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Antimicrobial garlic-derived diallyl polysulfanes: Interactions with biological thiols in *Bacillus subtilis*

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Abstract

Background: Diallylpolysulfanes are the key constituents of garlic oils, known to exhibit broad spectrum anticancer and antimicrobial activity. Studies in vitro, and in mammalian cells, have shown they react, via thiol-polysulfane exchange, with their major low molecular weight thiol, glutathione. However, there are no detailed reports of diallylpolysulfane effects on other common thiol metabolites (cysteine and coenzyme A) or major thiol cofactors (e.g. bacillithiol) that many Gram positive bacteria produce instead of glutathione. *Methods:* Diallylpolysulfanes were individually purified then screened for antimicrobial activity against *Bacillus subtilis*. Their impact on thiol metabolites (bacillithiol, cysteine, coenzyme A, protein thiols allyl thiols//persulfides) in *B. subtilis* cultures were analysed, by HPLC. *Results:* Diallylpolysulfane bioactivity increased with increasing chain length up to diallyltetrasulfane, but then plateaued. Within two minutes of treating *B. subtilis* with diallyltrisulfane or diallyltetrasulfane intracellular bacillithiol levels decreased by ~90%. Cysteine and CoA were also affected but to a lesser degree. This was accompanied by the accumulation of allyl thiol and allyl persulfide. A significant level of protein-*S*-allylation was also detected. *Conclusions:* In addition to the major low molecular weight thiol, diallylpolysulfanes can also have an impact on other thiol metabolites and protein thiols. *General Significance:* This study shows the rapid parallel impact of polysulfanes on different biological thiols inside *Bacillus subtilis* alongside the concomitant generation of allyl thiols and persulfides.

1. Introduction

Garlic oils are known to exhibit broad spectrum antimicrobial activitiy [1, 2, 3, 4, 5, 6]. When garlic is crushed the rapid conversion of alliin to allicin is catalysed by the enzyme alliinase (Fig. 1). Depending on preparation and storage conditions alliin can undergo numerous secondary reactions to produce complex mixtures of allyl-sulfur degradation products. Steam distillation of crude garlic extracts is used to obtain garlic oils, which are predominantly a mixture of diallylpolysulfanes containing one to six sulfur atoms (DAS1 – DAS6) (Fig. 1) with DAS2-3 being the major components [7, 8] Previous studies of DAS1 to DAS4 have shown that antimicrobial activity increases with increasing sulfur chain length [1, 2, 3, 9]. To date, there have been no reports on the relative bioactivities of the minor garlic oil components DAS5 and DAS6.

The key modes of diallylpolysulfane bioactivity are proposed to be initiated by their intracellular reactions with major low molecular weight thiol cofactors (ie. Glutathione (GSH) in eukaryotes and Gram-negative bacteria) to generate allyl thiol (ASH) and allyl persulfides (AS2H, AS3H) (Fig. 1) [5]. GSH plays an important role in oxidative stress management and maintaining an intracellular reducing environment. As a consequence, decreased concentrations of low molecular weight thiols (e.g. GSH) compromises the ability to defend against oxidative stresses whilst, in parallel, the generation of allyl persulfides (AS2H, AS3H) could elevate the production of reactive oxygen species (e.g. hydrogen peroxide, superoxide) thereby increasing oxidative stress [10, 11,12, 13, 14]. Further thiol reactions with allyl persulfides may also liberate hydrogen sulfide (H_2S) [15]. There is also scope for diallylpolysulfanes to perturb protein function via thiol-polysulfane exchange reactions with exposed cysteine residues [16, 17]. Previous studies in cancer cell lines have demonstrated up to 50% depletion of total cellular thiol content upon exposure to DAS4 [14]. However, to our knowledge there have been no reports on the impact of diallylpolysulfanes on intracellular depletion of individual low molecular weight thiols or the intracellular production of allyl thiol/persulfides. Instead of GSH, many Gram positive bacteria produce alternative, functionally equivalent, low molecular weight thiols such as mycothiol (actinomycetes) [18, 19] and bacillithiol (BSH) (firmicutes) [20, 21, 22]. Using *Bacillus subtilis* as a model BSH-utilising bacteria, we compare the antimicrobial activities of DAS1 to DAS6. Time profile studies of DAS3 and DAS4 effects on BSH, cysteine (Cys) and coenzyme A (CoA)) as well the protein thiol pool is also explored.

Abbreviations: Bacillithiol (BSH); Cysteine (Cys); Coenzyme A (CoA); Diallyl disulfide (DAS2); Diallyl trisulfide (DAS3); Diallyl tetrasulfide (DAS4); Diallyl pentasulfide (DAS5); Diallyl hexasulfide (DAS6); Allyl thiol (ASH); Allyl persulfide (AS2H); Allyl hydrotrisulfide (AS3H)

Figure 1. Diallylpolysulfanes found in garlic oils (DAS1-DAS6) and their potential mode(s) of action stimulated by reaction with cellular thiols. *Typical proportions of different diallylpolysulfanes found in garlic oils.

2. Materials and methods

2.1 General

BSH was chemically synthesised.[23] Thiol-S-bimane standards of BSH, GSH, Cys, CoA and H_2S were prepared as previously described [23, 24]. Analytical HPLC was conducted on a Jasco HPLC system equipped with, a JASCO FP-2020 fluorescence detector and a Jasco UV-1575 UV-VIS detector. Optical density (OD600) measurements were recorded on an ultraspec 10 Cell Densitymeter (Amersham Biosciences) or a Fluostar Omega microplate reader (Labtech). *B. subtilis* CU1065 wild-type and *bshA*⁻ mutant strains were generously provided by Prof. John Helmann (Cornell University) [25]. Both strains were cultivated in Belitsky minimal media (BMM) at 37 °C. Media for the *bshA*⁻ mutant also contained lincomycin (25 mg/L) and erythromycin (1 mg/L).

2.1 Preparation of DAS2-DAS6

Pure DAS2 was prepared by vacuum distillation of technical grade material (to remove DAS3 contaminants). DAS3-DAS6 were prepared by modification of a previously described method [26] (see supporting information).

2.2 HPLC analysis of DAS2 – DAS6

DAS2-DAS6 were separated by HPLC on a HiChrom ACE-AR C18 4.6 x 250 mm, 5 μm column, equilibrated at 37°C and UV detection at 210 nm. An isocratic solvent system (90% MeOH and 10% H_2O) was used at a flow-rate of 1.5 mL/min. DAS2 to DAS6 eluted at 3.6, 4.6, 5.9, 7.9 and 10.2 min respectively.

2.3 Determination of minimal inhibitory concentration (MIC) against B. subtilis (wild-type and bshA⁻) on agar

DAS1-DAS6 were tested against wild type *B. subtilis* and the *bshA-* mutant at 10 different 2-fold serial dilutions ranging from 1024 to 2 μg/mL. 100-fold concentrated stock solutions of DAS were prepared in 10% EtOH (DAS1-DAS6 were dissolved in 100% EtOH first, then diluted down to 100-fold concentrated stocks containing 10% EtOH). BMM agar was prepared (sterile mineral supplements and Tris were added to the warm agar after autoclaving) and 2970 μL of agar was added into each petri dish (3 cm diameter). 30 μL of the DAS stocks were added and mixed into the agar by stirring with a pipette tip. As controls 30 μL of 10% EtOH or 30 μL of sterile H_2O or 4 mg/L final concentration of chloramphenicol were added to the agar. Plates were prepared in triplicates for each compound. After the agar plates were dried, 4 μL of liquid bacterial culture (grown to OD600=1 in BMM) were dropped onto each plate and then incubated at 37°C for 16 h. The plates were observed for growth and the lowest concentration that inhibited the growth of the bacteria after 16 h was reported as the MIC.

2.4 Determination of MIC values against B. subtilis (wild-type and bshA⁻) in liquid media

DAS1-DAS6 were tested against *B. subtilis* at 10 different 2-fold serial dilutions ranging from 1024 to 2 μg/mL. Cultures, grown to an OD600=1 in BMM media were diluted 100-fold into fresh minimal media. 180 μL of diluted culture was then mixed with 20 μL of 10-fold concentrated stock solutions of diallylpolysulfanes (dissolved in 10% EtOH in water) in a 96 well microplate. Each concentration was tested in triplicate. The plates were sealed with parafilm and bacteria were grown at 37°C at 350 rpm for 16 h. Subsequently the OD600 was measured in a 96 well plate reader while shaking at 400 rpm and using 50 flashes per well. Each plate was measured twice in succession and averages of the readings were taken. OD600 values ≥ 0.1 were interpreted as growth and OD600 values <0.1 were interpreted as no growth.

2.5 Growth inhibition of DAS2-DAS6 against B. subtilis (wild-type and bshA⁻) in liquid media

Tested concentrations against *B.subtilis* wild type and *bshA*⁻ mutant (μM): DAS2: 750, 500, 250, 100; DAS3: 250, 200, 150, 100, 50; DAS4: 100, 50, 25; DAS5: 50, 25; DAS6: 50, 25. Bacterial cultures were grown at 37 °C in BMM. Once they had reached OD_{600}=0.5, samples of 100-fold concentrated stock solutions of different

diallylpolysulfanes in EtOH were added. To the untreated control cultures 1% EtOH (final concentration) was added. Growth was then periodically monitored by measuring optical density (OD_{600}).

2.6 Bimane derivatisation of low molecular weight thiols and DAS metabolites in B. subtilis [23, 24]

Bacteria were grown at 37 °C in BMM media. Aliquots sufficient to yield ~5 mg of residual dry cell weight were transferred into 50 mL falcon tubes at different time points and centrifuged for 3 min at 12,000 g at 4°C. The cell pellet was re-suspended, transferred into a pre-weighed Eppendorf tube and centrifuged again (12,000 g, 4 °C, 3 min).* The supernatant was discarded before freezing the cell pellet in liquid N_2 and storing at -20°C until subsequent derivatisation was performed. Frozen cell pellets were vortexed vigorously in 120 μL of monobromobimane (mBBr) mix (20 mM Hepes pH 8, 50% CH_3CN, 2 mM mBBr) and then incubated at 60°C for 15 min in the dark. After cooling on ice, $MeSO_3H$ was added to a final concentration of 25 mM and the reactions were vortexed and centrifuged at 12,000 g for 5 min. The supernatant was filtered through a 0.2 μm membrane, and then diluted five-fold with 10 mM $MeSO_3H$ prior to analysis by HPLC. The pellets were dried overnight at 60°C and weighed to determine their residual dry weight (rdw). Values for HPLC quantified thiol and diallylpolysulfane metabolites were converted to μmol RSH/g rdw. For *B. subtilis*, a thiol quantity of 1 μmol/g rdw equates to a cellular concentration of 1 mM. [27] ** For the first thiol samples that were collected immediately after diallylpolysulfane addition*, the cell pellets were frozen in the original 50 mL falcon tubes for storage. These frozen cell pellets were subsequently vortexed in mBBr mix before transferring into pre-weighed Eppendorf tubes and then further processed as described above.

2.7 HPLC analysis of the S-bimane derivatives of BSH (BSmB) and Cys (CySmB)

BSmB and CySmB were analysed by HPLC on a HiChrom ACE-AR C18 4.6 x 250 mm, 5 μm, 100Å column, equilibrated at 37 °C with solvent A (0.25% v/v acetic acid and 10% MeOH, adjusted to pH 4 with NaOH). Samples were eluted with a gradient of solvent B (90% MeOH) at a 1.2 mL/min flow rate as follows: 0-5 min, 0% solvent B; 5-15 min, 0-20% solvent B; and 15-20 min, 20-100% solvent B, followed by re-equilibration and reinjection. Eluting analytes were detected by fluorescence with excitation at 385 nm and emission at 460 nm. BSmB and CySmB eluted at 11.8 min and 14.3 min, respectively

2.8 HPLC analysis of the S-bimane derivatives of CoA (CoAmB), ASH (ASmB), AS2H (AS2mB) and AS3H (AS3mB)

S-bimane adducts were separated by HPLC on a Hichrom ACE C8 column (4x 150 mm, 5 μm) using solvent A (10 mM tetrabutylammonium phosphate (TBAP) in 90% H_2O, 10% MeOH, adjusted to pH 3.4 with acetic acid) and solvent B (10 mM TBAP in 90% MeOH, 10% H_2O, adjusted to pH 3.4 with acetic acid). Samples were eluted with a gradient of solvent B at 1 mL/min as follows: 0-16 min, 10-60% B; 16-19 min, 60-100% B; then re-equilibration and re-injection. Eluting analytes were detected by fluorescence with excitation at 385 nm and emission at 460 nm. Retention times were 16.2 min (ASmB), 17.9 min (CoAmB), 18.7 min (AS2mB) and 20.6 min (AS3mB). An alternative solvent gradient for ASmB and AS2mB analyses was also used as follows: 0-16 min, 35-60% B; 16-19 min, 60-100% B; then re-equilibration and re-injection. Under these conditions retention times were 10.0 min (ASmB) and 13.7 min (AS2mB).

2.9 HPLC analysis of allyl-S-glutathione mixed polysulfanes (AGS2-AGS4)

This was achieved using isocratic conditions with 40% solvent B (90% MeOH, 10% H_2O, containing 0.25% acetic acid adjusted to pH 4 with NaOH) and solvent A (0.25% acetic acid in H_2O, pH4) at 1.2 mL/min using a HiChrom ACE-AR C_{18} 4.6 x 250 mm, 5 μm column, equilibrated at 37°C and UV detection at 220 nm. The retention times for AGS2 to AGS4 were 4.7 min, 9.7 min and 24.5 min respectively.

2.10 Detection of ASH bound to proteins as protein-S-allyl mixed di/polysulfanes

Bacterial cultures were grown at 37 °C in BMM. Once they had reached OD_{600}=0.5, DAS3 (100 μM) or DAS4 (92 μM) were added. Aliquots of the cell culture (30 mL) were transferred into 50 mL Falcon tubes at different

time points and centrifuged (12,000 g, 3 min, 4°C). The cell pellet was re-suspended and transferred into an Eppendorf tube, centrifuged again, and the supernatant was discarded. The cell pellet was vortexed vigorously in 120 µL of mBBr mix (20 mM Hepes pH 8, 50% CH_3CN, 2 mM mBBr), incubated in the dark (15 min, 60°C), cooled on ice, and centrifuged (12,000 g, 5 min, 4°C). The cell pellet was incubated on ice for 90 min in lysis buffer (100 mM sodium phosphate, pH 7.4, 1 mM EDTA, 3 mg lysozyme, 75U benzoase, 5 mM *N*-ethylmaleimide). After centrifuging (12,000 g, 30 min) the supernatant was collected and the protein precipitated by treatment with an equal volume of 10% trichloroacetic acid (15 min, 0 °C). The precipitate was isolated by centrifugation (12,000 g, 10 min) and washed three times with 5% trichloroacetic acid. The pellet was re-suspended in 100 µL of Hepes pH 8, 50% CH_3CN, 2 mM dithiothreitol and incubated at 60 °C for 15 min. After centrifuging (12,000 g, 5 min) mBBr (6 mM) was added to the supernatant which was incubated in the dark (60 °C, 15 min). After cooling on ice, $MeSO_3H$ was added to a final concentration of 25 mM before filtering through a 0.2 µm membrane, and diluting five-fold (10 mM $MeSO_3H$) prior to analysis by HPLC.

3. Results

3.1 Stability of DAS3 – DAS6

Samples of DAS3, DAS4, DAS5 and DAS6, were stable for at least several months (by HPLC and ^{1}H-NMR) when stored as pure oils at -20 °C. To evaluate their stability in solution, freshly collected fractions of HPLC-purified DAS3-6 in aqueous HPLC grade methanol were stored at room temperature and periodically re-analysed (by HPLC) (Fig 2). Under these conditions their stability decreases with increased polysulfide chain length (i.e. DAS6 < DAS5 < DAS4 < DAS3). DAS3 was completely stable for at least seven days (data not shown). For DAS4-DAS6 the loss of the original polysulfide was accompanied by the formation of other longer and shorter diallylpolysulfane molecules. After 10 hours the 2% loss of DAS4 is accompanied by the formation of traces of DAS3 and DAS5-6 (Fig. 2A, inset). DAS5 is clearly less stable with 30% being lost in just 6 hours accompanied by the formation of DAS3-4 and DAS6 (Fig 2B). Almost 80% of DAS6 is lost within the first 3 hours accompanied by DAS3-5 and traces of DAS7 formation (Fig 2C).
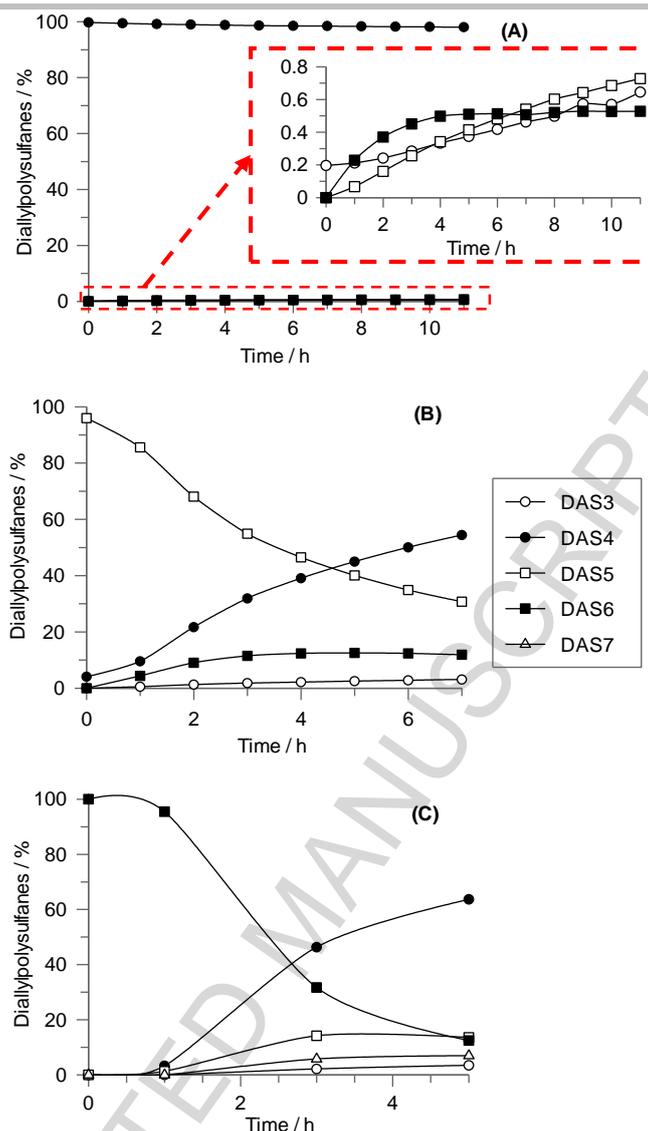
Figure 2. Stability profiles of (A) DAS4; (B) DAS5; (C) DAS6 in MeOH$_{(aq)}$ (90% vol.) at room temperature. Freshly collected fractions of HPLC-purified DAS3-6 were stored at room temperature and periodically re-analysed (by HPLC). (n=1)

These observed disproportionation reactions may proceed (albeit more slowly) in a similar manner to what occurs when DAS2 is reacted with liquid sulfur, at 120 ^{o}C, to prepare synthetic diallylpolysulfane mixtures [26]. Initial rearrangement to thiosulfoxide (**1**) (Scheme 1, step i) followed by nucleophilic reaction of (**1**) at any of the sulfur atoms on another diallylpolysulfane molecule would give an *S*-allylsulfonium intermediate (**3**) (step ii). Attack of the liberated hydropolysulfide (**2**) on the sulfonium allyl group of **3** would result in the initial disproportionation of two DAS4 molecules into DAS3 and DAS5 (Scheme 1, step iii). Such interconversions clearly proceed more rapidly with the longer (ie more reactive) diallylpolysufane chains whose internal sulfur-sulfur bonds are more labile [28]. In addition to the proposed thiosulfoxide mechanism (Scheme 1), traces of nucleophilic impurities in the HPLC solvents or on the surface of the glass storage vials could potentially contribute to the diallylpolysulfane isomerisation process [29].

When freshly purified samples of DAS3-6 were each incubated overnight with 10 mol% of GSH they all rearranged into complex mixtures of DAS2-6 (Fig. 3A) and mixed glutathione-*S*-allyl di-, tri- and tetra-sulfides (AGS2 – AGS4) (Fig. 3B). The presence of GSH likely accelerates the disproportionation process by the introduction of faster GSH-mediated thiol-polysulfide exchange mechanisms. This is particularly exemplified with DAS3, which doesn't rearrange in the absence of GSH. The formation of mixed di-, tri- and tetra-sulfide reaction products was also observed in all of the reaction mixtures. In the DAS3 reaction mixture, the formation

of the mixed tetrasulfide AGS4 must be the product of GSH reaction with the longer chain DAS4-6 molecules that are initially produced.
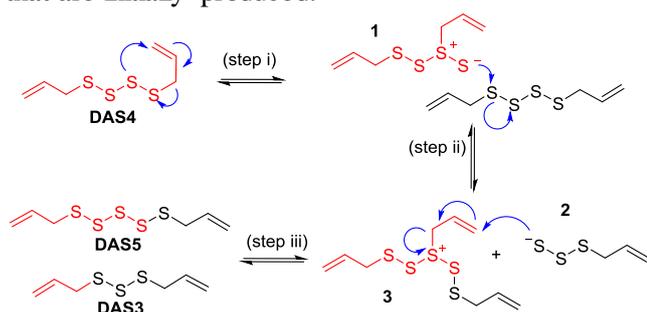
Scheme 1. Proposed mechanism for "disproportionation" of diallylpolysulfane molecules via a thiosulfoxide intermediate **1** (using DAS4 as an example)

Whatever the causes, DAS5 and DAS6 are clearly unlikely to remain as single molecular entities for the duration of any antimicrobial assays. This needs to be taken into consideration when evauliting their antimicrobial activity. To minimise thiol-mediated disproportionation of pure diallylpolysulfanes in the bioassays, all *B. subtilis* cultures were grown in Belitsky minimal media rather than LB media due to the variable thiol content of the latter, [30] (~35 uM by Ellman's assay (data not shown)).
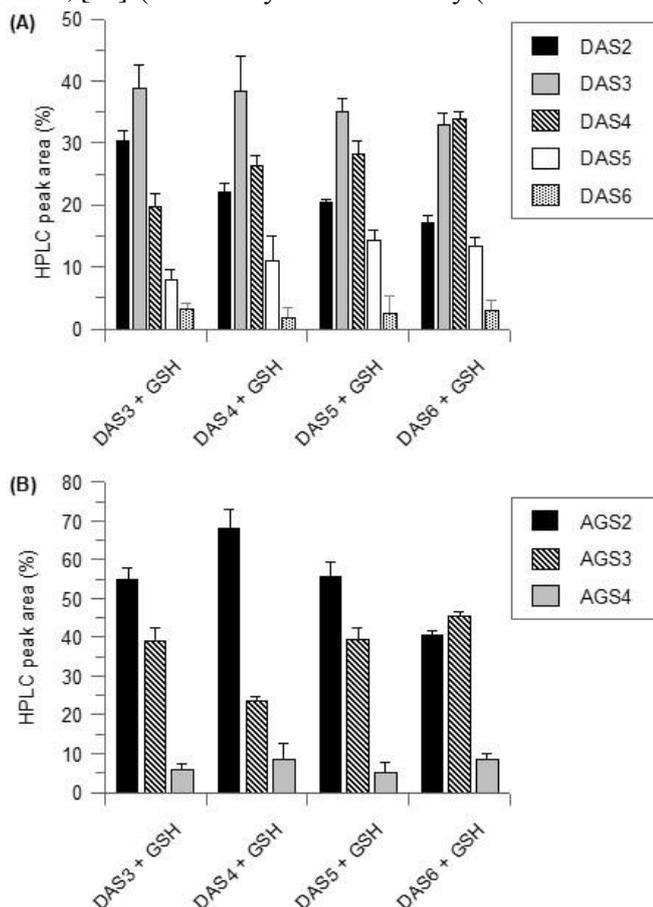
Figure 3. A: Relative distribution of different diallylpolysulfanes (DAS2-DAS6) and B: distribution of different glutathione-S-allyl mixed di-, tri-, and tetra-sulfanes (AGS2-AGS4) after overnight incubation of individual samples of DAS3 – DAS6 (100 mM) with GSH (10 mM) in Hepes buffer (25 mM, pH 8, 50% CH_3CN). The mean and standard deviation from two replicate experiments is shown.

3.2 DAS3-6 inhibition of B. subtilis growth and the influence of BSH

Instead of GSH, *B. subtilis* produces bacillithiol (BSH) as its major low molecular weight thiol [20], whose typical intracellular concentrations are 0.5-3 mM [31]. To explore the significance of BSH when *B. subtilis* are

exposed to diallylpolysulfanes, a BSH null-mutant (*bshA*⁻), deficient in the gene encoding first step in BSH biosynthesis has been knocked out, was also employed [25]. In both strains, growth inhibition studies on agar showed the efficacy increased with increasing polysulfane chain length, up to DAS5 (Table 1). DAS6 was less potent than DAS5, but still more effective than DAS4. The *bshA*⁻ mutant was more susceptible to DAS3-6 than the wild-type, suggesting that BSH is implicated in protection (or recovery) from diallylpolysulfane toxicity. Similar trends were observed when MIC measurements were carried out in liquid media (Table 1).

Table 1. MIC values for DAS1-DAS6 against *the B. subtilis* wild-type and the *bshA*⁻ mutant on agar and in liquid media. Experiments were carried out in triplicate.

Compound	MIC (µg/mL) (on agar)		MIC (µg/mL) (in minimal media)	
	wild-type	*bshA*⁻	wild-type	*bshA*⁻
DAS1	>1024	>1024	>1024	>1024
DAS2	>1024	>1024	>1024	>1024
DAS3	512-1024	256-512	>1024	1024
DAS4	32-64	16-32	32-64	16-32
DAS5	4-8	1-2	16-32	4-8
DAS6	16-32	2-4	16-32	4-8

Treatment of liquid cultures of wild-type *B. subtilis,* grown to early exponential phase, with DAS2-6 resulted in an immediate lag in growth whose recovery rate was concentration dependent (Fig. 4 and supporting information). The longer polysulfides were also the most potent (i.e.DAS6~DAS5~DAS4>DAS3>DAS2). Compared to the wild-type, the *bshA*⁻ mutant took at least twice as long to recover from the same levels of diallylpolysulfane exposure.
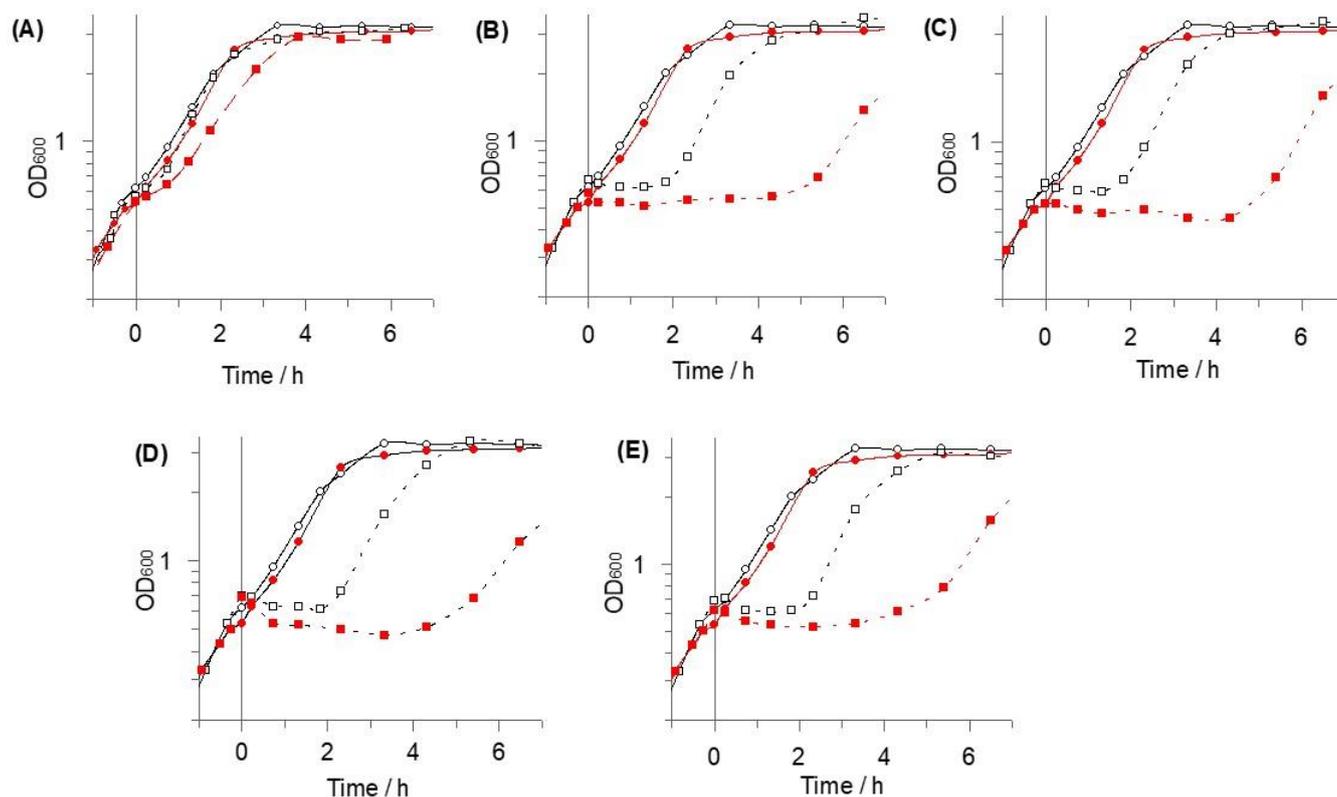
Figure 4. Growth comparison of *B. subtilis* wild-type and *bshA*⁻ mutant after treatment with (A) DAS2 (250 μM); (B) DAS3 (150 μM); (C) DAS4 (50 μM); (D) DAS5 (50 μM); (E) DAS6 (50 μM). DAS were added at OD_{600} ~0.5 (normalised to time = 0 for all graphs). All cultures contained 1% final concentration of ethanol and were grown in Belitsky Minimal Media. Key: Wild-type (black), *bshA*⁻ (red), normal growth (solid lines), growth with DAS2-DAS6 added (dotted lines)

3.3 Effects of DAS3 and DAS4 on cellular low molecular weight thiols

In light of the rapid rearrangement of pure DAS5 & DAS6 into complex diallylpolysulfane mixtures, subsequent studies were restricted to DAS3 and DAS4. In wild-type *B. subtilis* within two minutes of initial exposure to DAS3 (100 μM) the intracellular levels of BSH were almost fully depleted, whilst Cys levels decreased by ~50%, and the measured changes in CoA were not statistically significant (Fig. 5B-D). This was accompanied by the appearance of a 600 μM intracellular concentration of ASH (Fig. 5E). It is worth noting that these initial thiol metabolite analyses were performed immediately after addition of DAS3 into the media. The two minute time point reflects the time that the cells were exposed to DAS3 (during centrifugation) prior to trapping of the residual thiol pool by addition of monobromobimane to the cell pellet. Thirty five minutes post-exposure the BSH and Cys levels had recovered and the concentration of ASH had reduced to 250 μM. Despite the rapid recovery of low molecular weight thiol levels, bacterial growth did not resume until 2 hours after initial DAS3 exposure when the intracellular ASH and extracellular diallylpolysulfane levels were <10 μM (Fig. 5A and 5E).
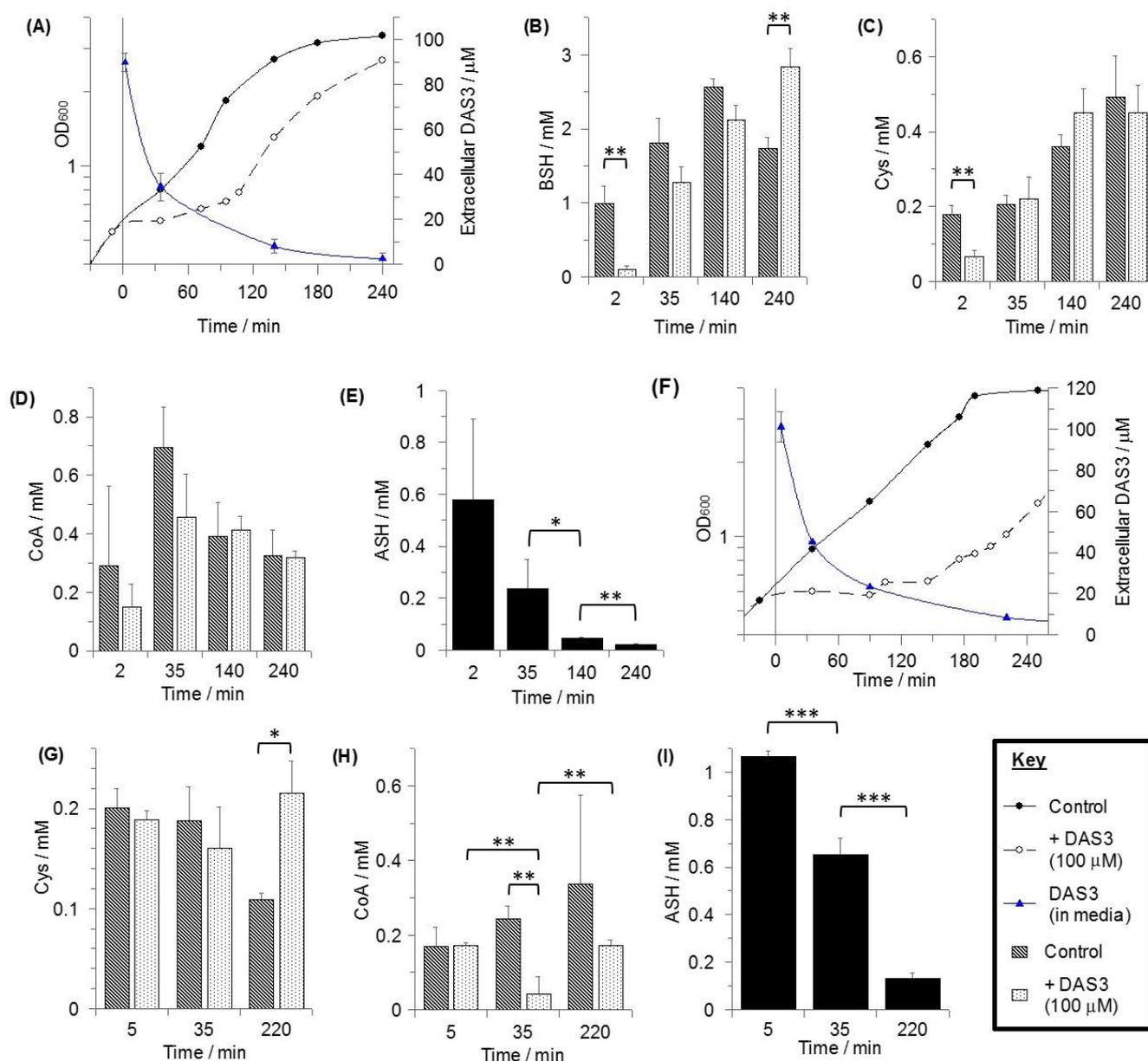
Figure 5. Thiol levels in wild-type (A-E) and BSH deficient (*bshA⁻*) *B. subtilis* (F-I) after treatment with DAS3 (100 μM). Wild-type: (A) Growth curves and extracellular DAS3; (B) BSH; (C) Cys; (D) CoA; (E) ASH. *bshA⁻* : (F) Growth curves and extracellular DAS3; (G) Cys; (H) CoA; (I) ASH. Triplicate cultures were split in two and DAS3 was added to half of them at OD_{600} = 0.6 (normalised to time = zero). Significant differences, calculated using the *t* test are indicated as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

In the *bshA⁻* mutant within the first few minutes of DAS3 addition, there was no notable depletion of Cys (Fig. 5G). CoA levels were initially unaffected, but a 3-4 fold depletion was observed at the 35 minute time point (Fig. 5H). The rates of extracellular DAS3 depletion and intracellular ASH formation/depletion were comparable in both the BSH deficient mutant and the wild type (Fig. 5E and 5I).

When the wild type was exposed to DAS4 BSH and Cys levels were initially depleted in a similar manner to that observed with DAS3 (Fig. 6B-C). In addition to the formation of ASH, high levels of allyl persulfide (AS2H) were also detected, but none of the hydrotrisulfide (AS3H) (Fig. 6E-F).
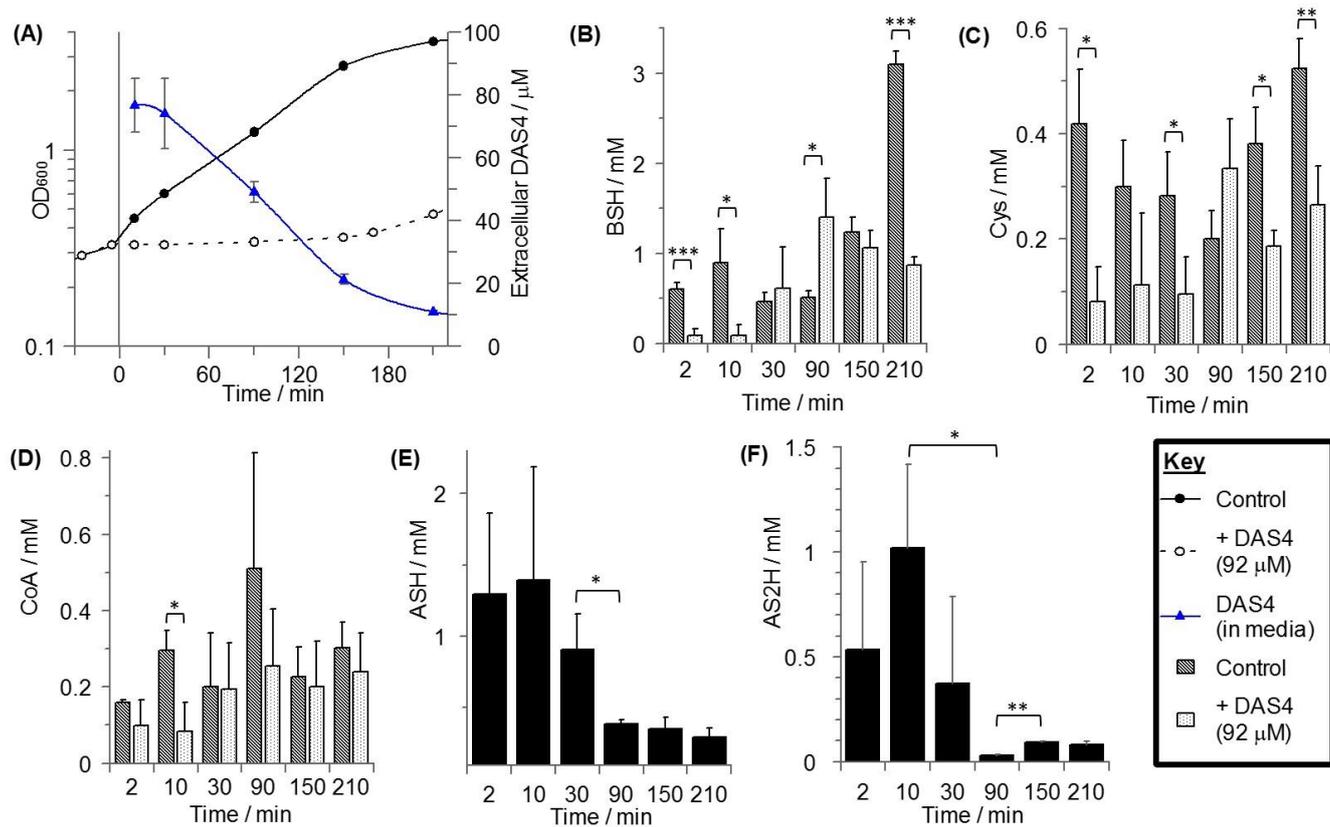
Figure 6. Thiol levels in wild-type *B. subtilis* after treatment with DAS4 (92 μM). (A) Growth curves and extracellular DAS4; (B) BSH; (C) Cys; (D) CoA; (E) ASH and AS2H. Triplicate cultures were split in two and DAS4 was added to half of them at OD_{600} = 0.35 (normalised to time = zero). The mean and standard deviation from three replicate experiments is shown. Significant differences, calculated using the *t* test are indicated as follows: * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001

3.4 Diallylpolysulfanes react with protein thiols

In the *bshA*[-] mutant, even in the absence of BSH, exposure to DAS3 showed comarable levels of ASH production to those seen in the wild-type (Fig. 5I and 5E). Accessible protein thiols could also potentially react with diallylpolysulfanes and contribute towards ASH/AS2H generation. In recent years the role of protein thiols contributing to the intracellular thiol redox pool has become apparent [32] with protein thiols representing up to 70% of the reduced thiol equivalents in *B. Subtilis* [33]. The formation of any protein-S-allyl mixed di/polysulfides was therefore investigated. After initial treatment with DAS3 or DAS4, the soluble protein content was isolated from *B. Subtilis* lysates under under non-reducing conditions. These were subsequently treated with dithiothreitol followed by bimane labelling and HPLC analysis of any liberated low molecular weight thiols. Under these conditions, within minutes of wild-type *B. Subtilis* being exposed to DAS3 or DAS4, ASH was detected, but not any BSH, Cys or CoA (Fig. 7).
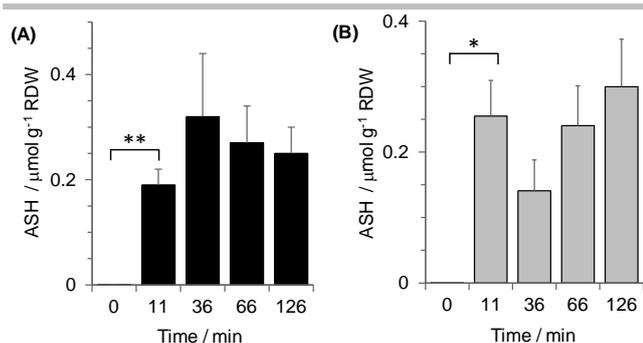
Figure 7. ASH liberated from proteins in wild-type *B. subtilis* after exposure to DAS3 (100 μM) or DAS4 (92 μM). RDW = Residual Dry Weight. The mean and standard deviation from two replicate experiments is shown. Significant differences, calculated using the *t* test are indicated as follows: * $P < 0.05$; ** $P < 0.01$.

4. Discussion

The correlation of increased antibacterial activity with increased diallylpolysulfane chain length (up to DAS4) is in agreement with what has previously been reported [1, 2, 9]. The results herein further extend this correlation to show that it plateaus around DAS5 (Table 1, Fig. 4). This could be due to decreased stability of longer chain polysulfanes for which there is a notable transition between DAS4 and DAS5-6 (Fig. 2).

In the absence of BSH (which facilitates redox homeostasis in *B. subtilis*) [21], the increased sensitivity to DAS3-6 suggests that BSH plays an important (but not critical) role in the recovery from diallylpolysulfane stress (Table 1, Fig. 4). However when BSH is present it may also contribute to the thiol-polysulfane exchange reactions that initiate diallylpolysulfane stress (Fig. 5, Fig. 6). Within two minutes of exposure to DAS3 and DAS4 the substantial depletion of BSH accompanied by the appearance of ASH and AS2H reflects the facile uptake of these molecules into *B. subtilis* and their immediate impact on BSH as well as a lesser effect on the depletion of Cys and CoA (Fig. 5, Fig. 6). Compared to Cys (pK_s = 8.38) and CoA (pK_s = 9.83), the microscopic thiol pKa value for BSH is more acidic (pK_s = 7.97) [31]. This means that in *B. subtilis*, with an intracellular pH of 7.7, the thiolate concentrations of BSH are at least an order of magnitude greater than those of Cys or CoA [31]. This could account for its preferential consumption upon exposure to diallylpolysulfanes. BSH depletion could compromise the bacteria's ability to cope with oxidative stress. The impact of diallylpolysulfanes on cellular thiol levels evidently differs in different organisms. For example, previous studies of HCT116 colon cancer cell lines only showed a 20% depletion of total soluble thiol content after 5 minutes exposure to 40 μM DAS4 which gradually increased to 50% after 2 hours. This may reflect different rates of uptake in different cell types [14].

Protein thiols also appear to contribute towards ASH and AS2H generation via reaction with diallylpolysulfanes to form *S*-allylated proteins (Fig. 7). Recent proteomic studies have identified a number of high abundant proteins involved in primary metabolism, which are *S*-allylated when *E. coli* is challenged with the diallylpolysulfane precursor allicin (Fig. 1) [34]. In future it will be interesting to identify the protein targets of DAS3 and DAS4 in *B. subtilis*.

The initially depleted low molecular weight thiol levels all recover within 30 minutes of exposure to DAS3 or DAS4. This could be due to their enzyme catalysed regeneration by disulfide reductases or their regeneration from *de novo* biosynthesis. Previous *B. subtilis* transcriptome studies have shown that the genes for the biosynthesis of Cys (*cysK*) and BSH (*bshA-C*) as well as two bacilliredoxins (*yphB, yqiW*) are all upregulated within 5 minutes of exposure to disulfide stress (induced by 0.5 mM diamide) [35]. Following diallylpolysulfide treatment, the recovery of these thiols does not coincide with recovery of growth, which is delayed for much longer. This could be due to prolonged effects on *S*-allylated proteins. Bacilliredoxins, which enzymatically reduce *S*-bacillithiolated proteins [36, 37], are unlikely to work as effectively with *S*-allyl protein substrates.
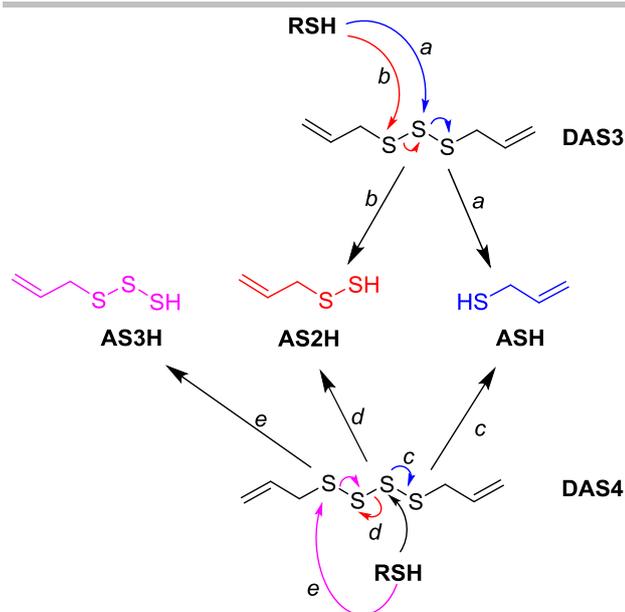
Scheme 2. Thiol-polysulfane exchange reaction pathways for generation of ASH, AS2H and AS3H from DAS3 and DAS4. Labels a-e refer to different thiol-polysulfane exchange reaction pathways that can occur.

A key difference between DAS3 and DAS4 treatments is the observed formation of ASH and AS2H with DAS4 (Fig. 6), but only ASH with DAS3 (Fig. 5). ASH can be generated by nucleophilic attack of the cellular thiol at the central S-atom of DAS3 (Scheme 2, path-a), whereas reaction at either of the peripheral S-atoms would generate AS2H (Scheme 2, path-b). Previous computational calculations predict that thiol-polysulfane exchange reactions at the peripheral S-atoms of DAS3 are kinetically more favourable [38]. However, only ASH production is observed in *B. subtilis* when exposed to DAS3. It could be that these computational studies do not reflect what occurs in the intracellular media of *B. subtilis*. Alternatively, any AS2H that is initially generated from DAS3 might be rapidly degraded to ASH via further thiol persulfide exchange reactions. Nucleophilic attack at either of the central S-atoms of DAS4 could generate both ASH and AS2H (Scheme 2, paths-c and d), whereas attack at the peripheral S-atoms would generate AS3H. The accumulation of AS2H with DAS4 treatment could be due to enhanced reactivity of DAS4 leading to rapid AS2H formation, so that it initially accumulates at a faster rate than it can be depleted. The potential for persulfides to further induce oxidative stress [13, 14] may contribute towards the greater biological activity of DAS4.

Interestingly, no hydrogen sulfide (H_2S) formation was observed following exposure to DAS3 and DAS4 even though its formation would be expected from thiol persulfide exchange reactions with any AS2H generated *in situ* [15]. A previous study has reported accumulation of intracellular H_2S in breast cancer MCF cells when treated with 100 μM DAS3 [15]. However, in another study no intracellular H_2S was detected in Human Embryo Kidney (HEK 293) cells exposed to 100 μM DAS3 unless the media was supplemented with Cys or GSH [39]. The authors suggested that the lack of H_2S accumulation could be due to the slow uptake of DAS3 such that H_2S metabolism is able to keep pace with the rate of GSH mediated H_2S formation, and that extracellular H_2S produced when there are thiols in the media diffuses into the cells and accumulates at a much faster rate. This proposition is further supported by another independent study where blocking of the exofacial thiols of red blood cells prior to treatment with garlic extracts lowered intracellular H_2S production by 75% [40]. The studies herein, using thiol-free minimal media, showed that, in *B. subtilis,* uptake of DAS3 and DAS4, and generation of ASH and AS2H occurs within minutes. This suggests that the rate of H_2S metabolism in *B. subtilis* maintains levels below the detection limits of these experiments.

For such structurally simple molecules their modes of action are likely to be very complex, as well as variable, across different organisms. The underpinning explanations for their modes of action via reactions with cellular thiols to generate persulfide species is primarily based upon *in vitro* experiments, and single time-point measurements of GSH, or timecourse measurement of total cellular thiol, depletion in mammalian cells. Herein, the concerted effects of diallylpolysulfanes on different biothiol sources inside an organism alongside the

intracellular generation of ASH and AS2H has been demonstrated. These thiol metabolite studies could serve as an initial benchmark against which the effects of diallylpolysulfanes on thiol metabolites in other (micro)organisms can be compared.

Conflict of interests

The authors declare that they have no conflicts of interests with the contents of this article

[1] S.M. Tsao, M.C. Yin, *In vitro* activity of garlic oil and four diallyl sulphides against antibiotic-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, J. Antimicrob. Chemother. 47 (2001) 665-670.

[2] E.A. O'Gara, D.J. Hill, D.J. Maslin, Activities of garlic oil, garlic powder, and their diallyl constituents against *Helicobacter pylori*, Appl. Environ. Microbiol. 66 (2000) 2269-2273.

[3] P. Rattanachaikunsopon, P. Phumkhachorn, Diallyl Sulfide Content and, Antimicrobial Activity against Food-Borne Pathogenic Bacteria of Chives (*Allium schoenoprasum*), Biosci. Biotech. Biochem. 72 (2008) 2987-2991.

[4] Z.M. Ross, E.A. O'Gara, D.J. Hill, H.V. Sleightholme, D.J. Maslin, Antimicrobial Properties of Garlic Oil against Human Enteric Bacteria: Evaluation of Methodologies and Comparisons with Garlic Oil Sulfides and Garlic Powder, Appl. Environ. Microbiol. 67 (2001) 475-480.

[5] U. Munchberg, A. Anwar, S. Mecklenburg, C. Jacob, Polysulfides as biologically active ingredients of garlic, Org. Biomol. Chem. 5 (2007) 1505-1518.

[6] A. Anwar, E. Gould, R. Tinson, J. Iqbal, C. Hamilton, Redox Modulation at Work: Natural Phytoprotective Polysulfanes From Alliums Based on Redox-Active Sulfur, Curr. Pharmacol Rep. 4 (2018) 397-407.

[7] A.G. Hile, Z. Shan, S.-Z. Zhang, E. Block, Aversion of European Starlings (Sturnus vulgaris) to Garlic Oil Treated Granules: Garlic Oil as an Avian Repellent. Garlic Oil Analysis by Nuclear Magnetic Resonance Spectroscopy, J. Agri. Food Chem. 52 (2004) 2192-2196.

[8] P. Satyal, J. Craft, N. Dosoky, W. Setzer, The Chemical Compositions of the Volatile Oils of Garlic (Allium sativum) and Wild Garlic (Allium vineale), Foods, 6 (2017) 63.

[9] S.M. Tsao, M.C. Yin, *In-vitro* antimicrobial activity of four diallyl sulphides occurring naturally in garlic and Chinese leek oils, J. Med. Microbiol. 50 (2001) 646-649.

[10] T. Chatterji, K. Keerthi, K.S. Gates, Generation of reactive oxygen species by a persulfide (BnSSH), Bioorg. Med. Chem. Lett. 15 (2005) 3921-3924.

[11] C. Jacob, A. Anwar, The chemistry behind redox regulation with a focus on sulphur redox systems, Physiol. Planta. 133 (2008) 469-480.

[12] A. Anwar, T. Burkholz, C. Scherer, M. Abbas, C.-M. Lehr, M. Diederich, C. Jacob, N. Daum, Naturally occurring reactive sulfur species, their activity against Caco-2 cells, and possible modes of biochemical action (vol 28, pg 251, 2008), J. Sulf. Chem. 29 (2008) 573-573.

[13] R. Munday, J.S. Munday, C.M. Munday, Comparative effects of mono-, di-, tri-, and tetrasulfides derived from plants of the allium family: Redox cycling in vitro and hemolytic activity and phase 2 enzyme induction in vivo, Free. Rad. Biol. Med. 34 (2003) 1200-1211.

[14] N.E.B. Saidu, R. Touma, I.A. Asali, C. Jacob, M. Montenarh, Diallyl tetrasulfane activates both the eIF2α and Nrf2/HO-1 pathways, Biochim. Biophys. Acta General, 1830 (2013) 2214-2225.

[15] D. Liang, H. Wu, M.W. Wong, D. Huang, Diallyl Trisulfide Is a Fast H2S Donor, but Diallyl Disulfide Is a Slow One: The Reaction Pathways and Intermediates of Glutathione with Polysulfides, Org. Lett. 17 (2015) 4196-4199.

[16] T. Hosono, T. Fukao, J. Ogihara, Y. Ito, H. Shiba, T. Seki, T. Ariga, Diallyl Trisulfide Suppresses the Proliferation and Induces Apoptosis of Human Colon Cancer Cells through Oxidative Modification of β-Tubulin, J. Biol. Chem. 280 (2005) 41487-41493.

[17] S. Kim, H.G. Lee, S.A. Park, J.K. Kundu, Y.S. Keum, Y.N. Cha, H.K. Na, Y.J. Surh, Keap1 Cysteine 288 as a Potential Target for Diallyl Trisulfide-Induced Nrf2 Activation, PLoS One, 9 (2014) 12.

[18] V.K. Jothivasan, C.J. Hamilton, Mycothiol: synthesis, biosynthesis and biological functions of the major low molecular weight thiol in actinomycetes, Natural Product Reports, 25 (2008) 1091-1117.

[19] R.C. Fahey, Glutathione analogs in prokaryotes, Biochim. Biophys. Acta General. 1830 (2013) 3182-3198.

[20] G.L. Newton, M. Rawat, J.J. La Clair, V.K. Jothivasan, T. Budiarto, C.J. Hamilton, A. Claiborne, J.D. Helmann, R.C. Fahey, Bacillithiol is an antioxidant thiol produced in Bacilli, Nat Chem. Biol. 5 (2009) 625-627.

[21] P. Chandrangsu, V.V. Loi, H. Antelmann, J.D. Helmann, The Role of Bacillithiol in Gram-Positive Firmicutes, Antiox. Redox Signal. 28 (2018) 445-462.

[22] V.R. Perera, G.L. Newton, K. Pogliano, Bacillithiol: a key protective thiol in Staphylococcus aureus, Exp. Re. Anti Inf. Ther, 13 (2015) 1089-1107.

[23] S.V. Sharma, V.K. Jothivasan, G.L. Newton, H. Upton, J.I. Wakabayashi, M.G. Kane, A.A. Roberts, M. Rawat, J.J. La Clair, C.J. Hamilton, Chemical and Chemoenzymatic Syntheses of Bacillithiol: A Unique Low-Molecular-Weight Thiol amongst Low G+C Gram-Positive Bacteria, Angew. Chem. Int. Ed. 50 (2011) 7101-7104.

[24] R.C. Fahey, G.L. Newton, B.J. William, W.G. Owen, Determination of low-molecular-weight thiols using monobromobimane fluorescent labeling and high-performance liquid chromatography, Methods Enzymol. Academic Press, Place Published, 1987, pp. 85-96.

[25] A. Gaballa, G.L. Newton, H. Antelmann, D. Parsonage, H. Upton, M. Rawat, A. Claiborne, R.C. Fahey, J.D. Helmann, Biosynthesis and functions of bacillithiol, a major low-molecular-weight thiol in Bacilli, Proc. Nat Acad. Sci. 107 (2010) 6482-6486.

[26] K. Wang, M. Groom, R. Sheridan, S. Zhang, E. Block, Liquid sulfur as a reagent: synthesis of polysulfanes with 20 or more sulfur atoms with characterization by UPLC-(Ag^+)-coordination ion spray-MS, J. Sulf. Chem. 34 (2013) 55-66.

[27] G. Bratbak, I. Dundas, Bacterial dry matter content and biomass estimations, Appl. Environ. Microbiol. 48 (1984) 755-757.

[28] T.L. Pickering, K.J. Saunders, A.V. Tobolsky, Disproportionation of organic polysulfides, J. Am. Chem. Soc. 89 (1967) 2364-2367.

[29] R. Steudel, The Chemistry of Organic Polysulfanes R−Sn−R (n > 2), Chem. Rev. 102 (2002) 3905-3946.

[30] G. Sezonov, D. Joseleau-Petit, R. D'Ari, *Escherichia coli* Physiology in Luria-Bertani Broth, J. Bacteriol. 189 (2007) 8746-8749.

[31] S.V. Sharma, M. Arbach, A.A. Roberts, C.J. Macdonald, M. Groom, C.J. Hamilton, Biophysical Features of Bacillithiol, the Glutathione Surrogate of Bacillus subtilis and other Firmicutes, Chembiochem, 14 (2013) 2160-2168.

[32] R.E. Hansen, D. Roth, J.R. Winther, Quantifying the global cellular thiol–disulfide status, Proc. Nat Acad. Sci. 106 (2009) 422-427.

[33] D.-C. Pother, M. Liebeke, F. Hochgrafe, H. Antelmann, D. Becher, M. Lalk, U. Lindequist, I. Borovok, G. Cohen, Y. Aharonowitz, M. Hecker, Diamide Triggers Mainly S Thiolations in the Cytoplasmic Proteomes of Bacillus subtilis and Staphylococcus aureus, J. Bacteriol., 191 (2009) 7520-7530.

[34] A. Müller, J. Eller, F. Albrecht, P. Prochnow, K. Kuhlmann, J.E. Bandow, A.J. Slusarenko, L.I.O. Leichert, Allicin Induces Thiol Stress in Bacteria through S-Allylmercapto Modification of Protein Cysteines, J. Biol. Chem. 291 (2016) 11477-11490.

[35] P. Nicolas, U. Mader, E. Dervyn, T. Rochat, A. Leduc, N. Pigeonneau, E. Bidnenko, E. Marchadier, M. Hoebeke, S. Aymerich, D. Becher, P. Bisicchia, E. Botella, O. Delumeau, G. Doherty, E.L. Denham, M.J. Fogg, V. Fromion, A. Goelzer, A. Hansen, E. Haertig, C.R. Harwood, G. Homuth, H. Jarmer, M. Jules, E. Klipp, L. Le Chat, F. Lecointe, P. Lewis, W. Liebermeister, A. March, R.A.T. Mars, P. Nannapaneni, D. Noone, S. Pohl, B. Rinn, F. Rugheimer, P.K. Sappa, F. Samson, M. Schaffer, B. Schwikowski, L. Steil, J. Stulke, T. Wiegert, K.M. Devine, A.J. Wilkinson, J.M. van Dijl, M. Hecker, U. Volker, P. Bessieres, P. Noirot, Condition-Dependent Transcriptome Reveals High-Level

Regulatory Architecture in Bacillus subtilis, Science, 335 (2012) 1103-1106.

[36] A. Gaballa, B.K. Chi, A.A. Roberts, D. Becher, C.J. Hamilton, H. Antelmann, J.D. Helmann, Redox regulation in Bacillus subtilis: The bacilliredoxins BrxA(YphP) and BrxB(YqiW) function in de-bacillithiolation of S-bacillithiolated OhrR and MetE, Antiox. Redox Signal. 21 (2014) 357-367.

[37] M. Imber, N.T.T. Huyen, A.J. Pietrzyk-Brzezinska, V.V. Loi, M. Hillion, J. Bernhardt, L. Tharichen, K. Kolsek, M. Saleh, C.J. Hamilton, L. Adrian, F. Grater, M.C. Wahl, H. Antelmann, Protein S-Bacillithiolation Functions in Thiol Protection and Redox Regulation of the Glyceraldehyde-3-Phosphate Dehydrogenase Gap in Staphylococcus aureus Under Hypochlorite Stress, Antiox. Redox Signal. 28 (2018) 410-430.

[38] Y.-R. Cai, C.-H. Hu, Computational Study of H2S Release in Reactions of Diallyl Polysulfides with Thiols, J. Phys. Chem. B. 121 (2017) 6359-6366.

[39] E.R. DeLeon, Y. Gao, E. Huang, K.R. Olson, Garlic oil polysulfides: H2S- and O2-independent prooxidants in buffer and antioxidants in cells, Am. J. Physiol. Regul. Integr. Comp. Physiol. 310 (2016) R1212-R1225.

[40] G.A. Benavides, G.L. Squadrito, R.W. Mills, H.D. Patel, T.S. Isbell, R.P. Patel, V.M. Darley-Usmar, J.E. Doeller, D.W. Kraus, Hydrogen sulfide mediates the vasoactivity of garlic, Proc. Nat Acad. Sci. 104 (2007) 17977-17982.

Highlights

1 Antibacterial activity of diallylpolysulfanes peaks at the diallylpentasulfide
2 Diallylpolysulfanes rapidly deplete bacillithiol in Bacillus subtilis.
3 Intracellular allyl thiol and allylpersulfide is also produced.
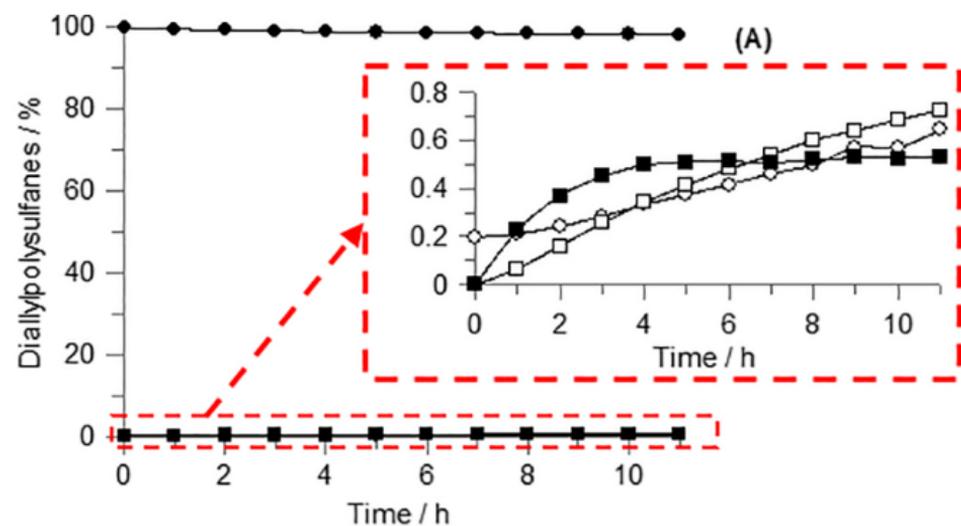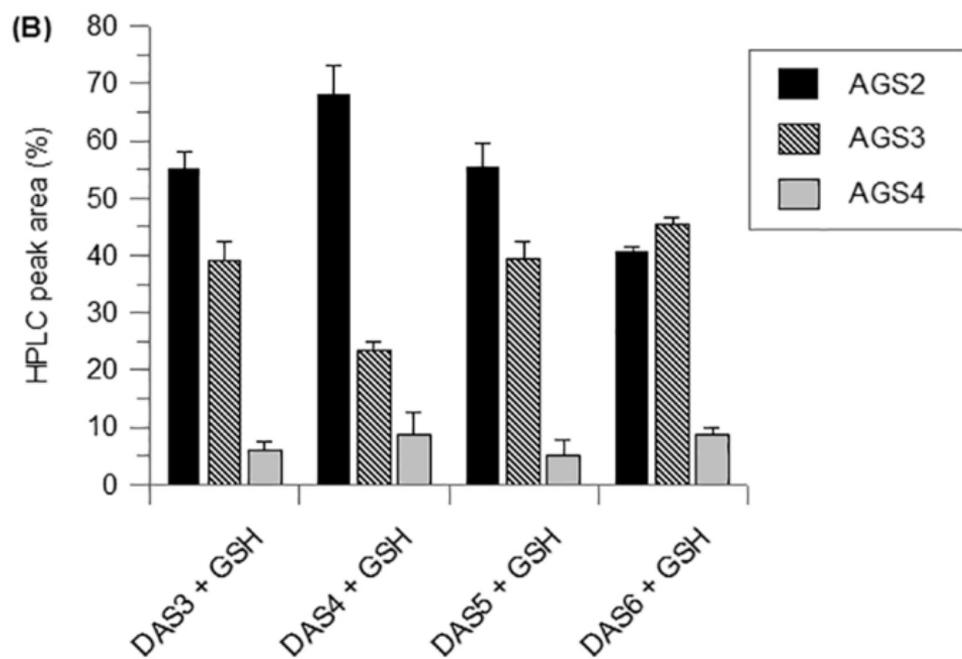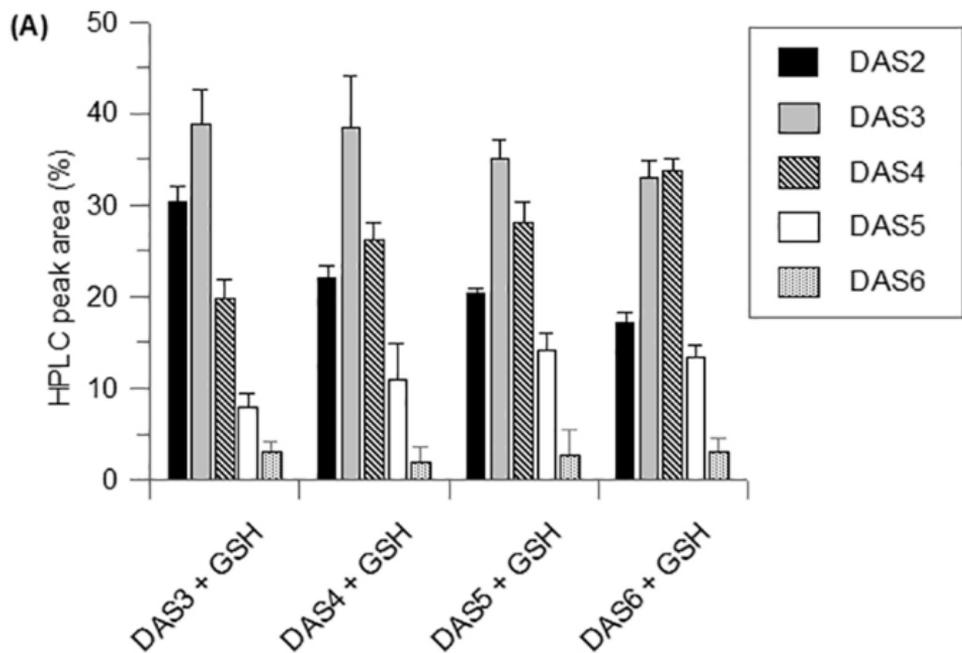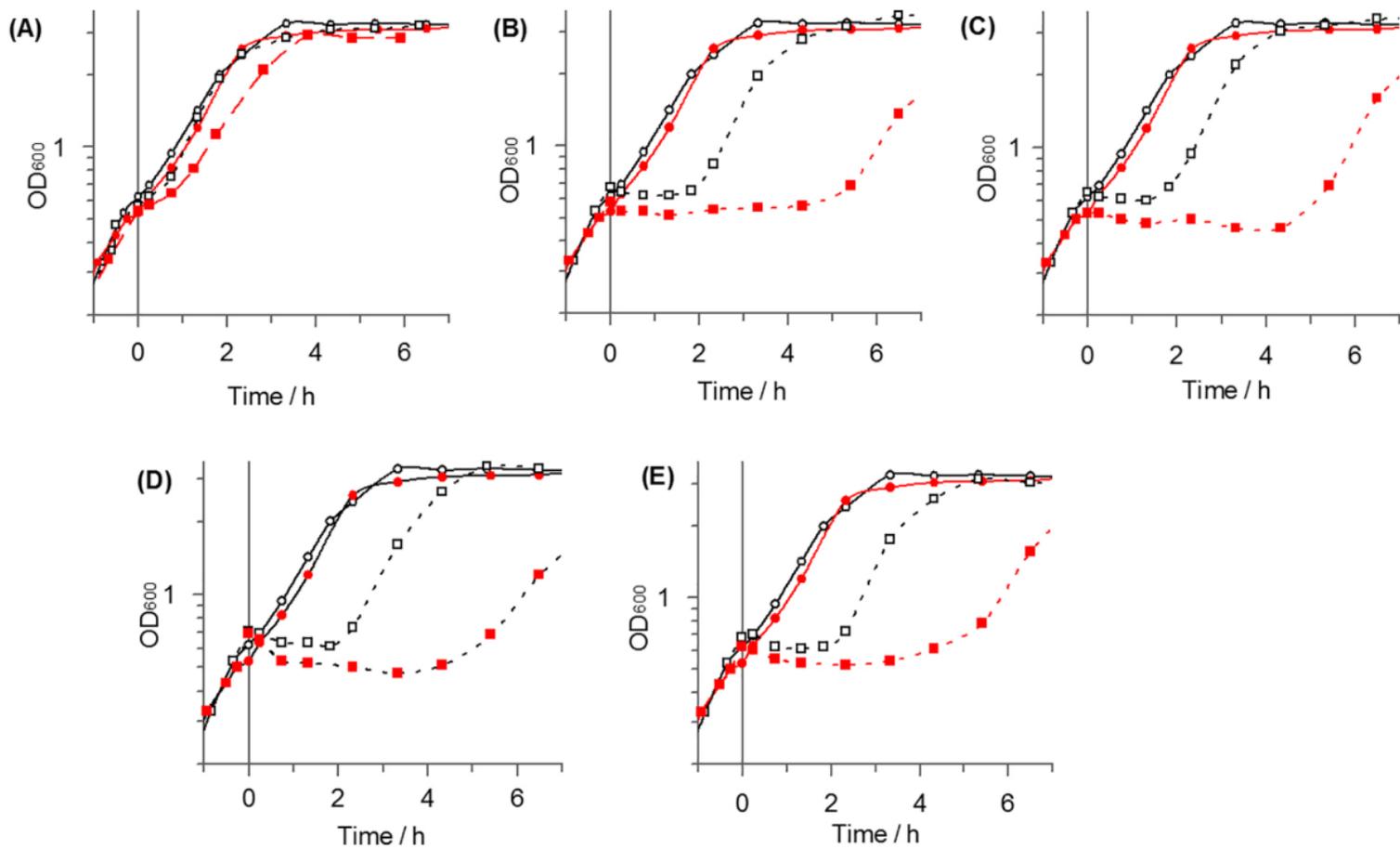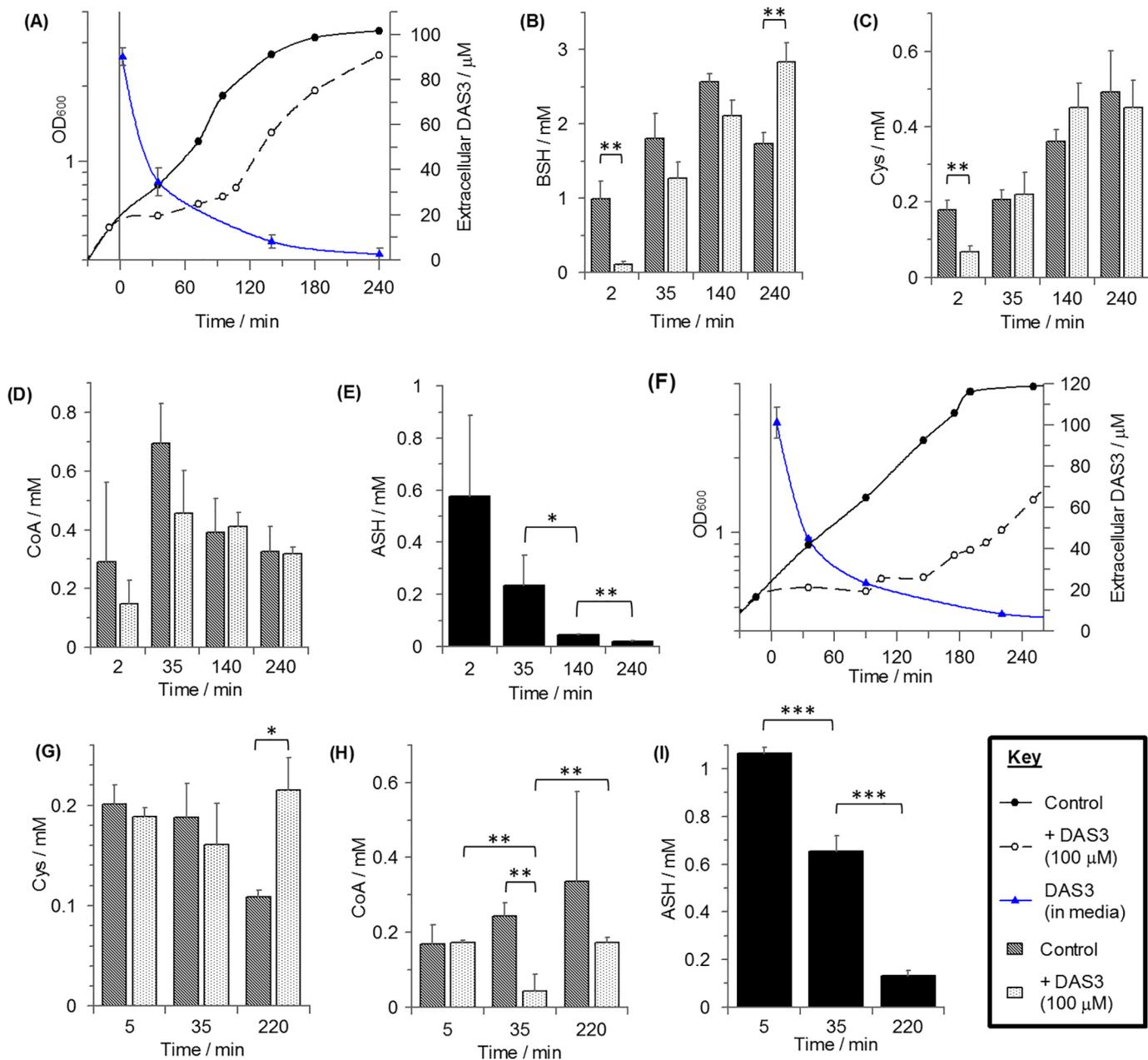4 Protein thiols also appear to be modified by diallylpolysulfanes
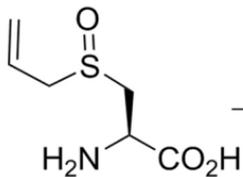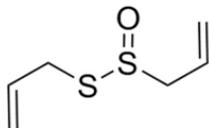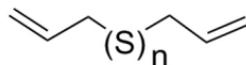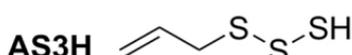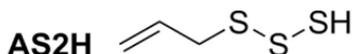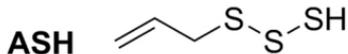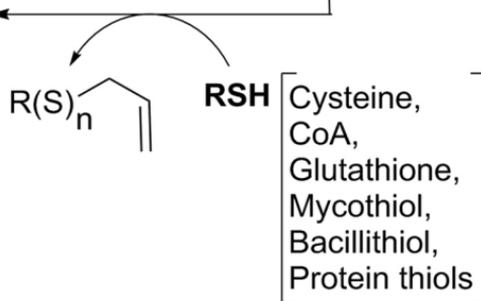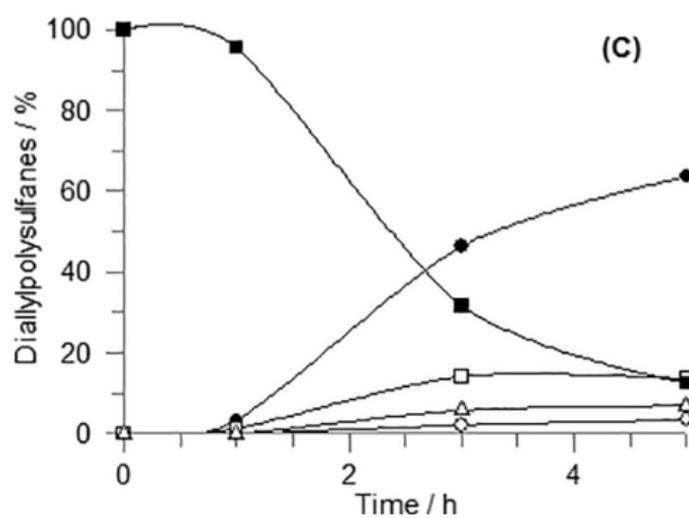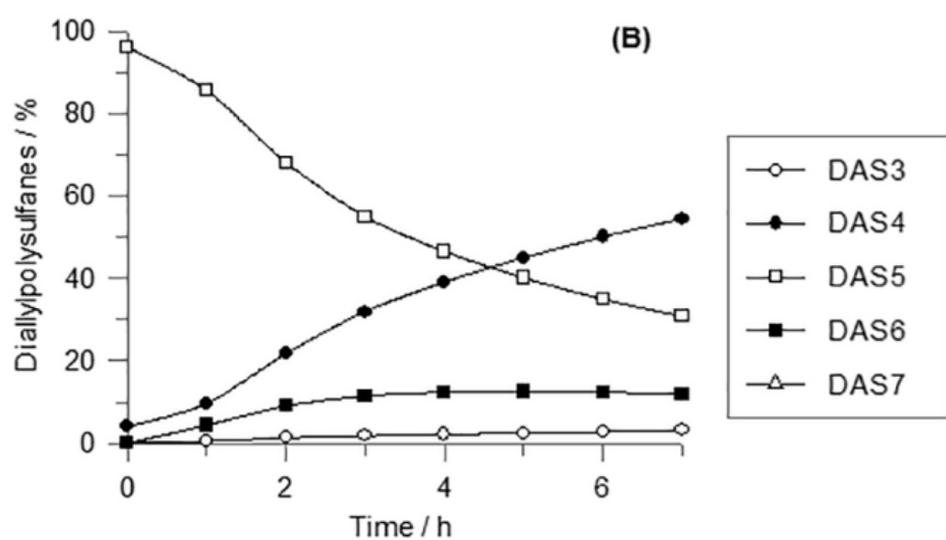
Figure 1

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