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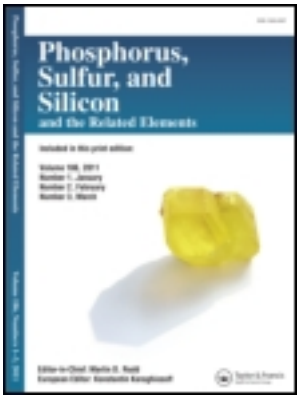

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Tetrasulfanes as Selective Modulators of the Cellular Thiolstat

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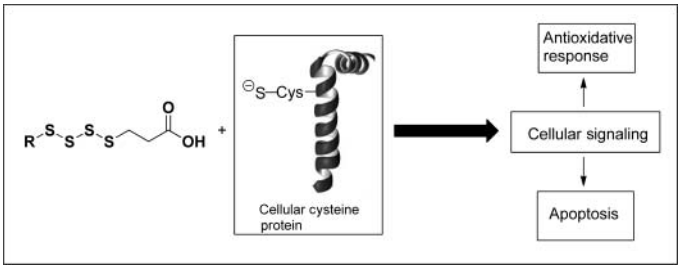
GRAPHICAL ABSTRACT



Abstract *Naturally occurring polysulfanes often exhibit a wide spectrum of biological activity, which results from their ability to react with, and hence modify cysteine residues in key proteins and enzymes of the cellular thiolstat. Such interactions frequently proceed via S-thiolation of cysteine residues and subsequent formation of Reactive Oxygen Species. Polysulfanes are highly effective, yet also surprisingly selective for cysteine residues and enable the control of numerous cellular processes ranging from an activation of antioxidant defenses to the induction of programed cell death. Unfortunately, the arsenal of natural tri- and tetrasulfanes is limited. Here, we showcase the synthesis of asymmetric tetrasulfanes, which exhibit an interesting nematocidal activity and represent a new generation of tailor-made polysulfanes with potential applications in the field of agriculture and medicine.*

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Keywords Cysteine; cellular thiolstat; nematodes; polysulfane; S-thiolation

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INTRODUCTION

Organic sulfur compounds (OSCs) form a class of natural compounds associated with an impressive chemical diversity, reactivity and—above all—wide range of biological activities, which include antioxidant, chemopreventive, antimicrobial, anti-inflammatory, and even anticancer activity.[1–11] At the same time, some of these OSCs occur in edible plants, such as garlic, onions, Shiitake mushrooms, asparagus, and mustard, and therefore have formed part of the human diet for centuries without any major adverse effects on human health.[12] Not surprisingly, certain OSCs, such as diallyltrisulfane (DATS) and diallyltetrasulfane (DATTS) from garlic, have recently attracted considerable interest as these compounds seem to possess a high, yet selective activity against bacteria, fungi, and certain cancer cells.[7,10,11,13,14–17,18–21]

This surprising combination of high activity and selectivity found in such chemically rather simple molecules can be rationalized using the model of the cellular thiolstat.[8,9,19,22,23] The latter represents a major intracellular regulatory network composed of redox sensitive cysteine proteins and enzymes, whose respective function and activity is in part controlled by the oxidation state of key cysteine residues. Oxidation of such proteins, which include β-tubulin, by compounds such as DATS or DATTS, usually occurs in form of S-thiolation, and may trigger major cellular response pathways, which can ultimately also lead to cell death via apoptosis.[24,25] During the last couple of years, a range of polysulfanes have therefore been synthesized and tested for biological activity, among them various tri- and tetrasulfanes, which usually are more reactive than the corresponding disulfanes, yet also chemically more stable than the higher (penta-, hexa- and hepta-) polysulfanes.[1,26] At the same time, the biological activity of nanoscopic sulfur (S_8)—which is easy to obtain and to handle and also does not smell—has been studied rather extensively, underlining the special (re-)activity of substances containing longer sulfur–sulfur chains.[10,11]

Here, we report our latest attempts to expand the scope of tetrasulfanes for biological applications by synthesizing *asymmetric* polysulfanes for possible agricultural and medical uses. These studies represent only the first step toward a wider range of new compounds and their biological activities, and hence are of a preliminary nature.

RESULTS AND DISCUSSION

As natural polysulfanes such as DATS and DATTS are associated with undesired properties (e.g., low solubility in aqueous media and an intense, arguably unpleasant smell), it is necessary to vary the side chains in order to "fine tune" the physical, chemical, and pharmacological properties of such compounds before they can be applied in practice. In the case of the tetrasulfanes, the synthetic method reported by Derbesy and Harpp in 1994 enables the rapid and effective synthesis based on the reaction of sulfur monochloride (S_2Cl_2) with a given thiol.[27] Whilst this method is fairly simple to employ, it traditionally results in symmetric tetrasulfanes, which are not always useful from a more biological perspective. We have therefore decided to expand the original method by Derbesy and Harpp to synthesize *asymmetric* tetrasulfanes (AS_4) in a one-pot synthesis employing S_2Cl_2 and *sequential* addition of equimolar amounts of *two different* thiols (Figure 1).[27] Our results obtained for compounds AS_4-1 and AS_4-2 demonstrate that this synthetic approach is fruitful as it results in the desired, i.e., asymmetric products, which can then be separated from the corresponding symmetric tetrasulfanes due to differences in the polarity of the three products formed.

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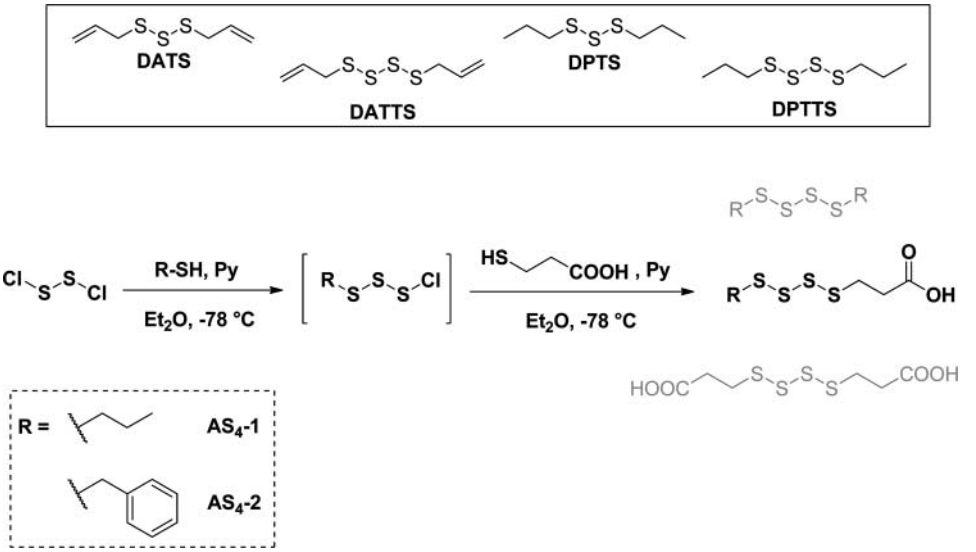

Figure 1 General scheme for the synthesis of asymmetric tetrasulfanes based on the method initially reported by Derbesy and Harpp in 1994. The insert shows the chemical structures of the lead compounds DATS and DATTS and the propyl analogues DPTS and DPTTS, which are found in garlic and onions, respectively.

To date, several biologically interesting AS_4 have already been synthesized employing this method, of which AS_4-1 (yield 19%) and AS_4-2 (yield 75%) are of particular interest and will be presented as part of this report: These compounds combine an acceptable lipophilicity with good solubility and stability in aqueous media, have virtually no smell, can be handled easily, and hence may be of interest for practical applications.

As redox activity is deemed to be key to biological activity, the redox behavior of AS_4-1 and AS_4-2 was studied using Cyclic Voltammetry in conjunction with a dropping mercury working electrode. As expected, AS_4-1 and AS_4-2 are both redox active. Their cathodic reduction potentials *E*pc were determined as −787 mV and −779 mV versus the Ag/AgCl reference electrode, respectively, which is comparable to the *E*pc of DATTS (−680 mV). Anodic oxidation potentials *E*pa were −560 mV for AS_4-1, −554 mV for AS_4-2, and −603 mV for DATTS, respectively. The midpoint $E_{1/2}$ potentials (often casually referred to as "redox potentials") were −674 mV, −667 mV, and −642 mV for AS_4-1, AS_4-2, and DATTS, respectively. Whilst such electrochemical studies using the dropping mercury electrode are not devoid of complications, they confirm that compounds such as AS_4-1 and AS_4-2 are redox active and possess redox properties comparable to the ones of DATTS.

We have then investigated the biological activity of AS_4-1 and AS_4-2 against the nematode *Steinernema feltiae*, which serves as a readily available, robust, and reproducible model of an agricultural pest. As Figure 2 indicates, both AS_4-1 and AS_4-2 possess a reasonable activity against *S. feltiae*. After 24 h, the viability of the nematode is decreased dramatically in the presence of both polysulfanes when compared to the DMSO control, with LD_{50} values for AS_4-1 and AS_4-2 in the range of 40 μM and 200 μM, respectively. The toxic effects observed for both compounds are statistically highly significant (compared to the DMSO control), and compare well with the one of DATTS (LD_{50} value around 150 μM).

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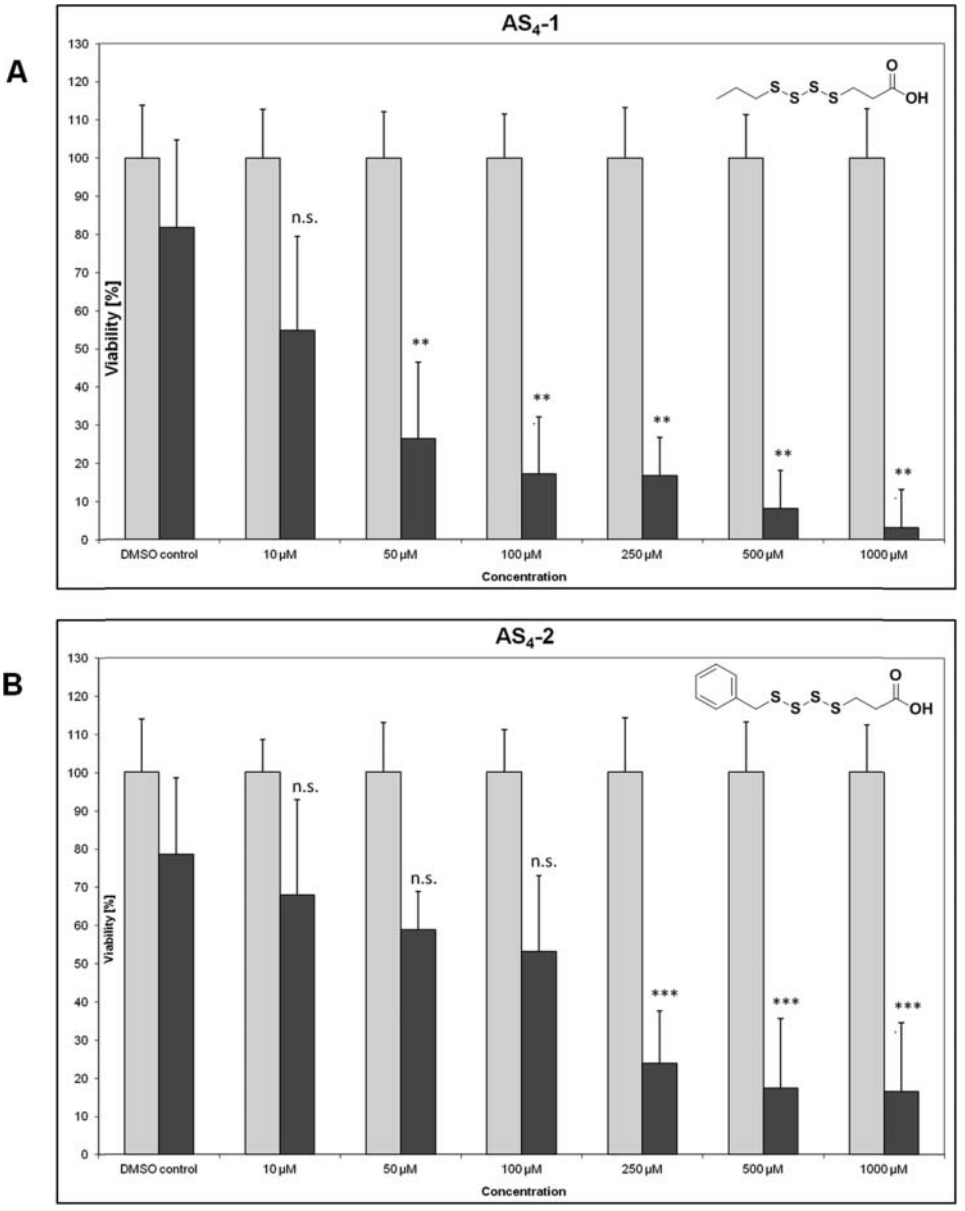

Figure 2 Activity of AS_4-1 and AS_4-2 against the nematode *S. feltiae* at 30 min (□) and after 24 h (■). Whilst most nematodes are still alive after 24 h in the DMSO control, there is a statistically highly significant reduction of the number of viable nematodes in the presence of AS_4-1 (Panel A) and AS_4-2 (Panel B), especially at concentrations above 50 μM. The difference between measurements were examined by using Student's *t*-test *versus* DMSO control, a value $p < 0.05$ was considered statistically significant. Symbols: n.s. = not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Data are depicted as mean ± SD ($n = 9$).

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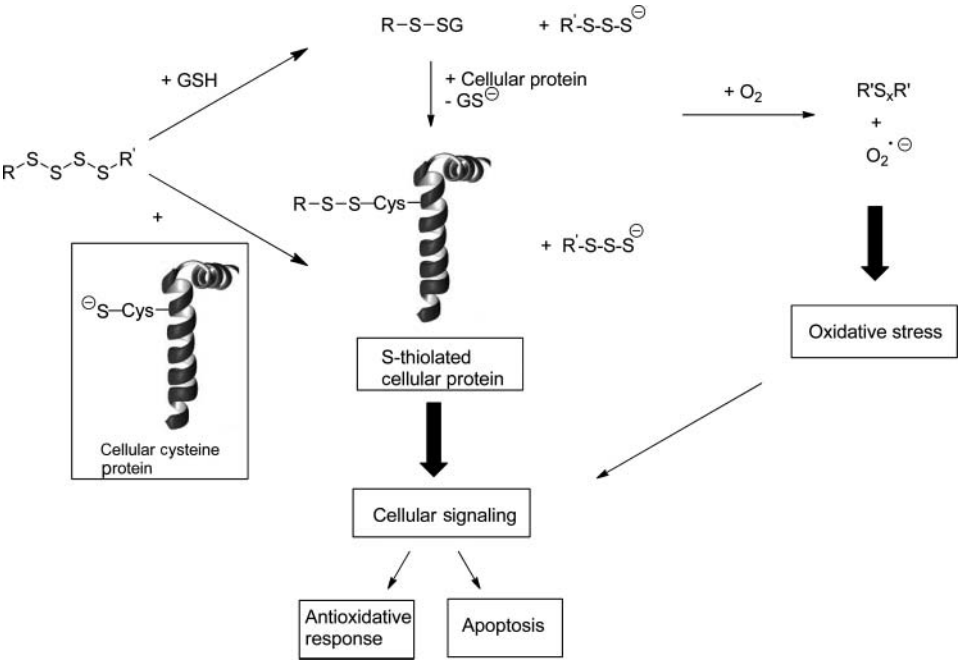

Figure 3 Selected aspects of the biochemical mode(s) of action of polysulfanes based on the established biochemical behavior of DATTS and related tetrasulfanes. In this model, the polysulfane reacts with key cellular proteins, modifying their (essential, regulatory) cysteine residues and hence changing their function or activity. Such modifications subsequently initiate cellular signaling and control cascades, which may result in a range of cellular responses. As a polysulfane-specific caveat, S-thiolation of proteins may not only occur by the direct reaction of the polysulfane with a cysteine thiol but may also proceed via initial formation of a mixed disulfide (RSSG) or GSSG, both of which can also S-thiolate proteins. Cysteine oxidation and S-thiolation may be enhanced further by the formation of superoxide radical anions ($O_2^{\bullet -}$) from O_2.

Our results show that it is possible to produce asymmetric tetrasulfanes with desired properties using a simple method based on S_2Cl_2 and mixtures of specifically selected thiols. Whilst this method necessarily also results in the formation of symmetric products (which may also be useful), the latter can be separated from the asymmetric compounds with comparable ease. In fact, it is possible to enhance the formation of the asymmetric product by adding the two thiol-containing educts *sequentially*, as the intermediate RS_3Cl, which is formed initially from RSH and S_2Cl_2, can be reacted with R'SH to the final product RS_4R'. This approach has been used quite successfully in the case of AS_4-2 (75% yield), and yields for the AS_4 compounds are generally quite acceptable, i.e., in the range of yields also encountered for most polysulfanes.

Whilst AS_4-1 and AS_4-2 are the first asymmetric tetrasulfanes we have explored, a wide range of asymmetric as well as symmetric tetrasulfanes can now be designed and synthesized, including multifunctional tetrasulfanes and amphiphilic structures. Asymmetric trisulfanes may also be considered, as the method of synthesis published in 1961 by Milligan et al. involves a two-step procedure, which provides scope for the employment of two different side-chain bearing educts.[28]

Indeed, from the perspective of pharmaceutical chemistry, asymmetric polysulfanes have several advantages: First, it is easier to fine-tune lipophilicity of the tetrasulfanes as

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the two side chains differ and individually can include specific lipophilic and hydrophilic groups. Whilst lipohilicity can also be controlled in symmetric tetrasulfanes, the fact that each function is added necessarily twice implies that control is more problematic—and as both chains are identical, such symmetric tetrasulfanes are often either too lipophilic or too hydrophilic. Symmetric derivatives of DATTS (calculated clog$P$ = 2.68), such as 1,4-dibenzyltetrasulfane (DBTTS) and 3,3′-dipropanoic acid tetrasulfane (DPSTTS), for instance, possess clog$P$ values of 4.64 and 0.71, respectively, which are both suboptimal from the perspective of Lipinski's rules. The asymmetric analogue AS_4-2, in contrast, possesses more amenable clog$P$ value of 2.76. Similarly, asymmetric polysulfanes enable the incorporation of different functional groups into *each* side chain. The modified method of Derbesy and Harpp also compares favorably to alternative synthetic methods for tetrasulfanes, such as the reaction of (symmetric or asymmetric) disulfanes with elemental sulfur. Whilst such "sulfur enrichment" of disulfanes also results in the corresponding tri- and tetrasulfanes, it is complicated by the appearance of a wide range of other (poly-)sulfane contaminants, which are difficult to separate.[26] And finally, S_2Cl_2 and thiols are readily available and larger scale syntheses of asymmetric tetrasulfanes for practical applications using this method can be considered.

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CONCLUSIONS

Whilst considerably more tests in diverse biological systems—and with a much wider range of compounds—are required, initial results indicate that the DATTS derivatives such as AS_4-1 and AS_4-2 retain redox activity and also aspects of the biological activity of DATTS (Fig. 3), yet lack some of the "undesired" properties associated with this natural product, such as its distinct smell or oily consistency. AS_4-2, for instance, is a non-smelling, yellow solid which, like DATTS, is active against *S. feltiae*, yet is considerably easier to handle. Ultimately, such compounds may combine sufficient chemical stability, solubility, and bioavailability with otherwise amenable physicochemical properties, and hence may also be superior to DATTS when it comes to agricultural or medical applications. Future studies will need to consider the spectrum of biological activity associated with such tailor-made polysulfanes in more detail, and also investigate their mode(s) of biological action.

EXPERIMENTAL

Synthesis of Polysulfanes (AS_4-1 and AS_4-2): General Procedure

To a solution of S_2Cl_2 (2.70 g, 20 mmol) in diethyl ether (50 mL), an equimolar amount of the respective mercaptane (20 mmol) and pyridine (1.58 g, 20 mmol) in diethyl ether (25 mL) was added dropwise for 1 h at −78 °C. The solution was kept at −78 °C for 45 min. Then, another equimolar solution of 3-mercaptopropionic acid (2.12 g, 20 mmol) and pyridine (1.58 g, 20 mmol) in diethyl ether (25 mL) was added dropwise for 1 h at −78 °C. The reaction mixture was stirred for another 1 h at −78 °C and then allowed to warm up to room temperature. Subsequently, the organic layers were washed with distilled water (three times) and dried over $MgSO_4$. After evaporating the solvent, the product was purified by column chromatography. **AS_4-1** was purified by column chromatography using CH_2Cl_2/methanol 9:1 v/v as eluent (R_f = 0.36). In case of **AS_4-2**, the compound was purified by column chromatography using CH_2Cl_2 as eluent (R_f = 0.38).

Nematode Assay

Steinernema feltiae was purchased from Sautter & Stepper GmbH (Ammerbruch, Germany) in the form of a soft cake and was used as a model organism for agricultural nematodes. Nematode suspensions were prepared in 250 mL of distilled water at 27 °C, compounds were dissolved in DMSO, and added to the nematode suspension, which was incubated at 27 °C. Viability readings were taken after 30 min and 24 h using a microscope (TR 200, VWR International, Belgium) at four-fold magnification. Each experiment was conducted in triplicate and on three separate occasions. In essence, the examination of each concentration of each compound was therefore carried out nine times in total.

ABBREVIATIONS

AS_4-1	3-(propyl tetrasulfanyl)-propanoic acid;
AS_4-2	3-(phenyl tetrasulfanyl)-propanoic acid;
DATS	diallyl trisulfane;
DATTS	diallyl tetrasulfane;
DBTTS	1,4-dibenzyltetrasulfane;
DPSTTS	3,3′-dipropanoic acid tetrasulfane;
DMSO	dimethyl sulfoxide;
GSSG	glutathione disulfide;
OSC	Organic Sulfur Compound;
S. feltiae	*Steinernema feltiae*

For the origin of the materials, the analytical methods used—including details of the nematode assay employed—and the analytical data for each compound synthesized (please consult the *Supplementary Materials*, available online).

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