Polysulfides as biologically active ingredients of garlic

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Garlic has long been considered as a natural remedy against a range of human illnesses, including various bacterial, viral and fungal infections. This kind of antibiotic activity of garlic has mostly been associated with the thiosulfinate allicin. Even so, recent studies have pointed towards a significant biological activity of trisulfides and tetrasulfides found in various *Allium* species, including a wide range of antibiotic properties and the ability of polysulfides to cause the death of certain cancer cells. The chemistry underlying the biological activity of these polysulfides is currently emerging. It seems to include a combination of several distinct transformations, such as oxidation reactions, superoxide radical and peroxide generation, decomposition with release of highly electrophilic S_x species, inhibition of metalloenzymes, disturbance of metal homeostasis and membrane integrity and interference with different cellular signalling pathways. Further research in this area is required to provide a better understanding of polysulfide reactions within a biochemical context. This knowledge may ultimately form the basis for the development of 'green' antibiotics, fungicides and possibly anticancer agents with dramatically reduced side effects in humans.

1. Introduction

For many centuries, empirical folk medicine has considered garlic and its products, such as garlic oils and powders, as powerful therapeutic agents. During the last 60 years, countless scientific studies have been conducted to confirm or refute the apparent health benefits ascribed to garlic.[1–3] As part of this research, various biologically active substances have been isolated from the different *Allium* species, such as garlic, onions and shallots. Many of these active ingredients contain sulfur.

Fig. 1 provides a necessarily incomplete overview of some of the biologically active sulfur species found in garlic. Among them, allicin has played the major role in garlic research. Today, most of the chemical and biochemical aspects of allicin formation and transformation processes are well established. Allicin is formed from the chemically rather unreactive sulfoxide precursor alliin in a reaction catalysed by the C–S-lyase enzyme alliinase.[1] Chemically speaking, allicin is a thiosulfinate, a reactive sulfur species which kills various bacteria, fungi, yeasts and even cancer cells.[1,2,4] Table 1 provides an incomplete list of key biological activities currently associated with both, allicin and diallylsulfides. Needless to say, the biological chemistry of allicin itself is a rapidly expanding and open area of research, and some of the latest developments in the field of organosulfur compounds from garlic, including allicin, have recently been reviewed by Tapiero and colleagues.[5]

The central role of allicin in garlic chemistry is presently being challenged by a number of findings which have confirmed antibiotic and anticancer activities for diallylsulfides similar or even superior to the ones of allicin.[6–14] During the last six years, several studies have demonstrated that diallyltrisulfide and diallyltetrasulfide, both occurring naturally in garlic as breakdown products of allicin, exhibit a wide spectrum of antibacterial, antifungal, antimicrobial and anticancer activity. Furthermore, Singh and colleagues could demonstrate in a series of experiments published in 2005 and 2006 that diallyltrisulfide was able to selectively attack DU145 and PC3 cancer cells in prostate cancer models, but not a normal prostate epithelial cell line (PrEC).[8,15–17]

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Table 1 A short and incomplete selection of antibacterial and antifungal activities of allicin and diallylsulfides. Where available, toxicity is given as minimum inhibitory concentration (MIC) in g ml^{-1}. Since values were compiled from several studies, which may have used different strains of organisms, a strict comparison is not always possible. Nonetheless, diallyltrisulfide and tetrasulfide are generally the most active among the diallylsulfides, with activities comparable to or even exceeding the activity of allicin. Please note that a whole range of various other biological activities, such as antimicrobial and pesticidal activity and repulsion of insects have also been associated with these garlic ingredients

Organism	Allicin	Diallylsulfide	Diallyldisulfide	Diallyltrisulfide	Diallyltetrasulfide
Helicobacter pylori[6]	6–12	2100–4100	100	13–25	3–6
Klebsiella pneumoniae[10]	—	96–104	72–80	40–48	20–24
Pseudomonas aeruginosa[10]	15[60]	80–88	64–72	32–36	12–16
Staphylococcus aureus[11]	15[60]	20	4	2	0.5
MRSA[11]	28[61]	32	12	8	2
Candida albicans[11]	0.8[60]	32	4	1	0.5
Aspergillus niger[11]	8–32[60]	40	8	2	1

Ute Münchberg (born 1981) studied Chemistry at the Friedrich Schiller University (Jena, Germany), completing her first degree (Diplom) in September of 2005. She joined the Jacob group in November 2005 to realise her long-standing interest in pharmacological chemistry. Ute is currently working on the synthesis of redox-catalysts with a twist, such as enzyme mimics able to single out and selectively kill cancer cells.

Awais Anwar (born 1975) obtained an MSc degree in Chemistry from the University of Peshawar in Pakistan (June 2000). He then studied at the Quaid-I-Azam University Islamabad for an advanced MPhil degree in Biochemistry, which he completed in February 2005. Awais then relocated to Germany where he worked on synthesis of naturally occurring spatozoates with Prof. Voelter at the Eberhard Karls University Tübingen (2004–2005). In November 2005, he joined the Jacob group at Saarland University as a PhD student. Coping well with the smell, Awais is currently investigating the chemical and biochemical properties of sulfur-containing natural products.

Susanne Mecklenburg (born 1980) studied Chemistry at the Technical University of Chemnitz (Germany), where she received her first degree (Diplom) in November 2003. Susanne then spent some time in the group of Prof. Lang (TU Chemnitz), where she synthesized new carbosilane- and carbosiloxane dendrimers. Discovering her interest in biological aspects of chemistry, she joined the Jacob group in May 2006 to work on multifunctional antioxidant catalysts mimicking natural human defence systems.

Claus Jacob (born 1969) studied Chemistry at the Universities of Kaiserslautern (Germany) and Leicester (UK), from where he received his BSc (Hons) degree in 1993. He then joined Prof. H.A.O. Hill FRS at the University of Oxford to carry out research in the area of bioinorganic Chemistry (DPhil in 1997). Between 1996 and 1999, Claus worked with Prof. B.L. Vallee at Harvard Medical School, where he investigated aspects of redox-controlled zinc trafficking (Feodor Lynen Fellow 1997–98 and BASF Research Fellow 1998–99). Returning to the UK, Claus took up a lectureship in Inorganic Chemistry at the University of Exeter, where he remained until 2005. As an act of rejuvenation, he exchanged his Senior Lectureship at Exeter for a Junior Professorship at Saarland University in 2005. His group is currently working on pharmaceutical aspects of redox agents, such as enzyme mimics, catalytic antioxidants and natural sulfur products.

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Awais Anwar

Susanne Mecklenburg

Claus Jacob

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?

These questions are far from trivial, and quick answers often do not withstand further scrutiny. Nonetheless, we can be almost certain that unlike allicin, whose ability to rapidly and indiscriminately modify thiol groups of peptides and proteins is well known,[18,19] diallyltrisulfide and diallyltetrasulfide are somewhat less reactive towards thiols. Still, they 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.

In the following sections, we will consider biochemical events associated with polysulfides from a chemical point of view. In doing so, we hope to encourage further, urgently needed research into the chemistry and biochemistry of natural and synthetic polysulfides and their possible application as antibiotic and anticancer agents.

2. Antibiotic activities associated with polysulfides

In order to appreciate the need for further natural polysulfide chemistry and biochemistry research, we must briefly consider some highlights of the emerging antibiotic activity of these agents.

Although the formation and transformation pathways of allicin and diallylsulfides in the (crushed) garlic clove are closely related (Fig. 1), compounds such as diallylsulfide, diallyldisulfide, diallyltrisulfide and diallyltetrasulfide have only recently attracted an adequate interest among researchers. In garlic chemistry, polysulfides may be seen as a 'second generation' of biologically active sulfur species, formed by decomposition of allicin, the

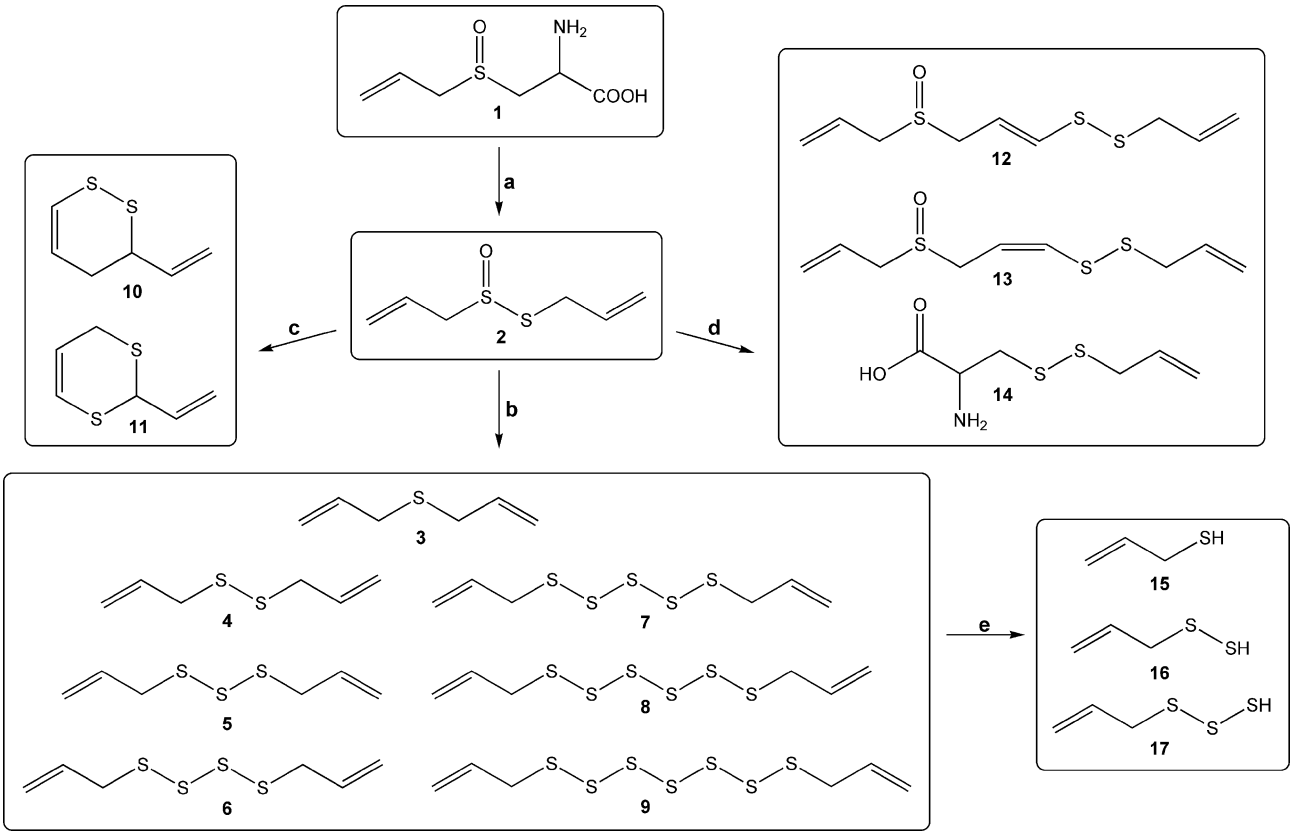

Fig. 1 Selection of biologically important sulfur agents found in garlic. Alliin (**1**) is enzymatically converted by the C–S-lyase alliinase to allicin (**2**), the compound commonly associated with the biological activity of garlic (reaction **a**). Decomposition and degradation of allicin results in a range of 'second generation' products. Significant concentrations of diallylsufide (**3**), diallyldisulfide (**4**), diallyltrisulfide (**5**) and diallyltetrasulfide (**6**) are frequently found in garlic extracts, such as garlic oils (reaction **b**). The presence of small amounts of diallylpentasulfide (**7**), diallylhexasulfide (**8**) and diallylheptasulfide (**9**) in garlic oils has occasionally been reported. Higher polysulfides may occur. Other decomposition products of allicin include the dithiins 3-vinyl-3,4-dihydro-1,2-dithiin (**10**) and 2-vinyl-2,4-dihydro-1,3-dithiin (**11**) (reaction **c**) and more complex chemical structures, such as *E*-ajoene (**12**) and *Z*-ajoene (**13**) (reaction **d**). Follow-on reactions of allicin and polysulfides with intracellular thiols result in additional sulfur species, such as *S*-allylmercaptocystein (**14**), *i.e.* thiolated cysteine and cysteine residues in peptides and proteins. Reduction of polysulfides, *e.g.* by GSH, result in allylmercaptan (**15**), allyl perthiol (**16**) and possibly allyl hydrotrisulfide (**17**) (reaction **e**). Each of these sulfur species exhibits its own chemical properties and biochemical activity.

initial 'antibiotic'. Allicin itself is a good biological defence chemical, since it rapidly, yet specifically reacts with cysteine residues in peptides and proteins, which may lead to a disruption of cellular function and cell death. Allicin, however, is also chemically unstable at room temperature and decomposes to various polysulfides and other compounds.

Aged garlic products, such as garlic oils and powders, therefore often contain only a fraction of the allicin found in freshly chopped or crushed garlic cloves. Instead, they contain considerable quantities of sulfides, mostly diallylsulfide, disulfide, trisulfide and tetrasulfide, all of which share with allicin the characteristic smell of garlic. In practice, the chemical composition of such preparations varies widely and critically depends on the processing procedure.† For instance, Maslin and colleagues found rather high concentrations of different diallylsulfides in a particular British garlic oil, including 106, 530, 115 and 43 mg g^{-1} mono-, di-, tri- and tetrasulfide, respectively.[6]

In contrast, Tsao and Yin have recently found just 1.18 mg g^{-1} diallyldisulfide in garlic oil and 0.94 mg g^{-1} in Chinese leek oil.[11] As in Maslin's sample, the disulfide was the most abundant of the four major diallylsulfides (RS_xR, x between 1 and 4), accounting for roughly half of the total diallylsulfide content. As expected, there was less diallyltrisulfide and tetrasulfide in the Chinese sample (0.75 and 0.37 mg g^{-1} in leek oil, respectively). Surprisingly, however, the diallylmonosulfide concentration in the Chinese sample was the lowest of the four sulfides with just 0.11 mg g^{-1}.

Higher polysulfides, such as the diallylpenta-, hexa- and heptasulfide have only sporadically been reported as constituents of garlic, and their concentrations are usually low. Maslin's team found 10.5 mg g^{-1} diallylpentasulfide and 0.14 mg g^{-1} diallylhexasulfide among further methyl allyl and dimethyl sulfides.[6] These findings confirm similar evidence of diallylpenta-, hexa- and heptasulfide occurrence in garlic and related extracts.[20–24]

† This may be a good reason why a potential future application of polysulfides in agriculture or medicine should be based on synthetic polysulfides, rather than processed plant material.

Biological activities of diallylsulfides confirmed to date vary considerably, and include, among others, antioxidant, antibacterial, antifungal and antimicrobial activities. Table 1 provides an overview of some of the toxic (antibiotic) activities, which are the focus of this Perspective.‡ For instance, Yin and colleagues have recently tested diallylsulfide, diallyldisulfide, diallyltrisulfide and diallyltetrasulfide against a variety of bacteria and fungi. They were able to show antibacterial activity of diallylsulfides against *Escherichia coli*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and methicillin-resistant *S. aureus*.[10,12] Antifungal activity was confirmed against *Candida albicans*, *Candida krusei*, *Candida glabrata*, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus*.[11]

Together, these toxicity studies give us a general idea as far as the biological activity of diallylsulfides is concerned. Here, and in most related studies dealing with the biological activity of polysulfides, toxicity is particularly pronounced for the tri- and tetrasulfide. In fact, the increase of antibiotic activity with increasing numbers of sulfur atoms is a trend observed in many biological assays, which has led to the insider's rule of thumb that "the more sulfur atoms in the polysulfide, the more active it is." For instance, Yin and colleagues identified the tetrasulfide as the most active diallylsulfide, with the trisulfide being around 4, the disulfide 8 and the monosulfide 40 times less active against *S. aureus* compared to the tetrasulfide (Table 1).[11]

Although most studies do not directly compare toxicity of polysulfides and allicin, some evidence points also towards a similar or even higher activity of the diallyltetrasulfide in contrast to allicin.§ As such, these findings demonstrate nicely that allicin is not necessarily the sole or most active 'antibiotic' in garlic. For instance, Maslin and colleagues compared the activities of polysulfides and allicin against *Helicobacter pylori*. Minimum inhibitory and minimum bactericidal concentrations of diallyltetrasulfide were similar to or less than the ones of allicin. As above, diallyltrisulfide was about half as active as the tetrasulfide (and allicin), while diallyldisulfide was at least 15 and diallylmonosulfide at least 300 times less active.[6]

Even so, the correlation between the number of sulfur atoms and antibiotic activity is more complicated. Based on the (sometimes vague) data available to date, the following picture emerges: firstly, the relationship between the number of sulfur atoms (x) and (selective) toxicity does not seem to be linear. While the monosulfide is often virtually inactive, the disulfide generally has some activity, which sharply increases when turning to the trisulfide. Although there is a further increase for the tetrasulfide, a plateau in activity seems to be reached with four sulfur atoms.

For instance, an early, unfortunately also very limited study on diallyl compounds from cabbage indicated that diallyltetrasulfide and diallylpentasulfide possess a comparable activity against *Saccharomyces cervisiae*.[20] Although a direct comparison with this activity is not possible, a preliminary study by Horie *et al.* from 1992 indicates that antioxidant activity sharply increases from tri- to tetrasulfide, but further increases to penta-, hexa- and heptasulfide are rather small.[21]

Clearly, such a trend in increasing activity is of great interest for the design and practical use of polysulfide agents as beneficial toxins, *e.g.* in medicine and agriculture, and there is an urgent need to investigate this matter further. On the other hand, it also sheds some light on the (bio-)chemical mechanisms possibly underlying biological activity. In essence, there seem to be two major breaks in the relationship between the number of sulfur atoms in the polysulfide and biological activity (toxicity), which point towards the emergence of qualitatively different 'chemistries'.

The first of the two breaks, when moving from the mono- to the disulfide, is easily explained: while monosulfides do not act as oxidants and cannot be reduced to thiols, disulfides are oxidants and can form thiols (RSH). The redox- and metal-binding chemistries of the thiol–disulfide pair provide the basis for many different biological events from which the monosulfide is excluded (see section 5).

The second break between di- and trisulfide is more difficult to explain. In essence, it may be associated with the perthiol (RSSH) chemistry, which is unique to tri- and higher sulfides (illustrated for diallyltetrasulfide in Fig. 2). Tetra-, penta-, hexa- and heptasulfides may form RSSH and even RS_xH ($x > 2$) faster and in higher yields than the trisulfide, yet there seems to be no major new 'chemistry' emerging when going from three to more sulfur atoms, with the possible exception of S_x release (see sections 4 to 6). The penta-, hexa- and heptasulfides may also be chemically less stable than the tri- and tetrasulfide, and therefore decompose before they can fully exert their activity *in vivo.*

3. 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 diallyltrisulfide. Although these studies must be considered as preliminary, they have provided insight into biological activities and rather unexpected regulatory cellular events associated with polysulfides.

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 and diallyltrisulfide as anticancer 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.[4,25] This approach employs a cancer cell selective antibody–alliinase hybrid and alliin as a 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 diallyltrisulfide may be stable enough to reach the tumour site without a delivery system. In addition, it may also selectively attack cancer cells without the

‡ Other activities, such as the protection of lipids from peroxidation, may be related to the lipophilicity and an (oxidised) polysulfide chemistry, and are discussed elsewhere.[21]

§ As will be discussed later, the similarity in biological activity of allicin and diallyltetrasulfide does not necessarily point towards a common mode of action.

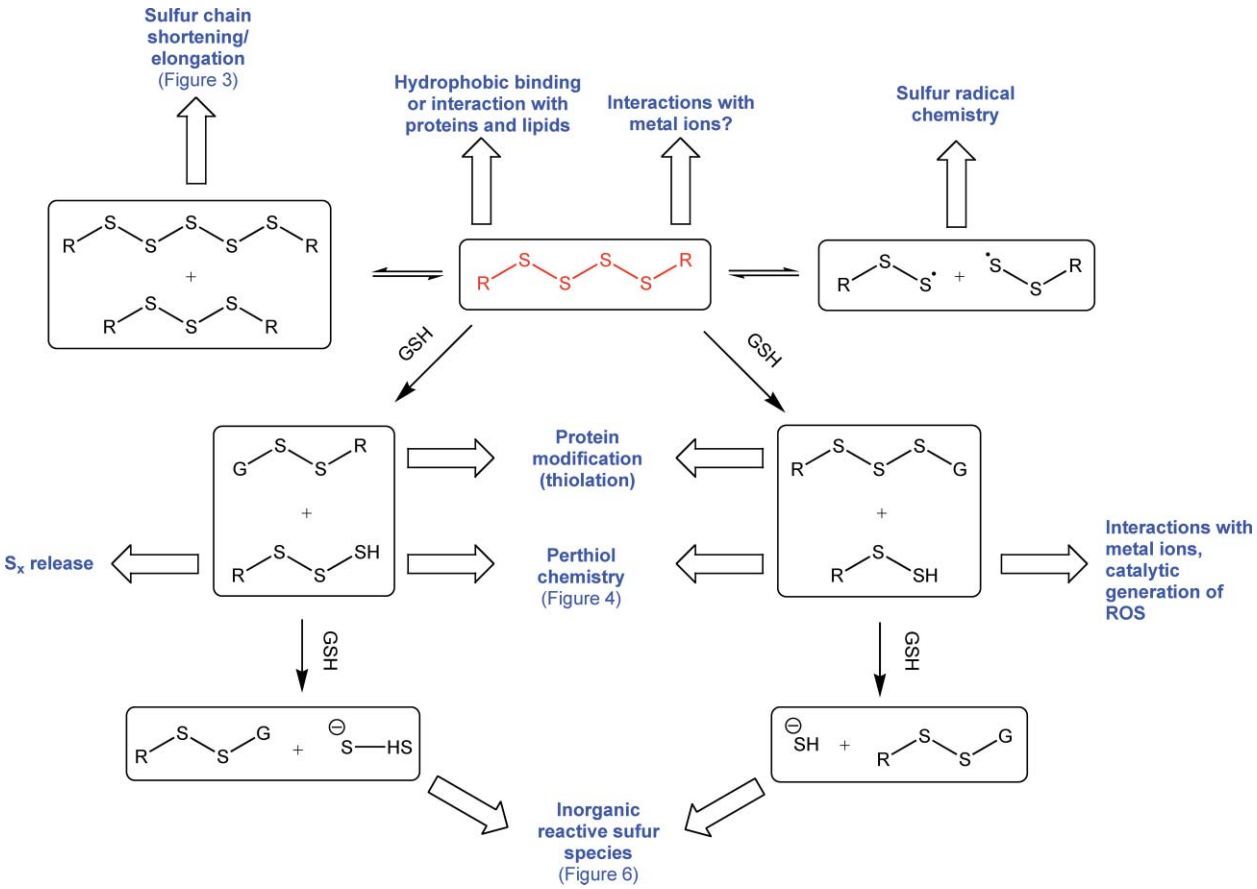

Fig. 2 Schematic overview of chemical reactions and biochemical actions associated with a polysulfide such as diallyltetrasulfide. Some biochemical effects, such as binding to hydrophobic parts in proteins and membranes or to metal ions, may be associated with the polysulfide itself. Other biological activities may be the result of polysulfide reaction products, such as thiols, perthiols and inorganic sulfur species. Please note that almost all biochemical effects (in blue) are in one way or another detrimental to living cells (see text for details).

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

In 2005, Yuan and colleagues reported an important link between diallyltrisulfide and cancer cell death.[14] 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^{-1}. 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 diallyltrisulfide with an IC_{50} value of 11.5 and 13.3 μM, respectively.[9] This effect was investigated further and found to be the result of diallyltrisulfide-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 diallyltrisulfide 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.[9]

The biochemical basis of the cytotoxic behaviour of diallyltrisulfide, which stands in stark contrast to its mono- and disulfide analogues, was also investigated by Singh and colleagues.[8,15–17] Research of this group demonstrated the ability of diallyltrisulfide (20–40 μM), but not diallylsulfide or diallyldisulfide (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 diallyltrisulfide-induced increase in intracellular levels of oxidative stress. Amazingly, cultured normal prostate epithelial cells (PrEC) were not affected by diallyltrisulfide, even at 40 to 80 μM concentrations.[8] Quite surprisingly, these findings point towards a selective toxicity of diallyltrisulfide in cancer cells, but not in the corresponding normal cells.

Although such studies will need to be confirmed and expanded in the future, they indicate that diallyltrisulfide may surpass allicin as far as chemical stability, toxicity and maybe even selective targeting are concerned. From a chemist's point of view, these rather interesting 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.

4. Polysulfides as multifacetted cytotoxins?

In analogy with allicin, 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.[9] 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.[26,27] 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 (Fig. 2). For instance, diallyltrisulfide is thought to thiolate β-tubulin and hence disturb the protein's biochemical function.[9] Similar thiolation reactions have been associated with calicheamine, which reacts with GSH.[28]

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 (see section 6).

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, which reacts with most thiols within seconds to minutes.[18,19] Nonetheless, diallyltetrasulfide and allicin 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.

¶ Presently available kinetic data on the reactivity of various diallylsulfides (and allicin) with diverse thiols, including protein thiols, does not allow us to project exactly how fast polysulfides may modify proteins *in vivo*. It is also not possible to judge if such a reaction would be relevant, assuming the presence of millimolar concentrations of GSH in most cells. GSH may 'mop up' most of these oxidants before they can target protein thiols.

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 (Fig. 2).

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.

4.1. 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 (see section 5).[13,28,29]

The case of diallyltetrasulfide 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.[13] 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.[3] RSSSH would, of course, open up an additional set of chemical reactions, such as S_2 and reductive S_2^{2-} release.

4.2. 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^{-1}, compared to 184 kJ mol^{-1} and 293 kJ mol^{-1} for the corresponding tri- and disulfides, respectively.[30,31] 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$^{\bullet}$).[32] 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

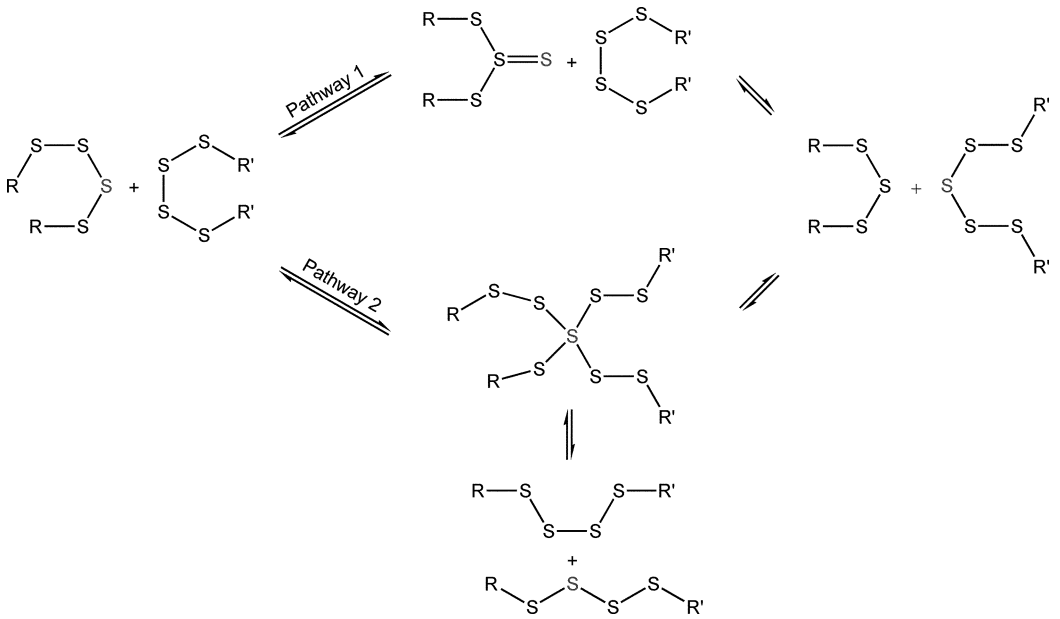

Fig. 3 'Sulfur transfer' between polysulfides resulting in simultaneous chain shortening/elongation, a process commonly observed for diallylpolysulfides. Pathway 1 proceeds *via* a thiosulfoxide intermediate, Pathway 2 *via* a tetra-coordinated sulfur species. Both pathways are debateable, yet may also be useful to explain apparent biochemical sulfur transfers, for instance from varacin. In essence, those pathways avoid the need to postulate the existence of a free S_x species.

dimethyltrisulfide and dimethyltetrasulfide, this type of reaction has been known for several decades.[31] 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 (see section 5).

4.3. 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.[33] 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*.[34] 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.

Both pathways are debateable. Neutral S_x species ($x = 2$, 3) are known to form from elemental sulfur and sulfur compounds at high temperature,[32,35–37] 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 (Fig. 3).[3,38] 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.[39]

4.4. Hydrophobic interactions

Not all explanations concerning the biological activity of polysulfides 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.[3] To date, it is unclear how such polysulfides behave once they encounter cellular membranes, cytosolic components, DNA or metal ions.

4.5. 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.

For instance, Steudel and colleagues have recently calculated bond energies for a set of lithium-dimethylsulfide complexes. Binding energies increase from dimethylsulfide to dimethylpentasulfide.

* This does not imply that the allyl-function in diallyltri- and tetrasulfide is unimportant. Several studies indicate a higher biological activity for allyl *versus* propyl analogues. Reasons behind this may include electronic effects associated with the allyl group and chemical reactivity, metabolic conversions or physical properties. Interestingly, diallyl-compounds are commonly associated with garlic, dipropyl-compounds with onions.

The maximum coordination numbers also increase, from 1 (for the monosulfide), 2 (for the di- and trisulfide) to 3 (for the tetra- and pentasulfide).[40] Is it possible that similar metal binding events inside living cells lead to a disturbance of intracellular metal homeostasis or enzyme inhibition *via* 'adventitious' binding to active site metal ions?

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.[41] Since this effect is comparable to the one of cyanide, one wonders if adventitious binding of dimethyldisulfide, or rather its reduced form, thiomethane (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^- (see sections 5 and 6), 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.

5. A central role for perthiols?

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*.[13,29] Indeed, RS_xH species ($x \geq 2$) 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.

The previous section has already discussed RS_xH formation in the context of trisulfide and tetrasulfide reduction in the presence of a thiol, such as GSH. Indeed, a thiol–polysulfide exchange reaction necessarily results in at least one RS_xH species ($x \geq 2$), most often a perthiol.[13] Assuming that RS_xH species are the biologically active forms of polysulfides, and that their formation inside living cells is controlled by a reductive step, they somewhat resemble bioreductive agents, such as the anticancer drug mitomycin C, which is reductively activated in hypoxic areas of tumours.[42] Is bioreductive activation of a polysulfide to a perthiol the key to the apparent cancer cell selectivity observed for diallyltrisulfide by Singh and co-workers?

Currently available data does not yet allow us to answer this question. Nonetheless, it does allow us to gather a glimpse of the RS_xH chemistry, which *in vivo* is somewhat different from the one of thiols (illustrated for allyl perthiol in Fig. 4). First and foremost, perthiols are considerably more acidic when compared to the corresponding thiols. For instance, the pK_a of allylmercaptan is 9.9, while the pK_a of the corresponding perthiol is only around 8.5. In essence, this implies that reactions requiring the deprotonated form are likely to proceed considerably faster in the case of the perthiol as compared to the thiol.[13]

5.1. 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 oxyhaemoglobin, to form reactive oxygen species (ROS), such as the superoxide radical anion ($O_2^{\bullet -}$) and hydrogen peroxide H_2O_2).[13,29] 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 (Fig. 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. It may also in part explain selectivity for cancer cells since ROS levels in certain cancer cells are known to be closer to the critical threshold for cell death when compared to normal cells.

Not surprisingly, several researchers, such as Munday, Gates and their colleagues have considered this catalytic cycle as the main explanation of polysulfide toxicity.[13,29] ROS generation by diallyltrisulfide may also explain the findings of elevated levels of oxidative stress in cancer cells killed by this compound.[8] 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.[29,43]

Similarly, Munday and colleagues have studied the redox behaviour of diallylsulfides in the presence of GSH and cellular

†† A word of caution: there are often parallel trends between pK_a values and redox potentials of chemically similar species (*e.g.* RSH, RSSH, RSeH), *i.e.* more acidic species are also generally more reducing. Nonetheless, acidity of a compound is not a direct cause of particular redox behaviour.

‡‡ We use the expression pseudo-catalytic since the polysulfide at the beginning and the one at the end of each cycle are not identical. Nonetheless, *in vitro* studies have shown that such as system may perform several catalytic turnovers before it comes to a halt.

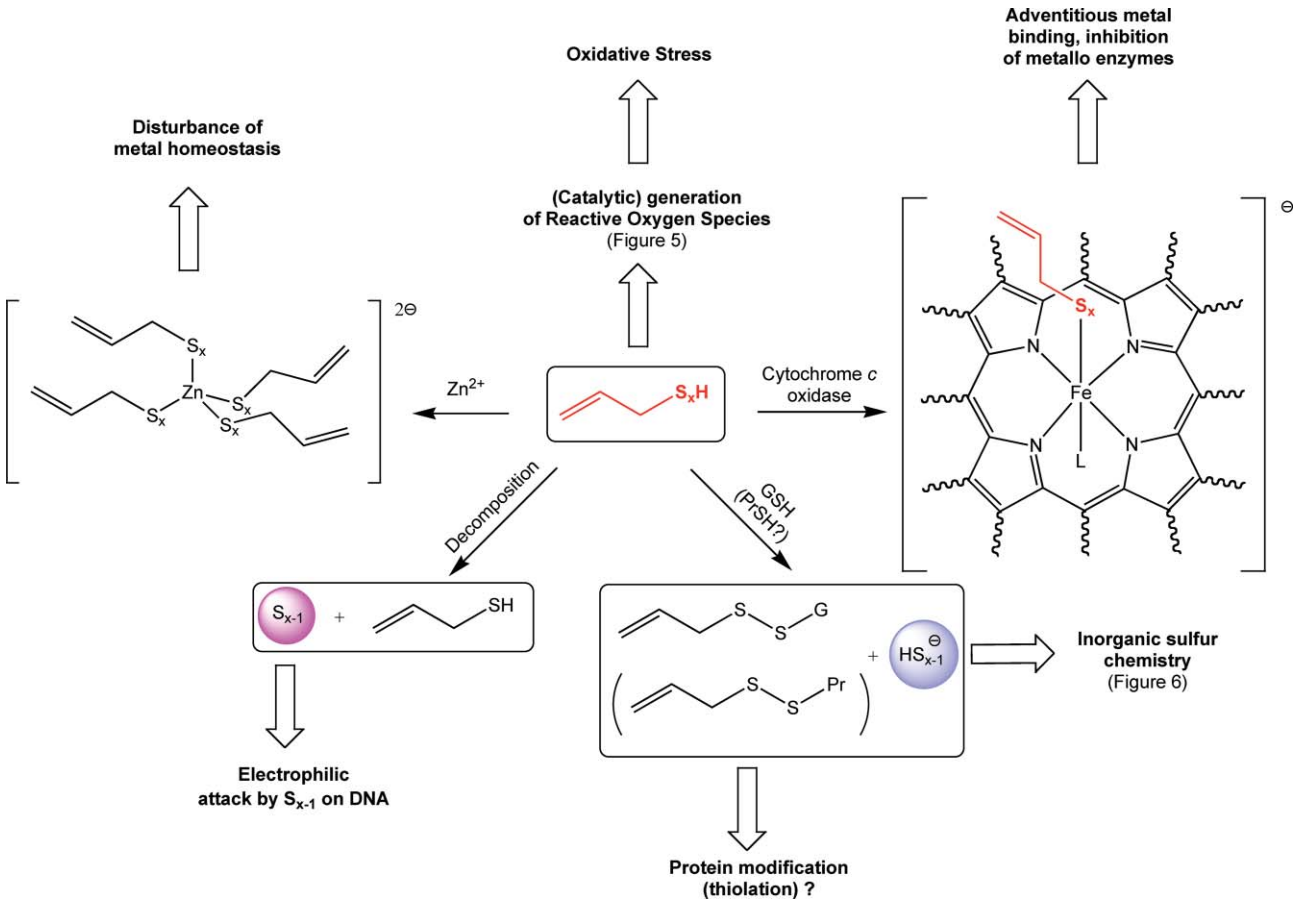

Fig. 4 Reactions of thiols and perthiols in an intracellular environment illustrated for allyl hydrosulfides. Thiols and perthiols can bind adventitiously to metal sites in proteins, such as cytochrome *c* oxidase, a key iron enzyme in energy metabolism known to be inhibited by a range of organic and inorganic sulfides. Similarly, thiols can interfere with the intracellular metal homeostasis by sequestering free metal ions, *i.e.* Zn^{2+}. While release of neutral polysulfides, such as S_3, is debatable, little is known about possible reactions with GSH and protein thiols (PrSH). Such reactions may lead to the thiolation and disturbance of protein function, as well as the formation of inorganic sulfur species.

oxidants, such as oxyhaemoglobin and methaemoglobin.[13] 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 an 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. Although a full discussion of these biochemical findings is beyond the scope of this Perspective, it reminds us that a living organism is highly complex and that besides simple chemical redox-processes, cellular signalling, protein expression, changes at the level of genes and regulation by posttranslational modification also need to be considered.

5.2. Metal binding

The chemistry of RS_xH species is not limited to their reaction with oxidants, such as dioxygen or haemoglobin. Like thiols, perthiols should be good ligands for (transition) metal ions such as zinc, copper and iron. Unfortunately, the perthiol coordination chemistry *in vitro* and *in vivo* has hardly been addressed to date and therefore remains speculative. One notable exception is an early paper by Sawahata and Neal, who have demonstrated that benzyl hydrodisulfide (Bn-SSH), but not benzyl mercaptan (Bn-SH), inhibits hepatic cytochrome P450.§§[44] The authors at the time went to a great length to explain this finding. Among other alternatives, such as cysteine thiolation *via* benzyl disulfide (Bn–S–S–Bn), they also speculated about a possible coordination of the perthiol to the haem iron at the active site.

§§ Ironically, this is the same perthiol compound Gates and colleagues used 23 years later to postulate that superoxide radical anion formation is the cause of polysulfide/perthiol toxicity in cells (see above).

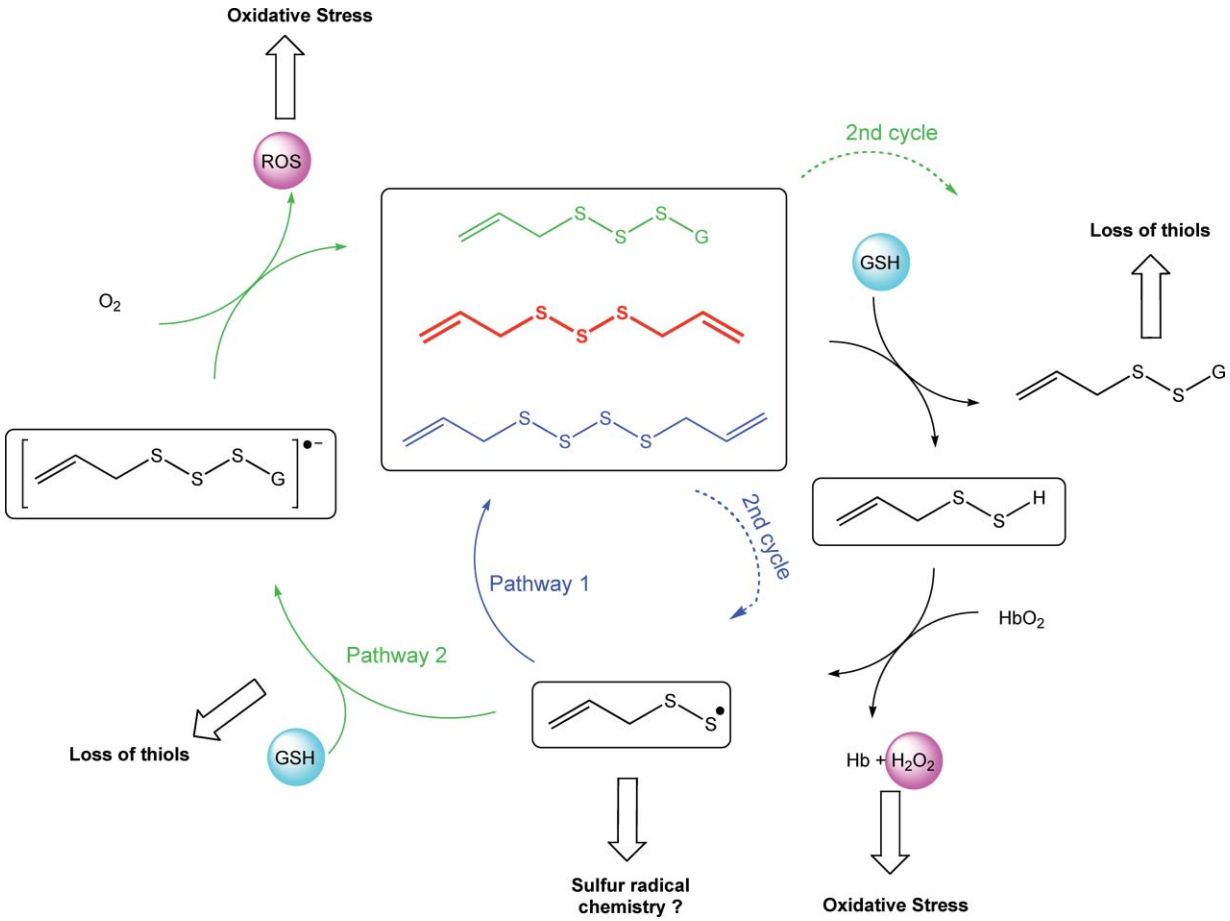

Fig. 5 The reaction of polysulfides in the presence of intracellular components GSH and O_2 as exemplified for diallyltrisulfide. Reduction by GSH in a thiol–trisulfide exchange reaction results in a mixed disulfide and a perthiol. The latter reacts with O_2 (bound to haemoglobin) to form a perthiyl radical and hydrogen peroxide. (Reduced varacin may react directly with O_2, *i.e.* in the absence of Hb, to generate $O_2^{\bullet -}$.) While H_2O_2 (and $O_2^{\bullet -}$) react further to cause oxidative stress, less is known about the fate of the sulfur-centred radical. Depending on its concentration, it may dimerise (Pathway 1) to regenerate a polysulfide, albeit not the same as at the start (here it forms a tetrasulfide). Alternatively, it may react with the large amounts of GSH in the cell (Pathway 2) to form a polysulfide radical anion, which may reduce O_2 to yield a reactive oxygen species (ROS) and regenerate a polysulfide. In any case, the regenerated polysulfides are able to enter a second cycle in a pseudo-catalytic process which is highly damaging to living cells. Please note that the stoichiometry of the individual reactions has not been included.

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.

Indeed, adventitious metal-complex formation is a major issue in enzyme regulation.[45] Numerous organic thiols are known to act as superfluous ligands in enzymes, either as regulators or inhibitors of enzyme activity. For instance, the activity of various matrix metalloproteases is regulated by cysteine ligands, which are either part of the pro-enzyme or of specific inhibitor proteins, and which bind to the active site zinc ion and therefore inhibit substrate binding.[46] Similarly, Goto and colleagues have found K_i values for the inhibition of metallo-β-lactamase from *Serratia marcescens* by mercaptoacetic acid and 2-mercaptopropionic acid (0.23 and 0.19 μM, respectively), which were around 20 times lower than the one for GSH (4.6 μM).[47] Other examples include the inhibition of angiotensin converting enzyme by captopril and the inhibition of carboxypeptidase A by D-cysteine.[48,49]

Rather than binding to a metal ion at the active site, thiols and perthiols may also form low molecular weight cytosolic complexes with zinc, copper and iron ions. This process would reduce the level of 'free' metal ions not bound to proteins and, under certain conditions, could prevent appropriate metal loading of *de novo* synthesised apo-metalloproteins, with adverse effects on protein function.¶¶ And finally, the ability of thiols to actively remove metal ions from the active site of metalloproteins, as observed for carboxypeptidase A and D-penicillamine,[49] might also be mirrored in the case of perthiols.

¶¶ It should be mentioned that decreasing the level of 'free' metal ions may also be beneficial, especially under conditions of oxidative stress, where an excess of labile iron causes redox-havoc in the cell. In those cases, chelators, including thiol agents, are used which exert their antioxidant effect by complexation of excess 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.

6. Inorganic sulfide anions

One of the perhaps 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.[50] This area of inorganic sulfur species has been reviewed by Toohey in 1989.[38] This review is a goldmine of information as far as the biological chemistry of S_x^{2-} species is concerned.

Unlike the neutral S_x species discussed earlier, 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. Not surprisingly, reductive release of polysulfide anions from varacin *in vitro* has been discussed by Chatterji and Gates, using mercaptoethanol as a reducing agent.[50] If such inorganic sulfur species are formed inside a living cell, what happens next?

During the last couple of years, it has become apparent that the biological role of sulfide anions has long been underestimated. Hydrogen sulfide is formed enzymatically in mammalian tissue by at least three enzymes, *i.e.* 3-mercaptopyruvate sulfurtransferase, cystathionine β-synthase and cystathionase.[51] Chemical formation pathways of H_2S may include the 'breakdown' and reduction of polysulfides.[50] In contrast, formation of H_2S_x ($x > 1$) *in vivo* is less certain.

Once formed, H_2S seems to affect a range of biochemical processes in the cell (Fig. 6). For instance, recent studies by Moore and colleagues have shown that hydrogen sulfide participates in intracellular signalling by influencing processes such as vasodilatation and inflammation.[52] 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 (such as mercaptoacetic acid, see above).

To underline the biological significance of H_2S, a number of biological responses towards H_2S are emerging at present. A recent paper in Science, for instance, has shown that "H_2S induces a suspended animation-like state in mice".[53] The authors explain this finding with the inhibition of the iron protein cytochrome *c* oxidase by the sulfide anion.

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.[54] 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).[55]

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 ($S_x^{\bullet}$), similar to the ones discussed for perthiols. Unlike organic thiols and perthiols, inorganic sulfide anions are also able to react with disulfide bonds in proteins, either by reduction of a disulfide to thiols, or by 'insertion' into the disulfide bond (Fig. 6). Such insertion reactions employing S_4^{2-} are used in synthetic organic chemistry to convert cyclic disulfides to trisulfides.[56] It is presently unknown if similar insertions also take place *in vivo*, and if they have any biochemical significance. Protein disulfides may therefore provide additional targets for polysulfide-derived sulfur species, which in turn may cause damage to the cell.

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-}.[38] Such cysteine modifications may inhibit enzymes, yet may also convey activity, as is the case in bovine liver rhodanese.[57]

In essence, this area of inorganic, reactive sulfur species in biology is still in its infancy. It provides ample opportunities for comprehensive bioorganic and 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.

7. Outlook

The previous sections have discussed a couple of recent developments in the field of polysulfides associated with the biological activity of garlic. 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 we have discussed in the previous sections 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.

For example, the reaction of diallyltrisulfide with thiols may, in the first instance, result in thiolated and therefore damaged proteins. At the same time, the perthiol product of this initial reaction would start generating ROS. Depending on the GSH content of the cell, these ROS may already be sufficient to trigger cell death. If GSH levels are higher, sulfide anions may be generated

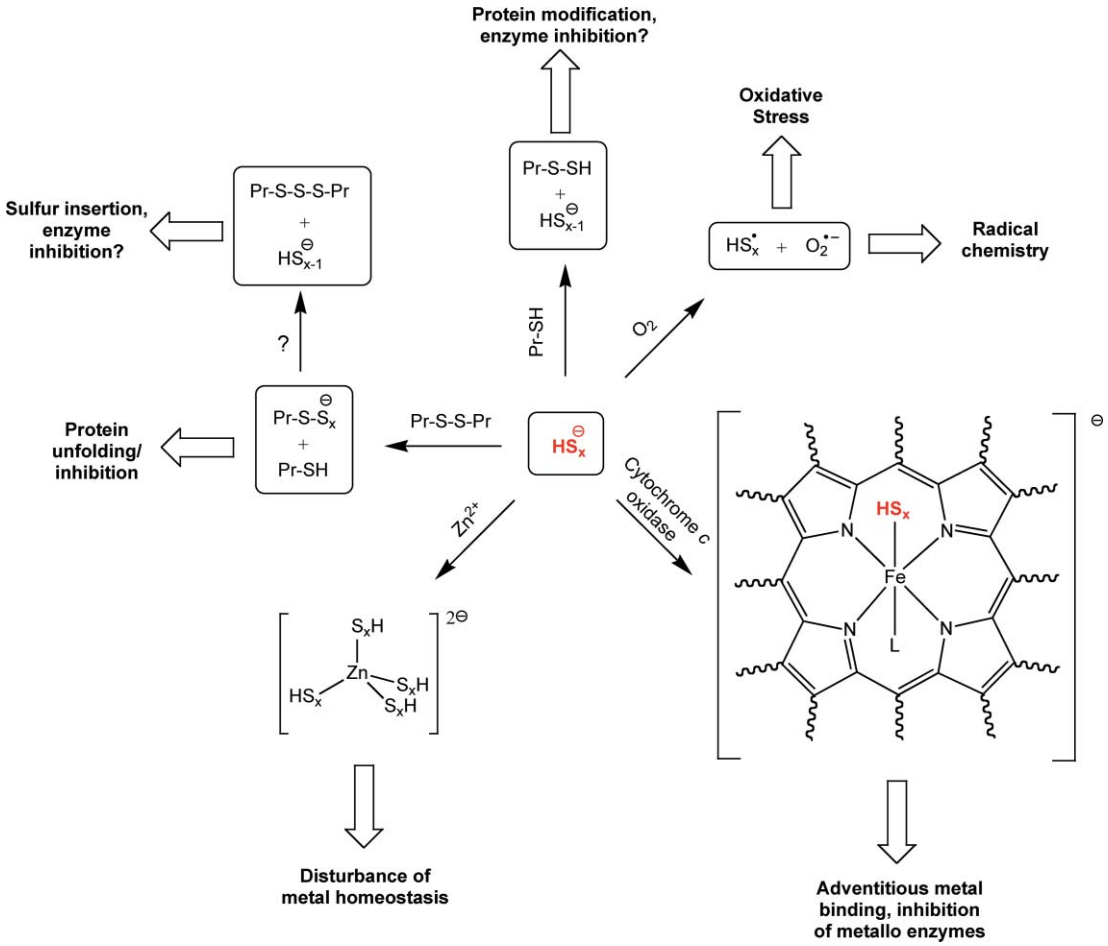

Fig. 6 Inorganic sulfide S_x^{2-} anions as biochemical signalling molecules. Although little is known about the chemistry of such anions inside the living cell, *in vitro* data point towards a range of possible interactions. Sulfide anions can inhibit enzymes by binding to active site metal ions, may disturb the intracellular metal homeostasis by sequestering free metal ions and may react with O_2 to form reactive oxygen species. While such reactions are also possible for organic thiols and perthiols, inorganic sulfide anions can also reductively break disulfide bonds and form perthiol sites in proteins, both processes which may result in loss of protein function.

which inhibit essential enzymes by acting as adventitious ligands, affecting structural disulfide bonds or converting cysteine thiols into perthiols.

Alternatively, diallyltrisulfide in high concentrations may simply change the permeability of phospholipid membranes of cells or 'dissolve' the skin of nematodes, which would subsequently die without the polysulfide conducting one single chemical reaction at all.

The likelihood of any of these scenarios most probably depends on the organism at hand. At the cellular level, aspects such as redox state and metal balance may decide which direction the polysulfide chemistry ultimately takes. In any case, polysulfides would hit certain cells in more than one way and therefore particularly hard, which may ultimately also explain the exceptional cytotoxic properties associated with them.

One of the main consequences of this diverse biological polysulfide chemistry is, of course, that the (bio-)chemical mechanisms of polysulfide toxicity are dramatically different from the ones of allicin. The latter is primarily an (unspecific) thiolation agent, whose reaction products include allyl sulfenic acid, itself also a thiolation agent, and disulfides.

Considering the chemical and biochemical complexity of polysulfide chemistry discussed here, 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 regarding the natural occurrence of diallylpenta-, hexa- and heptasulfide in plants and plant extracts, primarily garlic. Almost nothing is known about the chemical and metabolic stability or cytotoxicity of these compounds. Are they considerably more active than the tri- and tetrasulfide analogues or does biological activity level off once four sulfur atoms are reached? How stable are these compounds, and is S_3 release, as postulated for the pentasulfide varacin, also an issue as far as diallylpentasulfide is concerned?

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.[58,59] 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 play a role in the cytotoxicity of polysulfides.

Since such compounds are unstable and highly reactive under physiological conditions, they may only be present *in vivo* as transient chemical species. Furthermore, sulfur has few spectroscopic properties. As a consequence, questions related to polysulfide chemistry *in vitro* and *in vivo* quickly lead to a rather difficult area of chemical sulfur research, which is twinned with an equally demanding set of biochemical studies.

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 us.** 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.

There is also a real potential to use tri- and tetrasulfides 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 (0.1 to 1% in water), 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 pose a health risk.

In conclusion, one may safely state that the field of biological polysulfide chemistry contains the right mixture of initial, sometimes preliminary facts on the one hand, and demanding research questions on the other, to stimulate many, sometimes open-ended research projects in the short and medium term. Most of these programmes will need to be carried out at the interface of chemistry with other disciplines. Clearly, this area of chemistry not only smells of garlic, but also of considerable excitement and success.

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** This may not be entirely true: certain mammals can be poisoned by polysulfides from garlic and onion. Furthermore, Munday and colleagues have recently investigated possible harm to humans.[13]

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