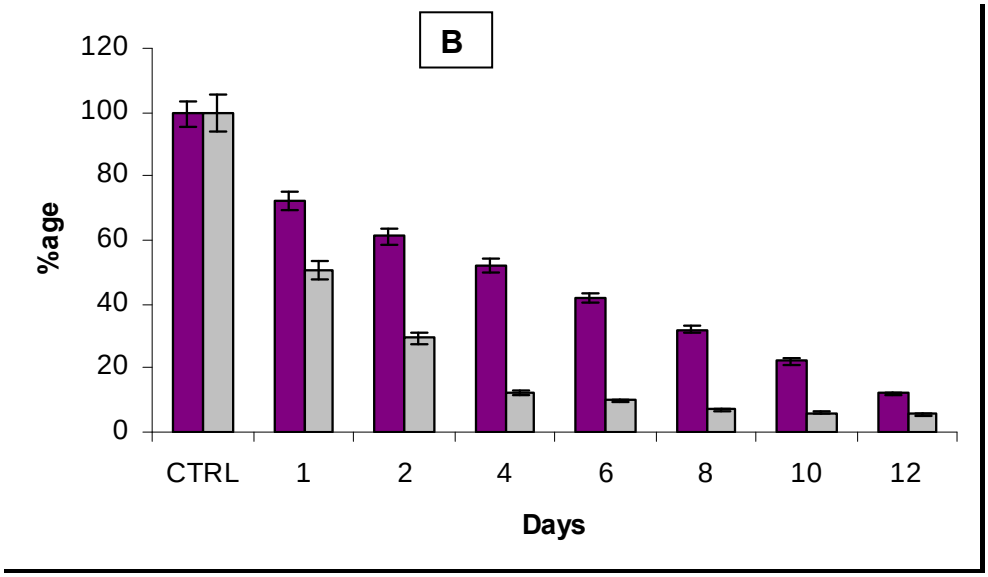


Figure 5.17 Stability profile of diallylpentasulfide **(11)** [**Panel A**] and diallylhexasulfide **(12)** [**Panel B**]. Bars in light color represent samples in light while dark colour represent samples in dark

diallyltetrasulfide **(10)** and pentasulfide, respectively *i.e*. both appear to formally "lose" one sulfur atom. The fate of lost sulfur is still unclear. There is some evidence that higher polysulfides (heptasulfides etc.) may be formed. More likely, however, is the formation of elemental sulfur (S_8). After longer incubation, the decomposition products formed initially further change their composition and produce trisulfides and disulfides.

Figure 5.18 shows decomposition of pentasulfide when the sample was placed at room temperature in light as well as in the dark. In course of 24 h, the pentasulfide sample placed in light was decomposed 50% to produce 20% tetrasulfide **(10)** and 14% hexasulfide **(12)**. Both of these species produced appeared to interact further with remaining pentasulfide to produce other species.

Interestingly, the decomposition of the diallylhexasulfide **(12)** occurred more rapidly when compared to the pentasulfide. Within 24 h, it decomposed to form 30% to diallylpentasulfide **(11)** and 10% to diallyltetrasulfide **(10)**. This process of "sulfur exchange" between the polysulfides continues until a stable equilibrium is reached. Please note that elemental sulfur may also be expelled, in a reaction resulting the reverse of the polysulfide synthesis process described in section 5.1.

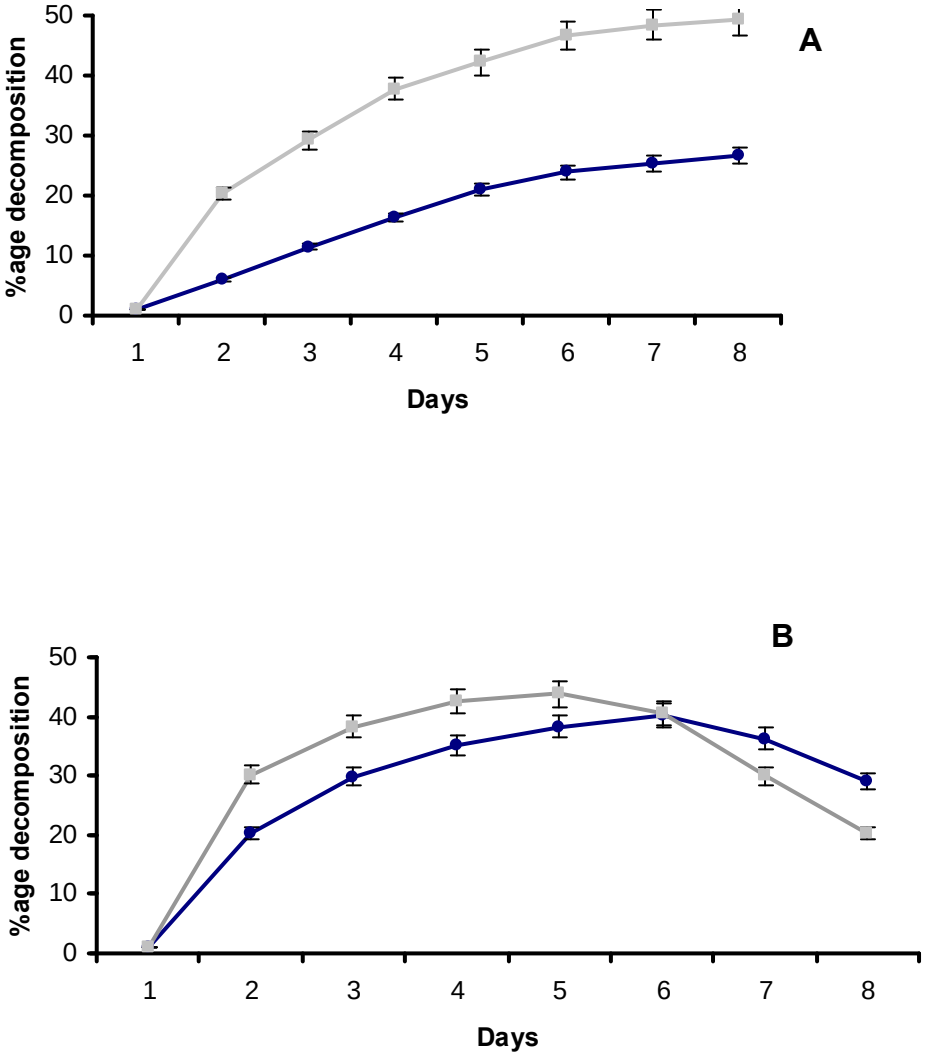


Figure 5.18 Formation of DATTS **(10)** as a decomposition product of diallylpentasulfide **(11)** at room temperature (Panel A); formation of diallylpentasulfide **(11)** as a decomposition product of diallylhexasulfide **(12)** (Panel B)**;** (in light (■), in dark (●))

Chapter 6

Discussion

The main aim of this study was to gain better understanding of the chemical reactions and possible biochemical mode(s) of action which may explain the biological activity of dialk(en)yl polysulfides, most notably their cytotoxicity against certain cancer cells and their use as green pesticides.

The Caco-2 and U937 cell culture studies have confirmed previous findings by Jakubikova, Seki, Singh and their colleagues. While monosulfides **(7)** and **(25)** are virtually non-toxic at the concentrations used, the DADS **(8)** and DPDS **(26)** exhibit some toxicity. Toxicity increases significantly for the DATS **(9)** and DPTS **(27)** and somewhat further for the DATTS **(10)** and DPTTS **(28)**. This pattern of increasing (cytotoxic) activity with increasing sulfur chain length has been observed in a range of different studies in the past, not only for cancer cells, but also for bacteria, fungi and yeast [4]. It has been confirmed in cancer cells and also in nematodes. Interestingly, the correlation between biological activity and sulfur chain length does not appear to be linear. It rather exhibits two distinct increases in activity – the first apparent increase at the disulfide and the second at the trisulfide.

This finding is particularly intriguing, since it reflects clear changes in the redox behavior of the compounds involved: disulfides, in contrast to monosulfides, are oxidizing agents able to modify cellular thiols via a thiol/disulfide exchange reaction. Although DADS **(8)** and DPDS **(26)** may only be a very weak oxidants, at a concentration of 700 µM they may still oxidize significant amounts of thiols in peptides, proteins and enzymes. This widespread oxidation of thiol groups may subsequently lead to a (small) toxic effect.

A similar change of biological activity, and, at the same time, 'chemistry', occurs when moving from the disulfides to the trisulfides. As already mentioned in the section 2.3, reduction of a trisulfide by a thiol initially leads to the formation of a mixed disulfide and a perthiol (RSSH). The latter is considerably more reducing when compared to the thiol (RSH), and may well trigger the formation of $O_2^{\bullet-}$ radicals and OS. This difference in 'chemistry' between the disulfides (**(8)** and **(26))** and trisulfides (**(9)** and **(27)**) may explain the sharp increase in cytotoxicity observed in the Caco-2 cell culture. The tetrasulfides (**(10)** and **(28)**), on the other hand, could react with a thiol to form either RSSH or RSSSH, depending on the site of nucleophilic attack. RSSSH may be somewhat more reactive compared to RSSH, yet that difference would only be gradual and not fundamental. Without an emerging 'new chemistry', DATTS **(10)** and DPTTS **(28)** may therefore be somewhat - but not dramatically - more active than DATS **(9)** and DPTS **(27)**.

There is considerable experimental support for the involvement of perthiol or hydropolysulfide chemistry in the biological activity of DATS **(9)** and DPTS **(27)** as well as DATTS **(10)** and DPTTS **(28)**, which will be discussed in more detail below. Interestingly, the low toxicity of 1,9-decadiene indirectly also supports this notion, since at the concentrations used, it rules out a decisive *physical* effect of the polysulfides on the cells. Nonetheless, the initial idea that long chain compounds such as DATS **(9)** (and DPTS **(27)**) as well DATTS **(10)** (and DPTTS **(28)**) may act as organic, 'solvent-like' toxins, such as their analogues, 1,8-nonadiene and 1,9-decadiene, is not too far fetched. We have noticed, for instance, that DATTS **(10)** dissolves certain types of plastic. It may well attack, disrupt or even dissolve cellular membranes, especially if used at higher concentrations. In theory, such compounds may also attach to hydrophobic parts of proteins and enzymes, with subsequent unfolding and/or inhibition of the protein or enzyme affected.

The experimental findings reported in this thesis, however, point against these purely physical modes of action in Caco-2 cells at a concentration of 700 μM. Although the tertiary structures and solvent-like properties of the sulfur and carbon analogues may differ, and therefore complicate a direct comparison between DATTS **(10)** and DPTTS **(28)** and 1,9-decadiene **(29)**, the rather low activity of the long chain hydrocarbons in the

cell culture studies points against lipophilic interactions as sole causes of cytotoxicity. This makes an explanation based on sulfur redox chemistry ever more likely.

Apart from the activity ranking of different sulfides, which provides some basic insight into possible modes of action, the Caco-2 cell culture studies have also confirmed a comparable activity between DATTS **(10)**, DPTTS **(28)**, allicin **(3)** and allylisothiocyanate **(62)**. It should be pointed out that the recent study by Jakubikova and Sedlak has found a lower 'ranking' for allicin **(3)**. In contrast, most studies available to date rank the cytotoxic activity of allicin **(3)** as equal or even higher compared to the one of DATS **(9)** and DATTS **(10)**. Ultimately, cell culture results are likely to depend heavily on the precise culturing conditions, such as thiol content of the incubation media, incubation times etc. The ranking of activities obtained as a part of this thesis should therefore also be seen as dependent on the experimental conditions applied. Although these three compounds may be classified as chemically related reactive sulfur species, their chemistry and mode(s) of action differ considerably. This has been confirmed in reaction kinetics studies using different biological assays as well as in the electrochemical studies.

As already mentioned, the activity of the DATS **(9)**, DPTS **(27)** and DATTS **(10)**, DPTTS **(28)** may well be related to radical generation, OS formation and subsequent cell death. There is also scope for H_2S release from these compounds, which may increase their biological activity even further. The thiosulfinate allicin **(3)**, on the other hand, is known to react rapidly with thiol groups in proteins and enzymes, thus inhibiting their function and catalytic activity (**Figure 6.1**). This inhibitory action obviously has dramatic effects on the biochemical processes within the cell, which ultimately also results in cell death. Allylisothiocyanate **(62)**, on the other hand, is a good electrophile which reacts with thiols to form thiocarbamates and with amines to form thiourea derivatives (**Figure 6.1**) [149]. Both reactions may involve amino acid residues in proteins and enzymes. As for allicin **(3)**, allylisothiocyanate **(62)** may therefore act as a protein and enzyme inhibitor which disrupts various essential biochemical processes in the cell and hence leads to cell death. In contrast to allicin, the reactivity of allylisothiocyanate is not limited to thiols, but also includes amines, which may enhance the reactivity of the isothiocyanate and also its associated cytotoxicity.

The concentration of sulfur compounds employed in the assays, *i.e.* 700 μM, is, of course, rather high. From a pharmacological point of view, the results presented here therefore do not imply that compounds such as DATTS **(10)**, DPTTS **(28)**, allicin **(3)** or allylisothiocyanate **(62)** may be good anticancer agents effective against Caco-2 cells. In other cells such as U937, DATTS **(10)** showed a very good activity at a very low concentration of 10 μM. In fact, the studies employing Caco-2 cells were not intended to find new agents against colon cancer, but to compare the cytotoxic activity of various sulfur compounds. The Caco-2 cell line was chosen as a robust model which would tolerate reasonable concentrations of toxic agents to allow a clear 'ranking' of activities. Lower concentrations of sulfur compounds, such as 50 and 100 μM, were also studied (results not shown). The results obtained at these concentrations reflect the increases of activity with increasing sulfur chain length, albeit at higher cell survival rates. DATTS **(10)**, for instance, reduced cell survival to 81% when used in a 100 μM concentration. It is possible that some of the more reactive sulfur compounds, such as allicin **(3)**, will react with components present in the incubation medium and/or cytosol and hence lose some of their reactivity before they reach their targets. This effect has been observed before and among others counts against the systemic (drug) application of allicin **(3)**. It may also explain why high concentrations are required in the assays.

Within the context of possible anticancer, cytotoxic agents, it is also worth mentioning that a recent study has found an apparent antioxidant activity of DATTS **(10)** when used in a kidney cell line 'poisoned' with Cd^{2+}. In this case, the ability of polysulfides, and more likely, their reduced counterparts (thiols, perthiols and hydropolysulfides), to bind and hence sequester toxic metal ions may dominate their biological behavior. Furthermore, the ability of RSSH or RSSSH to act as reductant may also, under certain circumstances, result in an antioxidant effect.

The precise behavior of such multi-talented redox and metal binding agents *in vivo* is clearly complicated and often depends on the cell line and test conditions employed. The presence of a strong reducing agent in cells is a double-edged sword. On the one hand, it may act as effective antioxidant, reducing various oxidative stressors. On the other hand, it may trigger a cascade of reactive oxygen species by reducing O_2 to $O_2^{\bullet-}$. This matter has already been discussed for $GSSG^{\bullet-}$ and may also apply to RSSH and RSSSH.

With respect to the fact that dialk(en)yl polysulfides are widely reported for their effects on various types of cancers [3, 53, 56, 150-154], but that they were up to date quite rarely investigated for their potency in the treatment of human leukemia [51, 155-159], the compounds efficacy in this area was elucidated using U937 cells as a model. Comparing activities of the compounds and quantifying changes to nuclear morphology, DATTS **(10)** and DPTTS **(28)** were more potent than the corresponding disulfides and trisulfides.

Interestingly, we found that DATTS **(10)** was able to induce selectively apoptosis in the U937 cancer cells under the same conditions without affecting viability of their healthy analogues *i.e.* primary monocytes and leukocytes from human blood [134]. Induction of apoptosis in U937 cells by DATTS **(10)** occured in a caspase-dependent manner, since the complete inhibition of caspases by the pan-caspase inhibitor z-VAD completely prevented any pro-apoptogenic ability of DATTS **(10)**. Moreover, apoptosis was characterized by cleavage and activation of caspase-3, 8 and 9, thus implying an involvement of the two main apoptotic pathways, extrinsic and intrinsic. Importanly, caspase-8 and -9 inhibitors were able to counteract the action of DATS **(9)** and DATTS **(10)**. DATTS **(10)** might be able to induce both apoptotic pathways independently, since no cross interaction between the two pathways- generally mediated by the truncation of Bid [137]- was observed.

Further studies are necessary to completely elucidate a role for caspase-8 and -9 upstream to caspase-3. Some pieces of evidence however, together with literature data strongly suggest an upstream role, especially for caspase-9. Indeed, DATTS **(10)** induced the release of cytochrome *c* and a rapid loss of the mitochondrial membrane potential, both factors required upstream to the activation of caspase-9 [160-162]. The release of cytochrome *c* occured at very early times of incubation, already at 4 h, whereas the activation of caspase-3 started to become significant after 8h. Nonetheless, the strong activation of caspase-3/7, as detected at 16 h of DATTS **(10)** treatment by the enzymatic activity assay, cannot exclude that other caspases could also be involved.

Focusing on the mitochondrial pathway, mediators of the pro-apoptotic mitochondrial events were the DATTS -activated pro-apoptotic proteins Bax and Bak. Nonetheless, the inducer of the activation of Bax and Bak remains a matter of discussion. On the one hand, it is well established that the anti-apoptotic proteins Bcl-2 and Bcl-xL control the state of activation of the two proteins [163]. On the other hand, BH3-only

proteins, like Bad, are considered to be activators of Bax and Bak [164]. DATTS **(10)** seems to act on both sides leading to the downregulation of the protein level of Bcl-xL and the inactivation of Bcl-2, as well as preventing the phosphorylation of Bad [141]. The early downregulation of Bcl-xL at 4 h of treatment, occurring concomitantly with Bak activation and cytochrome *c* release, points towards a main role for Bcl-xL in triggering apoptosis, with Bcl-2 and Bad inactivation playing a possible role in late reinforced pro-apoptotic pathways triggered by DATTS **(10)**.

As a part of the present study, it was demonstrated that DATTS **(10)** induces G2/M phase accumulation. The pieces of evidence are consistent with the hypothesis that the blockage of cell progression induced by DATTS **(10)** occurs more likely in mitosis rather than in the G2 phase. The first evidence for this is obtained from the microscopic analysis, which revealed an accumulation of cells with nuclei presenting a prophase-like pattern. In agreement with this observation is the fact that these same nuclei present the phosphorylated form of histone H3, which accumulated especially during mitotic chromatin condensation [146]. Concomitant with the increased number of cells with phosphorylated histone H3 is the increase of cyclin B1 expression, as expected in the case of an accumulation of cells prior to metaphase [148]. After 8 h of treatment with DATTS **(10)**, the percentage of cells with highest cyclin B1 content and positive to phosporylated histone H3 corresponds to a maximum of about one third of cells. This correlates well with doubling of U937 cells of around 20 h [165]. At longer times of incubation, apoptotic cells start to accumulate and both parameters (cyclin B1 and high H3P) become detectable. This is in line with previous studies showing that both proteins are targets of caspases [166].

Intringuingly, the complex CDK1/cyclin B1 is known to prevent caspase-9 activity by phosphorylation, thus protecting mitotic cells against apoptosis [166]. In view of this, the accumulation of these factors at early times of incubation might explain the delayed timing of caspase-9 activation with respect the release of cytochrome *c* that we have observed. In turn, this may imply a last attempt of cells to progress in the cell cycle and avoid apoptosis.

At the moment, the precise causes leading to a possible mitotic arrest have not been elucidated fully. Interestingly, some histone deacetylase inhibitors (HDACs) are known to cause mitotic arrest in the prometaphase step [167]. The contrast drugs affecting the microtubule apparatus typically cause arrest in the metaphase [168]. Moreover, an ability to inhibit histone deacetylase has been reported for certain garlic-derived organosulfur

compounds such as DADS **(8)**, AM **(6)**, SAC **(4)** [169]. It may therefore be worthwhile to investigate a potential role of DATTS **(10)** as histone deacetylase inhibitor.

Taken together, the evidence obtained in U937 cell culture makes DATTS **(10)** a very favourable candidate in search for new anticancer agents specific for leukaemia [170, 171].

At the same time, formulations of garlic-derived compounds have also attracted a commercial interest as 'green' pesticides, for instance products developed and marketed by ECOspray Ltd. (UK). Recent initiatives by the pesticide regulatory departments of European and North American governments have also stimulated renewed interest in biopesticide technologies to replace much more toxic synthetic pesticides from the market with more benign natural products. The literature is abundant with studies on screening for microorganisms with attributes of biopesticidal activity, however, very few of the authors have considered formulating the microorganisms with commercial applications in mind. A concerted plan in formulation development for biopesticides by multi-disciplinary teams is required to optimize biopesticide yield, efficacy, storage stability and delivery for this technology to evolve and meet today's agricultural demands.

The studies with the homologous series of diallyl polysulfides point towards a sharp increase in nematicidal activity as soon as the trisulfide is reached. This activity is irrespective of nematode species. The effect of polysulfides is consistent across the species studied and confirms similar studies in the literature. The results also indicate a large variation in sensitivity to DATS **(9)**, DATTS **(10)** and diallylpentasulfide **(11)** within species. *P. redivivus* appears to be very sensitive to diallylpentasulfide **(11)**, while *S. feltiae* is more resistant. In contrast, *S. feltiae* appears to be much more sensitive to DATS when compared with *P. redivivus.* The dilution series studies with DATTS have confirmed the nematicidal activity of this molecule, with both *P. hermaphrodita* and *S. feltia*.

Of particular interest from this study is the evidence that diallylpentasulfide **(11)** is also nematicidal. This is the first time where such an activity of the pentasulfide has been demonstrated. The work undertaken here provides evidence that DATS **(9)**, DATTS **(10)** and diallylpentasulfide **(11)** are all nematicidal molecules found in typical garlic oils.

Subsequently, different polysulfide formulations were prepared as "synthetic garlic oil" and tested in field trials by EcoSpray Ltd. UK. The field tests of garlic oil-coated

granules demonstrated that this material is comparable to commercial synthetic carbamate nematicides in reducing nematode root damage to carrots and parsnip. The results obtained (data not shown) confirmed that these formulations can act as natural based 'green' nematicides.

The electrochemical studies indicate that there is a general decrease in the reduction potentials (of the oxidized mono-, di-, tri and tetrasulfides) and the oxidation potentials (of reduced sulfides) with increasing sulfur chain length. As already mentioned, in Cyclic Voltammetry, the reduction potential is a property of the oxidized form of the compound, while the oxidation potential refers to the corresponding reduced species. Both are a result of the electrochemical method used, are closely related to each other and can be used to calculate the electrochemical potential $E^{0'}$, which in essence is the average of the two values.

This implies that the biological activity of tri- and tetrasulfides is probably not the result of an aggressive thiol oxidation chemistry, but is linked to the reducing power of the reduced form(s) of the polysulfides, *i.e.* RSSH and RSSSH. This observation corresponds to previous reports which have shown that perthiols are more reducing compared to thiols, due to (donating) electronic effects of the 'additional' sulfur atoms and subsequent increased electron density at the terminal sulfur atom.

Since Cyclic Voltammetry using a mercury electrode is not an ideal method to investigate sulfur compounds due to inherent and therefore unavoidable adsorption phenomena, further investigations will be necessary to confirm this rather interesting redox behavior of polysulfides. Nonetheless, the measurements presented here allow a preliminary comparison of potentials and also indicate that the potentials are strongly pH dependent. pH dependence of the oxidation and reduction potentials itself is interesting, since it implies that the disulfides (**(8)**, **(26)**), trisulfides (**(9)**, **(27)**)- and tetrasulfides (**(10)**, **(28)**) are more difficult to reduce at higher pH, *i.e.* are less oxidizing at pH 7.4, yet their reduced forms are stronger reducing agents at physiological (and higher) pH.

The notion of trisulfides (**(9)**, **(27)**) and tetrasulfides (**(10)**, **(28)**) as weak oxidants on their own, but - in their reduced form(s) - as good reductants, has been confirmed in the *in vitro*

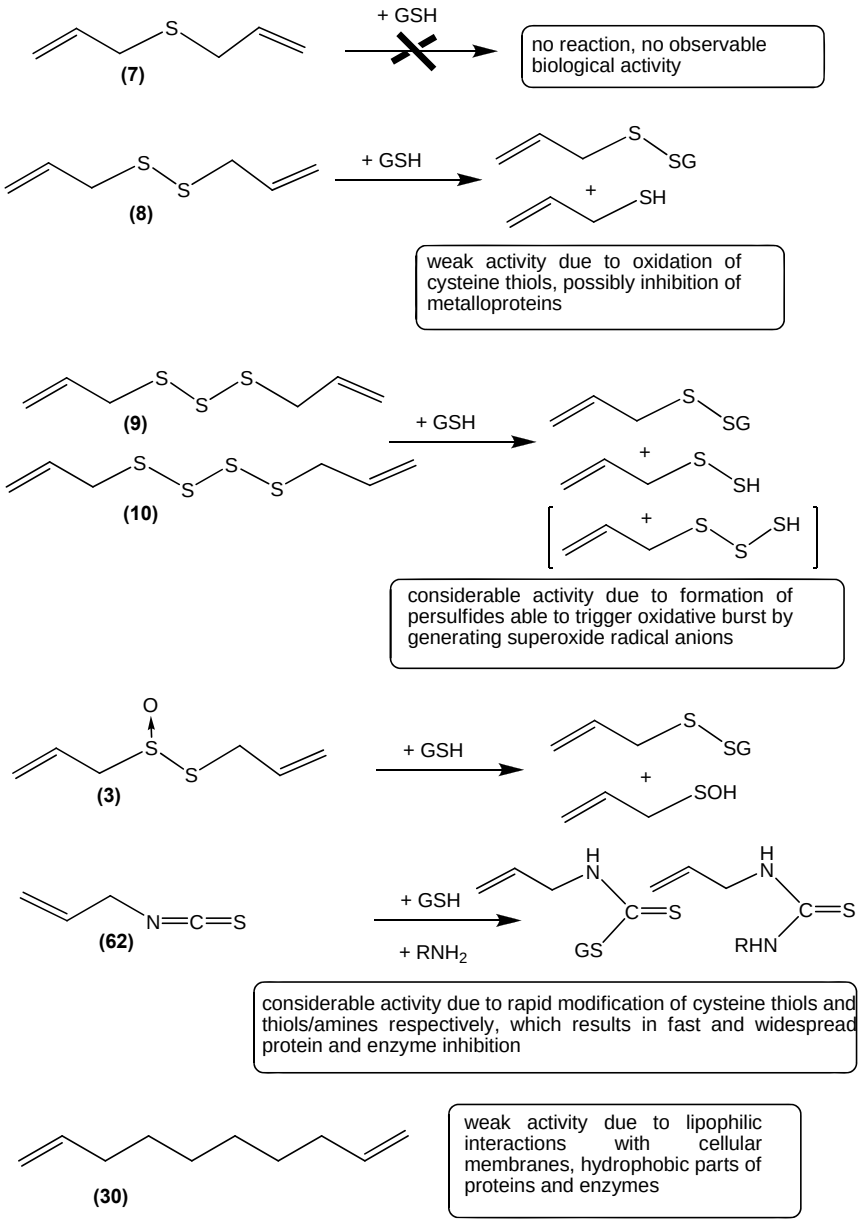


Figure 6.1 Summary of chemical reactions likely to be associated with the biological activity of various natural sulfur compounds (and their controls). For simplicity, GSH is used as a representative thiol, since it is abundant in millimolar concentrations in most human cells. This does not exclude other reactive thiol targets, such as redox-sensitive cysteine residues in proteins and enzymes, or dihydrolipoic acid. The diallylsulfide **(7)** does not react with thiols. Although it is also redox active (it can be oxidized to sulfoxide or sulfone), this redox chemistry does not play a significant role here. In contrast, the diallyldisulfide **(8)** contains a redox active sulfur-sulfur bond. It is a weak oxidant able to modify thiols in peptides, proteins and enzymes via thiol/disulfide exchange reactions. The reaction shown here for diallyldisulfide is the most common, but does not rule out alternative reactions, such as GSH attacking at the α-carbon or olefinic carbon of the disulfide with subsequent release of RSSH. Diallyltrisulfide **(9)** and diallyltetrasulfide **(10)**, on the other hand, may modify thiol groups and generate $O_2^{\bullet-}$ as part of a chemistry based on perthiols (RSSH) and hydrotrisulfide (RSSSH). While the chemical reactivity of allicin **(3)** is mostly associated with an attack of GSH at the S-atom, it too may react with GSH via several avenues, for instance by reacting at the α-carbon with subsequent release of allyl-S-SOH. Not surprisingly, the situation *in vivo* is highly complex and the reactions shown here do not exclude other chemical and biochemical processes, such as H_2S based signalling, interactions with metal ions or multiple reactions occurring simultaneously.

PhSH, 4-MP and NBT assays. As the PhSH and 4-MP assays illustrate, DATS **(9)**, DPTS **(27)**, DATTS **(10)** and DPTTS **(28)** are clearly not (strong) oxidants. In sharp contrast, they show a considerable activity in the NBT assay indicative of reducing action and possibly $O_2^{\bullet-}$ formation. It must be emphasized, however, that the NBT assay is not absolutely conclusive. It is, in theory, also possible that a chemical species other than $O_2^{\bullet-}$ reduces NBT, for instance RSSH or RSSSH. The effects of SOD on this reaction count against such an alternative explanation. In any case, and this is the key message in this context, the combination of tri- and tetrasulfides with GSH triggers a distinct chain of chemical reactions which is distinctively different from the monosulfide and disulfides.

Since this activity is triggered by GSH and affected by the enzyme SOD, a model for the (bio)chemical mode of action emerges, which follows the 'superoxide radical anion hypothesis' developed previously by the research groups of Munday and Gates (**Figure 6.1**) [19, 90].

In this model, GSH reduces the di-, tri- and tetrasulfides to thiol, perthiol and - possibly in the case of tetrasulfide - to hydrotrisulfide (RSSSH). This exchange reaction is probably slow and may account for the initial 'lag phase' frequently observed in the NBT assay. RSSH and RSSSH then react with O_2 to form $O_2^{\bullet-}$. The formation of $O_2^{\bullet-}$ from O_2 in the presence of GSH may be catalytic, although the studies presented here do not provide detailed information regarding this aspect of the reaction. It must be pointed out, however, that such studies are somewhat compromised by the fact that the reaction may proceed *via* different avenues and, in the end, may only be pseudo-catalytic.

Furthermore, it is still not clear if this chain of reactions requires catalytic amounts of redox active metal ions, such as iron or copper ions, and at which point. This aspect also requires further investigation. In any case, $O_2^{\bullet-}$ formation triggers a burst in ROS species which leads to the formation of OS and subsequent cell death.

The thiol formed from disulfides (**(8)**, **(26)**), on the other hand, is not able to trigger $O_2^{\bullet-}$ formation, yet may still exhibit some cytotoxic activity, for instance by adventitiously coordinating to metal ion centers in proteins and enzymes. At higher concentrations, disulfides (**(8)**, **(26)**) may therefore well be toxic to some organisms, such as cockroaches, probably by acting as oxidant or via RSH. Ultimately, diallyldisulfide **(8)** may also liberate a perthiol when attacked at the α-carbon or olefinic carbon, but these reactions are less

apparent when compared to the thiol/polysulfide exchange processes described above for DATS **(9)** and DATTS **(10)**.

Together with the cell culture studies, the electrochemical investigations and *in vitro* assays provide further support for the 'superoxide radical anion hypothesis'. This does not imply, of course, that alternative explanations, such as H_2S release and subsequent signaling, should be ruled out upfront [57]. The latter has recently attracted considerable interest among biochemists and together with $^{\bullet}NO$ and CO, H_2S is now firmly considered as the third gaseous signaling molecule. In fact, chemistry provides several avenues for H_2S release from DATS **(9)**, DATTS **(10)** and even DADS **(8)**. Since H_2S, HS^- and S^{2-} are highly reactive (and reducing) sulfur species, they may well interact with a variety of biomolecules, including metalloproteins and proteins containing disulfide bonds. Such reactions may not always be antioxidative, but, due to the reductive activation of redox systems, could also result in cytotoxic effects (hydrogen sulfide can also react with several reactive oxygen species and $^{\bullet}NO$, a chemistry which has hardly been explored to date). The relationship between polysulfides, hydrogen sulfide (bio)chemistry and subsequent pro- and antioxidant effects will need to be investigated further in the future. Ultimately, the biochemical mode(s) of action may well depend on the specific cell type (bacteria, fungi, yeast, various types of cancer) and status of the cell affected (*e.g.* OS). Furthermore, agents such as DATS **(9)**, DPTS **(27)**, DATTS **(10)** and DPTTS **(28)** may affect the biochemistry of a cell in more than one way, and this interplay of different reactions may also depend on the specific cell and its current status.

Chapter 7

Conclusion & Outlook

In summary, the cell culture, nematicidal effects, electrochemical and *in vitro* assays have provided further evidence for an 'unusual', yet interesting redox chemistry of natural polysulfides *in vitro* and *in vivo*. Further investigations will be required, of course, to explore in more detail the various possible chemical and biochemical reaction pathways of polysulfides in different organisms and cell types - and under various circumstances, such as OS or metal poisoning. Such studies may involve other cell types or a comparison of cells in the absence and presence of (external or internal) stressors.

Garlic, onions, leek and related *Allium* vegetables have been used as a source of medicines in many cultures and throughout history. These plants contain a wide range of sulfur agents with distinct chemical reactivity, biochemical profiles and associated biological activities against micro-organisms and cancer. As we have seen, allicin and its 'follow-on' products are among the numerous sulfur compounds found in these plants. These RSS exhibit a wide range of cancer chemopreventive activities, such as simple chemical antioxidant activities, induction of Phase II enzymes, modulation of cellular signalling pathways, interference with histone deacetylation and induction of apoptosis.

From a chemist's perspective, chemically rather simple molecules such as diallyltrisulfide and diallyltetrasulfide seem to be connected with a rather extensive and quite complicated network of different (bio-)chemical formation and transformation, signalling and control pathways. Although many of the reactions discussed as part of this thesis may ultimately only play a minor role in the biochemistry of polysulfides, a combination of several different reactions, rather than just one specific transformation, is

likely to be the source of the (selective) toxicity of the polysulfides found in garlic, onion and other sources.

Within this context, there is an urgent need for more appropriate electrochemical methods to deal with sulfides, in particular polysulfides. The use of Cyclic Voltammetry with mercury or similar metal electrodes is limited by adsoprtion phenomena, which complicate the voltammograms and their interpretation. Alternative methods may be required to enable measurements of reliable, absolute potentials, rather than just a relative comparison of signals.

Similarly, the *in vitro* studies conducted here should only be seen as an entry point for wider investigations. Many assays available to date are indirect and often unspecific. Improvements of such assays may hold the key to our future understanding of sulfur redox behavior *in vivo*. This applies particularly to assays which can be conducted in cell culture, such as fluorescent assays indicative of redox changes inside the cell (*e.g.* the 2',7'-dichlorodihydrofluorescein diacetate assay) [125].

Future research may also evaluate in earnest the therapeutic potential of polysulfides, or their practical use as 'green' pesticides. Ultimately, even if diallyltrisulfide **(9)** and diallyltetrasulfide **(10)** may not be ideal candidates for the development of new drugs or pesticides, they may still provide an important lead for the design of more effective agents based on the general polysulfide chemotype. Considering the chemical and biochemical complexity of polysulfide chemistry, it should be no surprise that this area of research provides ample opportunities for future studies at the interface of chemistry with biochemistry, biology, medicine and drug design. For instance, there is only vague evidence yet regarding the natural occurrence of diallylpenta- **(11)**, hexa- **(12)** and heptasulfide **(13)** in plants and plant extracts, primarily garlic.

Similarly, the biological chemistry of oxidised polysulfides is virtually unexplored. While it is known that hydrogen peroxide can convert disulfides to thiosulfinates and thiosulfonates, similar reactions with tri-and tetrasulfides have not been studied within a biochemical context. There is some evidence, however, that such polysulfide-S-oxides are formed by peroxide-oxidation of polysulfides. Once generated, they seem to be rather unstable and decompose rapidly to form polysulfides and SO_2 [172,

173]. It may well be that in addition to polysulfide reduction and RSSH formation, an opposite redox-event, *i.e.* polysulfide oxidation and subsequent SO_2 release, could pay a role in the cytotoxicity of polysulfides.

Here, pro-oxidant therapy is increasingly gaining the interest of researchers struggling with multi-resistant bacteria and cancer cells. Thiosulfinates and thiosulfonates provide a good lead, since these agents are selective, yet effective oxidants of thiol groups and might be suitable weapons against a range of microbes, bacteria, fungi and even cancer cells with a disturbed redox-balance. While the application of these agents is often compromised by sacrificial thiols, such as GSH, the use of a two-component substrate-enzyme systems, such as recently realised in an antibody-directed enzyme prodrug therapy (ADEPT) approach, might overcome these limitations and allow effective, target selective application of thiosulfinates.

The notion of enzymatic activation can also be extended to the glucosinolate-myrosinase system, which is found in numerous plants. Since certain bacteria and fungi also possess myrosinase enzymes, one might envisage glucosinolate-based pro-drugs targeted against such organisms.

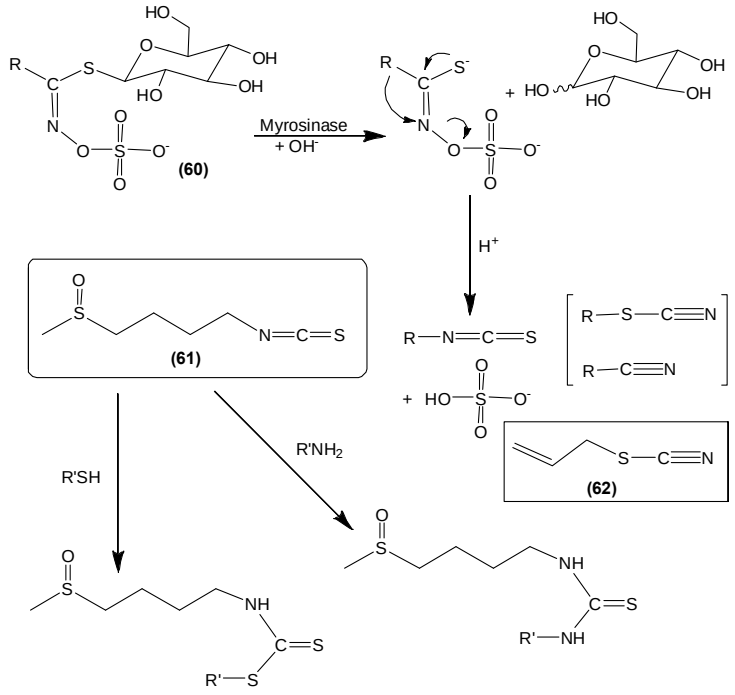


Figure 7.1 Myrosinase-catalysed conversion of an inactive glucosinolate **(60)** to a reactive isothiocyanate (sulforaphane, **(61)**). One very common isothiocyanate is Mustard oil **(62)**. Isothiocyanates react readily with thiol and amines, which might explain their cytoxic activity [4].

The latter would themselves from the active isothiocyanates which in turn would kill them in a process reminiscent of 'suicide'. Among them, sulforaphan **(61)** has recently attracted interest because of its suspected anticancer affects. In the future, two-component approaches based on an inactive pro-drug and an activation mechanism inside the target cell are likely to attract massive interest, since they are among the few strategies promising high target selectivity. For example, glucophasatin from *Raphanus sativus* sprouts has been considered as a potent antioxidant able to counteract H_2O_2 and the effects of oxidative stress (OS) [4].

While the chemical and biochemical properties of thiosulfinates and isothiocyanates are now fairly well understood, the field of natural polysulfides is still hardly explored.

Apart from basic research into the mode(s) of action of natural polysulfides, future studies may also pay attention to the pharmacological properties of natural polysulfides. Let's just for a second forget about the smell of these compounds and their association with folk medicine. The diallyl- and dipropylsulfides discussed here are rather active agents which kill a wide range of organisms harmful to humans; yet they do not cause too much harm to humans. As a consequence, such agents may be useful for therapeutic purposes, *e.g.* against bacterial infections, fungi and possibly even against certain types of cancer cells. Their complicated spectrum of likely modes of action also makes it highly unlikely that bacteria could develop resistance against such a combination of cellular insults. At the same time, these compounds are lipophilic and readily diffuse through cellular membranes. These properties make them ideal drug candidates as far as drug delivery and cellular uptake are concerned.

It is also safe to use tri-, tetra and pentasulfides in agriculture. Whilst active against various pests, there is no danger of contaminating the food chain, since these compounds can be used at low concentrations, decompose after a while and are metabolised by the plant or animal consuming them. In the end, only small concentrations would reach the human consumer, at which point they would rather 'spice up' the food item than posing a heath risk.

Another major area of research is emerging in the context of drug development is the production of hydrogensulfide. Within this context, we find several approaches designed to control intracellular levels of hydrogen sulfide. On the one end, hydrogen sulfide releasing drugs, possibly coupled to NSAIDs, form a major line of investigation. Since hydrogen sulfide release from such agents needs to be controlled tightly in the complex environment of the human body (excess hydrogen sulfide is toxic!), the development of such drugs poses a massive challenge for chemists, and only a few leads are available to date [174], [107]. At the other end, there are also attempts to lower the levels of hydrogen sulfide, for instance by inhibiting cystathionine β-synthase (CBS) and cystathionine β-lyase (CSE). Then again, currently available inhibitors of these enzymes are marred with problems, such as low selectivity for CBS or CSE. The development of specific CBS inhibitors (*e.g.* for the treatment of Down's syndrome) or CSE inhibitors is still a fruitful, yet difficult area of research.

Nonetheless, progress in the area of reactive sulfur species bodes well for the future. It has opened up a new and exciting field of research which brings together chemists, biochemists, biologists, pharmacologists, medical researchers and natural products experts to investigate difficult chemical and biochemical processes and to propose innovative new leads for the development of food supplements, drugs and 'green' pesticides. It is therefore save to assume that garlic, onions and the likes will continue to provide ample opportunities for multidisciplinary research and product development in the future, as well as tasty and - probably healthy - dishes for people not afraid of the odour associated with them.

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List of Publications

1. Cerella C, Scherer C, Cristofanon S, Henry E, Anwar A, Busch C, Montenarh M, Dicato M, Jacob C and Diederich M. Cell cycle arrest in early mitosis and induction of caspase-dependent apoptosis in U937 cells by diallyltetrasulfide (Al2S4)**,** *Apoptosis* 2009, 14 (5), 641-54.

2. **Anwar A**, Burkholz T, Scherer C, Abbas M, Lehr CM, Diederich M, Jacob C. Naturally occurring reactive sulfur species, their activity against Caco-2 cells, and possible modes of biochemical action, *Journal of Sulfur Chemistry* 2008, 29, 3 & 4, 251 – 68.

3. Jacob C, **Anwar A**, Burkholz T. Perspective on recent developments on sulfur containing agents and hydrogen sulfide signaling, *Planta Medica* 2008, 74, 1580-92.

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5. Münchberg U, **Anwar A**, Mecklenburg S, Jacob C. Polysulfides as biologically active ingredients of garlic *Organic & Bimolecular Chemistry* 2007, 21, 5(10), 1505-18.

6. Hayat S, Atta-ur-Rahman, Choudhary MI, Khan KM, Perveen S, Shah ST, **Anwar A**, Anwar MU, Bayer E, Voelter W. Synthesis and biological evaluation of isomeric derivatives of naturally occurring spatozoate *Arzneimittelforschung* 2006, 56 (5), 351-8.

7. Zaidi JH, Naeem F, Ambreen N, Khan KM, Pang YP, Cusack B, Richelson E, **Anwar A** & Voelter W. Pyrrole-Based Partial Peptidic Mimic of Neurotensin (8-13): Design and Synthesis *Letters in Organic* Chemistry, 2005, 3 (1), 21-24.

8. Khan KM, Shahzad SA, Rani M, Ali M, Perveen S, **Anwar A** and Voelter W. Synthesis of 5-Substituted-1,3,4-Oxadiazole-2(3*H*)-Thiones Under Microwave Irradiation *Letters in Organic Chemistry* 2006, 3, 286-8.

Book Chapters

- Jacob C & **Anwar A**. Sulfides in *Allium* Vegetables, *Chemoprevention of Cancer and DNA Damage by Dietary Factors*. 2009, Willey VCH Verlag GmbH, Weinheim, Germany.

Under Preparation

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Poster presentations/ Conferences

- "Towards Multifunctional Redox Catalysts with sensor/effector properties", International Symposium on Medicinal Chemistry 2006, Frankfurt University, Germany.

- "Sulfur containing Natural Products as Potent and Selective Cytotoxic Agents", Symposium on Chemistry and Biology of Bioactive Natural Products, September 19-21, 2007, Kaiserslautern, Germany.

- Workshop on "Cell Culture and *In Vitro* Models for Drug Absorption and Delivery", March 1 -10, 2006, Department of Biopharmaceutics and Pharmaceutical Technology, University of Saarland, Germany.