Journal of Sulfur Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713926081

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To cite this Article Anwar, Awais , Burkholz, Torsten , Scherer, Christiane , Abbas, Muhammad , Lehr, Claus-Michael , Diederich, Marc and Jacob, Claus(2008) 'Naturally occurring reactive sulfur species, their activity against Caco-2 cells, and possible modes of biochemical action', Journal of Sulfur Chemistry, 29: 3, 251 — 268
To link to this Article: DOI: 10.1080/17415990802195623
URL: http://dx.doi.org/10.1080/17415990802195623

Journal of Sulfur Chemistry
Vol. 29, Nos. 3–4, June–August 2008, 251–268

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Naturally occurring reactive sulfur species, their activity against Caco-2 cells, and possible modes of biochemical action

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(*Received 5 February 2008; final version received 7 May 2008*)

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 aging, antibiotic resistant bacteria, 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 and diallyltetrasulfide from garlic, 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. In many cases, the biological activity of these compounds is well established, but the underlying causes for this activity are hardly known. As part of our investigations, we have now confirmed the activity of diallyltrisulfide and diallyltetrasulfide against the fairly 'robust' Caco-2 colon cancer cell line. At the concentrations used, the activity observed for tri- and tetrasulfide is considerably higher than that of disulfide, while monosulfide is virtually inactive. Controls with the long chain carbon analog 1,9-decadiene count against solely lipophilic effects of diallyltetrasulfide, and together with the 'ranking' of activity, point toward a 'special' sulfur redox chemistry that emerges when shifting from di- to trisulfide. This special reactivity of polysulfides has previously been associated with certain oxidizing properties of the polysulfides. The electrochemical studies and thiol oxidation assays conducted as part of this study, 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: the latter seems to indicate dioxygen reduction to the superoxide radical anion, although other reductive events or H_2S release cannot be ruled out at this point. 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, for instance 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 that is clearly associated with many natural polysulfides.

Keywords: diallyltrisulfide; diallyltetrasulfide; electrochemistry; radical formation; cancer

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ISSN 1741-5993 print/ISSN 1741-6000 online

DOI: 10.1080/17415990802195623
http://www.informaworld.com

1. Introduction

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. Aging societies, for instance, have an increased need for disease preventive drugs, such as antioxidants able to fight oxidative stress (OS) that occurs naturally in aging organisms and has been associated with a range of diseases, including inflammatory diseases and possibly cancer (*1*). Furthermore, several bacterial strains resistant to classical antibiotics have developed and pose a serious problem, often as part of hospital-acquired infections (*2*). 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. 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', which exhibit antimicrobial activity, yet pose virtually no damage to the eco-system and human health.

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 to medical and agricultural research.[1] 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 (*3*). This area of research is as diverse as the different sulfur compounds found in nature. A few selected highlights serve to illustrate the current state-of-art of this sulfur chemistry in biology and medicine.

In vivo sulfur is highly abundant in most organisms. A 70 kg adult human male, for instance, contains around 140 g of sulfur, which is a similar amount compared to potassium. While sulfur itself is therefore clearly not a rare element in the human body, some of the reactive sulfur species we will discuss below are transient species and only occur in small concentrations at a time (*4*). The extraordinary biochemical behavior of sulfur and sulfur compounds is the result of sulfur's enormous flexibility in oxidation states, 'chemotypes'[2], and types of (redox) reactions it can take part in (*5*). Most of the underlying biological chemistry of sulfur is comparably novel, sometimes less than 5-years old, and may be considered as 'extreme' from the perspective of traditional biochemistry, which has usually focused on thiols, disulfides, and sulfate as the most familiar sulfur modifications found in living organisms. Not surprisingly, these lesser known, highly reactive, often transient, and frequently unstable sulfur chemotypes reflect a fairly recent field of biological sulfur chemistry, which may be seen as the biological counterpart to some of the other 'extreme' sulfur chemistries that are discussed as part of this *Special Issue*.

The redox chameleon sulfur can occur in around 10 different formal oxidation states *in vivo*, ranging from -2 (in H_2S, RSH) to $+6$ (in SO_4^{2-}) and including fractional formal oxidation states, such as -1.5 in the disulfide radical anion ($RSSR^{\bullet-}$) (*6*). The resulting 'chemotypes' of sulfur include thiols, sulfides, disulfides, sulfite, sulfate, hydrogen sulfide, S-nitrosothiols, thiyl radicals, disulfide radical anions, disulfide radical cations, sulfenic acids, sulfinic acids, sulfonic acids, thiosulfinates, thiosulfonates, trisulfides, tetrasulfides, pentasulfides, hexasulfides, perthiols, perthiyl radicals, hydropolysulfides, and hydropolysulfide radicals, to name just the most common, but by far not all sulfur species found in living organisms. Most of these sulfur species are highly reactive and, as far as their redox behaviour is concerned, can participate in one- and two-electron transfers, hydride transfer, radical reactions, and various exchange reactions associated with a change in formal oxidation state(s), such as the thiol/disulfide exchange reaction. In many ways, the human peroxiredoxin (Prdx) enzymes nicely summarize and illustrate this perhaps less usual

sulfur chemistry. These enzymes fulfill their biological tasks of H_2O_2 removal and redox sensing by employing one or two active site cysteine residues that alter between the thiol, disulfide, sulfenic acid, sulfinic acid, sulfinic phosphoryl ester, and thiosulfinate chemotype (*7, 8*).

Among the various reactive sulfur species discussed in the previous paragraph, polysulfides or, chemically more correctly, polysulfanes[3], have recently attracted considerable interest (*9*). Polysulfides occur naturally in a variety of plants, mushrooms and algae. Among them, the pentasulfide varacin (from *Lissoclinum vareau*) is perhaps best known among pharmaceutical researchers, since it exhibits an interesting antibiotic profile (*10*). Similarly, the cytotoxic activity of the antibiotic compound leinamycin (from *Streptomyces* sp.) and the anticancer agent calicheamicin $\gamma 1$ (from *Micromonospora echinospora*) are also in part associated with trisulfide chemistry (*11–13*). Other polysulfides, such as lenthionine and 1,2,3,4,5,6-hexathiepane are found in Shiitake mushrooms (*3*). The perhaps richest and most interesting sources of polysulfides with suspected biological activity are plants of the genus *Allium*, most notably garlic and onion (*14*). The cloves of garlic contain a diverse range of mono-, di-, tri-, tetra-, and higher polysulfides, including diallyltrisulfide and diallyltetrasulfide, which will be discussed in more detail as part of this manuscript (Figure 1). These compounds seem to form as follow-on products of allicin, a highly reactive, antimicrobial thiosulfinate synthesized enzymatically in garlic cloves from the sulfoxide alliin in the presence of the C–S-lyase enzyme alliinase (*15*). Similar substances also occur in onions (*14*).

The various biological activities of such polysulfides have been known for several decades. It has long been suspected that garlic and onion possess antimicrobial activity, and research has shown

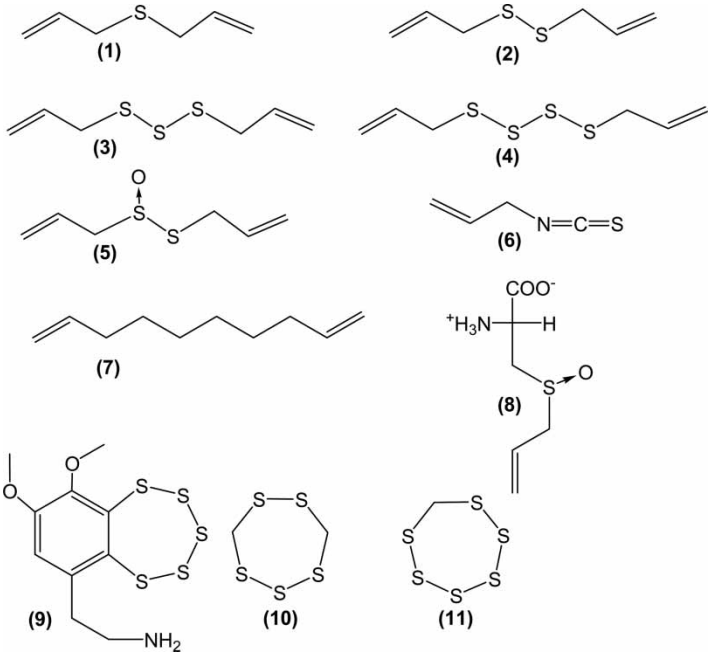

Figure 1. Natural sulfur agents discussed in this manuscript. Diallylsulfide **(1)**, diallyldisulfide **(2)**, diallyltrisulfide **(3),** and diallyltetrasulfide **(4)** are found in the cloves of garlic. They are believed to be follow-on or breakdown products of the highly aggressive thiosulfinate allicin **(5)**. The latter is synthesized 'on demand' from the sulfoxide alliin **(8)** via an enzymatic reaction involving the C–S-lyase alliinase. Allicin forms an integral part of the garlic host defence against various microorganisms. Allylisothiocyanate **(6)** is found in the seeds of mustard and related plants. Its chemical reactivity and biological activity are comparable to the ones of allicin. The carbon analog of diallyltetrasulfide, 1,9-decadiene **(7)** serves as a 'sulfur-free' control. Polysulfide chemistry may also play a role in the case of varacin **(9)** from *L. vareau* and polysulfides isolated from Shiitake mushrooms, *i.e.* lenthionine **(10)** and 1,2,3,4,5,6-hexathiepane **(11)**, as well as caliceamicin $\gamma 1$ (chemical structure not shown).

that the antibacterial, antiviral, and antifungal effects sometimes ascribed to garlic are partially due to diallyldisulfide, diallyltrisulfide, and diallyltetrasulfide. Interestingly, recent research by Xiao and Antosiewicz has also found cancer cell selective cytotoxic activity of diallyltrisulfide against prostate cancer cells PC-3, but not against normal prostate cancer cells PrEC (*16, 17*). These findings which are supported by several related cancer cell studies from China, Japan, and the Slovak republic, have widened the spectrum of biological activities and possible applications associated with polysulfides from garlic (*18–20*). At the same time, formulations of garlic-derived compounds have also attracted a commercial interest as 'green' pesticides, for instance produced and marketed by ECOspray Ltd. (UK).

In most cases, the chemical and biochemical basis of the observed polysulfide activity against microbes and cancer cells is somewhat murky. During the last decade, various mechanisms have been proposed, which explain some, but often not all of the chemical, biochemical, and biological aspects known to be associated with compounds such as diallyltrisulfide and diallyltetrasulfide (*21*). The perhaps most common explanation for the observed cytotoxic activity of polysulfides is their ability to oxidize thiol groups via a thiol/polysulfide exchange reaction in analogy to the well-known thiol/disulfide exchange reaction (*19*). This notion is supported by evidence of post-translational cysteine thiol modification in proteins as a result of diallyltrisulfide activity, for instance β-tubulin thiolation in the human colon cancer cell lines HCT-15 and DLD-1, when cells were treated with diallyltrisulfide (*19*). This explanation assumes, however, that polysulfides are good oxidants, which – possibly selectively – modify reactive cysteine residues in proteins and enzymes in the presence of a large excess of GSH. This hypothesis heavily relies on the oxidizing power of diallyldi-, tri-, and tetrasulfides, which will be investigated as part of this manuscript.

In general, thiol/disulfide and similar exchange reactions are slow and often require high concentrations of reaction partners, while the biological activity of polysulfides manifests itself at low concentrations of polysulfide and within a rather short period of time. Oxidative exchange reactions may therefore not fully explain the biological activities observed. These have led some researchers to consider alternative modes of action. The activity of varacin, for instance, has recently been associated with the release a highly reactive, electrophilic S_3 molecule (*22*). This is an interesting hypothesis, since S_3, in analogy with ozone (O_3), may well explain the unusually high reactivity and biological activity associated with certain polysulfides. Experimental evidence for the existence of such an 'extreme' sulfur species under physiological conditions, however, is still sparse, and warrants further investigations. In any case, the release of S_3 is only likely in the case of a pentasulfide (or compounds with even longer sulfur-sulfur chains). It is not easily applicable to diallyltrisulfide or diallyltetrasulfide and may not explain their respective biological activities.

Another explanation for the biological activity of (poly)sulfides has emerged recently. It involves more or less complete reduction of the sulfur–sulfur moiety, for instance by GSH, with subsequent release of H_2S (HS^-) (*23*). The latter is now known to fulfill important regulatory and signaling tasks in the cell and may, in part, explain the (selective) cytotoxic activity of diallyltrisulfide and diallyltetrasulfide. Interestingly, release of H_2S from diallyldisulfide has also been postulated (*23*). This reaction may proceed via a substitution at the α-carbon of the disulfide, or, in the case of the diallyldisulfide (but not the dipropyldisulfide analog), via attack of GSH at the olefinic carbon with double-bond migration. In both cases, allylperthiol is formed (alongside a mixed allyl-glutahionyl-monosulfide), which is then attacked by a second equivalent of GSH to yield a mixed disulfide and H_2S.

The possibly most interesting explanation proposed so far is based on a sequence of reactions that involves perthiols[4] (RSSH) and hydropolysulfides (RS_xH, $x \geq 3$) (*12, 24*). In this model, a (slow) thiol/polysulfide exchange reaction between polysulfide and a reducing thiol (such as GSH) forms a perthiol or hydropolysulfide and a mixed disulfide (RSSG). The perthiol and hydropolysulfide are considered as being highly reducing, and may react with O_2 to form a

perthiyl ($RSS^{\bullet}$) or hydropolysulfide radical ($RSSS^{\bullet}$) and the superoxide radical anion, $O_2^{\bullet -}$. It should be noted that this reaction is apparently catalyzed by redox active metal ions, such as Cu^{+} or Fe^{2+}, for instance in the form of hemoglobin (*12, 24*). Inside the cell, $O_2^{\bullet -}$ is rapidly converted to H_2O_2 by superoxide dismutase (SOD) enzymes. H_2O_2 may react further with Fe^{2+} or Cu^{+} ions to form the highly toxic hydroxyl radical, $HO^{\bullet}$ (*25*). In any case, $O_2^{\bullet -}$ formation could trigger an oxidative burst in the cell affected, which, in turn, may lead to cell death. The $RSS^{\bullet}$ or $RSSS^{\bullet}$ radical formed as a part of this process may ultimately dimerize to regenerate a polysulfide or react with GSH to form $RSSSG^{\bullet -}$ or $RSSSSG^{\bullet -}$, reducing species that may react with O_2 to yield another equivalent of $O_2^{\bullet -}$ and RSSSG or RSSSSG, respectively. Since a polysulfide is regenerated, the process is sometimes thought to be (pseudo-)catalytic (*12*).

Although this 'superoxide radical anion hypothesis' may explain most biochemical observations available to date – including the increased cysteine oxidation in proteins such as β-tubulin – the underlying chemistry is little understood. To gain further insight into the different (bio-)chemical modes of action of diallyltrisulfide and diallyltetrasulfide, this manuscript will explore the aspects of polysulfide redox behaviour. We will first compare the biological activity of the diallylmono-, di-, tri-, and tetrasulfides in the Caco-2 cell culture model, using allicin and mustard oil as sulfur-based natural product benchmarks. These studies are also designed to 'enter' the tetrasulfide into cancer cell research, for instance by expanding the recent work of Jakubikova and Sedlak (*20*). We will then consider the notion of polysulfides as oxidizing agents using electrochemical methods. This will be followed by various *in vitro* assays to investigate the redox reactions that may possibly explain the biological activity observed in Caco-2 cells and other models. It should be emphasized from the outset that such studies are necessarily of a preliminary nature and may, at best, point toward one (bio-)chemical model or another. It is not our intention to arrive at an ultimate decision regarding the mode of action of polysulfides in various, often dramatically different organisms.

2. Results

2.1. *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 toward toxic agents, which makes them ideal for rapid screening and comparison of toxic and less toxic agents (*20*). Then again, 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 Figure 2. While various concentrations of agents between 150 nM and 1.4 mM were tried, a distinction of activity was best possible at 700 μM. Compounds employed at lower concentrations had little effect (80–90% cell survival even at 100 μM), whilst experiments at higher concentrations were marred by solubility problems of these fairly lipophilic compounds. While 700 μM diallylsulfide is virtually non-toxic against the Caco-2 cells (90% cell survival), there is a measurable toxic effect of diallyldisulfide when employed at the same concentration (80% cell survival). Nonetheless, this effect is small compared to the effects caused by diallyltrisulfide and diallyltetrasulfide (57% and 36% cell survivals at 700 μM polysulfide, respectively). In order to compare these results to previous studies, allicin and allylisothiocyanate 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 diallyltetrasulfide (36%) was comparable to the ones of allicin (34%) and allylisothiocyanate (35%).[5]

Diallyltrisulfide and diallyltetrasulfide are fairly lipophilic compounds. Their biological activity may therefore, at least in principle, arise from hydrophobic interactions – for instance with

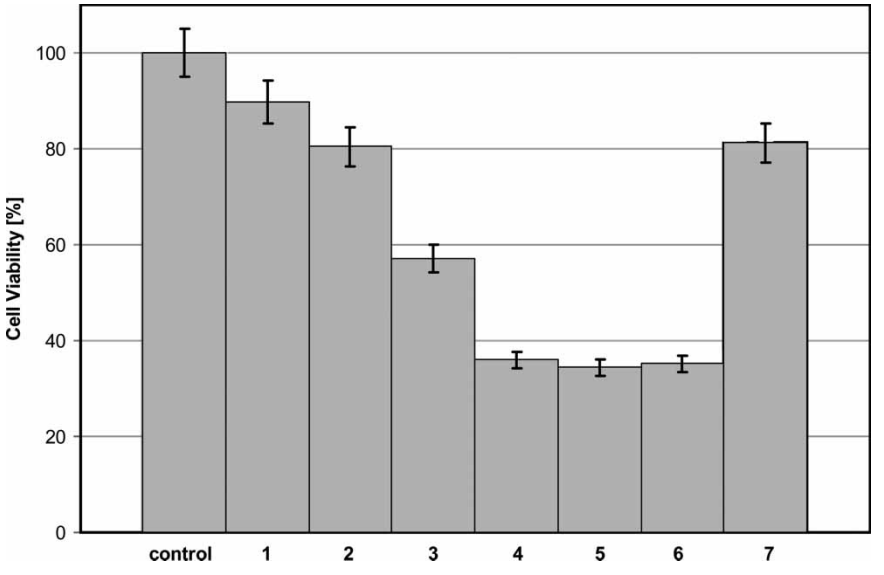

Figure 2. Viability of cultured Caco-2 colon cancer cells in the presence of various natural sulfur compounds and controls (given at 700 μM for comparison). While diallylsulfide **(1)** has only marginal effects on the cell survival, diallyldisulfide **(2)** reduces cell viability by approximately 20%. A considerably more significant reduction is observed for diallyltrisulfide **(3)** and diallyltetrasulfide **(4)**, with 43% and 64% reduction of cell survival, respectively. Diallyltetrasulfide exhibits a cytotoxicity comparable to one of the benchmark compounds allicin **(5)** and allylisothiocyanate **(6)**. In contrast, the carbon analog of diallyltetrasulfide, *i.e.* 1,9-decadiene **(7)**, 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. All experiments were performed in triplicate.

cellular membranes and/or proteins and enzymes – and not necessarily from the chemical reactivity associated with sulfur–sulfur bonds. After all, diallyltrisulfide and diallyltetrasulfide are analogs 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 showed 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 of diallyltrisulfide and diallyltetrasulfide. This was investigated further using the electrochemical method of cyclic voltammetry.

2.2. *Electrochemical properties of diallyldi-, tri- and tetrasulfide*

Diallyldi-, tri-, and tetrasulfides 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.

The electrochemical results obtained are listed in Table 1. 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. Diallyldisulfide, trisulfide, and tetrasulfide, on the other hand, exhibited at least two reduction and oxidation signals, of which one reduction signal (Epc) and one oxidation signal (Epa) were dominant (Table 1 and Figure 3).[6] Both of these major signals seem to result from a thermodynamically quasi-reversible electron transfer process of electrochemically active

Table 1. Summary of cytotoxic activity, electrochemical and *in vitro* results of diallylsulfides **(1)–(4)** and benchmark/control compounds. The increase of biological activity observed for **(1)–(4)** in the Caco-2 cell culture assays corresponds to a decrease in electrochemical potentials to more negative values (shown here for pH 7.4) and an increase of apparent reactivity in the NBT assay in the presence of GSH. Experimental details are provided in the text. For more details see also Figures 2, 3, and 4.

	Cell survival	*E*pc	*E*pa	PhSH		NBT (10^{-9} ms^{-1})	
Compound	(in %)	(mV)	(mV)	(10^{-9} ms^{-1})	4-MP (10^{-9} ms^{-1})	Without SOD	With SOD
Diallylsulfide **(1)**	92	–	–	0	0	0.37	0
Diallyldisulfide **(2)**	80	−590	−463	0	0	1.75	0
Diallyltrisulfide **(3)**	57	−657	−591	0	0.84	19.74	8.62
Diallyltetrasulfide **(4)**	36	−680	−603	0	2.81	39.77	11.32
Allicin **(5)**	34			10.58	17.40	n.d.	n.d.
Mustard oil **(6)**	35			3.68	5.61	n.d.	n.d.
1,9-decadiene **(7)**	81	–	–	–	–	–	–

n.d. = not determined.

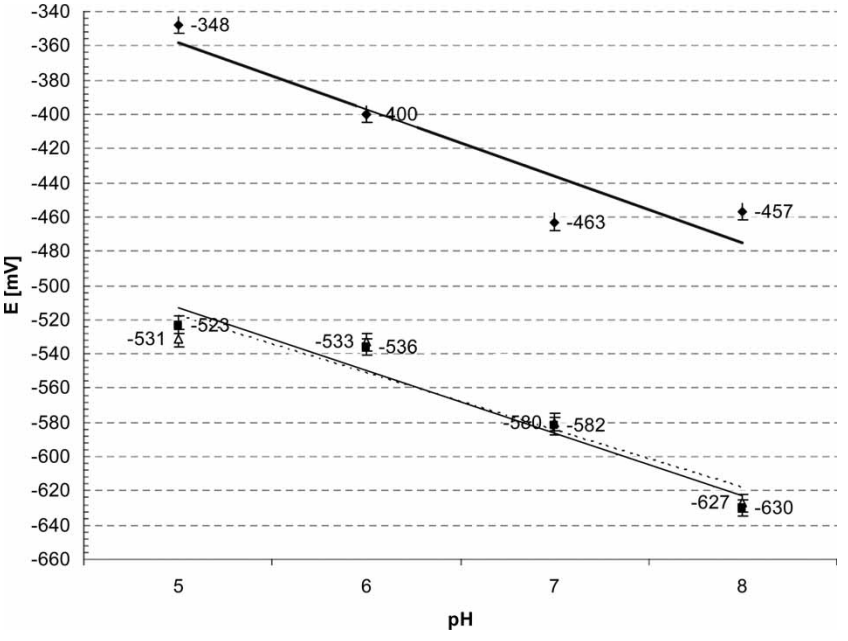

Figure 3. The influence of sulfur chain length and pH on the oxidation potential *E*pa of the diallylsulfides under investigation. While diallylsulfide shows no reduction or oxidation signal, the diallyldisulfide (♦), diallyltrisulfide (■), and diallyltetrasulfide (Δ) exhibit two major, distinct, pH-dependent oxidation and reduction currents (*E*pa and *E*pc, respectively). The considerable more positive potentials measured for GSH and diallyldisulfide compared to the tri- and tetrasulfides 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 behaviour is particularly pronounced at physiological and alkaline pH.

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 that complicate the voltammograms and allow only a very limited interpretation of the signals and their parameters (see below). Nonetheless, a few interesting trends can be observed.

First of all, the reduction signal *E*pc generally becomes more negative with the 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 diallyldisulfide, diallyltrisulfide, and diallyltetrasulfide exhibit Epc values of −590, −657, and −680 mV, respectively (Table 1). These Epc values for the main reduction signals are fairly negative, compared, for instance, to the measured value for the reduction of GSSG (Epc = −463 mV). Based on this initial electrochemical estimate of reduction potentials, it is therefore unlikely that the diallyltrisulfide or diallyltetrasulfide *on their own* behave as strong oxidants, as may have been suggested by some cell culture studies (*19*).

Interestingly, the oxidation potential Epa observed for the (reduced) di-, tri-, and tetrasulfides 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, at 200 mV/s). In comparison, the Epa value for GSH under the same experimental conditions is −442 mV. In essence, this implies that the reduced forms of the tri- and tetrasulfides, *i.e.* most likely RSSH or RSSSH, are considerably more reducing than GSH and the reduced form of diallyldisulfide, *i.e.* allylmercaptan (RSH), and may well reduce O_2 to $O_2^{\bullet -}$. This finding is in agreement with the literature reports describing lower pKa values for RSSH compared to RSH, and an associated higher reactivity as reducing agents (see below) (*24*).

The electrochemical studies also confirm a strong pH dependence of the oxidation and reduction potentials for the di-, tri-, and tetrasulfides, which generally shift toward more negative potentials with increasing pH. The oxidation potentials, Epa, for instance, shift around 100 mV to more negative values between pH 5.0 and 8.0, which results in a ΔEpa/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 toward a certain control of redox behaviour by pH.

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 linear correlation between oxidation and reduction peak current (Ipa and Ipc, 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.

2.3. *Thiophenol, 4-mercaptopyridine and nitrotetrazolium blue assays*

The electrochemical studies may only provide a preliminary insight into the apparent redox behaviour 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 (*26*). The thiophenol (PhSH) assay measures the oxidation of PhSH to the disulfide PhSSPh in methanolic solution, while the corresponding 4-mercaptopyridine (4-MP) assay operates in buffered aqueous media (*27*). The former is ideally suited to study the redox behaviour of water-insoluble compounds, while the 4-MP assay reflects physiological conditions more accurately (*e.g.* aqueous media, pH 7.4).

The results of these assays are summarized in Table 1. 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 (positive) 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 diallyldisulfide nor diallyltrisulfide or diallyltetrasulfide showed any significant disulfide formation (PhSSPh or dipyridinedisulfide, respectively). These results support the findings of the electrochemical studies. Neither diallyltrisulfide nor diallyltetrasulfide on their own are strong oxidants; 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 and the polysulfides.

It was therefore decided to employ the 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 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 4 and summarized in Table 1. Neither diallylsulfide, diallyldisulfide nor diallyltrisulfide or diallyltetrasulfide react with NBT in the absence of GSH. Interestingly, GSH, which itself does not react with NBT either, seems to 'trigger' the reduction of NBT in the presence of diallyltrisulfide and diallyltetrasulfide, but not in the presence of diallylsulfide or diallyldisulfide (Figure 4a).

These results are highly revealing. First of all, there is a clear difference in 'chemistry' between the disulfide on the one hand, and the tri- and tetrasulfides on the other. Secondly, the tetrasulfide is clearly the most 'active' of the compounds. Thirdly, the reaction needs a trigger, such as GSH, that is also reflected by an initial 'lag' phase in the reaction frequently observed for the tetrasulfide. This observation accounts for the formation of a reactive intermediate, such as RSSH or RSSSH. Therefore, the reduced form of diallyldisulfide, *i.e.* allylmercaptan (RSH), was also investigated and found to be inactive in this assay, either in the absence or presence of GSH. And fourthly, the reaction observed for diallyltrisulfide and diallyltetrasulfide 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 4b and Table 1). 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 diallyltrisulfide and diallyltetrasulfide with NBT by SOD points indeed toward a chemical process that 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.[7]

Interestingly, NBT reduction by diallyltrisulfide and diallyltetrasulfide 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 diallyltrisulfide and diallyltetrasulfide 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). This notion is supported by the electrochemical potentials of HS^-, which under the conditions used (mercury electrode, Ag/AgCl counter electrode, buffered solution at pH 7.4) were around -1 mV for Epa and -298 mV for Epc. This makes HS^- a more modest reducing agent when compared to the reduced forms of diallyltrisulfide and diallyltetrasulfide.

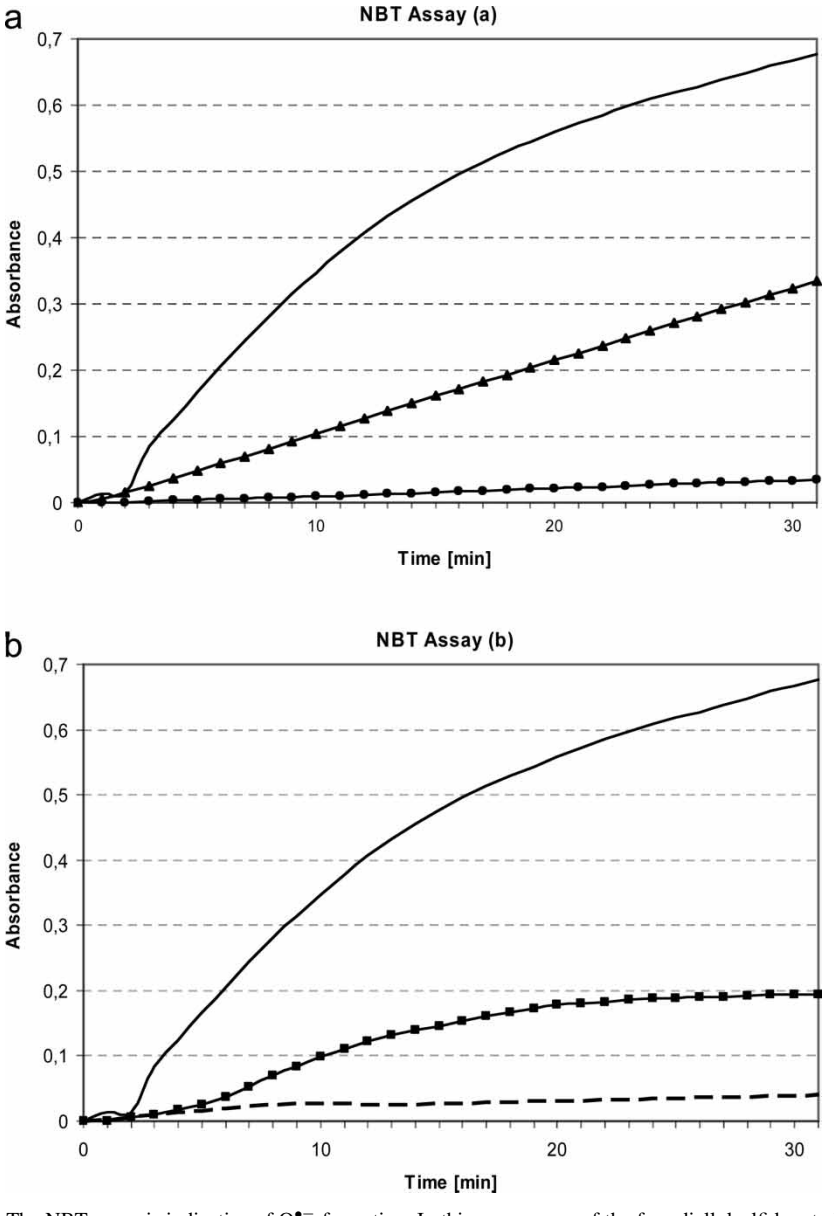

Figure 4. The NBT assay is indicative of $O_2^{\bullet -}$ formation. In this assay, none of the four diallylsulfides studied shows any significant activity on its own. In the presence of GSH, however, diallyltrisulfide **(3)** (–▲–) and diallyltetrasulfide **(4)** (—) cause the reduction of the NBT dye, while diallyldisulfide **(2)** (–●–) (and diallylsulfide, not shown) remain inactive (a). The activity of the diallyltrisulfide and diallyltetrasulfide depends critically on the presence of the reducing agent GSH, and is affected by SOD. While diallyltetrasulfide **(4)** and NBT do not react directly (– –), the significant reaction in the presence of GSH (—) is partially inhibited by SOD (–■–) (b).

3. Discussion

The main aim of our investigations has been a better understanding of the chemical reactions and possible biochemical mode(s) of action, which may explain the biological activity of diallyltrisulfide and diallyltetrasulfide, most notably their cytotoxicity against certain cancer cells.

The Caco-2 cell culture studies have confirmed previous findings by Jakubikova, Seki, Singh and their colleagues. While diallylsulfide is virtually non-toxic at the concentrations used, the disulfide exhibits some toxicity, which increases significantly for the trisulfide and somewhat further for the tetrasulfide. 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 (*3*). Interestingly, the correlation between biological activity and sulfur chain length does not seem to be linear. It 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 behaviour of the compounds involved: Disulfides, in contrast to monosulfides, are oxidizing agents able to modify cellular thiols via a thiol/disulfide exchange reaction. Although diallyldisulfide may only be a very weak oxidant at a concentration of 700 μM, it 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 disulfide to the trisulfide. As already mentioned in the introduction, 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, a process potentially catalyzed by redox active transition metal ions. This difference in 'chemistry' between the disulfide and trisulfide may explain the sharp increase in cytotoxicity observed in the Caco-2 cell culture. The tetrasulfide, 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. Without an emerging 'new chemistry', the tetrasulfide may therefore be somewhat – but not dramatically – more active than the trisulfide.

There is considerable experimental support for the involvement of perthiol or hydropolysulfide chemistry in the biological activity of diallyltrisulfide and diallyltetrasulfide, which will be discussed in more detail below. Interestingly, the comparably 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 diallyltrisulfide and diallyltetrasulfide may act as organic, solvent-like toxins, such as their analogs, 1,8-nonadiene and 1,9-decadiene, is not too far fetched; diallyltetrasulfide dissolves certain types of plastic and 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, however, point against these possible modes of action in Caco-2 cells at a concentration of 700 μM. Although the tertiary structure and solvent properties of the sulfur and carbon analogs may differ, and therefore complicate a direct comparison between diallyltetrasulfide and 1,9-decadiene, 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 diallyltetrasulfide, allicin, and allylisothiocyanate.[8] Although these three compounds may be classified as chemically related reactive sulfur species, their chemistry and mode(s) of action differ considerably.

As already mentioned, the activity of the trisulfide and tetrasulfide 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, on the other hand, is known to react rapidly with thiol groups in proteins and enzymes, thus inhibiting their function and catalytic activity (Figure 5). This inhibitory action obviously has dramatic effects on the biochemical processes within the cell, which ultimately also results in cell death. Allylisothiocyanate, on the other hand, is a good electrophile that reacts with thiols to form thiocarbamates and with amines to form thiourea derivatives (Figure 5) (*28*). Both reactions may involve amino acid residues in proteins and enzymes. As for allicin, allylisothiocyanate 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. This may enhance the reactivity of the isothiocyanate and also its associated cytotoxicity further.

The concentration of sulfur compounds employed in the assays, *i.e.* 700 μM, is, of course, rather high.[9] From a pharmacological point of view, the results presented here therefore do not imply that compounds such as diallyltetrasulfide, allicin or allylisothiocyanate may be good anticancer agents effective against Caco-2 cells. In fact, our study was not intended to find new agents against colon cancer, but to compare the cytotoxic activity of various sulfur compounds. As mentioned before, 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 also reflect the increase of activity with increasing sulfur chain length, albeit at higher cell survival rates. Diallyltetrasulfide, for instance, reduced cell survival to 81% when used in a 100 μM concentration (see Section 2).

Within the context of possible anticancer, cytotoxic agents, it is also worth mentioning that a recent study has found an apparent antioxidant activity of diallyltetrasulfide when used in a kidney cell line 'poisoned' with Cd^{2+} ions. 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 behaviour. Furthermore, the ability of RSSH or RSSSH to act as reducing agents may also, under certain circumstances, result in an antioxidant effect.[10] The precise behaviour of such a multi-talented redox and metal binding agents *in vivo* is clearly complicated and often depends on the cell line and test conditions employed.

While the studies presented here have not necessarily identified new leads for novel anticancer agents, they have shed some light on the possible mechanism(s) by which trisulfides and tetrasulfides exert their biological activity. The differences in activity observed in the Caco-2 cell culture model (and elsewhere) are reflected in the oxidation and reduction potentials of the sulfides as well as in the thiol oxidation and NBT assays.

The electrochemical studies indicate that there is a general decrease in the reduction potentials (of the oxidized forms of the mono-, di-, tri-, and tetrasulfides) and the oxidation potentials (of the reduced forms of these sulfides) with increasing sulfur chain length.[11] This implies that the biological activity of diallyltrisulfide and diallyltetrasulfide 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 behaviour 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 di-, tri-, and tetrasulfides 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.

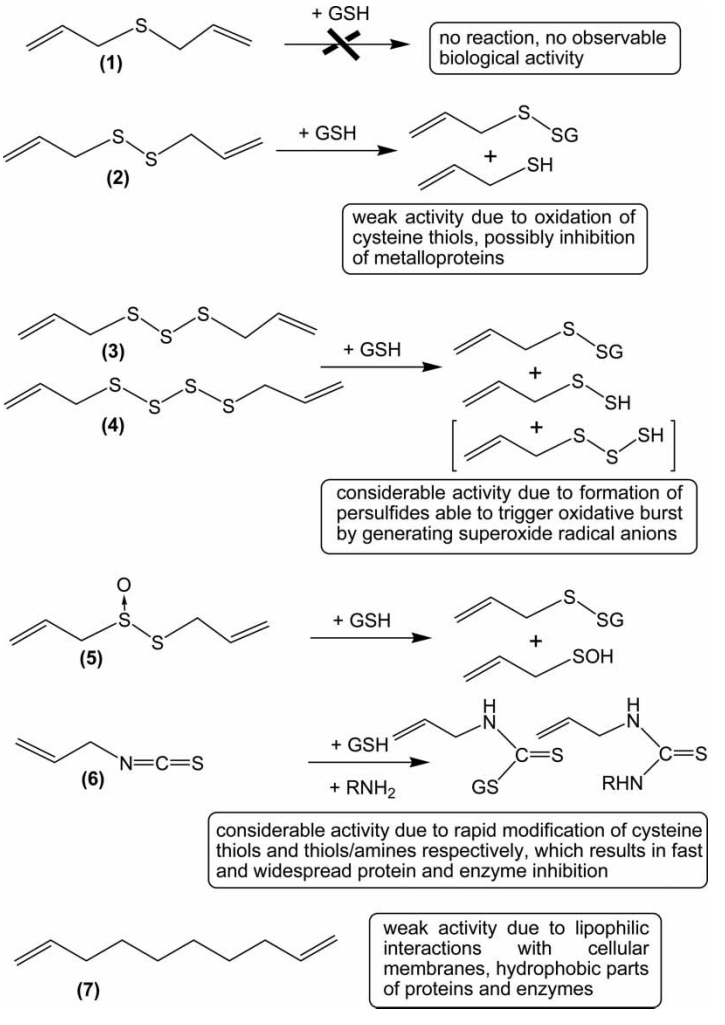

Figure 5. Summary of chemical reactions likely to be associated with the biological activity of various natural sulfur compounds (and their controls). For simplicity, GSH is used as a representative thiol, since it is abundant in millimolar concentrations in most human cells. This does not exclude other reactive thiol targets, such as redox-sensitive cysteine residues in proteins and enzymes, or dihydrolipoic acid. The diallylsulfide **(1)** 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 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 **(3)** and diallyltetrasulfide **(4)**, on the other hand, may modify thiol groups and generate $O_2^{\bullet-}$ as a part of a chemistry based on perthiols (RSSH) and hydrotrisulfide (RSSSH). While the chemical reactivity of allicin **(5)** 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 the 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 signaling, interactions with metal ions or multiple reactions occurring simultaneously.

The notion of diallyltrisulfide and diallyltetrasulfide as weak oxidants on their own, but – in their reduced form(s) – as good reductants, has been confirmed in the *in vitro* PhSH, 4-MP and NBT assays. As the PhSH and 4-MP assays illustrate, the trisulfide and tetrasulfide 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.[12] 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 Chatterji (*12, 24*) (Figure 5).

In this model, GSH reduces the di-, tri-, and tetrasulfide 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.[13] Furthermore, it is still not clear whether this chain of reactions itself 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 reactive oxygen species that leads to the formation of OS and subsequent cell death.

The thiol formed from diallyldisulfide, 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, diallyldisulfide may therefore well be toxic to some organisms, such as cockroaches, probably by acting as an oxidant or via RSH. Ultimately, diallyldisulfide may also liberate a perthiol when attacked at the α-carbon or olefinic carbon (see introduction), but these reactions are less apparent when compared to thiol/polysulfide exchange processes described above for the diallyltrisulfide and diallyltetrasulfide.

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 (*23*). 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 diallyltrisulfide, diallyltetrasulfide, and even diallyldisulfide. 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 cancer types) and status of the cell affected (*e.g.* presence of OS). Furthermore, agents such as diallyltrisulfide and diallyltetrasulfide 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.

4. Conclusions

In summary, the Caco-2 cell culture, 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. Future research may also evaluate in earnest the therapeutic potential of polysulfides, or their practical use as 'green' pesticides. Ultimately, even if diallyltrisulfide and diallyltetrasulfide 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 polysulfide chemotype.

At the same time, 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 that 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 behaviour *in vivo*. This applies particularly to assays that 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) (*29*).

Nonetheless, recent 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.

5. Experimental

5.1. *Materials*

Diallylsulfide, diallyldisulfide, allylisothiocyanate (mustard oil), allylmercaptan, glutathione (GSH), thiophenol (PhSH), 4-MP, NBT, Super Oxide Dismutase (Cu,Zn-SOD), sodium hydrogen sulfide, potassium dihydrogen phosphate, potassium hydrogen phosphate, and EDTA were purchased from Sigma-Aldrich, Munich (Germany). Diallylsulfide, diallyldisulfide, and allylmercaptan were distilled before use. Deionised water (Millipore, 18.2 MΩcm) was used unless stated otherwise.

Diallyltrisulfide was synthesized from allylchloride, sodium thiosulfate, and sodium sulfide according to the method of Milligan *et al.* (*30*). The compound was purified by vacuum distillation (at 1 mbar). Diallyltetrasulfide was synthesized from allylmercaptan and sulfur chloride (S_2Cl_2) as described by Derbesy and Harpp and purified by column chromatography (3% chloroform in petrol ether (40–65 °C)) (*31*). Allicin was obtained by oxidation of diallyldisulfide with H_2O_2 according to the method of Lawson and Wang (*32*). It was purified using silica gel chromatography (5% ethyl acetate in petrol ether (40–65 °C)). Analytical data obtained for the compounds (GC-MS, ^{1}H-NMR, ^{13}C-NMR) was in accordance with literature values. The purity of compounds was confirmed by GC-MS and HPLC.

5.2. *Caco-2 cell culture*

Cell viability of cultured Caco-2 cells was measured using the standard MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. 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 h 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, no antibiotics)) for up to 48 h at different concentrations (150 nM–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.

5.3. *Cyclic voltammetry*

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 a 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 measured between −900 and 0 mV *vs.* Ag/AgCl, with three full cycles per experiment. Unless stated otherwise, potentials and currents were obtained from the third cycle. As a 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}.

5.4. *Thiophenol and 4-MP assays*

All spectrophotometric assays were performed using a Cary 50 *Biospectrophotometer* from Varian Inc. The thiophenol (PhSH) assay measures the oxidation of PhSH to PhSSPh (*26*). It is frequently used to monitor thiol-specific oxidation reactions and catalytic oxidation events, for instance, in the form of a simple glutathione peroxidase (GPx) assay. PhSH was dissolved in methanol to a final concentration of 0.5 mM. The assay was initiated by the 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 every minute. All measurements were performed in triplicate. Zero order rates were calculated between 0 and 30 min.

The 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 solution (*27*). 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. The final concentration of 4-MP was 1 mM. 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.

5.5. *NBT assay*

The 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 that 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 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 the addition of GSH (0.5 mM final concentration). A series of controls, such as NBT together with GSH or sulfur compounds together 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 1 unit). All experiments were performed in triplicate. Zero order rates were calculated between 0 and 30 min.

Acknowledgements

The authors would like to thank David Sadler Bridge and Murree Groom (both ECOspray Ltd. (UK)) and Prof. Eric Block (University at Albany, State University of New York, US) for many helpful discussions and advice on the manuscript. This work was supported financially by the University of Saarland, the German Academic Exchange Service (DAAD), the Ministry of Economics and Science of Saarland, the Ministry of Culture and Education of Luxembourg, and the Foundation for Scientific Cooperation between Germany and Luxembourg.

Notes and References

1. The label 'natural' in medicine and pesticide development therefore provides not (only) a social or political advantage, but 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 a part of effective natural host defense systems for millions of years.
2. The expression 'chemotype' is an occasion used in biochemistry and pharmacology to denote a specific set of agents that share a common functional group.
3. For chemists, the 'sulfane' nomenclature is preferable since it follows the UPAC rules, and reserves 'sulfide' and 'polysulfide' for ionic compounds. Since the names for compounds such as diallyltrisulfide are established and widely known among (bio)chemists, we will use this more traditional terminology here.
4. Some authors refer to the 'perthiol' RSSH also as (hydro-)persulfide. We will use the expression 'perthiol' to indicate its close relationship with thiol, RSH.
5. An attempt to estimate IC_{50} values for these compounds in Caco-2 cell culture was made. The IC_{50} values of diallylsulfide and diallyldisulfide are well above 1 mM. The IC_{50} value of diallyltrisulfide appears to be close to 750 μM, while the IC_{50} values of diallyltetrasulfide, allicin, and allylisothiocyanate appear to fall within a similar range of between 500 and 700 μM.
6. 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 tetrasulfides to a thiol, perthiol, hydropolysulfide or another species, while *E*pa reflects the oxidation of such a reduced species to the di-, tri-, tetrasulfides (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.
7. 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 as completely O_2 free, and O_2 is also generated/'recycled' when $O_2^{\bullet-}$ reacts with and reduces NBT.
8. It should be pointed out that the recent study by Jakubikova and Sedlak has found a lower 'ranking' for allicin. In contrast, most studies available to date rank the cytotoxic activity of allicin as equal or even higher compared to the one of diallyltrisulfide and diallyltetrasulfide. 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. Our own ranking of activities should therefore also be seen as dependent on the experimental conditions applied.
9. It is possible that some of the more reactive sulfur compounds, such as allicin, will react with components present in the incubation medium and/or cytosol, and hence lose some of their reactivity before they reach their intracellular targets. This effect has been observed before and among others counts against the systemic (drug) application of allicin. It may also explain why high concentrations of compounds are required in the assays.
10. The presence of a strong reducing agent in a cell is a double-edged sword. On the one hand, it may act as an 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 (and also to HS^-).
11. As already mentioned, in cyclic voltammetry, the reduction potential *E*pc is a property of the oxidized form of the compound, while the oxidation potential *E*pa refers to the corresponding reduced species. Both potentials are the 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.
12. 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 disulfide chemistry.

13. Detailed investigations of eventual catalytic processes are underway. 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. These complications have already been mentioned briefly in the introduction.

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