JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

A Tissue Homogenate Method To Prepare Gram-Scale *Allium* Thiosulfinates and Their Disulfide Conjugates with Cysteine and Glutathione

Guodong Zhang*,† and Kirk L. Parkin*

Department of Food Science, Babcock Hall, University of Wisconsin—Madison, 1605 Linden Drive, Madison, Wisconsin 53706, United States

Supporting Information

ABSTRACT: The health benefits of *Allium* vegetables are widely attributed to the enzyme-derived organosulfur compounds called thiosulfinates (TS). However, the lack of a suitable method to prepare TS in good yields has hampered the evaluation of their biological activities. This paper describe a simple enzymatic method using *Allium* tissue homogenates as a reaction system to prepare gram-scale TS, including those enriched in 1-propenyl groups, which are particularly difficult to obtain. This method is simple, easy to scale up, and requires no column purification step, making it suitable for practical large-scale production of *Allium* TS. The prepared TS were further utilized to prepare the disulfide conjugates with cysteine and glutathione (CySSR and GSSR, R = methyl, ethyl, propyl, 1-propenyl, and allyl), which are the presumptive metabolites of TS. Among all of the *Allium* CySSR and GSSR conjugates, the newly prepared glutathione conjugate with 1-propenyl TS, GSSPe, showed the most potent effect to induce quinone reductase (QR, a representative phase II enzyme) in murine hepatoma cells (Hepa 1c1c7) and inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated macrophage cells (RAW 264.7).

KEYWORDS: *Allium, thiosulfinate, alliinase, thiosulfinate conjugates, S-1-propenylmercaptocysteine (CySSPe), S-1-propenylmercaptoglutathione (GSSPe)*

INTRODUCTION

Epidemiological studies support that a diet rich in *Allium* vegetables is correlated with reduced risks of many human diseases.[1,2] The health benefits of *Allium* vegetables are widely attributed to the alliinase-transformed organosulfur compounds called thiosulfinates (TS) and related organosulfur species (chemical structures of representative TS are shown in Figure 1).[3] For example, allicin, which is a major TS derived from garlic, has been reported to have a variety of health-promoting effects including anti-inflammation, up-regulation of phase II enzymes, antiproliferation and induction of apoptosis, cardioprotection via relaxation of pulmonary arteries and inhibition of platelet aggregation, and antibacterial activity.[3] TS and related organosulfur compounds are unstable in vivo and are conjugated with cysteine (Cys) and glutathione (GSH) to generate putative metabolites named *S*-alk(en)ylmercaptocysteine (CySSR) and *S*-alk(en)ylmercaptoglutathione (GSSR), respectively (chemical structures of CySSR and GSSR are shown in Figure 1).[4−7] These presumptive metabolites of TS are widely believed to contribute to the health-promoting effects of *Allium* vegetables.[7] For example, *S*-allylmercaptocysteine (CySSA), which is the cysteine disulfide conjugate with allicin, has been shown to exert potent anticancer activities in vitro and in vivo and has been marketed as a dietary supplement for a decade.[8−10]

Having a simple and reliable method to prepare TS in good yields would help to investigate their biological activities and potential therapeutic applications. However, until now there is no satisfactory method to prepare TS. Direct solvent extraction of crushed *Allium* tissues generates a complex mixture. Chemical synthesis of TS is difficult; in particular, the 1-propenyl TS, which are the major TS species in onions, are very challenging to prepare.[11,12] An alliinase-based enzymatic method is very attractive; however, it can generate only semipreparative quantities due to the very low yields of this enzymatic reaction.[13,14] The reasons for the low reaction yields include (1) the enzymatic reaction reportedly stopping after a limited number of cycles because alliinase is inactivated by its own product TS;[14] (2) the instability of both alliinase and its product TS; and (3) the major impurities in the synthetic alliinase substrates, *S*-alk(en)yl-L-cysteines (CySR), are inhibitors of alliinase.[15] Recently an immobilized alliinase method was reported to continuously produce TS via separation of the newly generated TS from alliinase enzyme; however, the procedure was complicated and time-consuming.[14] Until now there is no report of the use of alliinase enzyme for large-scale preparation of TS.

For preparation of CySSR and GSSR, in our previous study we have reported the chemical synthesis of non-1-propenyl conjugates CySSR and GSSR (R = methyl, ethyl, propyl, and allyl).[16] However, this multistep organic synthesis method was difficult to carry out and cannot prepare the disulfide conjugate of 1-propenyl TS (CySSPe and GSSPe, the chemical structures are shown in Figure 1). The 1-propenyl TS are the major TS species in some *Allium* vegetables such as onions[3] and have

Received: December 18, 2012
Revised: March 4, 2013
Accepted: March 6, 2013
Published: March 6, 2013

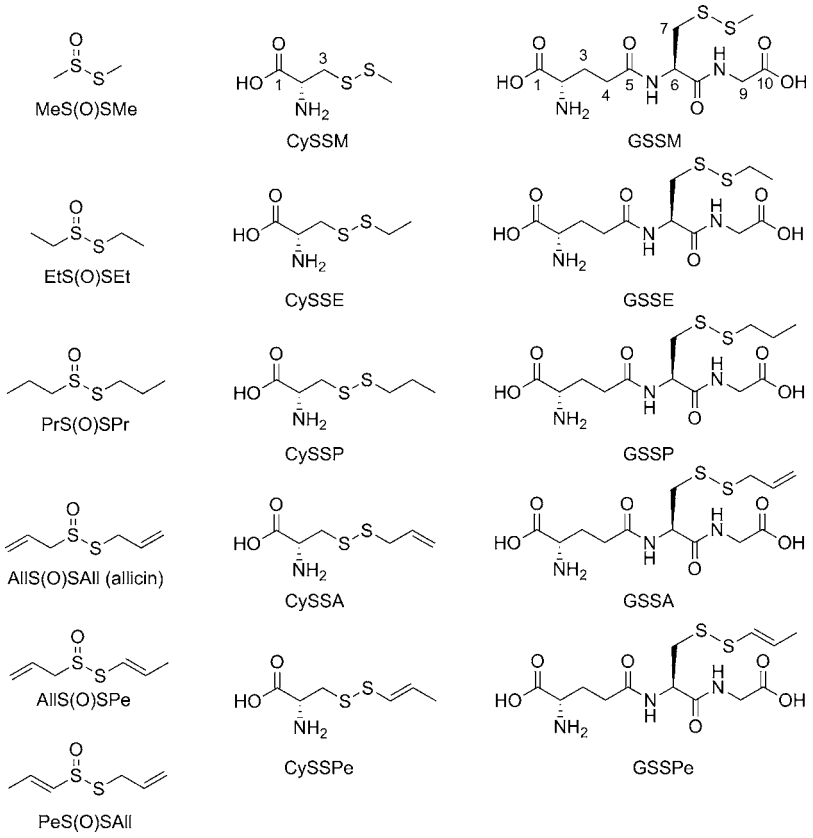


Figure 1. Chemical structures of TS, CySSR, and GSSR in this study.

been shown to have the most potent phase II enzyme inducing activities among major *Allium* TS species.[17] Therefore, the biological activities of CySSPe and GSSPe may be critical to our understanding of the health benefits of onion and other *Allium* vegetables.

In this paper, we describe a simple enzymatic method using *Allium* tissue homogenates to prepare gram-scale TS, including those enriched in 1-propenyl groups, which are particularly difficult to obtain. The prepared TS were further utilized to prepare CySSR and GSSR (R = methyl, ethyl, propyl, and allyl) via a one-step process without any chromatography purification process and CySSPe and GSSPe via a one-step process with HPLC purification.

MATERIALS AND METHODS

Materials and General Experimental Procedures. Chemicals were obtained from Sigma-Aldrich (Milwaukee, WI, USA), and solvents for extraction or HPLC analysis were purchased from Fisher Scientific (Fair Lawn, NJ, USA) unless otherwise noted. (±)-*S*-Alk(en)yl-L-cysteine sulfoxides (ACSO), including MCSO, ECSO, PCSO, and 2-PeCSO, were prepared as described in our earlier paper.[13] Cell culture medium and supplements were from Invitrogen (Carlsbad, CA, USA). Garlic and onion were purchased from a local retailer in Madison, WI, USA. NMR data were collected on Varian Unity-Inova 400 and 500 MHz NMR spectrometers (Analytical Instrumentation Center, School of Pharmacy, UW—Madison). High-resolution ESI-MS was collected on an Agilent ESI-TOF mass spectrometer (Mass Spectrometry/Proteomics Facility, Biotechnology Center, UW—Madison).

Preparation of Gram-Scale Non-1-propenyl TS. Peeled garlic (100 g) in 20−90 mL of water was homogenized using a kitchen blender for 1−2 min at 4 °C, and the garlic homogenate was then incubated at 20−22 °C for 30 min−1 h and filtered through a cheesecloth; the filtrate was rapidly extracted with equal amounts of ethyl acetate (EtOAc) twice. Three to four grams of synthetic ACSO (R = methyl, ethyl, propyl, and allyl) dissolved in 10 mL water was added into the resulting aqueous layer with a total reaction volume of ∼100 mL. The reaction mixture was incubated at room temperature for 40−60 min, and the formed TS were then extracted with an equal amount of CH_2Cl_2. The CH_2Cl_2 layer was dried over anhydrous $MgSO_4$ and evaporated to dryness under a gentle nitrogen stream at 20−22 °C to obtain the final product. The dried TS were used directly for HPLC analysis or preparation of CySSR and GSSR without any purification.

To check the purity of the prepared TS, the CH_2Cl_2 extract was analyzed on a 250 × 4.6 mm, 5 μm, silica gel column (Supelco, Bellefonte, PA, USA) eluted with a gradient of 2-propanol in hexane from 2:98 (v/v) held for 6 min to 10:90 for the next 10 min followed by a 9 min hold, with a flow rate of 1.8 mL/min and the detector set at 254 nm.[18] The structures of the prepared TS were confirmed by NMR and MS analysis; ^{1}H NMR data of TS were reported in our previous study.[13]

Tissue Reaction To Enrich 1-Propenyl TS. White onions (200 g) were cut into two pieces for each onion bulb and heated in 100 mL of boiling water for 4−5 min. After the heat-inactivated onion tissues were cooled, they were homogenized with 10 g of peeled fresh garlic for 1−2 min at 4 °C. The homogenized mixture was incubated at room temperature for 40−60 min and filtered through a cheese cloth; the filtrate was extracted with 100 mL of CH_2Cl_2. The CH_2Cl_2 extract was dried over anhydrous $MgSO_4$ and evaporated to dryness under a gentle nitrogen stream at room temperature. HPLC analysis was carried out using the conditions as described above.

Preparation of Non-1-propenyl CySSR and GSSR (R = Methyl, Ethyl, Propyl, and Allyl). The prepared TS were reconstituted in water, 1.5−2.0 M Cys or GSH dissolved in water was added (1 M TS reacts with 2 M Cys or GSH, thus, extra TS were used in the reaction), and the reaction mixture was stirred at room temperature for 2 h. After the reaction, the reaction product was dried using a rotary evaporator under vacuum or a freeze-dryer, and the

dx.doi.org/10.1021/jf4003818 | J. Agric. Food Chem. 2013, 61, 3030−3038

dried reaction mixture was then washed with CH_2Cl_2 to remove residual TS; no purification step was required. The ^1H NMR data of CySSR and GSSR were reported in our previous study.[16]

Preparation of CySSPe and GSSPe. The above prepared 1-propenyl-contaning TS were reconstituted in water, 1.5−2.0 M Cys or GSH dissolved in water was added, and the reaction mixture was stirred at room temperature for 1−2 h. The reaction mixture was purified by HPLC to obtain pure CySSPe and GSSPe using a 250 × 10 mm C18 HPLC column (Phenomenex, Torrance, CA, USA) eluted with 35% methanol in water with 0.5% acetic acid, flow rate of 3.2 mL/min, and detection at 254 nm.

The structure of CySSPe was confirmed by comparing its NMR and MS data with a previous paper.[19] ^1H NMR of CySSPe (D_2O, 500 MHz): δ 4.35 (dd, J = 4.0, 8.0 Hz, 1H, H-2), 3.37 (dd, J = 4.0, 15.5 Hz, 1H, H-3α), 3.21 (dd, J = 8.0, 15.0 Hz, 1H, H-3β), 6.17 (m, 2H, H-4 and H-5), 1.81 (m, 3H, H-6). HRESIMS, m/z, $[M + H]^+$ at 194.0308 (calcd 194.0309), $[M - H]^-$ at 192.0166 (calcd 192.0153).

The structure of GSSPe was confirmed by a series of 1D and 2D NMR, including ^1H, ^{13}C, COESY, HMQC, and HMBC. ^1H NMR of GSSPe (D_2O, 500 MHz): δ 3.82 (tr, J = 6.5 Hz, 1H, H-2), 2.19 (qr, J = 7.5 Hz, 1H, H-3α), 2.20 (qr, J = 7.5 Hz, 1H, H-3β), 2.56 (tr, J = 7.5 Hz, 1H, H-4α), 2.58 (tr, J = 7.5 Hz, 1H, H-4β), 4.72 (dd, J = 4.5, 9.5 Hz, 1H, H-6), 3.01 (dd, J = 9.5, 14.5 Hz, 1H, H-7α), 3.27 (dd, J = 4.5, 14.5 Hz, 1H, H-7β), 3.97 (s, 2H, H-9), 6.12 (m, 2H, H-11 and H-12), 1.78 (br d, J = 5.0 Hz, 3H, H-13). ^{13}C NMR (D_2O, 125 MHz): δ 17.7 (C-13), 26.4 (C-3), 31.6 (C-4), 38.4 (C-7), 42.0 (C-9), 53.0 (C-6), 54.2 (C-2), 123.5 (C-12), 133.5 (C-11), 172.9−175.1 (C-1, C-5, C-8, and C-10). HRESIMS, m/z $[M + H]^+$ at 380.0933 (calcd 380.0950), $[M - H]^-$ at 378.0802 (calcd 378.0794).

Quinone Reductase (QR) Induction Assay. A bioassay based on Hepa 1c1c7 cells (ATCC, Rockville, MD, USA) was used to assess QR induction essentially as described earlier.[20] Hepa 1c1c7 cells (5×10^3 cells/well) were plated in 96-well plates in 200 μL of MEM supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37 °C in 5% CO_2 in air and allowed to attach for 24 h. The medium was then replaced with fresh MEM containing test compounds for 48 h. A standard assay cocktail was prepared, and the QR activity was determined by measuring the absorbance of the reduced tetrazolium dye over a 10 min period at 490 nm. The cell viability was determined by crystal violet assay at 610 nm. The degree of QR induction was calculated as the ratio of QR activity in the treated (induced) sample relative to the untreated control sample. The concentration required for doubling the specific activity of QR relative to nontreated control cells was used as an indicator of inducer potency, expressed as CD values.

Nitric Oxide (NO) Assay. Mouse macrophage RAW 264.7 cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 15% fetal bovine serum with antibiotics (a mixture of penicillin and streptomycin) at 37 °C in 5% CO_2 in a 96-well plate (5×10^5 cells/well) for 24 h. The medium was then replaced with fresh medium containing test compounds and 1 μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA). After 24 h of treatment, a 100 μL sample was taken from the culture supernatant of each well and mixed with an equal amount of freshly prepared Griess reagent and incubated for 10 min, and then the optical density was measured at 542 nm. The cell viability was measured by a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at 550 nm.[21] IC_{50} values for NO inhibition were determined using XLfit software (IDBS, Surrey, UK).

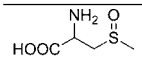
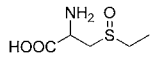
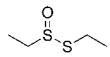
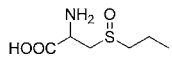
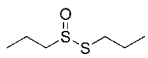
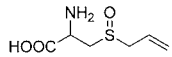
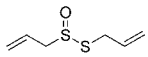
Western Blot. Hepa 1c1c7 and RAW 264.7 cells were plated in 6-well plates and allowed to attach for 24 h and then treated with test compounds for 24−48 h. Cell protein samples (50 μg) were resolved using 10% SDS−polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% skim milk solution in Tris-buffered saline Tween-20 (TBST) for 1 h and then incubated with primary antibodies against inducible nitric oxide synthase (iNOS) and QR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 3% skim milk in TBST buffer at 4 °C overnight. After three washings with TBST buffer, the blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at 20−22 °C and washed three times with TBST buffer. The secondary antibody on the blot was detected by a chemiluminescence reagent (Pierce, Rockford, IL, USA) coupled to autoradiography using Kodak BioMax film.

Statistics. Results are expressed as the mean ± SD of at least three separate experiments, each with three to four replicates for each condition assessed. Group comparisons were carried out using Student's t test. P values of <0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Preparation of Gram-Scale Non-1-propenyl TS (RSS(O)R, R = Methyl, Ethyl, Propyl, and Allyl). To achieve gram-scale preparation of TS using alliinase enzyme, we developed a simple method for large-scale preparation of crude alliinase. After garlic tissues were homogenized and incubated to allow the conversion of endogenous garlic ACSO to TS, the garlic homogenate was washed with an organic solvent such as EtOAc. After EtOAc washing, alliinase enzyme in the resulting aqueous layer was found to be still active. Addition of gram-scale synthetic (±)-ACSO (MCSO, ECSO, PCSO, or 2-PeCSO) into the aqueous layer generated the corresponding pure TS after 30 min−1 h of incubation at room temperature with a yield of 42−99% (Table 1 shows the yields

Table 1. Yields and Scales of Reactions To Convert ACSO to TS

ACSO Species	Weight of ACSO	Generated TS	Weight of TS	Yield (molar basis)
 MCSO	3.26 g	 MeS(O)SMe	0.5 g	42%
 ECSO	3.55 g	 EtS(O)SEt	1.0 g	67%
 PCSO	2.00 g	 PrS(O)SPr	0.6 g	65%
 2-PeCSO	4.00 g	 AllS(O)SAll (allicin)	2.0 g	99%

and scales of the reactions). The yields are calculated on the basis of the dry weight of the final product (note volatile losses of TS during solvent evaporation). A relative yield of 2-PeCSO (yield = 99%) > ECSO (yield = 67%) ≈ ECSO (yield = 65%) > MCSO (yield = 42%) was obtained (Table 1), consistent with the substrate selectivity of alliinase enzyme.[13] The almost complete conversion of (±)-2-PeCSO to allicin (yield = 99%) indicated the slow and recalcitrant (−)-2-PeCSO in the synthetic substrate was also converted by the crude alliinase. Without any purification, the prepared TS were found be to essentially without impurity as assessed by HPLC (Figure 2) and ^1H NMR analysis (data not shown). The structures of the prepared TS were confirmed by comparing the NMR data with our previous results.[13]

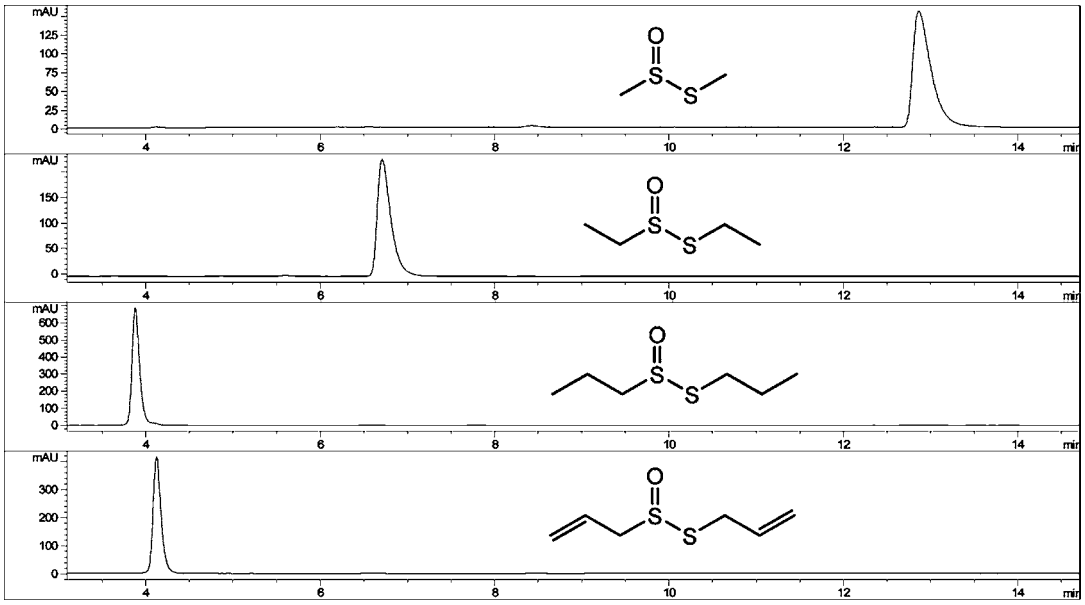


Figure 2. HPLC analysis of TS prepared by the washed garlic homogenate method.

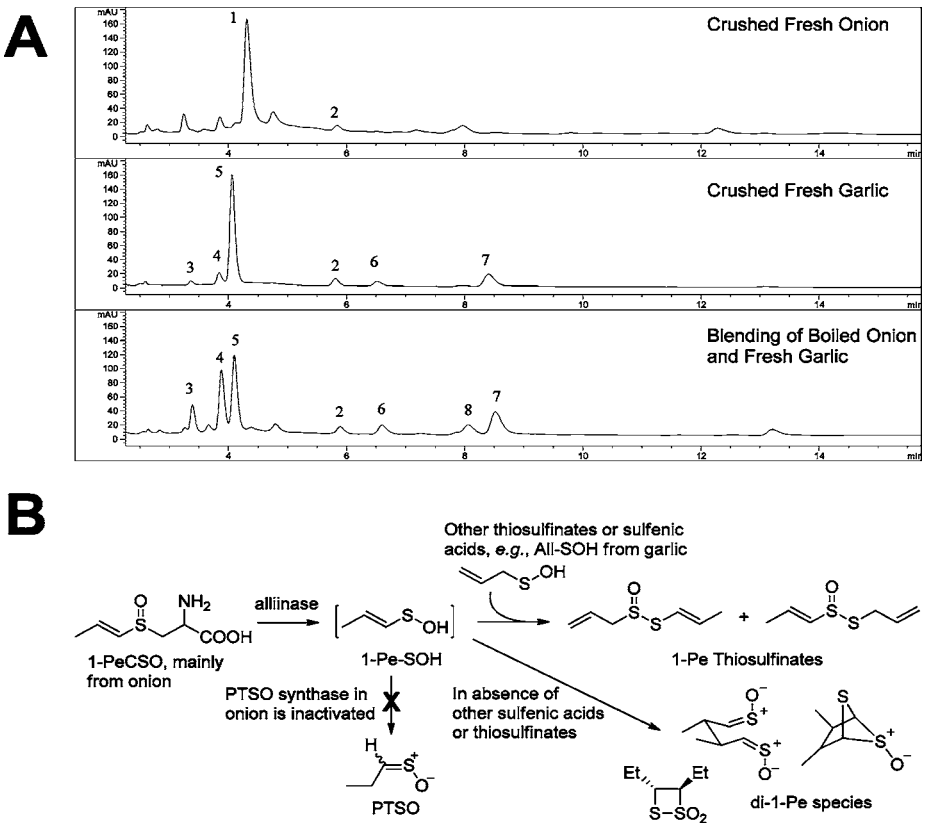


Figure 3. (A) HPLC analysis of organosulfur profile of crushed fresh onions (top panel), fresh garlic (middle panel), and the blend of boiled onion and fresh garlic (bottom panel). Tissue blending increased the proportion of 1-propenyl TS (peaks 3, 4, and 8) without scarifying the total yield. Peaks: 1, PTSO; 2, benzyl alcohol as internal standard; 3, AllSS(O)Pe; 4, AllS(O)SPe; 5, AllSS(O)All (allicin); 6, MeSS(O)All; 7, MeS(O)SAll; 8, MeS(O)SPe. (B) Proposed mechanism for the enrichment of 1-propenyl TS. Two key points for the enrichment of 1-propenyl organosulfur compounds are that (i) inactivation of PTSO synthase in onion (via heating) diminished the production of PTSO and (ii) the enzyme-generated 1-Pe-SOH needs to be rapidly captured by other sulfenic acids; otherwise they would react with each other to form di-1-propenyl TS (PeSS(O)Pe), which would rapidly rearrange to form cyclized sulfur compounds.

Alliinase is a major enzyme in *Allium* tissues; for example, it comprises ~10% of total protein in garlic.[22,23] It catalyzes the carbon−sulfur (C−S) bond cleavage of ACSO to form sulfenic acids (RSOH), which rapidly react with each other to generate homologous (RS(O)SR) or heterologous (RS(O)SR′) TS.[3] However, using purified alliinase enzyme, the yields of this enzymatic reaction were low, and only semipreparative quantities of TS were obtained.[13,14] Here we have developed

dx.doi.org/10.1021/jf4003818 | J. Agric. Food Chem. 2013, 61, 3030−3038

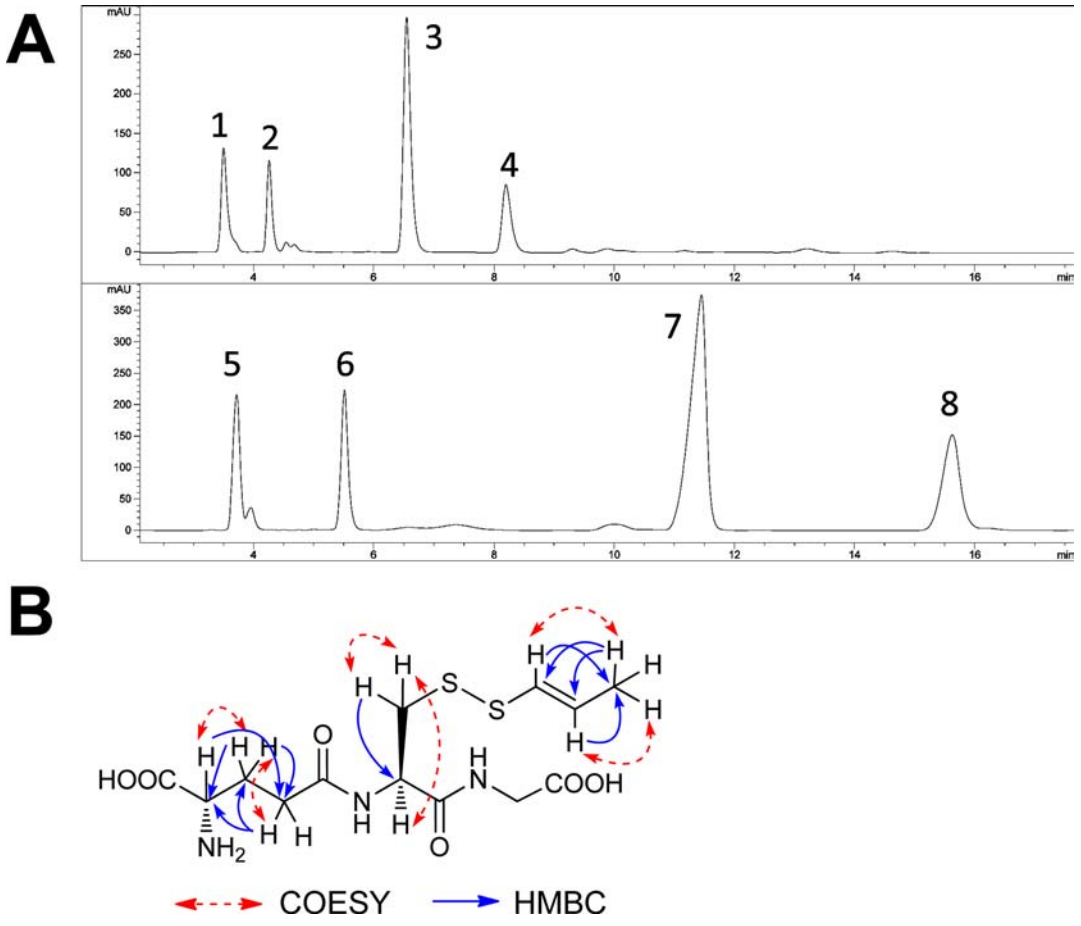


Figure 4. (A) HPLC analysis of reaction product of Cys or GSH with tissue enriched 1-propenyl TS (analytical C18 HPLC was eluted by 30% methanol/water solution with 0.5% acetic acid, FR = 0.8 mL/min, detection at 254 nm). Peaks: 1, Cys; 2, CySSM; 3, CySSA; 4, CySSPe; 5, GSH; 6, GSSM; 7, GSSA; 8, GSSPe. (B) Selected COESY and HMBC correlations of GSSPe.

a simple method to prepare a large amount of active alliinase enzyme. Compared with a previous method such as $(NH_4)_2SO_4$ precipitation, which took 1−2 days, this method can prepare a large amount of crude alliinase in 30 min−1 h; thus, this is very easy to scale-up. Due to the simple operations, a large amount of alliinase enzyme can be used in the reaction system, leading to high reaction yields in gram-scale reactions (Table 1). During the preparation of the crude alliinase, endogenous TS and other compounds soluble in organic solvent from garlic were removed by the first step organic solvent washing (using EtOAc). Thus, the second-step organic solvent extraction (using CH_2Cl_2) after the enzymatic reaction recovered only the newly formed TS derived from the exogenously added synthetic ACSO. This leads to high purity of the generated TS, even without any purification step (Figure 2). For each reaction, the gram-scale enzymatic conversion can be completed in 30 min−1 h; the short reaction time minimizes the decomposition of relatively unstable TS.

Tissue Reaction To Enrich 1-Propenyl TS (1-PeS(O)SR or 1-PeSS(O)R). *S*-1-Propenyl-L-cysteine sulfoxide (1-PeCSO) is the dominant ACSO species in some *Allium* vegetables such as onions.[24] Upon tissue crushing, 1-PeCSO is converted by alliinase to form *S*-1-propenyl sulfenic acid (1-Pe-SOH), which has at least three fates: (1) 1-Pe-SOH is converted to a lachrymatory factor propanethial *S*-oxide (PTSO) via the catalytic activity of PTSO synthase;[25] (2) 1-Pe-SOH reacts with itself to form di-1-propenyl disulfide *S*-oxide (1-Pe-S(O)S-1-Pe), which quickly rearranges to form cyclized sulfur compounds;[12,26] (3) 1-Pe-SOH reacts with other sulfenic acids (such as Me-SOH, Pr-SOH, and 2-Pe-SOH) to form heterologous 1-propenyl TS (1-Pe-S(O)S-R and R-S(O)S-1-Pe).[12,26] Among all TS, 1-propenyl TS are the most challenging to prepare because they are present in low abundance in onions or garlic; the organic synthesis involves multistep synthesis, which is very difficult.[12,26] The washed garlic homogenate method described above cannot be used to prepare 1-propenyl TS; addition of 1-PeCSO or a combination of 1-PeCSO and another ACSO to the washed garlic homogenate generated only the cyclized sulfur compounds dervied from di-1-propenyl disulfide *S*-oxide (data not shown).

Here we developed a tissue reaction method to enrich 1-propenyl TS, using mixed blending of fresh garlic and heat-inactivated onion tissues. Figure 3A shows the HPLC analysis of the organosulfur compounds derived from fresh onion (top panel), fresh garlic (middle panel), and a mixture of fresh garlic and heat-inactivated onion tissues (bottom panel). PTSO (peak 1) is the dominant organosulfur compound from fresh onion; the other organosulfur compounds are minor. Allicin (peak 5) is the dominant organosulfur compound from fresh garlic, no PTSO was observed, and 1-propenyl TS (peaks 3 and 4) comprise <5% of the total garlic TS. Using the mixed blending strategy, the proportion of 1-propenyl TS (peaks 3, 4, and 8) increased to 50% of the total TS, whereas no PTSO formation

dx.doi.org/10.1021/jf4003818 | J. Agric. Food Chem. 2013, 61, 3030−3038

was observed. The total TS yields of the mixture were similar to that of garlic tissues (data not shown).

Figure 3B shows the fate and flux of the *S*-1-propenyl group in reaction processes to explain the enrichment of 1-propenyl species. PTSO formation requires PTSO synthase, which exists in onion but not in garlic;[25] thus, PTSO formation in the mixture was diminished by inactivation of onion enzyme. 1-PeCSO in onions was converted by the garlic alliinase to form 1-Pe-SOH, which reacted with other sulfenic acids (mostly 2-Pe-SOH derived from garlic) to form the heterologous 1-propenyl TS (the bottom panel in Figure 3A shows high levels of AllSS(O)Pe and AllS(O)SPe from the mixture). Garlic tissue contains a high amount of alliinase and no PTSO synthase; also, it contains a high amount of 2-PeCSO, which will be rapidly converted to All-SOH to capture onion-derived 1-Pe-SOH. These properties make garlic a suitable candidate for tissue reactions.

Preparation of CySSR and GSSR (R = Methyl, Ethyl, Propyl, and Allyl). Our previous study has reported the chemical synthesis of non-1-propenyl CySSR and GSSR using the reaction of Cys or GSH with dithiophosphoric alk(en)yl disulfides;[16] however, the multistep chemical synthesis was difficult to carry out. Here we prepared CySSR/GSSR (R = methyl, ethyl, propyl, and allyl) by the reaction of prepared pure TS with Cys or GSH in water. Without any chromatography purification, >95% purity of CySSR and GSSR was obtained; ^{1}H NMR analysis showed the only impurity in the reaction product was unreacted Cys or GSH. Using TS to prepare the disulfide conjugates has the following advantages: (1) the reaction requires only the TS and the thiol compound (no catalyst is needed); (2) the reaction is rapid and almost complete; (3) TS are well soluble in many solvents from water to CH_2Cl_2, so they can be used to react with hydrophilic or lipophilic thiol compounds; and (4) an extra amount of TS is used in the reaction, and the residue TS can be easily removed by evaporation or solvent washing (no chromatography purification is required).

Preparation of CySSPe and GSSPe. 1-Propenyl TS are major species in fresh onion tissues;[3] thus, the biological effects of the 1-propenyl mixed disulfide conjugate derivatives (CySSPe and GSSPe) may be key to understanding the health benefits of onion. However, our previous method could not be used to prepare CySSPe and GSSPe because 1-propene-1-thiol is not available to prepare the synthetic intermediate dithiophosphoric 1-propenyl disulfide using the synthetic strategy in our previous study.[16] Using a modified synthesis strategy, we have synthesized the dithiophosphoric 1-propenyl disulfide (Supporting Information, Scheme S1). However, the reaction of dithiophosphoric 1-propenyl disulfide with Cys or GSH failed to generate CySSPe or GSSPe (data not shown). This result suggests that a new method is needed to prepare CySSPe and GSSPe.

Here we prepared CySSPe and GSSPe by the reaction of Cys or GSH with the enriched 1-propenyl TS prepared from the above mixture. Figure 4A shows a typical HPLC profile of the reaction product. For the reaction with Cys or GSH, four peaks were observed on HPLC, and they were confirmed to be unreacted starting material Cys or GSH and the methyl, allyl, and 1-propenyl disulfide conjugates with Cys or GSH. The identities of peaks 1−3 and 5−7 were confirmed by coelution with synthesized standards. Peaks 4 and 8 were purified by semipreparative HPLC and confirmed to be CySSPe and GSSPe, respectively, by NMR and MS analysis. The structure of CySSPe was confirmed by comparing its NMR and MS data with previous results.[19] GSSPe was not prepared before, and the structure of GSSPe was confirmed by a series of 1D and 2D NMR techniques including ^{1}H, ^{13}C, COESY, HMQC, and HMBC (the NMR data of GSSPe are shown in Table 2; selected COESY and HMBC correlations are shown in Figure 4B).

Table 2. ^{1}H (500 MHz), HMBC (125 MHz), and ^{13}C (125 MHz) NMR Data for *S*-1-Propenylmercapto-L-glutathione (GSSPe) in D_2O[a]

		HMBC		
position	δ_H	^2J	^3J	δ_C
1				175.1 or 172.9
2	3.82 (tr, 6.5)	C-1, C-3	C-4	54.2
3	2.19 (qr, 7.5)	C-2, C-4		26.4
	2.20 (qr, 7.5)			
4	2.56 (tr, 7.5)	C-3		31.6
	2.58 (tr, 7.5)			
5				175.1 or 172.9
6	4.72 (dd, 4.5, 9.5)			53.0
7	3.01 (dd, 9.5, 14.5)	C-6		38.4
	3.27 (dd, 4.5, 14.5)			
8				175.1 or 172.9
9	3.97 (s)			42.0
10				175.1 or 172.9
11	6.12 (m)		C-13	133.5
12	6.12 (m)	C-13		123.5
13	1.78 (br, 5.0)	C-12	C-11	17.7

[a] J in hertz in parentheses.

In a typical tissue reaction described above, one heat-inactivated onion bulb (~300 g) and fresh garlic (~15 g) generate ~0.2 g of enriched 1-Pe TS mixture, which can be used to prepare 0.5−1.0 g of Cys or GSH conjugate mixture, which has defined compositions containing ~20% CySSPe or GSSPe. Compared with a previous multiple-step chemical synthesis of CySSPe,[19] this vegetable blending strategy is a very simple and cost-effective method to prepare mixed or pure 1-propenyl disulfide conjugates.

CySSPe and GSSPe Have the Most Potent Biological Activities among *Allium* CySSR/GSSR Conjugates. Chemoprevention refers to the use of agents to suppress tumor progression, leading to reduced risk of cancers.[27] Two signaling pathways have been shown to play a key role in the action mechanisms of many chemopreventive agents: (1) induction of phase II detoxification enzymes such as QR and glutathione *S*-transferase, which are involved in the detoxification of environmental carcinogens to inhibit the formation of ultimate electrophilic and carcinogenic species; and (2) inhibition of inflammation, including the production of inflammatory mediators and cytokines, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2. Chronic inflammation is widely believed to be correlated with increased cancer risks.[28]

Our previous study has shown that CySSR and GSSR (R = methyl, ethyl, propyl, and allyl) have potent effects to induce QR activities in Hepa 1c1c7 cells and inhibit LPS-induced NO formation in RAW 264.7 cells.[16] Here we further found that CySSA and GSSA induced the expression of QR protein in Hepa 1c1c7 cells and inhibited LPS-induced iNOS expression in RAW 264.7 cells (Supporting Information, Figure S1),

dx.doi.org/10.1021/jf4003818 | J. Agric. Food Chem. 2013, 61, 3030−3038

supporting the effects of CySSR and GSSR are at the protein level. However, the effects of CySSPe and GSSPe are unknown. Here we tested the biological activities of the newly prepared 1-propenyl conjugates CySSPe and GSSPe (Figure 5A–D).

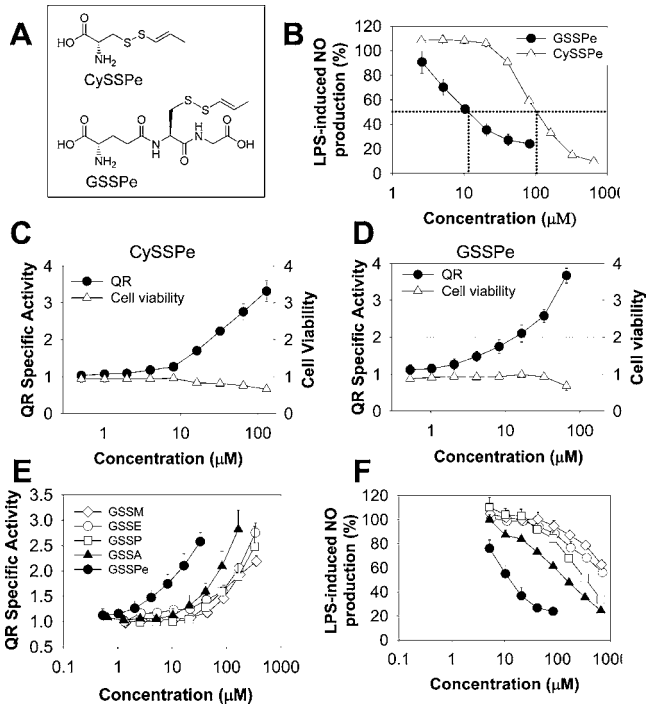


Figure 5. Effects of CySSR and GSSR on induction of QR and inhibition of LPS-induced NO formation: (A) chemical structures of CySSPe and GSSPe; (B) LPS-induced NO assay of CySSPe and GSSPe in RAW 274.7 cells; (C) QR assay of CySSPe; (D) QR assay of GSSPe; (E) structure–activity relationships (SAR) of GSSR for QR induction; (F) SAR of GSSR conjugates for LPS-induced NO formation. The results are expressed as the mean ± SD of three independent experiments with four duplicates in each experiment.

Among all of the *Allium* CySSR and GSSR conjugates, GSSPe showed the most potent effects to inhibit LPS-induced NO formation in RAW 264.7 cells with an IC_{50} value = 12.6 ± 1.1 μM (Figure 5B) and induce QR specific activity with a CD value = 14.9 ± 0.8 μM (Figure 5D). CySSPe had a potent effect with a CD value = 31.1 ± 8 μM (Figure 5C); however, it had a weak NO inhibitory effect with IC_{50} = 120.8 ± 1.8 μM (Figure 5B). For QR induction, the CD values of CySSPe and GSSPe were comparable to those of 1-propenyl TS, which have CD values of ~10 μM.[17] These result suggest that CySSPe and GSSPe had potential cancer preventive and anti-inflammatory effects.

Structure–Activity Relationships (SAR) of CySSR and GSSR Conjugates. The CD and IC_{50} values of CySSR and GSSR are shown in Table S1 in the Supporting Information. Comparison of the biological activities of CySSR and GSSR (R = methyl, ethyl, propyl, allyl, and 1-propenyl) revealed that the R- group plays a key role in the potency of the conjugates. For GSSR conjugates, an order of potency of GSSPe > GSSA > GSSP ≈ GSSE ≈ GSSM was observed for both QR and NO assays (the dose responses of all GSSR conjugates for QR and NO assays are shown in Figure 5E,F). For CySSR conjugates, a similar trend was also observed except CySSPe showed a weak NO inhibitory effect.

Previous study has shown that many cancer preventive compounds activate phase II enzymes or inhibit inflammation via covalent modification of important signaling proteins such as Keap1 for phase II enzyme induction[29] or p65 for NF-κB-mediated inflammation signaling.[30] CySSA has been shown to covalently interact with protein thiols.[31,32] A recent study showed that it disrupted microtubule polymerization via covalent modifications of cysteine residues of microtubule.[8] Therefore, it is likely that CySSR and GSSR exert their biological activities via direct interactions with cysteine residues of Keap1 or p65 proteins. The relative potencies of CySSR and GSSR may be due to varied reactivity of the conjugates with protein thiols. More studies are required to characterize the mode of actions of CySSR and GSSR.

β-Lyase-Dependent Mechanism May Not Be Involved in Biological Activities of CySSR. A previous study has shown that CySSA is an efficient substrate of β-lyase, which cleaves CySSA to generate highly redox-active persulfide species (RSS), leading to modulation of redox signaling (proposed mechanism is shown in Figure 6A).[33] To test

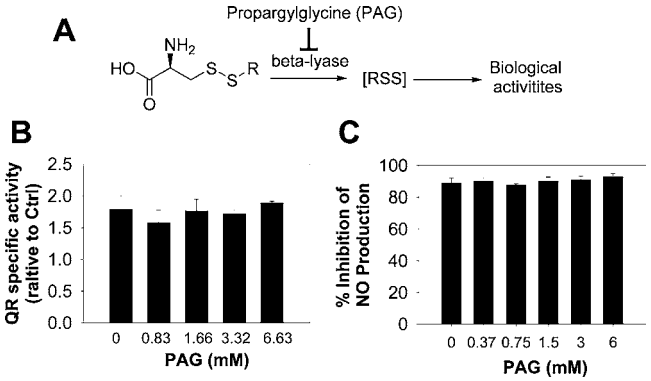


Figure 6. β-Lyase inhibitor propargylglycine (PAG) has no effect on activity of CySSA in vitro. (A) The previously proposed mechanism of β-lyase in the activation of CySSR to form redox active RSS species shows that propargylglycine (PAG) is a chemical inhibitor of β-lyase. (B) Hepa 1c1c7 cells were treated with a combination of CySSA (0.17 mM) and varied doses of PAG. After 48 h of treatment, QR activities were assayed. (C) RAW 264.7 cells were treated with a combination of CySSA (0.09 mM) and varied doses of PAG and then stimulated with 1 μg/mL LPS. After 24 h of treatment, the concentration of nitrite in the medium was analyzed. The results are expressed as the mean ± SD of three independent experiments with four duplicates in each experiment.

whether the β-lyase-dependent mechanism was involved in the QR inducing and NO inhibitory effects of CySSA, the cells were treated with a combination of CySSA and varied doses of propargylglycine (PAG), a chemical inhibitor of β-lyase.[34] As shown in Figure 6B,C, co-addition of PAG (0.37–7 mM) had no effect on CySSA-induced QR induction (Figure 6B) or NO inhibition (Figure 6C), suggesting the β-lyase pathway was not involved in the QR inducing and NO inhibitory effects of CySSA.

In summary, here we demonstrate a simple enzymatic method for gram-scale preparation of *Allium* TS, as well as their putative metabolites CySSR and GSSR. To our best knowledge, this is the first study to use alliinase enzyme for gram-scale preparation of TS. The prepared CySSR and GSSR conjugates have potent effects to induce phase II enzymes and inhibit

dx.doi.org/10.1021/jf4003818 | J. Agric. Food Chem. 2013, 61, 3030–3038

inflammation in vitro, which may contribute to the health-promoting effects of *Allium* vegetables.

■ ASSOCIATED CONTENT

Supporting Information

Additional scheme, table, and figure. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: (K.L.P.) klparkin@wisc.edu or (G.Z.) gdzhang@ucdavis.edu.

Present Address

†Department of Entomology and Comprehensive Cancer Center, University of California, Davis, One Shield Avenue, Davis, CA, 95616, USA.

Funding

This work was supported by the University of Wisconsin, College of Agricultural and Life Sciences, in part through an Agricultural Research Station Hatch grant (WIS01184) and the American Institute for Cancer Research (project 10A050).

Notes

The authors declare the following competing financial interest(s): K.P. and G.Z. have applied for a U.S. patent for the tissue homogenate method: Process of preparing conjugates of Allium organosulfur compound with amino acids, peptides and proteins, US 20110082282, assigned to Wisconsin Alumni Research Foundation.

■ ABBREVIATIONS USED

ACSO, (±)-*S*-alk(en)yl-L-cysteine sulfoxide; Cys, cysteine; CySR, *S*-alk(en)yl-L-cysteine; CySSR, *S*-alk(en)ylmercapto-L-cysteine; CySSM, *S*-methylmercapto-L-cysteine; CySSE, *S*-ethylmercapto-L-cysteine; CySSP, *S*-propylmercapto-L-cysteine; CySSA, *S*-2-propenylmercapto-L-cysteine; CySSPe, *S*-1-propenylmercapto-L-cysteine; EtOAc, ethyl acetate; GSH, glutathione; GSSR, *S*-alk(en)ylmercapto-L-glutathione; GSSM, *S*-methylmercapto-L-glutathione; GSSE, *S*-ethylmercapto-L-glutathione; GSSP, *S*-propylmercapto-L-glutathione; GSSA, *S*-2-propenylmercapto-L-glutathione; GSSPe, *S*-1-propenylmercapto-L-glutathione; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PTSO, propanethial *S*-oxide; QR, quinone reductase or NAD(P)H:quinone oxidoreductase; SAR, structure and activity relationships; TS, thiosulfinates

■ REFERENCES

(1) Galeone, C.; Pelucchi, C.; Levi, F.; Negri, E.; Franceschi, S.; Talamini, R.; Giacosa, A.; La Vecchia, C. Onion and garlic use and human cancer. *Am. J. Clin. Nutr.* **2006**, *84*, 1027−1032.

(2) Sengupta, A.; Ghosh, S.; Bhattacharjee, S. Allium vegetables in cancer prevention: an overview. *Asian Pac. J. Cancer. Prev.* **2004**, *5*, 237−245.

(3) Rose, P.; Whiteman, M.; Moore, P. K.; Zhu, Y. Z. Bioactive *S*-alk(en)yl cysteine sulfoxide metabolites in the genus *Allium*: the chemistry of potential therapeutic agents. *Nat. Prod. Rep.* **2005**, *22*, 351−368.

(4) Teyssier, C.; Siess, M. Metabolism of dipropyl disulfide by rat liver phase I and phase II enzymes and by isolated perfused rat liver. *Drug Metab. Dispos.* **2000**, *28*, 648−654.

(5) Jin, L.; Baillie, T. A. Metabolism of the chemoprotective agent diallyl sulfide to glutathione conjugates in rats. *Chem. Res. Toxicol.* **1997**, *10*, 318−327.

(6) Germain, E.; Chevalier, J.; Siess, M. H.; Teyssier, C. Hepatic metabolism of diallyl disulphide in rat and man. *Xenobiotica* **2003**, *33*, 1185−1199.

(7) Miron, T.; Rabinkov, A.; Mirelman, D.; Wilchek, M.; Weiner, L. The mode of action of allicin: its ready permeability through phospholipid membranes may contribute to its biological activity. *Biochim. Biophys. Acta−Biomembranes* **2000**, *1463*, 20−30.

(8) Xiao, D.; Pinto, J. T.; Soh, J.; Deguchi, A.; Gundersen, G. G.; Palazzo, A. F.; Yoon, J.; Shirin, H.; Weinstein, I. B. Induction of apoptosis by the garlic-derived compound 3*S*-allylmercaptocysteine (SAMC) is associated with microtubule depolymerization and c-Jun NH_2-terminal kinase 1 activation. *Cancer Res.* **2003**, *63*, 6825−6837.

(9) Shirin, H.; Pinto, J. T.; Kawabata, Y.; Soh, J.; Delohery, T.; Moss, S. F.; Murty, V.; Rivlin, R. S.; Holt, P. R.; Weinstein, I. B. Antiproliferative effects of *S*-allylmercaptocysteine on colon cancer cells when tested alone or in combination with sulindac sulfide. *Cancer Res.* **2001**, *61*, 725−731.

(10) Howard, E. W.; Ling, M.; Chua, C. W.; Cheung, H. W.; Wang, X.; Wong, Y. C. Garlic-derived *S*-allylmercaptocysteine is a novel in vivo antimetastatic agent for androgen-independent prostate cancer. *Clin. Cancer Res.* **2007**, *13*, 1847−1856.

(11) Lawson, L. D.; Wang, Z. J. Low allicin release from garlic supplements: a major problem due to the sensitivities of alliinase activity. *J. Agric. Food Chem.* **2001**, *49*, 2592−2599.

(12) Block, E.; Bayer, T.; Naganathan, S.; Zhao, S. *Allium* chemistry: synthesis and sigmatropic rearrangements of alk(en)yl 1-propenyl disulfide *S*-oxides from cut onion and garlic. *J. Am. Chem. Soc.* **1996**, *118*, 2799−2810.

(13) Shen, C.; Parkin, K. L. In vitro biogeneration of pure thiosulfinates and propanethial-*S*-oxide. *J. Agric. Food Chem.* **2000**, *48*, 6254−6260.

(14) Miron, T.; SivaRaman, H.; Rabinkov, A.; Mirelman, D.; Wilchek, M. A method for continuous production of allicin using immobilized alliinase. *Anal. Biochem.* **2006**, *351*, 152−154.

(15) Schwimmer, S.; Ryan, C. A.; Wong, F. F. Specificity of L-cysteine sulfoxide lyase and partially competitive inhibition by *S*-alkyl-l-cysteines. *J. Biol. Chem.* **1964**, *239*, 777−782.

(16) Zhang, G.; Li, B.; Lee, C.; Parkin, K. L. Cysteine and glutathione mixed-disulfide conjugates of thiosulfinates: chemical synthesis and biological activities. *J. Agric. Food Chem.* **2010**, *58*, 1564−1571.

(17) Block, E.; Gillies, J. Z.; Gillies, C. W.; Bazzi, A. A.; Putman, D.; Revelle, L. K.; Wang, D.; Zhang, X. Allium chemistry: microwave spectroscopic identification, mechanism of formation, synthesis, and reactions of (*E*,*Z*)-propanethial *S*-oxide, the lachrymatory factor of the onion (*Allium cepa*). *J. Am. Chem. Soc.* **1996**, *118*, 7492−7501.

(18) Block, E.; Naganathan, S.; Putman, D.; Zhao, S. H. *Allium* chemistry: HPLC analysis of thiosulfinates from onion, garlic, wild garlic (ramsoms), leek, scallion, shallot, elephant (great-headed) garlic, chive, and Chinese chive. Uniquely high allyl to methyl ratios in some garlic samples. *J. Agric. Food Chem.* **1992**, *40*, 2418−2430.

(19) Hiramitsu, T.; Sakamoto, K. 2-Amino-2-carboxyethyl *trans*-1-propenyl disulfides as antitumor agents and their preparationJpn. Kokai Tokkyo Koho 89-19476, 1990; 4.

(20) Prochaska, H. J.; Santamaria, A. B. Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. *Anal. Biochem.* **1988**, *169*, 328−336.

(21) Granger, D. L.; Taintor, R. R.; Boockvar, K. S.; Hibbs, J. B., Jr. Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction. *Methods Enzymol.* **1996**, *268*, 142−151.

(22) Ellmore, G. S.; Feldberg, R. S. Alliin lyase localization in bundle sheaths of the garlic clove (*Allium sativum*). *Am. J. Bot.* **1994**, *81*, 89−94.

(23) Van Damme, E. J. M.; Smeets, K.; Torrekens, S.; Van Leuven, F.; Peumans, W. J. Isolation and characterization of alliinase cDNA clones from garlic (*Allium sativum* L.) and related species. *Eur. J. Biochem.* **1992**, *209*, 751−757.

(24) Thomas, D. J.; Parkin, K. L. Quantification of alk(en)yl-L-cysteine sulfoxides and related amino acids in alliums by high-

performance liquid chromatography. *J. Agric. Food Chem.* **1994**, *42*, 1632−1638.
(25) Imai, S.; Tsuge, N.; Tomotake, M.; Nagatome, Y.; Sawada, H.; Nagata, T.; Kumagai, H. Plant biochemistry: an onion enzyme that makes the eyes water. *Nature* **2002**, *419*, 685−685.
(26) Block, E. The organosulfur chemistry of the genus *Allium* − implications for the organic chemistry of sulfur. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 1135.
(27) Surh, Y. J. Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer.* **2003**, *3*, 768−780.
(28) Coussens, L. M.; Werb, Z. Inflammation and cancer. *Nature* **2002**, *420*, 860−867.
(29) Dinkova-Kostova, A. T.; Massiah, M. A.; Bozak, R. E.; Hicks, R. J.; Talalay, P. Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 3404−3409.
(30) García-Piñeres, A. J.; Castro, V.; Mora, G.; Schmidt, T. J.; Strunck, E.; Pahl, H. L.; Merfort, I. Cysteine 38 in p65/NF-κB plays a crucial role in DNA binding inhibition by sesquiterpene lactones. *J. Biol. Chem.* **2001**, *276*, 39713−39720.
(31) Miron, T.; Listowsky, I.; Wilchek, M. Reaction mechanisms of allicin and allyl-mixed disulfides with proteins and small thiol molecules. *Eur. J. Med. Chem.* **2010**, *45*, 1912−1918.
(32) Rabinkov, A.; Miron, T.; Mirelman, D.; Wilchek, M.; Glozman, S.; Yavin, E.; Weiner, L. *S*-Allylmercaptoglutathione: the reaction product of allicin with glutathione possesses SH-modifying and antioxidant properties. *Biochim. Biophys. Acta−Mol. Cell Res.* **2000**, *1499*, 144−153.
(33) Cooper, A. L.; Pinto, J. Aminotransferase, L-amino acid oxidase and β-lyase reactions involving L-cysteine *S*-conjugates found in allium extracts: relevance to biological activity? *Biochem. Pharmacol.* **2005**, *69*, 209−220.
(34) Abeles, R. H.; Walsh, C. T. Acetylenic enzyme inactivators. Inactivation of γ-cystathionase, in vitro and in vivo by propargylglycine. *J. Am. Chem. Soc.* **1973**, *95*, 6124−6125.