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Isotope Dilution Mass Spectrometry for the Quantification of Sulfane Sulfurs

Chunrong Liu[1,4], **Faya Zhang**[2], **Gerhard Munske**[3], **Hui Zhang**[2], and **Ming Xian**[1]

Ming Xian: mxian@wsu.edu

[1]Department of Chemistry, Washington State University, Pullman, WA 99164, USA

[2]Department of Pharmaceutical Sciences, Washington State University, Pullman, WA 99164, USA

[3]Center for Reproductive Biology Molecular Biology and Genomics Core, Washington State University, Pullman, WA 99164, USA

[4]Research Institute of Science and Technology, Central China Normal University, Wuhan, 430079, China

Abstract

Sulfane sulfurs are one type of important reactive sulfur species. These molecules have unique reactivity that can attach reversibly to other sulfur atoms and exhibit regulatory effects in diverse biological systems. Recent studies have suggested that sulfane sulfurs are involved in signal transduction processes regulated by hydrogen sulfide (H_2S). Accurate and reliable measurements of sulfane sulfurs in biological samples are thus needed to reveal their production and mechanisms of actions. Herein we report a convenient and accurate method for the determination of sulfane sulfurs concentrations. The method employs a triphenylphosphine derivative (**P2**) to capture sulfane sulfurs as a stable phosphine sulphide product **PS2**. The concentration of **PS2** was then determined by isotope dilution mass spectrometry, using a $^{13}C_3$-labelled phosphine sulfide **PS1** as the internal standard. The specificity and efficiency of the method were proved by model reactions. It was also applied in the measurement of sulfane sulfurs in mice tissues including brain, kidney, lung, liver, heart, spleen, and blood.

Keywords

Reactive sulfur species; Isotope dilution assay; sulfane sulfur; mass

Correspondence to: Ming Xian, mxian@wsu.edu.

The reactions between triphenylphosphine derivatives and sulfane sulfurs were studied.
Based on these reactions, a convenient isotope dilution mass assay for sulfane sulfur detection was developed.
The method was used in the determination of sulfane sulfur concentrations in mice tissues.

Introduction

Reactive sulfur species (RSS) are a family of sulfur-containing molecules that widely exist in biological systems. Representative examples are biothiols (such as cysteine and glutathione), S-modified cysteine adducts (such as S-nitrosothiols and sulfenic acids), hydrogen sulphide (H_2S), persulfides, and polysulfides. Many of these species play important roles in physiological and pathophysiological processes.[1-3] Among them, reactive sulfane sulfurs have received increased attention as some of these compounds are suggested to be involved in signal transduction processes mediated by H_2S.[4-12] Sulfane sulfur refers to a sulfur atom with six valence electrons but no charge (represented as S^0).[13-16] Biologically relevant sulfane sulfur compounds include persulfides (R-S-SH), hydrogen polysulfides (H_2S_n, n>1), polysulfides (R-S-S_n-S-R), as well as protein-bound elemental sulfur (S_8) (Figure 1). H_2S and sulfane sulfurs are interchangeable, therefore they always coexist. From a chemistry perspective, sulfane sulfurs can be the metabolites of H_2S; meanwhile they can be the precursors of H_2S. Because of this property, some mechanisms of actions that were originally attributed to H_2S may actually be mediated by sulfane sulfurs. For example, recent work by Kimura and co-workers suggests that H_2S-derived sulfane sulfurs may be the actual signaling molecules.[8,10] Moreover it has been long appreciated that sulfane sulfurs have unique regulatory effects in diverse biological systems: post-transcriptional modification of transfer RNA, synthesis of sulfur-containing cofactors and vitamins, activation or inhibition of enzymes, etc.[4]

Despite the increased interest in sulfane sulfurs, fundamental questions regarding their production and functions remain to be clarified. To this end, accurate and reliable measurements of sulfane sulfurs in biological samples are needed. Currently, several methods for sulfane sulfur detection have been reported (Figure 2). The traditional method, i.e. cyanolysis, is based on the reaction between sulfane sulfurs and cyanide (CN^-) under basic pH (8.5∼10) to form thiocyanate (SCN^-), which can be further converted to ferric thiocyanate and measured by its characteristic UV absorbance at 460 nm.[17] Another method is to convert sulfane sulfurs into H_2S, upon treating with reductants like DTT. The resultant H_2S can be determined by chromatography methods or trapped with monobromobimane, and then analyzed to deduce the concentrations of sulfane sulfurs.[13] It should be noted that monobromobimane-based methods have been used to detect individual sulfane sulfur species,[18] as well as the whole sulfane sulfur pool.[19] Recently specific fluorescent probes, such as SSP2, were developed by our laboratory.[20] This method is based on a sulfane sulfur-mediated cyclization to turn-on fluorescence signals. It allows the detection of sulfane sulfurs by convenient and non-destructive fluorescent measurements.

In order to develop other new and efficient methods for sulfane sulfur detection we have initiated a program to study the reactions of sulfane sulfurs. It is known that triarylphosphines (PAr_3) can rapidly react with sulfane sulfur species to form triarylphosphine sulfides ($S{=}PAr_3$).[21] However this reaction has not been well-appreciated in sulfane sulfur detection. One report by Sörbo et al employed a gas chromatographic method analyzing $S{=}PAr_3$ generated from sulfane sulfurs in tissue samples.[22] This work indicated the possibility of using this reaction for sulfane sulfur detection, while the method requires complicate chromatographic pretreatments of samples. We envisioned that isotope

dilution mass spectrometry would be a useful application of the reaction for sulfane sulfur detection. Isotope dilution is a widely applied method for analyzing chemical substances in biological samples.[23-26] The method comprises the addition of isotope-enriched substances (as internal standards) to samples containing non-isotope labelled analytes. Unlike traditional analytical methods, which rely on signal intensities, isotope dilution utilizes the ratios between internal standards and analytes to determine the concentrations. Another advantage of mass spectrometry is that the method does not need complex sample extraction and separation; therefore it is easy for operation.[27] Because of these advantages, isotope dilution has been applied in the quantification of proteins, nucleosides (derived from DNA or RNA), vitamins, and other bioactive molecules.[24-29] Yet the applications on sulfane sulfurs have not been reported.

Herein we proposed an isotope dilution strategy for sulfane sulfurs (Figure 3). We expected that triarylphosphine reagents could effectively react with sulfane sulfurs in biological samples to form phosphine sulfide **A**. After the reaction was completed, isotope-labelled phosphine sulphide **B** (exampling as ^{13}C isotopes) would be spiked into the sample as internal standards. **A** and **B** should show identical chemical/physical properties (such as solubility, extraction efficiency, sensitivity to ionization in mass, etc.). When the mixture was subjected to mass analysis, the comparison of mass peaks' intensities corresponding to **A** and **B** should allow us to determine the concentration of **A**, and in turn determine the original concentration of sulfane sulfurs.

Materials and Methods

Chemicals

All solvents and chemicals were reagent grade. L-Alanine, thionyl chloride and Fmoc-Ala-OH-$^{13}C_3$ were purchased from Sigma–Aldrich; 4-dimethylaminopyridine and HOBt were purchased from Acros Organics; DCC, methyl iodide, and piperidine were purchased from Alfa Aesar. 2-(Diphenylphosphino)benzoic acid **3** was prepared according to a literature protocol.[30]

Synthesis of phosphine P1 and P2

Phosphine **P2** was prepared from L-alanine methyl ester through a standard coupling procedure with 2-(diphenylphosphino)benzoic acid **3** in 54% yield.[31] ^1H NMR (300 MHz, $CDCl_3$) δ 1.27 (d, J = 7.2 Hz, 3H), 3.72 (s, 3H), 4.64 (t, J = 7.5 Hz, 1H), 6.54 (d, J = 6.9 Hz, 1H), 6.95 (m, 1H), 7.41-7.25 (m, 12H), 7.64 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 168.4, 137.2, 137.1, 136.8, 134.4, 134.3, 134.0, 133.9, 130.6, 129.1, 129.0, 128.9, 128.8, 128.1, 128.0, 52.7, 48.6, 18.6; ^{31}P NMR δ: -8.6; HRMS m/z 392.1428 $[M+H]^+$; calcd for $C_{23}H_{23}NO_3P$: 392.1416; IR 3269, 3067, 2946, 1723, 1567, 1465, 1249, 1053, 747; mp 123-124 °C.

The ^{13}C-labeled phosphine **P1** was synthesized from ^{13}C-labeled alanine methyl ester using the same procedure for **P2**. The ^{13}C-labeled alanine methyl ester was prepared from Fmoc-Ala-OH-$^{13}C_3$ in 2 steps: esterification with methyl iodide[32] and deprotection to remove the Fmoc group.[33] The overall yield was 23%. ^1H NMR (300 MHz, $CDCl_3$) δ 1.06 (m, 1.5H),

1.49 (m, 1.5H), 3.72 (d, *J* = 3.9 Hz, 3H), 4.39 (m, 0.5H), 4.86 (m, 0.5H), 6.54 (d, , *J* = 6.6 Hz, 1H), 6.95 (m, 1H), 7.60-7.25 (m, 12H), 7.66 (m, 1H); ^{31}P NMR δ: -8.6; MS (ESI$^+$) m/z 433.3 (M+K$^+$). IR 3270, 3057, 2946, 1728, 1565, 1460, 1249, 1052, 747; mp 124-125 °C.

Sulfane sulfur compounds

Elemental sulfur and sodium tetrasulfide were purchased from Alfa Aesar. Sodium tetrathionate was purchased from Sigma–Aldrich. Other sulfane sulfur compounds such as **5**, **6**, **7,** and sodium disulfide were prepared using methods described previously.[18] Compound **7** was a 1:1 mixture of trisulfide and tetrasulfide.

Time-dependent reactions of P2 with sulfane sulfurs

To a solution of **P2** (29.4 mg, 0.075 mmol) in CH_3CN (20.0 mL) and phosphate buffer (5.0 mL, 100 mM, pH = 7.4) was added **7** or S_8 (0.025 mmol). The mixture was stirred at rt. 0.5 mL of the mixture was taken out at different time intervals (0, 0.25, 0.5, and 3 hours) and monitored by ^{31}P NMR. The results showed that the reaction was completed within 15 min.

Reactions of P2 with sulfane sulfur species

General protocol: to a solution of **P2** (9.8 mg, 0.025 mmol) in CH_3CN (20.0 mL) and phosphate buffer (5.0 mL, 100 mM, pH 7.4) was added elemental sulfur (0.8 mg, 0.025 mmol). The mixture was stirred for 3 h at r.t. and then diluted with CH_2Cl_2 (50 mL). The organic layer was seperated, dried by $MgSO_4$, and concentrated. Purification by flash column chromatography afforded compound **PS2** as a white solid (9.7 mg, 92% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15 (d, *J* = 7.5 Hz, 3H), 3.69 (s, 3H), 3.86 (m, 1H), 7.16 (m, 1H), 7.35-7.60 (m, 8H), 7.75-7.91 (m, 5H), 8.08 (d, *J* = 6.3 Hz, 3H);^{13}C NMR (75 MHz, $CDCl_3$) δ 172.9, 167.8, 133.3, 133.2, 132.8, 132.7, 132.3, 132.2, 132.0, 131.9, 131.8, 131.5, 131.4, 131.2, 130.5, 130.3, 128.9, 128.8, 128.7(6), 128.7(1), 52.5, 49.3, 17.2; ^{31}P NMR δ 45.1; MS (ESI$^+$) m/z 446.0 (M+Na$^+$); IR 3240, 3050, 2950, 1740, 1650, 1520, 1440. mp 86-87 °C.

Compound **PS1** was synthesized using the same procedure for **PS2**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.94 (m, 1.5H), 1.36 (m, 1.5H), 3.64 (m, 0.5H), 3.69 (d, *J* = 3.6 Hz, 3H), 4.10 (m, 0.5H), 7.16 (dd, *J* = 15, 7.5 Hz, 1H), 7.35-7.60 (m, 8H), 7.76-7.91 (m, 5H), 8.08 (d, *J* = 3.9 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.4 (d, *J* = 61.5 Hz, 1C, $^{13}CO_2Me$), 133.4, 132.8, 132.7, 132.2, 131.9, 131.8, 131.6, 131.4, 130.5, 130.3, 128.9, 128.8, 128.7(7), 128.7(1), 52.5, 49.3 (dd, *J* = 61.5, 33.7 Hz, 1C, NH^{13}CH), 17.6 (d, *J* = 40.5 Hz, $^{13}CH_3$); ^{31}P NMR δ: 45.1, MS (ESI$^+$) m/z 449. 1 (M+Na$^+$). IR 3250, 3050, 2940, 1660, 1650, 1520, 1430. mp 87-88 °C.

Control experiments

To the solution of **P2** (9.8 mg, 0.025 mmol) in CH_3CN (20.0 mL) and phosphate buffer (5.0 mL, 100 mM, pH = 7.4) was added cysteine **8** or GSSG (0.025 mmol). The mixture was stirred at rt. 0.5 mL sample was removed from the mixture at different time (0, 2, 4, 6, 8, and 24 h) and monitored by ^{31}P NMR. No reaction was observed. After 24 h, CH_2Cl_2 (50.0 mL) was added into the solution to extract the products. The organic layers were separated, dried

by $MgSO_4$, concentrated, and purified column chromatography. Unreacted **P2** was recovered in 91-94% yields.

To the solution of **P2** (9.8 mg, 0.025 mmol) or **PS2** (10.8 mg, 0.025 mmol) in CH_3CN (20.0 mL) and phosphate buffer (5.0 mL, 100 mM, pH = 7.4) was added DTT (0.025 mmol). The mixture was stirred at rt. 0.5 mL sample was removed from the mixture at different time (0, 2, 4, and 8 h) and monitored by ^{31}P NMR. No reaction was observed After 8 h, CH_2Cl_2 (50.0 mL) was added into the solution to extract the products. The organic layers were separated, dried by $MgSO_4$, concentrated, and purified column chromatography. Unreacted **P2** and **PS2** were recovered in 95% and 96%, respectively. The protocol described above was also applied to test the reaction between **PS2** and H_2O_2, the stability of **P2** and **PS2** in solutions. The results were shown in text.

Preparation of stock solutions

P2: 19.6 mg of **P2** was dissolved in 10.0 mL CH_3CN to provide the stock solution. [**P2**] = 5.0 mM.

PS1: 10.6 mg of **PS1** was dissolved in 25.0 mL CH_3CN to provide the stock solution.

[**PS1**] = 1.0 mM.

Compound **7**: 26.6 mg of **7** was dissolved in 100.0 mL CH_3CN to provide the stock solution. [**7**] = 0.5 mM, [sulfane sulfur] = 1.0 mM.

General procedure for mass analysis

A model experiment was carried out to verify the effectiveness of isotope dilution mass spectrometry assay in the quantification of sulfane sulfurs. A series of solutions of 7 were prepared in a mixed solvents of acetonitrile and phosphate buffer (4/1). The final sulfane sulfur concentrations were 0, 1, 2.5, 5, 7.5, and 10 μM, respectively. Then phosphine **P2** (100 μM) was added to trap the sulfane sulfurs. After 3 h the internal standard **PS1** (5 μM) was spiked into each sample and solutions were subjected to MALDI-TOF/TOF mass analysis.

1. A series of compound **7** stock solutions (0, 1, 2.5, 5.0, 7.5, 10.0 μL) were placed in microcentrifuge tubes (2 mL) and diluted to 0.2 mL with phosphate buffer (100 mM, pH = 7.4).
2. 0.78 mL of acetonitrile was added.
3. 20 μL of **P2** stock solution (5.0 mM) was added.
4. The tubes were put on a Fisher Scientific Pulsing Vortex Mixer for 3 hours.
5. 5 μL of **PS1** stock solution (1.0 mM) was added to each tube.
6. 2.5 μL of sample was added to 2.5 μL of fresh dihydroxybenzoic acid matrix (concentration: 10 mg/mL water). This mixture was centrifuged, mixed, then re-centrifuged.

7. Approximately 0.3 μL of the sample/matrix was added to three spots on the stainless steel sample plate. The plate was put under vacuum and dried.
8. The sample plate was loaded onto a Sciex 4800 MALDI TOF/TOF Analyzer. The MS reflector positive acquisition method supplied by the manufacture was modified to collect data in the mass range of 455 to 475 with a delayed extraction time of 300 nsec. The edge bias laser pattern was used because of the DHBA matrix.
9. The laser intensity was adjusted to keep the signal intensity of the highest peak in the spectra below 10,000 in the accumulated 400 spectra. A total of 1600 spectra were collected in groups of 400 if each set of 400 was below 10,000. This was done to keep the sometimes much larger 464 peak from affecting the intensity of the 465 peak.
10. All peaks in the spectra were labeled with mass and area. The relative intensity of the 465/462 peak was calculated from the displayed areas for the two masses. The correct mass is the lower mass peak when two peaks were seen for either the 462 or 465 peak. This assignment of these peaks was confirmed by accurate mass determination when internal calibration was used in spectra collected in the 200 to 1400 mass ranges.

Animals

Female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, WA), and single housed in Wegner Hall Vivarium, at Washington State University. Mice were euthanized via overdose of isoflurane. Blood, brain, spleen, lung, liver, heart and kidney were collected. The animal protocol was approved by the Institutional Animal Care and Use Committee at Washington State University.

Preparation of blood and organ samples

1. Mice were euthanized via overdose of isoflurane.
2. 500 μL of blood were collected by cardiac puncture and placed in a microcentrifuge tube.
3. 0.1 mL of heparin solution (100 mg in 1.83 mL of phosphate buffer) was added.
4. The blood samples were centrifuged at 800 *g* for 3 min.
5. Plasma and red blood cell samples were collected with micropipette.
6. Different organs including spleen, liver, lung, heart, brain and kidney were extracted separately.
7. The freshly extracted organs were weighted, placed in 15.0 mL tubes, to which was added phosphate buffers (100 mM, PH = 7.4) (organs/phosphate buffer = 1 g/4 mL).
8. Organ samples were homogenized by an OMNI International TH-287 homogenizer.

9. The samples were stored at -78 °C.

Measurements of sulfane sulfers in animal specimens

1. 0.2 mL of organ homogenates were placed in microcentrifuge tubes (2.0 mL).
2. 0.78 mL of acetonitrile was added.
3. 20 μL of **P2** stock solution (5.0 mM) was added.
4. The tubes were put on a Fisher Scientific Pulsing Vortex Mixer for 3 hours.
5. 5 μL of **PS1** stock solution (1.0 mM) was added.
6. The mixtures were centrifuged at 800 g for 3 min.
7. The supernatants were removed by micropipettes.
8. 2.5 μL of supernatants were added into 2.5 μL of fresh dihydroxybenzoic acid matrix (concentration: 10 mg/mL water). This mixture was centrifuged, mixed, then re-centrifuged.
9. 0.3 μL of the sample/matrix was added to three spots on the stainless steel sample plate. The plate was put under vacuum and dried.
10. The sample plate was loaded onto a Sciex 4800 MALDI TOF/TOF Analyzer. The MS reflector positive acquisition method supplied by the manufacture was modified to collect data in the mass range of 455 to 475 with a delayed extraction time of 300 nsec. The edge bias laser pattern was used because of the DHBA matrix.
11. The laser intensity was adjusted to keep the signal intensity of the highest peak in the spectra below 10,000 in the accumulated 400 spectra. A total of 1600 spectra were collected in groups of 400 if each set of 400 was below 10,000. This was done to keep the sometimes much larger 464 peak from affecting the intensity of the 465 peak.
12. All peaks in the spectra were labeled with mass and area. The relative intensity of the 465/462 peak was calculated from the displayed areas for the two masses. The correct mass is the lower mass peak when two peaks were seen for either the 462 or 465 peak. This assignment of these peaks was confirmed by accurate mass determination when internal calibration was used in spectra collected in the 200 to 1400 mass ranges.

Results and Discussion

Synthesis of triarylphosphine reagents P1 and P2

In order to introduce isotopes on triarylphosphines, we decided to prepare a ^{13}C-labelled phosphine **P1** (Figure 4). Three ^{13}C atoms were installed for the purpose of better distinguishing the peaks of analyte and internal standard in mass spectrum. As alanine-$^{13}C_3$ **1** is commercially available, it was selected as the starting material and converted into ^{13}C-labelled phosphine **P1** in three steps. **P1** should react with sulfane sulfurs to produce **PS1**, which is the internal standard in this study (*vide infra*). Meanwhile, the non-isotope labelled

sulfane sulfur trapping reagent **P2** was prepared from alanine methyl ester **4** through similar synthetic steps.

Model reactions of phosphine reagents with sulfane sulfurs

With phosphine **P2** in hand, it was first needed to ensure the reagent could effectively trap sulfane sulfur species. To this end, we tested the reactions between **P2** and two representative sulfane sulfurs (elemental sufur S_8 and a cysteine polysuflide **7**). In this study, 3 equiv. of **P2** vs 1 equiv. of sulfane sulfurs were used. The reactions were monitored by ^{31}P NMR. As shown in Figure 5, three peaks were observed: δ 45.1 (**PS2**), δ -8.6 (**P2**), and δ 2.7 (phosphate buffer). The ratios between δ 45.1 and δ -8.6 were used to determine the progress of the reactions. In both cases, the reactions were found to be fast and completed within 15 min.

We next determined the effectiveness of the reaction toward diffrent sulfane sulfur compounds including small molecule polysulfides/perthiols (**5**, **6**, **7**), elemental sulfur (S_8), polythionate, and hydrogen polysulfides (Na_2S_2 and Na_2S_4). These substrates were employed to mimic biological sulfane sulfurs. Reaction time was prolongened to 3 hours to ensure the reaction was completed. As shown in Figure 6, all reactions worked well and the desired phosphine sulfide **PS2** was obtained in high isolated yields (84% – 94%), indicating that **P2** was effeicent in capturing sulfane sulfurs. The internal standard, $^{13}C_3$-labeled phosphine sulfide **PS1**, was also obtained from the reaction between **P1** and elemental sulfur in excellent yield under the same conditions.

Control reactions

If our proposed method is used for biological sulfane sulfur detection, two criteria should be met: 1) phosphine **P2** should not react with other sulfur species (such as disulfides and biothiols) to form phosphine sulfide, and 2) the product **PS2** (and **PS1**) should be stable during sample preparation and analysis. To address these concerns, we tested the reactions of **P2** in the presence of a model disulfide (GSSG) and a thiol derivative **8**. Again ^{31}P NMR was used to monitor the reaction (Figure 7). **P2** was found to be un-reactive toward these species as no new peak was observed after 24 hours. In addition, we also tested DTT, a commonly used reductant, in this study. No reaction was observed for DTT either (data not shown).

The stability of the phosphine sulfide **PS2** was next studied. **PS2** was found to be stable in the presence of thiols (such as DTT) and oxidants (such as H_2O_2). ^{31}P NMR assay results were shown in Figure 8. **PS2** was also found to be stable in a wide range of pH (2 ∼ 10) (data not shown).

Method validation

To ensure the proposed isotope dilution mass analysis can accurately determine the concentration of **PS2**, a linear calibration curve covering the concentration range (0.0, 1.0, 2.5, 4.0, 5.0, 7.5, 15, and 25.0 μM) was obtained. 5.0 μM of the internal standard **PS1** was added into each sample. Linear regression was calculated with nonweighting and non-zero-forced, and the linear equation was obtained: $y=0.198x-0.0062$ ($r^2=0.9997$). Over the entire

concentration range of the calibration curves, the mean observed percentage deviation of back-calculated concentrations was between 1.5 and 5.0 % with an imprecision (CV) of <10%.

To understand the sensitivity of the method, we determined the limit of quantification (LOQ) and the limit of detection (LOD). LOQ was defined as the lowest concentration that could be reliably and reproducibly measured with values for accuracy and intra- and interday imprecision (coefficient of variation (CV) <20%). LOQ of this method was determined to be 0.20 μM, based on direct measurements of a series of diluted **PS2** calibration solutions. LOD, defined as the lowest concentration that gave a signal-to-noise ratio of at least 3, was 0.07 μM.

Trapping and quantifying sulfane sulfurs by mass spectrometry

Next we decided to verify the effectiveness of isotope dilution mass spectrometry assay in the quantification of sulfane sulfurs. A model experiment was carried out (Figure 9, the detailed experimental protocols were described in Materials and Methods). Cysteine polysulfide **7** was employed as the model of sulfane sulfurs. A series of solutions of **7** were prepared in a mixed solvent of acetonitrile and phosphate buffer (4/1). The final sulfane sulfur concentrations were 0, 1, 2.5, 5, 7.5, and 10 μM, respectively. Then phosphine **P2** (100 μM) was added into each sample and the reactions were kept at rt. After 3 h the internal standard **PS1** (5 μM) was spiked into each sample and solutions were subjected to MALDI-TOF/TOF mass analysis. As expected we observed very distinct mass peaks of **PS2** (462.1, $M+K^+$) and **PS1** (465.1, $M+K^+$). The intensity ratios of these two peaks allowed us to deduce the concentrations of **PS2** from the spiked concentration of **PS1**. As summarized in Table 1, the measured concentrations of **PS2** were 0, 0.99, 2.54, 5.0, 7.34, and 10.3 μM, which correlated well to the initial sulfane sulfur concentrations of compound **7**. These results demonstrated that the phosphine based isotope dilution method was effective in the determination of sulfane sulfur concentrations.

Quantification of sulfane sulfurs in tissue samples

Finally we applied this method to determine sulfane sulfur concentrations in mice tissues. The protocol is illustrated in Figure 10 and described in the Materials and Methods. The results were shown in Table 2. Plasma and erythrocytes were found to have relatively low sulfane sulfur concentrations (4.7-13.1 nmol/g and 2.3-3.7 nmol/g), while other organs maintained higher concentrations. The average concentrations were 57.0 nmol/g (liver), 150.9 nmol/g (kidney), 46.0 nmol/g (brain), 61.8 nmol/g (heart), and 56.1 nmol/g (spleen). The concentrations of sulfane sulfur in lung were a bit lower (20.8 nmol/g). These data were comparable to reported values.[13]

Conclusion

In summary, we reported in this study a convenient isotope dilution mass assay for sulfane sulfur detection. The strategy was based on a selective reaction between triphenylphosphine derivatives and sulfane sulfurs. The specificity and efficiency of the method were proved by

model reactions. It was also applied in the measurement of sulfane sulfurs in mice tissues including brain, kidney, lung, liver, heart, spleen and blood.

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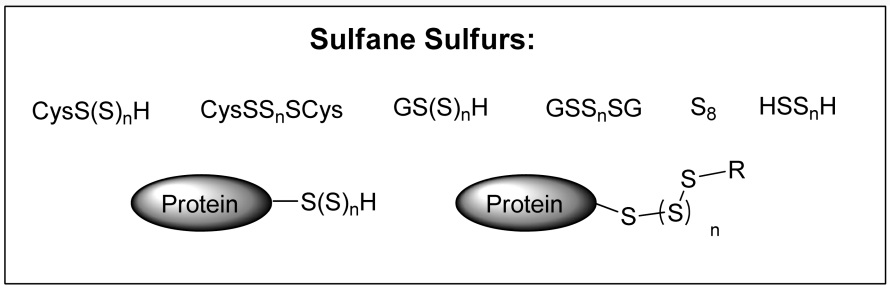

Figure 1. The pool of biological sulfane sulfurs

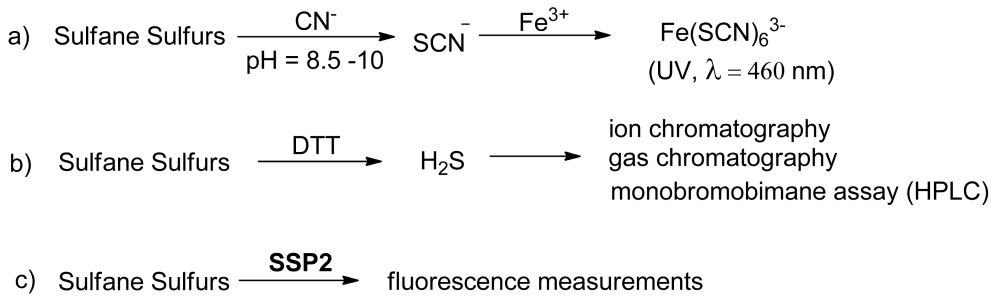

Figure 2. Current methods for sulfane sulfur detection

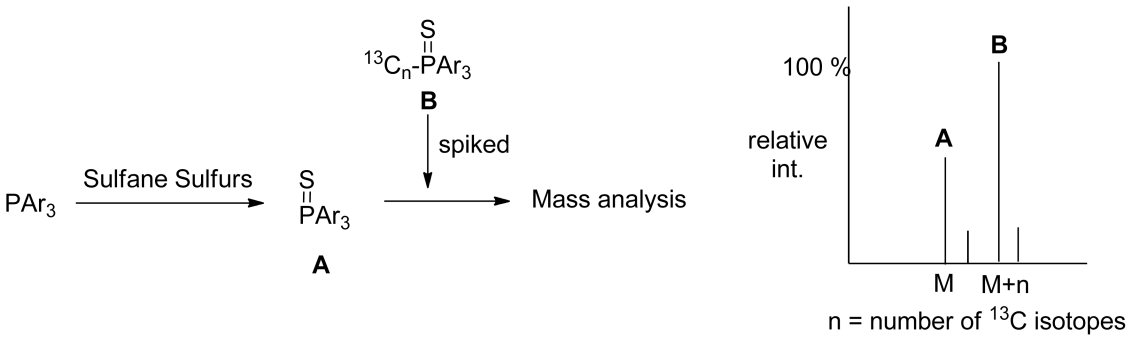

Figure 3. Proposed isotope dilution mass spectrometry method for sulfane sulfur detection

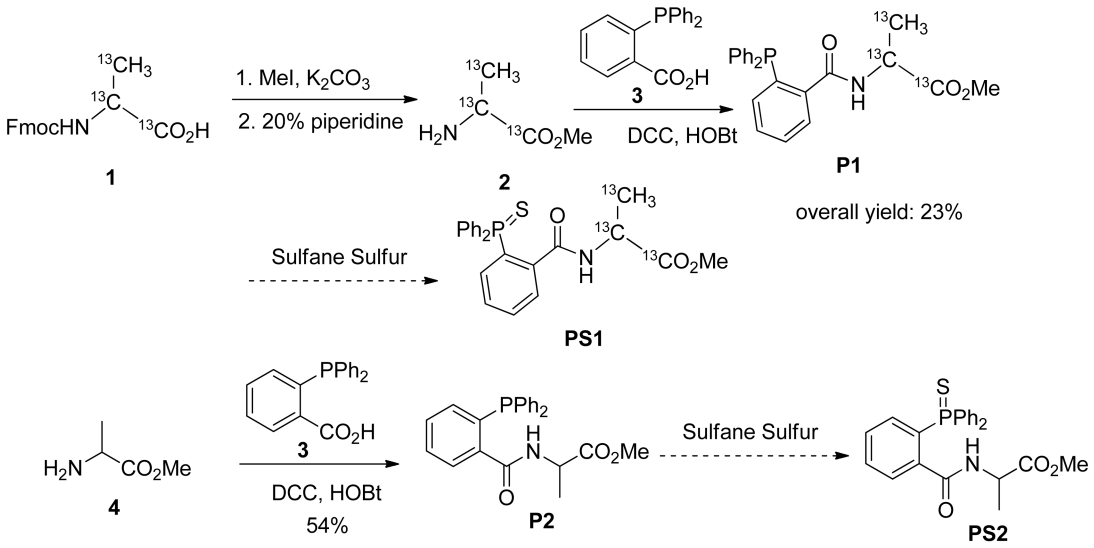

Figure 4. Preparation of triarylphosphine reagents

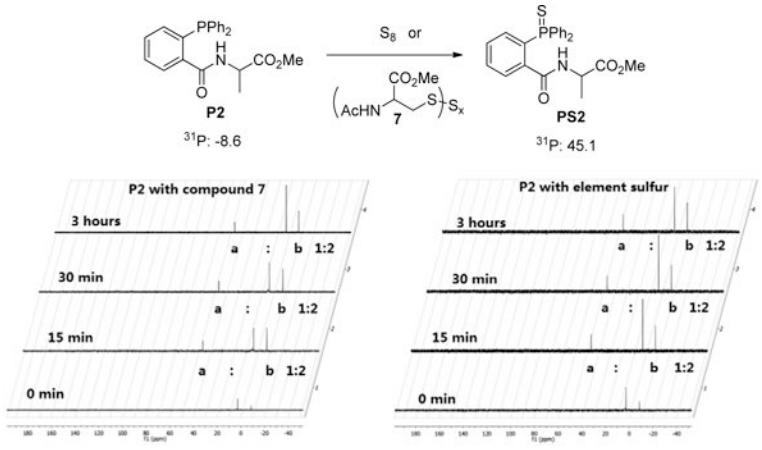

Figure 5. Time-dependent reaction progress of P2 in the presence of sulfane sulfurs

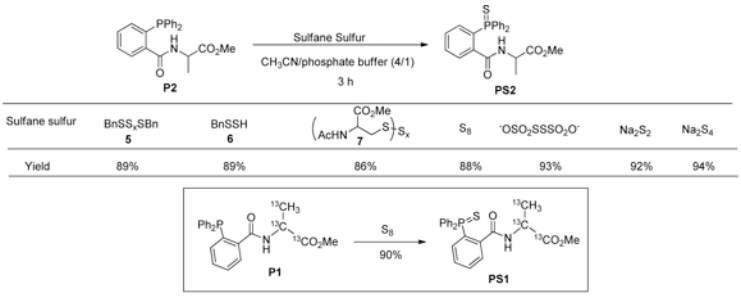

Figure 6. The reactions between P2 and sulfane sulfur compounds

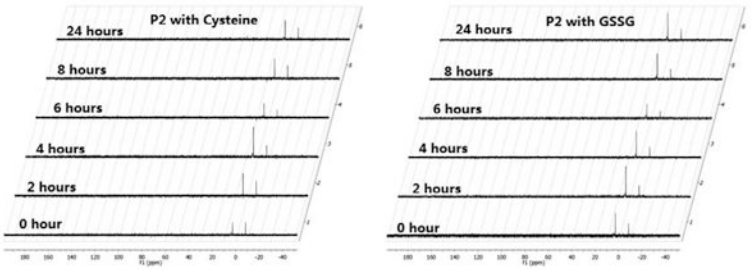

Figure 7. Test the reactivity of P2 toward thiols and disulfides

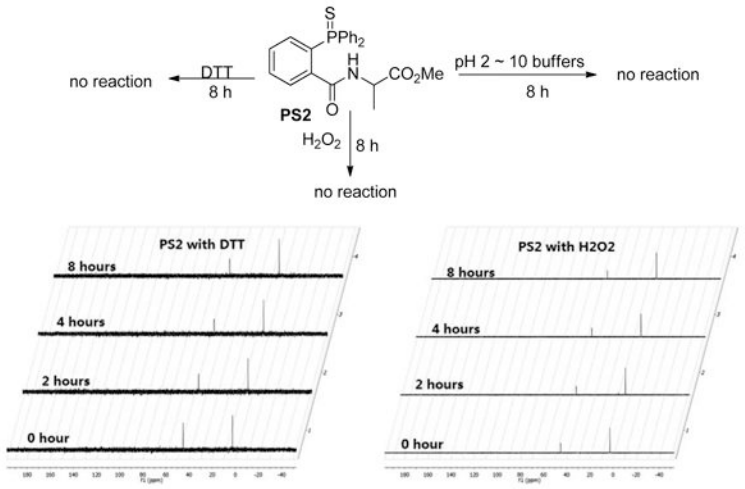

Figure 8. Stability test of PS2

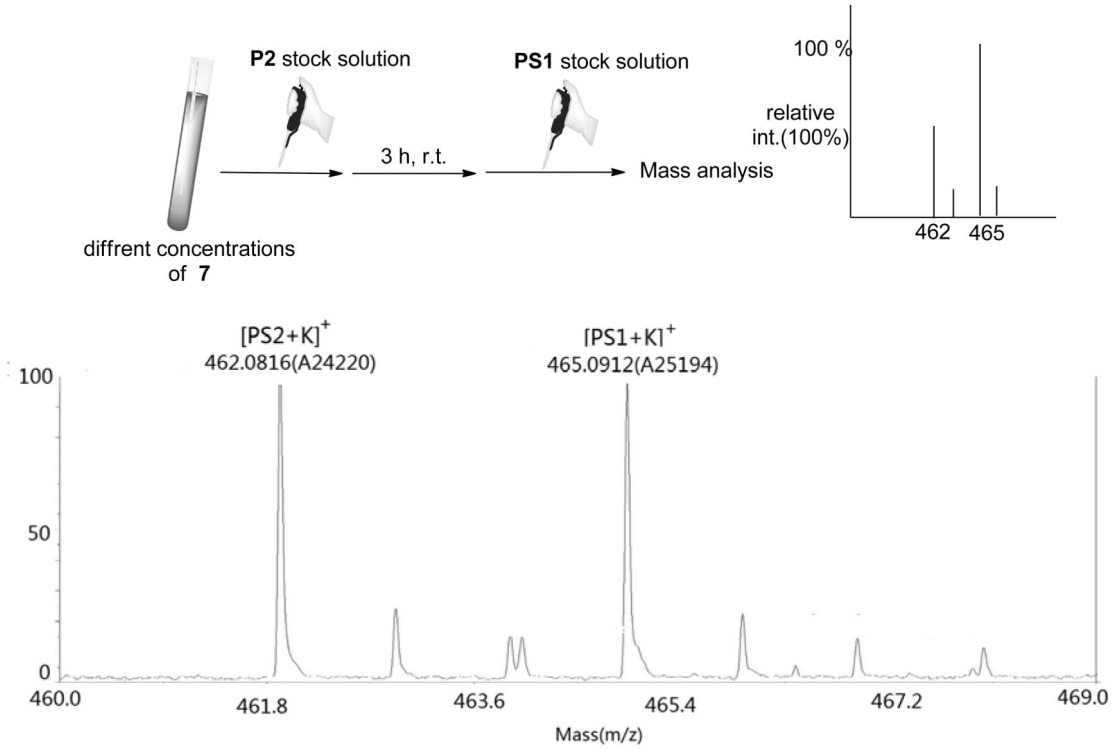

Figure 9.

schematic descriptions of the isotope dilution mass protocol and a representative mass spectrum.

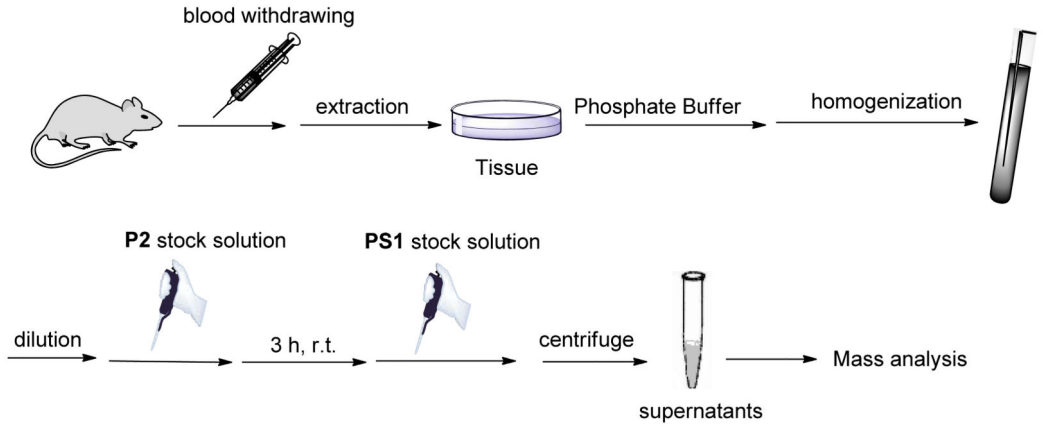

Figure 10.
The general procedure for the quantification of sulfane sulfur in mice tissues.

Table 1

A model study to quantify sulfane sulfurs

Initial sulfane sulfur concentration of 7 (μM)	**0**	**1**	**2.5**	**5**	**7.5**	**10**
ratio of **PS1/PS2**	0	5.03±0.2	1.96±0.07	1.00±0.01	0.68±0.04	0.48± 0.01
Measured sulfane sulfur concentration (μM)	0	0.99±0.05	2.54±0.10	5.00 ±0.01	7.34 ± 0.44	10.3±0.3

Table 2

Sulfane sulfur concentrations in tissues

Sulfane sulfurs ($^{nmol}/_{9}$)	Plasma	Erythrocytes	Liver	Kidney	Brain	Heart	Lung	Spleen
Mouse								
1	13.1	2.8	52.5	125.0	43.5	62.8	20.8	60.9
2	-	-	53.4	148.8	38.1	66.1	19.6	54.8
3	6.5	2.3	58.6	148.8	42.5	45.2	16.2	48.8
4	4.7	3.7	63.3	181.1	59.8	73.1	24.5	60.0
average	**8.1**	**2.9**	**57.0**	**150.9**	**46.0**	**61.8**	**20.3**	**56.1**