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ARTICLE

Cysteine and Glutathione Mixed-Disulfide Conjugates of Thiosulfinates: Chemical Synthesis and Biological Activities

GUODONG ZHANG,[†] BIN LI,[‡] CHEN-HSIEN LEE,[†,§] AND KIRK L. PARKIN*,[†]

[†]Department of Food Science, Babcock Hall, University of Wisconsin—Madison, 1605 Linden Drive, Madison, Wisconsin 53706 and [‡]College of Food Science and Technology, South China Agricultural University, Guangzhou, People's Republic of China. [§] Present address: Department of Food Science and Biotechnology, 250 Kuo Kuang Road, National Chung Hsing University, Taichung 402, Taiwan.

The chemical syntheses of cysteine (CYS) and glutathione (GSH) mixed -disulfide conjugates (CySSR, GSSR, respectively) of mercapto residues representing most of the R groups of thiosulfinates (R = methyl, ethyl, propyl, and allyl) are described. Gram-scale conjugates were prepared as >98% pure preparations, with 80% reaction yield for each of the two seminal synthesis steps, with structures confirmed by ^{1}H NMR and high-resolution MS analyses. These conjugates are derivatives of thiosulfinates that may be evolved in processed foods, in the digestive tract, and through in vivo metabolism. The prepared conjugates were found to be able to induce quinone reductase (QR, a representative phase II enzyme) in murine hepatoma cells (Hepa 1c1c7) and to inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated macrophage cells (RAW 264.7), indicating they have potential cancer preventive and anti-inflammatory activities. Among the prepared conjugates, the allyl conjugates of CYS and GSH, *S*-allylmercaptocysteine (CySSA) and *S*-allylmercaptoglutathione (GSSA), showed the most potent activity regarding QR induction and NO production inhibition. The conjugates with saturated R groups were also active and conferred biological activity as cystine and oxidized glutathione exhibited no effects in these cellular assays.

KEYWORDS: ***Allium*; thiosulfinates; mixed-disulfide conjugates; *S*-allylmercaptocysteine; *S*-allylmercaptoglutathione; phase II enzyme induction; anti-inflammatory**

INTRODUCTION

The health benefits of *Allium* vegetables are widely attributed to or correlated with the enzyme-transformed organosulfur compounds called thiosulfinates (**1**) (**Figure 1**) because they have exhibited many biological activities (*1*−*3*). However, the rapid metabolism of thiosulfinates in situ and in vivo may indicate that their metabolic products may contribute to bioactivity or be the putative bioactive agents. Thiosulfinates can freely permeate cell membranes and rapidly react with glutathione (GSH) to form intracellular GSH mixed-disulfide conjugates (GSSR) (*4*). In extracellular fluids where cysteine/cystine is the dominant thiol (*5*), similar thiol−thiosulfinate chemical conjugations may occur. GSSR (R = propyl or allyl) species may also be derived from metabolism of other *Allium* organosulfur compounds such as dipropyl disulfide, diallyl disulfide, and diallyl sulfide through reactions mediated by cytochrome P450 or glutathione *S*-transferase (*6*, *7*) (**Figure 2**). In foods, thiosulfinates are produced in minced, but unheated, *Allium* vegetable tissues (*3*). In the presence of free thiols, including those in proteins, conjugation reactions can occur in foods containing thiosulfinates. Thiosulfinates are most stable at acidic pH in foods and of the stomach, while also persisting for several hours at duodenal pH (*1*, *3*, *8*). Thiosulfinates react more favorably in conjugation reactions with thiols with increasing pH toward neutrality (*9*), and in the gastrointestinal tract both cysteine and glutathione are made available by secretion into the lumen (*5*). In blood, thiosulfinates have a half-life of < 1 min, and the presumed fate is through reaction with thiols in plasma or blood cells (*3*).

Some thiosulfinate conjugates of cysteine (CYS) have demonstrable biological activities. For example, the *S*-allylmercaptocysteine (CySSA) derivative is believed to be a major bioactive principle in aged garlic extract and has antioxidant (*10*), antiproliferative (*11*), apoptosis-inducing (*12*), and antimetastatic activities (*13*). Aside from the studies on CySSA, there is a dearth of information on other alk(en)yl mixed disulfides of biologically relevant thiols, likely because they are not commercially available. Anticancer effects can be mediated through the antioxidant response element to afford up-regulation of phase II enzymes (*14*). Phase II enzyme activities, such as quinone reductase, glutathione-*S*-transferase, and UDP-glucuronyltransferase, are responsible for detoxifying carcinogens and facilitating their excretion from the body.

The current methods to prepare CYS/GSH mixed-disulfide conjugates of thiosulfinates rely mainly on the reaction of thiosulfinates and CYS/GSH (*4*, *9*, *15*). However, preparation of some thiosulfinates, particularly the heterologous and *S*-1-propenyl-containing species, by chemical synthesis is challenging (*3*, *16*). Enzymatic methods to prepare thiosulfinates have been described, but appear limited to small-scale preparations (*17*, *18*). Thiosulfinates are not available commercially,

*Corresponding author [e-mail klparkin@wisc.edu; telephone (608) 263-2011; fax (608) 262-6872].

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likely because they are relatively unstable (*8*, *19*). Thus, to facilitate studies on CYS/GSH mixed-disulfide conjugates that represent the range of derivatives that can be formed from *Allium* tissue constituents and through in vivo metabolism, it is necessary to adopt facile chemical methods for large-scale preparation of these conjugates. In this paper, we describe a simple combination of steps to synthesize cysteine and glutathione *S*-alk(en)yl disulfide conjugates (CySSR and GSSR, respectively) representative of many thiosulfinates common to *Allium* and chemotaxonomically related species. A dose-dependent assessment of cellular bioactivities of these conjugates related to cancer chemoprotection and anti-inflammatory effects is also provided.

MATERIALS AND METHODS

Materials and General Procedures. Chemicals were obtained from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) Chemical Companies. Solvents used for extraction or chromatography were purchased from Fisher Scientific (Chicago, IL). Cell culture media and adjuvants were from Invitrogen (Carlsbad, CA).

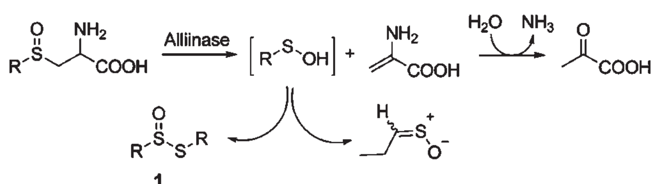


Figure 1. Organosulfur transformation in disrupted *Allium* tissues. Transient sulfenic acid (RSOH) can condense to form thiosulfinates (R−S(O)S−R′) of (dis)similar R groups or rearrange to form propanethial-*S*-oxide (PTSO, a tear-inducing agent, only when R = 1-propenyl).

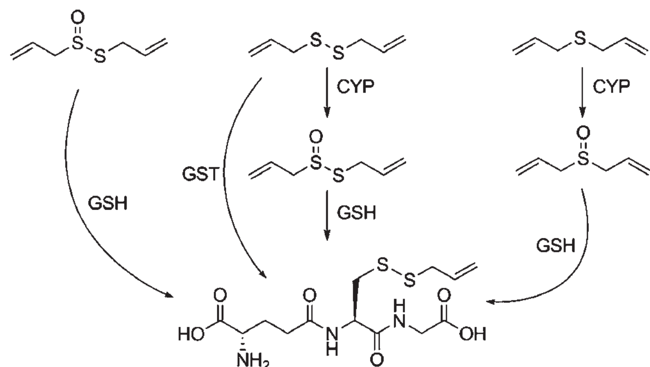


Figure 2. Metabolic transformation of organosulfur components derived from *Allium* tissues. CYP, cytochrome P450; GST, glutathione-*S*-transferase. Adapted from refs *6* and *7*.

Silica gel (60 Å, 230−400 mesh ASTM; Fisher Scientific, Fair Lawn, NJ) and C18 reverse phase silica gel (60 Å, 230−400 mesh ASTM; EMD Chemical Inc., Gibbstown, NJ) were used for flash column chromatography. NMR spectra were collected on a Varian Unity-Inova 400 and 500 MHz NMR spectrometer (Analytical Instrumentation Center, School of Pharmacy, University of Wisconsin (UW)—Madison). High-resolution ESI-MS analyses were conducted on an Agilent ESI-TOF mass spectrometer (Mass Spectrometry/Proteomics Facility, Biotechnology Center, UW—Madison).

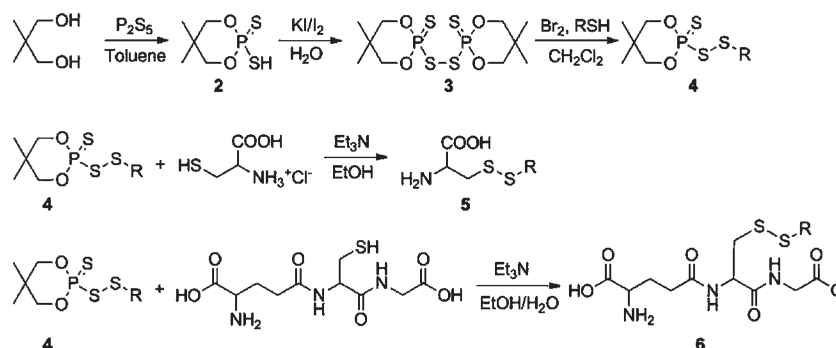
Synthesis of Bis(5,5-dimethyl-2-thiono-1,3,2-dioxaphosphorinanyl) Disulfide. The synthesis of bis(5,5-dimethyl-2-thiono-1,3,2-dioxaphosphorinanyl) disulfide (**3**) was carried out according to the methods described by Edmundson (*20*) (**Scheme 1**). 2,2-Dimethylpropane-1,3-diol (41.6 g) was added to a suspension of 44.8 g of P_2S_5 in 200 mL of toluene under reflux for 2−3 h. The product mixture was then subjected to solvent removal by vacuum rotary evaporation and the residue extracted with diethyl ether to obtain 5,5-dimethyl-2-thiolo-2-thiono-1,3,2-dioxaphosphorinane (**2**), which was used for the next step without further purification after vacuum rotary evaporation of ether. ^{1}H NMR of **2** (400 MHz, $CDCl_3$): δ 4.08 (s, 2H, $-CH_2O-$), 4.04 (s, 2H, $-CH_2O-$), 1.09 (s, 6H, CH_3-).

A volume of 20 mL of concentrated NH_4OH (28−30%) was then added dropwise to a solution of (**2**) in 300 mL of toluene with vigorous stirring, and the mixture was extracted with water (3 × 300 mL) to recover the NH_4^+ salt of **2**. Then, 50 g of I_2 dissolved in KI solution (100 g of KI in 400 mL water) was added portion-wise to the aqueous phase over 30 min with vigorous stirring. The aqueous-insoluble product (**3**) was extracted with CH_2Cl_2 (3 × 500 mL). The organic layers were combined, washed with water, dried over anhydrous $MgSO_4$, and vacuum rotary evaporated to afford the disulfide product (**3**), which was used for the next step without further purification. ^{1}H NMR of **3** (400 MHz, $CDCl_3$): δ 4.39 (d, J = 11.5 Hz, 2H CCH_2O), 4.38 (d, J = 11.0 Hz, 2H, CCH_2O), 4.04 (d, J = 11.0 Hz, 2H, CCH_2O), 3.99 (d, J = 11.0 Hz, 2H, CCH_2O), 1.30 (s, 6H, $C(CH_3)_2$), 0.96 (s, 6H $C(CH_3)_2$); APCIMS: $[M + H]^+$ 395.0 (calcd 394.97).

Synthesis of 5,5-Dimethyl-2-thiono-1,3,2-dioxaphosphorinanyl-2-disulfanylmethane. The synthesis of 5,5-dimethyl-2-thiono-1,3,2-dioxaphosphorinanyl-2-disulfanylmethane (**methyl-4**) was carried out according to modified methods described by Antoniow and Witt (*21*). Br_2 (4 g) was added to a solution of 10 g (**3**) in 100 mL of CH_2Cl_2 at −5 °C. The mixture was stirred at this temperature for 30 min, and 3.55 g of sodium methanethiolate (CH_3SNa) was then added, followed by stirring at 20−22 °C overnight. The resulting reaction mixture was washed three times with 1 vol of water. The organic phase was dried over anhydrous $MgSO_4$ and subjected to vacuum rotary evaporation to afford the target product (**methyl-4**). This derivative was directly used for the next step without further purification. For identification, a >98% pure **methyl-4** was obtained by silica gel column chromatography eluted with hexane/CH_2Cl_2 (1:1). ^{1}H NMR of **methyl-4** (500 MHz, $CDCl_3$): δ 4.12 (m, 4H, CCH_2O), 2.65 (s, 3H, SCH_3), 1.22 (s, 3H, CCH_3), 1.05 (s, 3H, CCH_3).

Synthesis of 5,5-Dimethyl-2-thiono-1,3,2-dioxaphosphorinanyl-2-disulfanylethane (Ethyl-4). The preparation of the ethyl form of **4** was similar to the above method, except that (i) ethanethiol was used instead of sodium methanethiolate and (ii) after the addition of ethanethiol, the reaction was stirred for 30 min instead of overnight. ^{1}H NMR of **ethyl-4**

Scheme 1. Synthesis of CYS and GSH Conjugates of Thiosulfinates (R = Methyl, Ethyl, Propyl, or Allyl)[a]



[a] To prepare CSSM/GSSM, CH_3SNa was used in reaction step 3 because CH_3SH is a gas at normal conditions. The chemical synthesis was carried out using methods adapted from refs *20* and *21*.

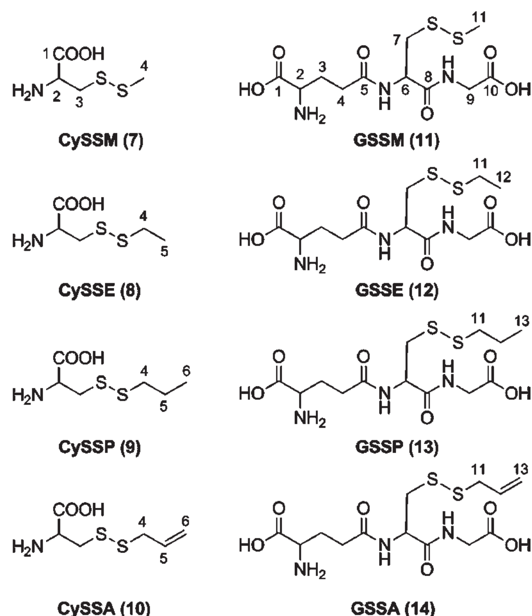


Figure 3. Chemical structures of prepared GSH and CYS conjugates of thiosulfinates. GSSM, *S*-methylmercaptoglutathione; GSSE, *S*-ethylmercaptoglutathione; GSSP, *S*-propylmercaptoglutathione; GSSA, *S*-allylmercaptoglutathione; CySSM, *S*-methylmercaptocysteine; CySSE, *S*-ethylmercaptocysteine; CySSP, *S*-propylmercaptocysteine; CySSA, *S*-allylmercaptocysteine.

(500 MHz, $CDCl_3$): δ 4.12 (m, 4H, CCH_2O), 3.02 (qr, J = 7.2 Hz, 2H, SCH_2CH_3), 1.37 (tr, J = 7.2 Hz, 3H, SCH_2CH_3), 1.21 (s, 3H, CCH_3), 1.05 (s, 3H, CCH_3).

Synthesis of 5,5-Dimethyl-2-thiono-1,3,2-dioxaphosphorinanyl-2-disulfanylpropane (Propyl-4). The propyl form of **4** was synthesized similarly to the method of the ethyl conjugate except propanethiol was used. ^{1}H NMR of **propyl-4** (500 MHz, $CDCl_3$): δ 4.12 (m, 4H, $-CCH_2O$), 2.98 (tr, J = 7.0 Hz, 2H, SCH_2CH_2), 1.75 (sex., J = 7.0 Hz, 2H, $CH_2CH_2CH_3$), 1.20 (s, 3H, CCH_3), 1.05 (s, 3H, CCH_3), 1.01 (tr, J = 7.0 Hz, 3H, CH_2CH_3).

Synthesis of 5,5-Dimethyl-2-thiono-1,3,2-dioxaphosphorinanyl-2-disulfanyl-2-propene (allyl-4). The allyl form of **4** was synthesized similarly to the described method of the ethyl conjugate except 2-propene-1-thiol was used. ^{1}H NMR of **allyl-4** (500 MHz, $CDCl_3$): δ 5.86 (m, 1H, $CH_2CH{=}CH_2$), 5.23 (m, 2H, $CH_2{=}CH-$), 4.11 (m, 4H, CCH_2O), 3.65 (d, J = 7.6 Hz, 2H, SCH_2CH), 1.20 (s, 3H, CCH_3), 1.05 (s, 3H, CCH_3).

Synthesis of Cysteine (CYS) Alkyl Disulfide Conjugates. The mixed-disulfide conjugates CySSR (**5**; **Scheme 1**) that were prepared are shown in **Figure 3** (**7**−**10**). For the propyl disulfide conjugate of CYS (CySSP, **9**), triethylamine (5.7 mmol) was combined with L-cysteine hydrochloride monohydrate (1.42 mmol) and (**propyl-4**) (2.83 mmol) in 15 mL of ethanol. The reaction mixture was vigorously stirred at 20−22 °C for 1−2 h. The solvent was removed by vacuum rotary evaporation, and the residue was washed with CH_2Cl_2 to remove the excess reactant (**propyl-4**) and the major byproduct (**2**). An alternative workup protocol after reaction involved filtering the product mixture to recover **5** (largely insoluble in ethanol) and washing the particulate material with cold ethanol to obtain the final product. In both cases, >80% pure CySSP was obtained (the only impurity shown on ^{1}H NMR was unreacted L-cysteine). A >98% pure sample of **9** was obtained by RP-18 column chromatography eluted with 50% methanol in water. Methyl (CySSM, **7**), ethyl (CySSE, **8**), and allyl (CySSA, **10**) conjugates of CYS were prepared according to the same method except that methyl, ethyl, and allyl forms of **4** were used.

Synthesis of Glutathione (GSH) Alkyl Disulfide Conjugates. The mixed-disulfide conjugates GSSR (**6**; **Scheme 1**) that were prepared are shown in **Figure 3**. Glutathione conjugates of propyl (GSSP, **13**), ethyl (GSSE, **12**), methyl (GSSM, **11**), and allyl residues (GSSA, **14**) were prepared as just described for the corresponding cysteine conjugates, except that GSH (1.40 mmol) replaced CYS in the reaction mixtures (**Scheme 1**). Similar purities of the GSSR species were obtained as reported for the CySSR species following purification by RP-18 flash chromatography using the conditions described in the preceding paragraph.

Phase II Enzyme Induction Assay. A bioassay based on cultured murine hepatoma cells (Hepa 1c1c7; ATCC, Rockville, MD) was used to assess QR induction as a phase II enzyme biomarker essentially as described in the original method (*14*). Hepa 1c1c7 cells were grown for 24 h in 96-well plates (5×10^3 cells/well) in 200 μL of minimal essential medium (MEM) supplemented with 10% fetal bovine serum (treated with activated charcoal to remove any traces of endogenous QR inducers), 100 U/mL of penicillin G, and 100 μg/mL of streptomycin at 37 °C in 5% CO_2 in air. The medium was replaced with fresh MEM containing test compounds (serially diluted 1:2 in MEM), and the cells were incubated for another 48 h.

After the culture medium was decanted, cells were lysed by adding 50 μL of saturated digitonin aqueous solution in 2 mM EDTA, pH 7.8, and then incubated at 37 °C for 30 min with gentle shaking. For the QR assay, a standard assay cocktail (containing the essential components of FAD, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and menadione) was prepared and added in a final volume of 200 μL to each well. QR activity was determined by measuring the absorbance of the reduced tetrazolium dye over a 10 min period using a Spectra Max plus optical microtiter plate scanner (Molecular Devices, Sunnyvale, CA) set at 490 nm.

A duplicate plate was prepared for cell protein (viability) assays and to facilitate relative enzyme specific activity calculations. The second plate was emptied of liquid contents, 100 μL of crystal violet (0.2% in 2% ethanol) was added to each well and incubated for 10 min at 20−22 °C, and finally the plates were rinsed under cold running water to remove excess stain. A 150 μL aliquot of 0.5% (w/v) SDS (prepared in 50% aqueous ethanol) was added to each well, and the plates were incubated for 1 h in a shaker oven at 37 °C to dissolve the dye. The optical density of each well was read at 610 nm. The absorbance values of no-cell blanks were subtracted from those of the controls and treated samples. The degree of staining (absorbance) with crystal violet was used as a measure of cell protein (density) and provided the basis for calculating changes in QR specific activity. QR specific activity was not determined in cases when loss of viability exceeded 50% as suggested (*14*).

For a given induced or control sample, QR specific activity was calculated using the linear portion of the reaction progress curve (typically 5 min) from the QR assay relative to the absorbance value (protein) of the crystal violet stain. The degree of QR induction was then calculated as the ratio of QR specific activity in the treated (induced) sample relative to the control sample. A "CD value" (concentration required to double the specific activity of QR) was interpolated from dose-dependent response plots for each conjugate tested and was used as an indicator of relative inducer potency. Untreated control cells were assigned a relative QR specific activity of 1.0.

Nitric Oxide (NO) Evolution Bioassay. Mouse macrophage cells (RAW 264.7, ATCC) were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen) medium supplemented with 15% fetal bovine serum with antibiotic (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, and the plate was incubated for another 24 h. A 100 μL sample of culture supernatant from each well was then combined with 100 μL of freshly prepared Griess reagent (equal volumes of 2% (w/v) sulfanilamide containing 4% (w/v) H_3PO_4 and 0.2% (w/v) naphthylethylenediamide) and incubated for 10 min, and then the absorbance was measured at 542 nm (*22*). The concentration of nitrite (evolved from NO) in the supernatant was calculated from a standard curve prepared with sodium nitrite dissolved in DMEM.

Cell viability was measured by the MTT assay (*23*). After a 100 μL sample had been taken from the cell culture supernatant for the NO assay, the residual medium was decanted, 100 μL of MTT dye (0.05 mg/mL in phosphate-buffered saline) was added to each well, and the cells were incubated for 1 h at 37 °C. Then the solution was decanted, and 200 μL of DMSO was added to each well and incubated for 10 min under gentle shaking at 37 °C to dissolve the dye. Relative cell viability was calculated by measuring the optical density at 550 nm with untreated control cells assigned a value of 1.0.

Statistical Analysis. Results are expressed as mean values ± SD for two or three separate experiments, each with two to three replicates for each condition assessed. Differences between responses were determined using a one-tailed *t* test, with a *P* value of <0.05 considered to be significant.

RESULTS AND DISCUSSION

Synthesis of CYS/GSH Mixed-Disulfide Conjugates of Thiosulfinates. The first stage of synthesizing the CYS/GSH disulfide conjugates of thiosulfinates (**5**, **6**) involved the preparation of the methyl, ethyl, propyl, and allyl disulfide conjugates of dithiophosphoric acid (**4**) according to protocols described for preparing other compounds (*20*, *21*). This stage posed no complications with typical yields of 70−80%, and derivatives of **4** appeared to be stable at 20−22 °C for several months. In previous papers, only hydrophobic thiol substrates were investigated for the preparation of unsymmetrical disulfides (*21*). In the present study, some modifications were necessitated by making use of the water-soluble thiol substrates CYS and GSH in the reaction. The second stage of the synthesis involved reaction of CYS or GSH with excess dithiophosphoric-alk(en)yl disulfides (**4**) in the presence of triethylamine as base in ethanol or ethanol−water as solvent. CYS/GSH conjugates (**5**, **6**) were generated with yields of ~80%, likely limited by the partial solubility of **4** in aqueous or polar organic media. The major byproduct of the reaction (**2**) was difficult to remove from the reaction product mixture, and it was difficult to resolve from the reaction products (**5**, **6**) under the RP-18 flash chromatography conditions used. It was found that evaporation of the ethanolic reaction medium followed by extracting the product mixture with CH_2Cl_2 circumvented this problem and removed the main contaminants (**2** and **4**), as well as other unidentified impurities in the reaction mixture. This afforded >80% pure CYS/GSH mixed-disulfide conjugate preparations (**7**−**14**), and the only impurity remaining after CH_2Cl_2 washing was identified as unreacted CYS or GSH. The utility of this washing/purification step allowed the use of a molar excess of **4** in the reaction to favor complete thiol conversion into the intended disulfide conjugates (**5**, **6**), whereas the original method used equimolar ratios of organic soluble **4** and thiols as a means to minimize residual byproduct and unreacted materials (*21*). Subsequently, >98% pure (assessed by peak integration by ^{1}H NMR) CYS/GSH mixed-disulfide conjugates (**7**−**14**) were obtained by a final RP-18 column chromatography purification step.

High-resolution ESI-TOF MS profiles data of the CySSR/GSSR derivatives (**7**−**14**; **Figure 3**) are summarized in **Table 1**. (ESI-TOF HR-MS spectra are available in the Supporting Information.) The molecular formulas of the prepared compounds were confirmed by high-resolution MS analysis. As an example, the reaction product of cysteine and sodium methyl mercaptoate exhibited the molecular ions *m/z* $[M + H]^+$ at 168.0153 and $[M - H]^-$ at 165.9996, consistent with the molecular formula of CySSM (**7**): $C_4H_9NO_2S_2$ (calcd $[M + H]^+$ *m/z* 168.0153, $[M - H]^-$ *m/z* 165.9996). Compared with the starting material cysteine (exact molecular weight of 121.0198), a +45.9877 Da fragment was observed, corresponding to the addition of the $-SCH_3$ group to form the disulfide bond.

^{1}H NMR spectral signals of the CySSR and GSSR derivatives in D_2O are summarized in **Tables 2**−**4**. The ^{1}H NMR signal assignments could be compared only for those species previously analyzed (*24*, *25*), which include CySSA (**10**), GSSA (**14**), and CYS and GSH, with differences identified in the balance of this section. No reports of ^{1}H NMR spectra could be found for the other conjugates (**7**−**9**, **11**−**13**). For CySSA (**10**), the two germinal protons of C-3 are adjacent to the chiral center C-2, and, thus, the H-3 protons should exhibit different chemical shifts and split each other with a germinal coupling constant of ^2J = 15.0 Hz. H-2 and H-3 protons should exhibit three groups of doublets of doublets signals at δ 4.10 (H-2, dd, *J* = 4.0, 8.5 Hz, 1H), 3.10 (H-3, dd, *J* = 8.5, 14.5 Hz, 1H), and 3.35 (H-3, dd, *J* = 4.0, 15.0 Hz, 1H) (**Table 3**). This contrasts with the previously reported (*24*) spectral characteristics of shifts of δ 4.07 (H-2, q) and 3.41 (H-3, dq). Also, the previously reported δ 3.19 (H-4, dq) appears to be a transposing error, as the published spectrum reveals a value close to our δ 3.45 (H-4, br d) signal, consistent with our results. As with CySSA (**10**), the two germinal protons of H-7 of GSSA (**14**) were interpreted as two doublets of doublets signals at δ 3.01 (H-7, dd, *J* = 9.5, 14.5 Hz, 1H) and 3.27 (H-7, dd, *J* = 4.5, 14.5 Hz, 1H) (**Table 4**) as opposed to the reported δ 5.88 (H-7, m) (*12*). We also interpret each of the ^{1}H NMR signals of H-3 and H-4 as having two chemical shifts (δ 2.19, 2.20 for H-3, tr; δ 2.57 and 2.58 for H-4, tr) instead of single shifts (δ 2.14 for H-3, q; δ 2.52 for H-4, q) as reported in ref *9* based on the conformational analysis of the glutathione skeleton (*25*). In addition, H-9 protons should exhibit a singlet signal because of the absence of coupling protons, instead of the doublet signal previously reported (*12*). The hydrochloride forms of CySSR were also prepared and characterized by NMR (**Table 2**), and the only major difference with

Table 1. High-Resolution ESI-TOF MS of CySSR/GSSR Species

compd	molecular formula	$[M + H]^+$	calcd $[M + H]^+$	$[M - H]^-$	calcd $[M - H]^-$
CySSM	$C_4H_9NO_2S_2$	168.0153	168.0153	165.9996	165.9996
CySSE	$C_5H_{11}NO_2S_2$	182.0303	182.0309	180.0155	180.0153
CySSP	$C_6H_{13}NO_2S_2$	196.0465	196.0466	194.0315	194.0309
CySSA	$C_6H_{11}NO_2S_2$	194.0300	194.0309	192.0170	192.0153
GSSM	$C_{11}H_{19}N_3O_6S_2$	354.0784	354.0794	352.0633	352.0637
GSSE	$C_{12}H_{21}N_3O_6S_2$	368.0942	368.0950	366.0790	366.0794
GSSP	$C_{13}H_{23}N_3O_6S_2$	382.1097	382.1107	380.0943	380.0950
GSSA	$C_{13}H_{21}N_3O_6S_2$	380.0944	380.0950	378.0912	378.0794

Table 2. ^{1}H NMR of Hydrochloride CYS Conjugates of Thiosulfinates (CySSR−HCl)[a]

position	CySSM	CySSE	CySSP	CySSA
2	4.30 (dd, 4.0, 8.5)	4.37 (dd, 4.0, 8.5)	4.36 (dd, 4.0, 8.0)	4.30 (dd, 4.0, 8.0)
3	3.15 (dd, 8.0, 15.0) 3.35 (dd, 4.0, 15.0)	3.21 (dd, 8.5, 15.0) 3.38 (dd, 4.0, 15.0)	3.15 (dd, 8.0, 15.0) 3.31 (dd, 4.0, 15.0)	3.14 (dd, 8.0, 15.0) 3.32 (dd, 4.0, 15.0)
4	2.42 (s)	2.82 (qr, 7.0), 2.81 (qr, 7.0)	2.72 (tr, 7.2) 2.73 (tr, 7.2)	3.39 (br d, 7.5)
5		1.34 (tr, 7.5)	1.66 (sex, 7.2)	5.87 (ddtr, 7.0, 10.0, 17.0)
6			0.92 (tr, 7.2)	5.20 (m)

[a] See **Figure 3** for position assignments.

Table 3. ^{1}H NMR of CYS Conjugates of Thiosulfinates (CySSR)[a]

position	CySSM	CySSE	CySSP	CySSA
2	4.12 (dd, 4.0, 8.5)	4.11 (dd, 4.0, 9.0)	4.11 (dd, 4.0, 9.0)	4.10 (dd, 4.0, 8.5)
3	3.13 (dd, 8.5, 15.0)	3.11 (dd, 9.0, 15.0)	3.10 (dd, 8.5, 14.5)	3.10 (dd, 8.5, 14.5)
	3.39 (dd, 3.5, 15.0)	3.35 (dd, 4.0, 15.0)	3.35 (dd, 4.0, 15.0)	3.35 (dd, 4.0, 15.0)
4	2.49 (s)	2.82 (qr, 7.0)	2.81 (tr, 7.5)	3.45 (br d, 7.5)
		2.81 (qr, 7.0)	2.80 (tr, 7.5)	
5		1.35 (tr, 7.5)	1.75 (tr, 7.0)	5.95 (ddtr, 7.0, 10.0, 17.0)
6			1.00 (tr, 7.5)	5.28 (m)

[a] See **Figure 3** for position assignments.

Table 4. ^{1}H NMR of GSH Conjugates of Thiosulfinates (GSSR)[a]

position	GSSM	GSSE	GSSP	GSSA
2	3.82 (tr, 6.0)	3.83 (tr, 6.0)	3.82 (tr, 6.0)	3.85 (tr, 6.5)
3	2.19 (qr, 7.0)	2.19 (qr, 7.0)	2.19 (qr, 7.0)	2.19 (qr, 7.0)
	2.20 (qr, 7.0)	2.20 (qr, 7.0)	2.20 (qr, 7.0)	2.20 (qr, 7.0)
4	2.58 (tr, 7.0)	2.56 (tr, 8.0)	2.56 (tr, 7.5)	2.57 (tr, 7.5)
	2.56 (tr, 7.5)	2.58 (tr, 8.0)	2.58 (tr, 7.5)	2.58 (tr, 7.5)
6	4.76 (dd, 4.0, 9.5)	4.76 (dd, 4.5, 9.5)	4.76 (dd, 5.0, 9.5)	4.75 (dd, 4.5, 9.5)
7	3.02 (dd, 9.5, 14.5)	3.00 (dd, 9.5, 14.5)	3.00 (dd, 9.5, 14.5)	3.01 (dd, 9.5, 14.5)
	3.30 (dd, 4.5, 14.5)	3.27 (dd, 4.0, 14.5)	3.27 (dd, 4.5, 14.5)	3.27 (dd, 4.5, 14.5)
9	3.97 (s)	3.98 (s)	3.95 (s)	4.01 (s)
11	2.46 (s)	2.77 (qr, 7.5)	2.76 (tr, 7.5)	3.42 (br d, 6.5)
12		1.32 (tr, 7.5)	1.72 (sex., 7.5)	5.93 (ddtr, 7.0, 10.0, 17.0)
13			0.99 (tr, 7.5)	5.25 (m)

[a] See **Figure 3** for position assignments.

spectra and corresponding CySSR species (**Table 3**) was a ~0.2 ppm downfield signal of H-2.

The bioavailability (absorption) of thiosulfinates approaches 100%, and they are metabolized rapidly in human blood in vitro, where the half-life is < 1 min (*15*). Thiosulfinates progressively decay over a 24 h period in *Allium* tissue macerates (*19*). The cellular membrane permeability and ability to react with thiols (*4*, *9*, *24*) are properties that can account for the short half-life of thiosulfinates in physiological milieu. GSSA and other GSSR species may be formed in the intestinal lumen following consumption of freshly minced *Allium* vegetables with foods rich in either GSH or GSH provided by biliary excretion (*5*). These observations implicate the formation of mixed-disulfide conjugates of thiosulfinates with biological/physiological thiols as representing major derivatives that form in foods in situ and in the gut or through metabolic processes in vivo. The relative importance of these conjugates in the diet is likely proportional to the relative abundance of thiosulfinates containing the alk(en)yl groups that form these conjugates. Methyl-containing thiosulfinates are most ubiquitous and found in virtually all species of *Allium* vegetables (around 30% in total thiosulfinates of onions, garlic, leek, and shallot, 86% in Chinese chive) (*19*). Ethyl-containing thiosulfinates are found in trace amounts (*26*), whereas propyl-containing thiosulfinates are present at levels of ~20% in onions and ~50% in shallot, leek, and scallion while being virtually absent in garlic. Allyl thiosulfinates are the dominate species in garlic (50−94%), and allicin is widely believed to be responsible for the health benefits of garlic (*1*, *19*). 1-Propenyl thiosulfinates are major species in fresh onion tissue macerates (*19*), and the biological effects of the 1-propenyl mixed-disulfide conjugate derivatives may be key to understanding the health benefits of onion. However, the method described in this paper cannot be used directly to prepare the conjugates of 1-propenyl thiosulfinates because the corresponding 1-propene-1-thiol (RSH, step 3 in **Scheme 1**) is not available.

QR Induction as a Marker for Phase II Enzyme Up-regulation. Previous studies have shown that many *Allium* organosulfur compounds, such as thiosulfinates and (poly)sulfides, can induce phase II enzymes (*27*, *28*), an important mechanism for conferring protection against cancer (*14*). It was of interest to determine if similar activities are associated with CYS and GSH mixed-disulfide conjugates CySSR/GSSR, which are the potential in vivo metabolic products of *Allium* organosulfur compounds. Representative dose-dependent responses for QR induction and cell viability for Hepa 1c1c7 cells treated with CySSE (**8**) and GSSA (**10**) are shown in **Figure 4**. Dose−responses for QR induction were considered to be valid for levels up to > 50% retention of cell viability because further losses in viability may confer intolerable increases in variability in the analysis (*14*). These dose−responses allowed for the determination of relative potency of the mixed-disulfide conjugates as the concentration required to double (CD value) QR

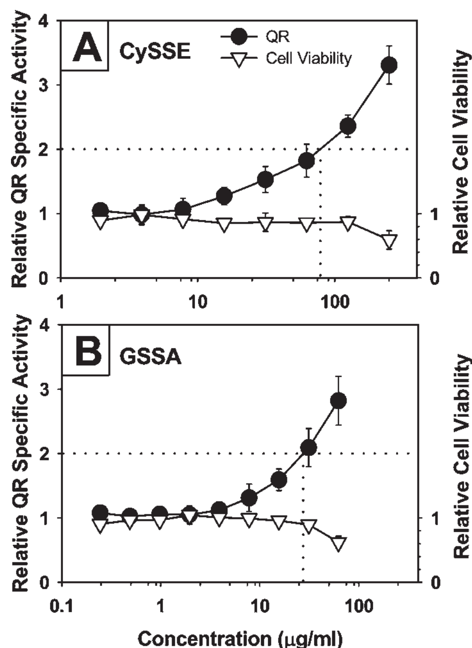


Figure 4. Quinone reductase (QR) induction in murine Hepa 1c1c7 cells by CySSE and GSSA. The potency of the QR inducer is indexed by a CD value, which is the concentration required to double QR specific activity interpolated from the response curves (indicated by broken lines). Results are expressed as mean values ± SD for relative QR specific activity.

Table 5. QR-Inducing Potency of CYS and GSH Conjugates of Thiosulfinates (CySSR/GSSR)

compd	CD value (μg/mL)	(μM)
CySSM	88.9 ± 7.6	(532)
CySSE	76.5 ± 20.3	(423)
CySSP	92.0 ± 13.0	(472)
CySSA	25.8 ± 8.9	(134)
GSSM	86.9 ± 5.9	(246)
GSSE	72.4 ± 17.0	(197)
GSSP	72.7 ± 9.3	(191)
GSSA	33.1 ± 9.9	(87.3)

specific activity in Hepa cells. All mixed-disulfide conjugates behaved qualitatively similarly and induced QR in Hepa 1c1c7 cells relative to noninduced controls ($P < 0.05$). CD values derived by interpolation of the dose−response curves are summarized in **Table 5**. The CYS and GSH mixed-disulfide conjugates with saturated R groups (methyl, ethyl, and propyl) showed similar trends in relative potency of methyl = ethyl = propyl < allyl based on micromolar CD values ($P < 0.05$), with the group of GSSR derivatives about twice as potent as the CySSR derivatives. Thus, the presence of the unsaturated R group in these conjugates conferred greatest potency to the mixed-disulfide conjugates. The mere presence of the R group in these conjugates also conferred QR-inducing activity because both cystine (CySSCy) and oxidized glutathione (GSSG) had no effect on relative QR specific activity and viability of Hepa cells relative to noninduced controls (data not shown) when tested over the same dose range as the conjugates.

The CD values of 100−500 μM exhibited for phase II enzyme (QR) induction by CySSR/GSSR species were several-fold greater (less potent) than the CD values of 30−100 μM reported for homologous thiosulfinates with the same R groups and 10−20 μM for 1-propenyl-containing thiosulfinates (*19*). A more recent study revealed allicin at 10−20 μM to up-regulate GSH and some phase II enzymes in vascular epithelial cells (*29*).

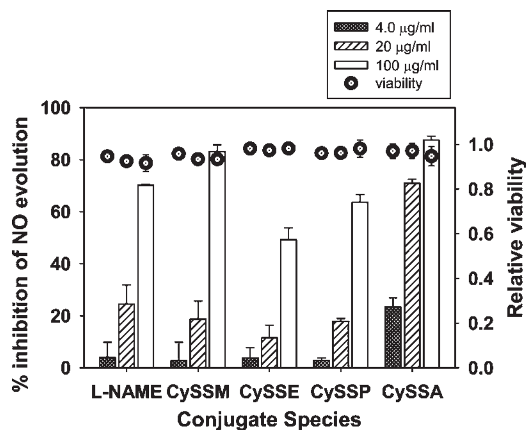


Figure 5. Dose-dependent inhibition of NO production in lipopolysaccharide-stimulated macrophage cells (RAW 264.7) by CySSR conjugates of thiosulfinates. L-NAME (N^G-nitro-L-arginine-methyl-ester) was used as a positive control in the experiment. The results are expressed as mean percent NO inhibition values ± SD of treated cells compared to control (untreated cells).

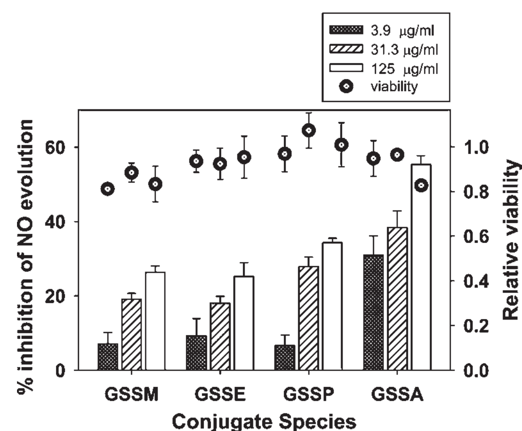


Figure 6. Dose-dependent inhibition of NO production in lipopolysaccharide-stimulated macrophage cells (RAW 264.7) by GSSR conjugates of thiosulfinates. The results are expressed as mean percent NO inhibition values ± SD of treated cells compared to control (untreated cells).

NO-Based Anti-inflammatory Assay. Allicin and ajoene (an α-sulfinyl disulfide from garlic) exert the anti-inflammatory effect of inhibiting inducible nitric oxide synthase (iNOS) expression in LPS-activated macrophages (*30*). In the present work, all CySSR/GSSR mixed-disulfide conjugates inhibited LPS-induced NO evolution in activated macrophages (**Figures 5** and **6**). Inhibition of NO production by the CySSR species exhibited a dose-dependent response qualitatively similar to N^G-nitro-L-arginine-methyl-ester (L-NAME) (**Figure 5**), a well-known inhibitor of iNOS activity. CySSA (**10**) was the most potent inhibitor among the conjugates ($P < 0.05$) with the saturated CySSR species (**7**−**9**) having similar potencies. IC_{50} values estimated from semilog plots (not shown) revealed values of 40 μM for CySSA, 300 μM for CySSM, 560 μM for CySSE, and 360 μM for CySSP. The viability of macrophages treated with all CySSR species was >94% over the dose range examined, indicating the magnitude of inhibition of NO production observed cannot be accounted for by loss of viable cells. Evaluation of the GSSR species revealed similar responses of macrophages (**Figure 6**) as observed for the CySSR species. GSSA (**14**) was a more potent inhibitor of NO production than the saturated GSSR species ($P < 0.05$), and an IC_{50} value of 240 μM for GSSA was estimated. As a group, the

other GSSR species were less potent at inhibiting NO evolution in the macrophage bioassay than the CySSR species ($P < 0.05$). The viability of macrophages treated with all GSSR species was >82% over the dose range examined, indicating that loss of viability could have only a marginal influence on NO production at doses where substantial inhibition occurred. CySSA (**10**)/GSSA (**14**) showed more potent NO inhibitory activity than the positive control L-NAME, a standard iNOS inhibitor (IC_{50} value estimated at 430 μM). CySSCy and GSSG had no effect on NO evolution and viability of macrophages when tested at up to 200 μg/mL relative to LPS-activated controls (data not shown).

As far as we are aware, ours is one of the first studies on in vitro anti-inflammatory effects of mixed-disulfide conjugates of CYS/GSH and thiosulfinates. The potency of CySSA (IC_{50} of 40 μM) was comparable to the corresponding thiosulfinate allicin (IC_{50} of 15−20 μM) (*30*) in terms of NO inhibition in RAW 264.7 cells. The other CySSR derivatives and GSSA were as much as an order of magnitude less potent, with IC_{50} values of 240−560 μM. No reports on the effects of thiosulfinates other than allicin (*29*) on NO evolution in cultured macrophages could be found to afford further comparisons. An expanded evaluation of other anti-inflammatory responses, such as modulation of pro-inflammatory cytokines/mediators such as PGE_2, or inflammation-mediating enzymes iNOS and COX-2 evoked by CySSR/GSSR and the corresponding thiosulfinates is warranted.

Structure−Activity Relationships among Mixed-Disulfide Conjugates. Both phase II enzyme induction and anti-inflammatory effects supported the existence of structure−activity relationships of CYS/GSH−thiosulfinate disulfide conjugates. Conjugates with unsaturated R groups (allyl) had more potent bioactivities compared to the ones with saturated R groups (methyl, ethyl, and propyl). The presence of the alk(en)yl groups also conferred bioactivity as the corresponding CySSCy and GSSG were inactive. Furthermore, with respect to phase II enzyme induction, the disulfide unit of the mixed-disulfide conjugates appears to be important as the CySA (*S*-allyl cysteine) derivative was previously found to induce, but not double, QR in the Hepa cell line at levels up to 25 mM (*31*). The same pattern of structure−activity relationships for these biological effects implies the existence of a general mechanism for the bioactivities of *Allium* compounds, with redox modulation being a likely feature (*32*, *33*). It would be interesting to prepare CYS/GSH disulfide conjugates of 1-propenyl thiosulfinates and study their bioactivities, as 1-propenyl thiosulfinates are more potent phase II enzyme inducers compared with other thiosulfinates (*19*, *27*).

One other interesting result was that GSSA was more potent than CySSA on a micromolar basis in QR induction, but the reverse was true for inhibition of NO evolution in activated macrophages. One feature that likely relates to these phenomena is the fate of these conjugates in cultured cells. CySSCy and CySSR mixed-disulfide conjugates are imported into cells by cystine-specific (xCT) and the L system (leucine and related neutral amino acids) transporters, respectively (*34*, *35*). Imported CySSCy and $CySSCH_2CH_2OH$ are reduced intracellularly to yield CYS to support GSH synthesis, and the corresponding RSH derived from $CySSCH_2CH_2OH$ is exported from the cell. Because multidrug-resistant protein transporters function to export glutathione *S*-conjugates, GSH, and GSSG from cells (*36*), importation of these chemotypes and the similarly structured GSSR species into cells would pose a futile cycle, and it can be expected that GSSR species would remain in the extracellular space until metabolized. Thus, it is likely that CySSR and GSSR accumulate and act in different (extra)cellular compartments when tested in vitro, but they may both affect redox modulation within that compartment. In vivo, the GSSR conjugates would most likely be formed intracellularly after consumption of thiosulfinates (*9*) or through metabolic activity (*6*, *7*) (cf. **Figure 2**).

Further evaluation of the biological effects of CySSR and GSSR, including in vivo studies, would help to determine if they could serve as vehicles for delivering *Allium*-related health benefits through dietary interventions.

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Supporting Information Available: High-resolution ESI-TOF MS of various compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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