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Diallyl Trisulfide Is a Fast H_2S Donor, but Diallyl Disulfide Is a Slow One: The Reaction Pathways and Intermediates of Glutathione with Polysulfides

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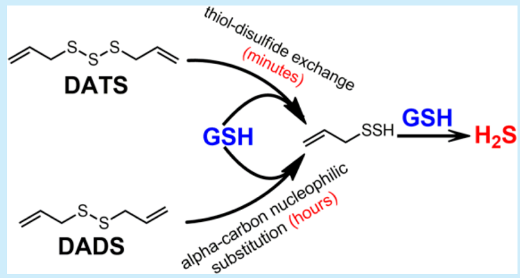
Supporting Information

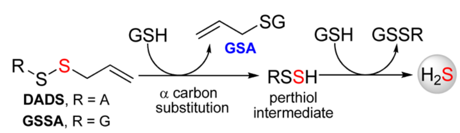
ABSTRACT: Diallyl trisulfide (DATS) reacts rapidly with glutathione (GSH) to release H_2S through thiol−disulfide exchange followed by allyl perthiol reduction by GSH. Yet diallyl disulfide (DADS) only releases a minute amount of H_2S via a sluggish reaction with GSH through an α-carbon nucleophilic substitution pathway. The results clarify the misunderstanding of DADS as a rapid H_2S donor, which is attributed to its DATS impurity.



H ydrogen sulfide (H_2S) is a gaseous signaling molecule that exerts important regulatory functions in cardiovascular, immune, gastrointestinal, endocrine, and nervous systems.[1] In mammalian tissues, H_2S is produced endogenously by four enzymes including cystathionine γ-lyase (CSE, EC 4.4.1.1), cystathionine β-synthase (CBS, EC 4.21.22), 3-mercapto-pyruvate sulfurtransferase (3-MST, EC 2.8.1.2), and cysteine aminotransferase (CAT, EC 2.6.1.3).[2] Because of their therapeutic potentials, a large number of H_2S donors have been reported.[3]

Diallyl trisulfide (DATS) and diallyl disulfide (DADS) are the two major organosulfides in garlic oil.[4] They are produced from the decomposition of allicin and have been studied extensively for their health benefits.[5] In 2007 Benavides and co-workers reported that DATS and DADS are donors of H_2S.[6] They showed that DATS and DADS could be converted into H_2S by human red blood cells or by rat aorta through a thiol, mainly glutathione (GSH), dependent mechanism. Moreover, the H_2S produced was able to relax rat aorta rings. Based on their observation that both DADS and DATS rapidly released H_2S when mixed with GSH, an α-carbon nucleophilic substitution mechanism was proposed to explain the rapid H_2S generation from DADS (Scheme 1). First, DADS reacts with GSH to generate S-allyl glutathione (GSA) and the key intermediate allyl perthiol (ASSH), which quickly reacts with GSH to produce *S*-allyl glutathione disulfide (GSSA) and H_2S. Furthermore, it was purposed that GSSA also could undergo α-carbon nucleophilic substitution and generate H_2S rapidly.[6] This mechanism has been widely accepted.[7] However, we have found and reported herein that DADS is not a rapid H_2S donor.

Scheme 1[a]



[a]A = allyl.

Addition of DATS (50 μM) to GSH (10-fold excess) solution led to an instant production of H_2S (Figure 1A). In contrast, addition of DADS (purified, 100 μM) to GSH (500

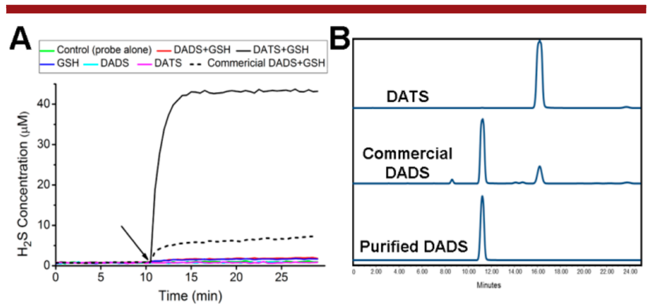


Figure 1. (A) H_2S releasing dynamics of the reaction between allylsulfides and GSH in PBS (10 mM, pH 7.4). Samples were added at the time shown by the arrow. Initial concentrations for each compound were DATS (50 μM), DADS (100 μM), commercial DADS (100 μM), and GSH (500 μM). (B) HPLC traces of DATS, commercial DADS, and purified DADS.

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μM) failed to produce any detectable H_2S. We applied an H_2S selective fluorescence probe to detect the H_2S in the reactions. The Cu(II) complex functionalized fluorescent probe (BCu) reacts with H_2S and leads to large fluorescence enhancement by 20-fold.[8] Yet, when DADS purchased from a commercial source (100 μM) was added to GSH (500 μM), H_2S production was observed. We suspected that the commercial DADS was a rapid H_2S donor because of its DATS impurity. Indeed, HPLC analysis of the sample revealed a significant amount of DATS (around 10%) in the commercial DADS (Figure 1B).

Mechanistic studies revealed that DATS and DADS reacted with GSH mainly through a thiol−disulfide exchange reaction instead of α-carbon nucleophilic substitution. The reaction between DADS (Figure 2A) or DATS (Figure 2B) with 20 equiv (20 mM) of GSH for 20 min gave nearly identical products. The major products were oxidized glutathione (GSSG), GSSA (Figure S2), allyl mercaptan (ASH), and DADS, which could be formed due to oxidation of ASH. However, the expected product GSA from α-carbon substitution was not observed. We also evaluated their reaction products of equal molar ratio (Figure 2C, D). For DADS, the products were the same as those from reaction at a 1:20 molar ratio. In contrast, the products from DATS were remarkably different. While ASH was not observed, we have detected GSSG, GSSA, and a number of new peaks including *S*-allyl glutathione trisulfide (GS_3A, retention time at 35 min), and series of allyl polysulfides A-(S)$_n$-A (n = 2−6). Corresponding G-(S)$_n$-A (n = 3−6) were detected by LC-MS (Figures S3−S6). The products observed above were consistent with the thiol−disulfide exchange reaction.[9]

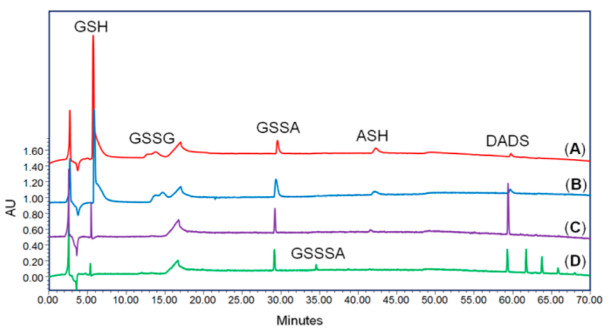
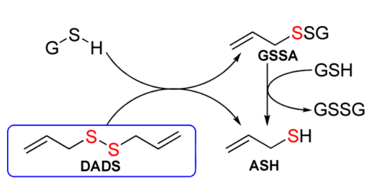


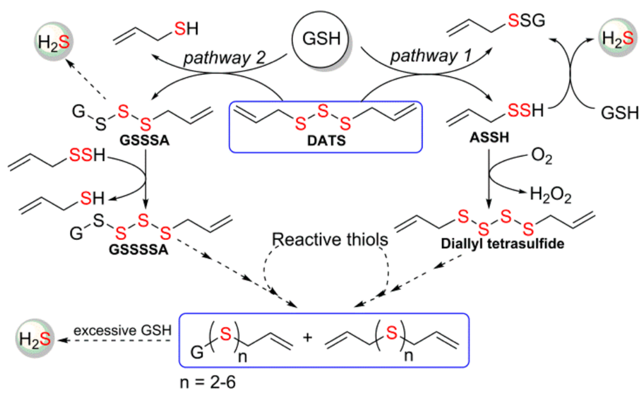
Figure 2. HPLC chromatograms of the products of the reactions of (A) DADS (1 mM) + GSH (20 mM), (B) DATS (1 mM) + GSH (20 mM), (C) DADS (1 mM) + GSH (1 mM), and (D) DATS (1 mM) + GSH (1 mM) at 210 nm. A = allyl.

Our results indicate that, in the presence of GSH, DADS can react rapidly with GSH via thiol−disulfide exchange to generate ASH and GSSA. The latter may undergo a similar thiol−disulfide exchange with another GSH, producing ASH and GSSG (Scheme 2). However, these reactions do not give rise to H_2S. For DATS (Scheme 3), there are two possible thiol−disulfide exchange reaction pathways. Pathway 1 is the nucleophilic attack of GSH on allylic sulfur generating GSSA and ASSH, with the latter possibly releasing H_2S upon reduction by GSH; pathway 2 is the nucleophilic attack of GSH on the central sulfur atom of DATS leading to ASH and GS_3A. These together with formed AS_2H and reactive thiols such as GSH in the system may undergo further metathesis and redox reaction to form polysulfides GS_nA, and AS_nA as observed in Figure 2D. However, when an excessive amount of GSH was available, these compounds could eventually be reduced by GSH to give H_2S.

Scheme 2

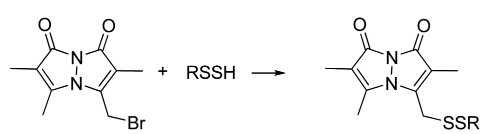


Scheme 3



We found that an α-carbon substitution reaction did take place slowly between DADS and GSH. The first evidence for this was the existence of perthiol intermediates (GSSH and ASSH) in the reaction mixture between DADS and GSH. Highly reactive perthiols are an emerging class of bioactive compounds,[10] as they have other reaction patterns such as nucleophilic addition to alkyne while releasing an elemental sulfide atom.[11] A structurally characterized tritylhydrodisulfide (TrtSSH) releases sulfide upon reduction or disproportionates upon deprotonation to donate elemental sulfur.[12]

The intermediates of the reaction between DADS/DATS and GSH were trapped by monobromobimane (mBBr).[13]



To preserve reactive perthiol species, excessive amounts of DATS/DADS (6 mM) were reacted with GSH (1 mM) in PBS (pH 7.4) at 37 °C for 15 min under anaerobic conditions, and then monobromobimane was added. The resulting mixture was subjected to LC-MS (ESI/APCI) analysis. In the case of DATS (Figure 3A), GS-bimane (peak 1, Figure S12) and GSS-bimane (peak 4, Figure S14) were detected, indicating GSH and GSSH were present. In addition, GSSA and GS_3A were detected (Figures S13, S15). For the intermediates containing an allyl group, we found AS-bimane (peak 11), ASS-bimane (peak 12), AS_3-bimane (peak 13), and AS_4-bimane (peak 14) (Figures S18−S21), which were from AS_nH (n = 1−4). When purified DADS (free of DATS) was used in the reaction (Figure 3B) there was no GS_3A, AS_3-bimane, or AS_4-bimane detected. A compound (peak 15) appeared at a similar retention time as ASS-bimane (peak 12), but its mass spectrum did not match with AS_5-bimane (Figure S25). The GSS-bimane (Figure S23) and ASS-bimane (Figure S26) could be detected by LC-MS at very low intensities. The existence of these two compounds

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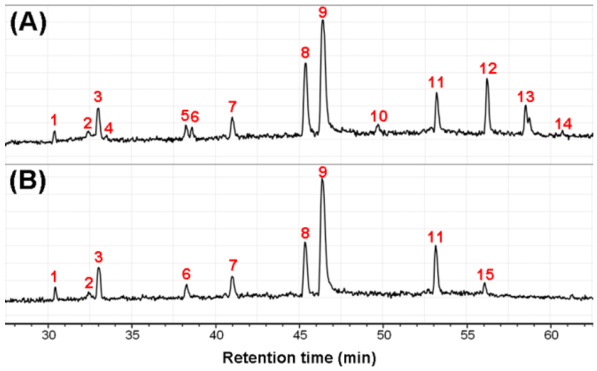


Figure 3. LC-MS-ESI (cationic mode) spectrum of (A) DATS (3 mM) + GSH (0.5 mM) + mBBr (2.5 mM), (B) DADS (3 mM) + GSH (0.5 mM) + mBBr (2.5 mM). Peaks were identified as (1) GS-bimane, (2) impurity, (3) GS_2A, (4) GSS-bimane, (5) GSSSA, (6) i.p., (7) i.p., (8) monochlorobimane, (9) monobromobimane (mBBr), (10) unknown, (11) AS-bimane, (12) AS_2-bimane, (13) AS_3-bimane, (14) AS_4-bimane, and (15) unknown.

confirmed that GSSH and ASSH were generated during the reaction between DADS and GSH.

Slow accumulation of GSA in the reaction between DADS and GSH further supports the sluggish α-carbon substitution reaction. When DADS (1 mM) and GSH (10 mM) were mixed for 1.5 h in PBS at 37 °C, a small GSA peak (Figure S8) was detected at 23 min, along with a diallyl sulfide (DAS) peak at 56 min (Figure 4). The GSA and DAS peaks grew slowly over

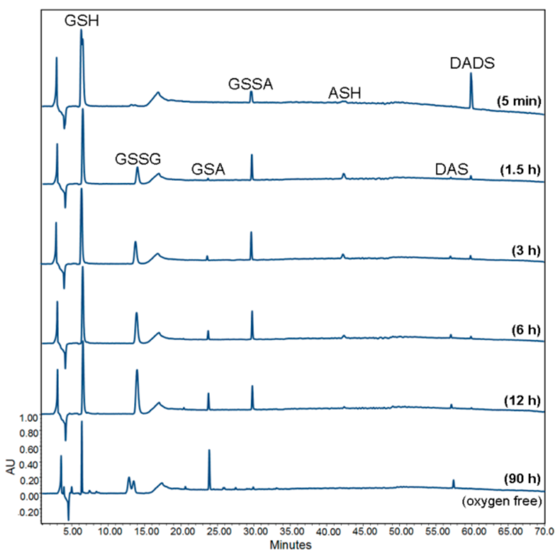
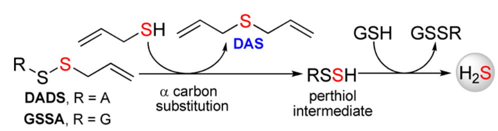


Figure 4. HPLC chromatograms (210 nm) of the products of the reactions of DADS (1 mM) + GSH (10 mM) in PBS (pH 7.4) at 37 °C at different time points.

time with the decreasing concentration of GSH, GSSA, ASH, and DADS. Since thiols are fairly sensitive to oxidation, the reaction in an anaerobic environment was carried out for 90 h to minimize oxidation during the long period of incubation (Figure 4). Under such conditions, the GSSA, ASH, and DADS peaks were barely detectable, while GSSG, GSA, and DAS peaks were the major ones. It is worth noticing that after 12 h, a small peak at the 20 min retention time was observed, which is likely to be a product from the oxidation of GSSG by H_2O_2. In accordance, incubating GSSG with H_2O_2 (Figures S9, S10) gave rise to the same product. The residual oxygen in the system may account for such a reaction.

Taken together, our results suggest besides fast thiol–disulfide exchange reaction between GSH and DADS, α-carbon nucleophilic substitution also occurs but slowly. When the reaction time was short, reversible thiol–disulfide exchange was dominant. As the reaction time prolongs, these products would be irreversibly converted into α-carbon nucleophilic substitution products. In addition, the production of DAS in the reaction mixture suggested that ASH also participated in an α-carbon nucleophilic substitution with either DADS or GSSA and generated H_2S (Scheme 5).

Scheme 5



Because of the reactive nature of H_2S, the direct quantification of H_2S generated from DADS was problematic under aeriated media (e.g., cell culture media) using fluorescent probes. H_2S generation from DADS via ASSH would result in accumulation of either GSA or DAS, which was shown to be inert toward GSH (Figure S27); therefore, the amount of H_2S produced could be estimated from the total amount of GSA and DAS.

When DADS (1 mM) and 10 equiv of GSH were mixed in PBS (10 mM, pH 7.4) and incubated at 37 °C, the sum of GSA and DAS generated was around 0.08 mM after 1.5 h and increased to 0.52 mM after 12 h (Figure 5A). The formation

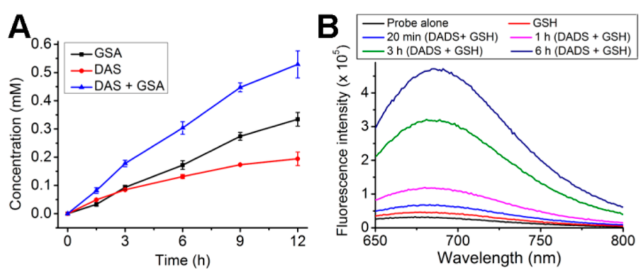


Figure 5. (A) Accumulation of GSA and DAS in the DADS (1 mM) + GSH (10 mM) reaction in PBS (10 mM, pH 7.4) at 37 °C. (B) Fluorescence response of H_2S probe (BCu, 20 μM) toward the reaction mixture of DADS (100 μM) + GSH (1 mM) over time (λ_{ex} = 620 nm).

rate of H_2S was estimated (from the slope of the linear fit of the blue line in Figure 5A). Since the concentration of GSH is in large excess, the α-substitution reaction rate is roughly pseudo-first-order to GSSA and DADS (the sum of the two concentrations should be equal to that of the DADS in the beginning of the reaction). Therefore, the pseudo-first-order rate constant is estimated to be $(1.24 \pm 0.04) \times 10^{-5}$ s^{-1}. The slow formation rate of H_2S during this process was confirmed by the fluorescence assay. When the H_2S probe (BCu, 20 μM) was added to a reaction mixture of DADS (100 μM) and GSH (1 mM) that had been mixed under argon (to prevent H_2S from oxidation) for 20 min in PBS at 37 °C, no obvious increase in fluorescence was observed, indicating little H_2S formed. If the reaction was allowed to continue for 6 h before the addition of the H_2S probe, a 20-fold gain in fluorescence

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intensity was observed (Figure 5B). Our result is in contrast to that of Benavides and co-workers, in which around 30−40 μM of H_2S was generated from the reaction between 100 μM of DADS and 2 mM GSH in less than 10 min.[6]

H_2S imaging studies were conducted to evaluate the H_2S releasing activity of DADS/DATS in cell lines. The probe (BCu) treated breast cancer MCF-7 cells were washed with PBS thrice and then treated with 100 μM DADS, DATS, or Na_2S for another hour.[8] As shown in Figure 6A, control or 100 μM DADS treated cells gave off weak fluorescence. Yet, cells treated with 100 μM DATS or Na_2S produce much stronger fluorescence. The fluorescence intensity per cell showed that DATS treatment significantly elevated the fluorescence intensity. In contrast, 100 μM DADS nearly had no effect (Figure 6B). These results demonstrated that DATS was a good H_2S donor in cell lines, while DADS was not.

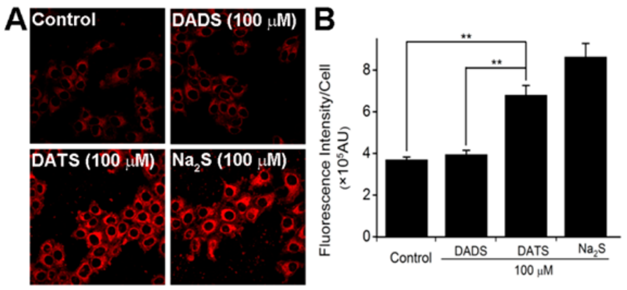

Figure 6. Fluorescence images of H_2S generated from DATS in cell line. MCF-7 cells were incubated with 20 μM H_2S probe BCu for 2 h and then washed and subjected to different treatments for another 1 h. (A) Confocal images of the cells, (λ_{ex} = 633 nm, λ_{em} > 650 nm). (B) Average fluorescence intensity per cell in different groups; data were represented as mean ± SEM, n = 3.

In conclusion, we found that DATS releases H_2S instantly through thiol−disulfide exchange with GSH in both chemical and biological systems. DADS releases H_2S sluggishly through α-carbon nucleophilic substitution and does not lead to significant H_2S formation in cell lines. The previous misunderstanding of DADS as a H_2S donor is mainly attributed to its DATS contamination in commercial samples. To avoid misleading results, we suggest that the purity of DADS should be carefully checked and clearly stated in all studies concerning its health promoting effects. Slow H_2S donors may be preferred for cardiovascular health promotion, as it may help to maintain H_2S concentrations at "healthy" low levels for a longer period of time. Rapid bursts of H_2S, on the other hand, may lead to toxic effects that could be utilized for anticancer or antibacterial applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01962.

Experimental details, LC-MS spectra, and supporting HPLC chromatogram (PDF)

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Notes

The authors declare no competing financial interest.

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