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S-Alk(en)ylmercaptocysteine: Chemical Synthesis, Biological Activities, and Redox-Related Mechanism

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ABSTRACT: *S*-Alk(en)ylmercaptocysteine (CySSR, R = methyl, ethyl, propyl, 1-propenyl, and allyl), which are the putative metabolites of *Allium* thiosulfinates, were chemically synthesized. CySSR, but not the corresponding monosulfide species *S*-alk(en)yl cysteine (CySR), were able to induce quinone reductase (QR, a representative phase II enzyme) in Hepa 1c1c7 cells and inhibit nitric oxide (NO, an inflammatory biomarker) formation in lipopolysaccharide (LPS)-activated RAW 264.7 cells. These results indicate the importance of the disulfide bond for the biological activities of CySSR. Glutathione (GSH) and *N*-acetylcysteine (NAC), but not other types of cellular antioxidants, suppressed multiple biological activities of CySSR in vitro. The inhibitory effects of GSH and NAC on the biological activities of CySSR were correlated with a glutaredoxin (Grx)-dependent intracellular reduction of CySSR to generate cysteine and RSH, which were secreted into the extracellular medium.

KEYWORDS: *allium, thiosulfinates, S-alk(en)ylmercaptocysteine, S-1-propenylmercaptocysteine, phase II enzyme, inflammation, glutaredoxin*

INTRODUCTION

Epidemiological studies have consistently shown that consumption of *Allium* vegetables (such as onions, garlic, chives, and leeks) is associated with reduced incidence of many human diseases.[1−4] The health benefits of *Allium* vegetables are widely attributed to the enzyme-evolved organosulfur compounds called thiosulfinates (TS).[5] TS and related organosulfur compounds are highly unstable in vivo and are rapidly metabolized to mixed disulfide conjugates with cellular cysteine and glutathione, named *S*-alk(en)ylmercaptocysteine (CySSR) and *S*-alk(en)ylmercaptoglutathione (GSSR) respectively (Figure 1).[5−9] CySSR and GSSR are proposed to be the active species which contribute to the health-promoting effects of *Allium* vegetables. Indeed, *S*-allylmercaptocysteine (CySSA, an active ingredient of aged garlic extract) has been marketed as a dietary supplement for decades and is a promising cancer chemoprotective agent with multiple biological effects, including antioxidant,[10] phase II enzyme inducing and anti-inflammatory,[11] apoptosis inducing,[12] antiproliferative,[13] and antimetastatic[14] effects. CySSA has been shown to covalently modify protein thiols.[15,16] A recent study shows that it disrupted microtubule polymerization via covalent modifications of cysteine residues of microtubule.[12] Therefore, CySSA could exert its biological activities via a redox-related mechanism.

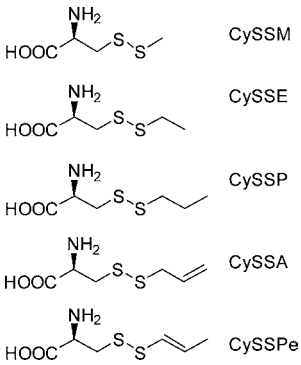

Figure 1. Chemical structures of CySSR in this study. Abbreviations: CySSM, *S*-methylmercaptocysteine; CySSE, *S*-ethylmercaptocysteine; CySSP, *S*-propylmercaptocysteine; CySSA, *S*-allylmercaptocysteine; CySSPe, *S*-1-propenylmercaptocysteine.

For the preparation of CySSR, we have reported the chemical synthesis of non-1-propenyl CySSR (R = methyl, ethyl, propyl, and allyl). However, this method cannot be used to prepare the cysteine conjugate with 1-propenyl TS, *S*-1-propenylmercaptocysteine (CySSPe).[11] 1-Propenyl TS are the dominate TS species of some *Allium* tissues such as onions, and are the most challenging TS to be chemically synthesized.[5,17] 1-Propenyl TS haven been shown to have the most potent phase II enzyme inducing activities among major *Allium* TS.[18] Therefore the biological activities of CySSPe could be critical to understand the health-promoting effects of onions. Here we report a novel chemical synthesis strategy to prepare CySSPe. The biological activities and redox-related mechanisms of CySSR (R = methyl, ethyl, propyl, allyl, and 1-propenyl) were also studied in cell cultures.

MATERIALS AND METHODS

Materials and Chemicals. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All solvents used for extraction or chromatography were purchased from Fisher Scientific (Hampton, NH). Hepa 1c1c7 and RW 264.7 cells were purchased from ATCC (Manassas, VA). Cell culture medium and supplements were from Invitrogen (Carlsbad, CA). NMR data were collected on Varian Unity-

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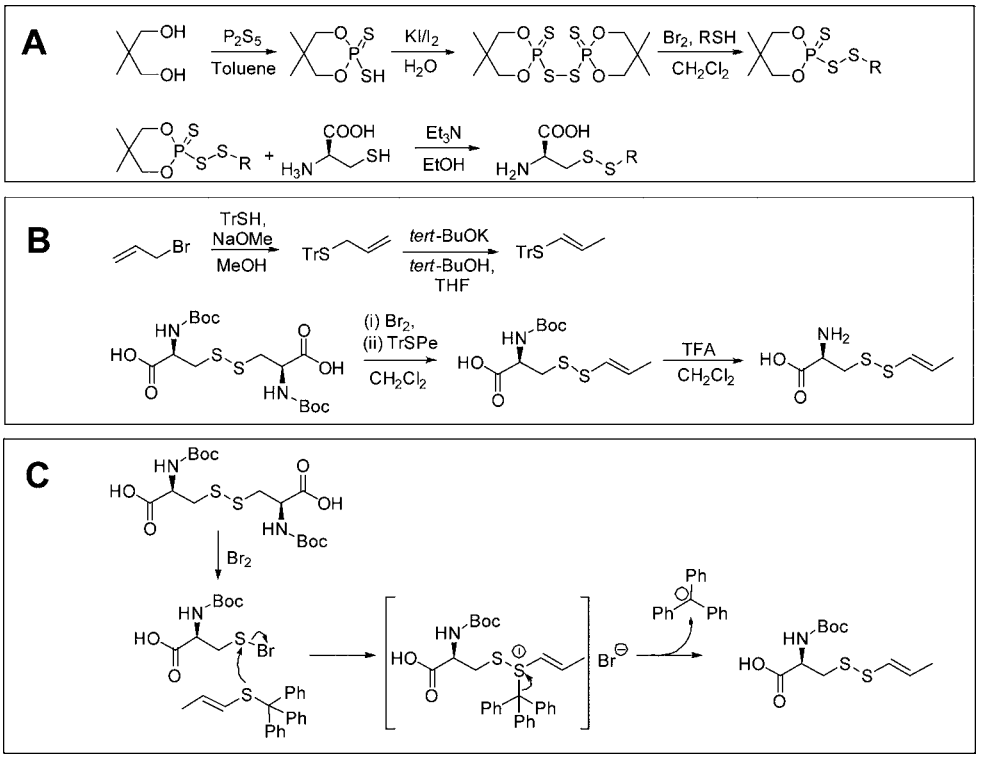
Scheme 1. Chemical Synthesis of CySSR[a]



[a](A) Chemical synthesis of non-1-propenyl *S*-alk(en)ylmercaptocysteine (CySSR, R = methyl, ethyl, propyl, and allyl). (B) Chemical synthesis of *S*-1-propenylmercaptocysteine (CySSPe). (C) Proposed mechanism for the reaction of *N*-Boc-cysteine sulfenyl bromide and TrSPe to generate Boc-CySSPe.

Inova 400 and 500 MHz NMR spectrometers (Analytical Instrumentation Center, School of Pharmacy, UW-Madison). High-resolution ESI-MS data were collected on an Agilent ESI-TOF mass spectrometer (Mass Spectrometry/Proteomics Facility, Biotechnology Center, UW-Madison).

Chemical Synthesis of Non-1-propenyl CySSR (R = Methyl, Ethyl, Propyl, and Allyl). The synthesis of CySSR (R = methyl, ethyl, propyl, and allyl) was carried out as described earlier.[11] As shown in Scheme 1, dithiophosphoric-alk(en)yl disulfides were prepared by the reaction of (5,5-dimethyl-2-thiono-1,3,2-dioxophosphorinanyl)sulfenyl bromide and RSH (R = Me, Et, Pr, and All, for R = Me, MeSNa was used) in CH_2Cl_2. CySSR were synthesized by the reaction of the corresponding dithiophosphoric-alk(en)yl disulfides with L-cysteine using Et_3N as a base in a mixed solution of ethanol and H_2O (50:50, vol:vol).

Chemical Synthesis of *S*-1-Propenylmercaptocysteine (CySSPe). Sodium (2 g) was added to 200 mL of methanol in a reflux apparatus, stirred until the sodium was totally dissolved, and 25 g of triphenylmethanethiol (TrSH) was added. The reaction mixture was refluxed for 1 h, and 10 mL of allyl bromide was added and refluxed for another 2 h. The solution was allowed to cool, and 19 g of particulate material, trityl allyl sulfide (TrSAll), was obtained by filtration using filter papers and was used directly for the next step without any purification. *tert*-BuOK (11 g) was added to a solution of 19 g of TrSAll in 100 mL of anhydrous tetrahydrofuran (THF) and 200 mL of *tert*-BuOH, stirred at 20−22 °C overnight. The solvent in the reaction mixture was removed by rotary evaporation, water and CH_2Cl_2 were added to the residue, and the product was extracted into the CH_2Cl_2 phase. Trityl 1-propenyl sulfide (TrSPe) was obtained as a white color powder by purification of the CH_2Cl_2 extract on a silica gel column eluted with 10% CH_2Cl_2 in hexane.

Bromine (0.13 mL) was added to a suspension of 1.2 g of Boc-cystine in 50 mL of CH_2Cl_2 at −80 °C, and stirred at −80 °C for 10−15 min. Then 1.7 g of TrSPe dissolved in 10 mL of CH_2Cl_2 was added, and the mixture was stirred for another 30 min at 20−22 °C. The reaction mixture was purified on a silica gel flash column eluted with 5% methanol in CH_2Cl_2 to provide 0.32 g (yield 20%) of Boc-CySSPe as a yellow oil. To remove the Boc group, 0.84 mL of trifluoroacetic acid (TFA) was added to a solution of 0.32 g of Boc-CySSPe in 0.84 mL of CH_2Cl_2 at 0 °C, stirred at 0 °C for 1.5 h. Solvent was removed from reaction mixture under vacuum, and the residue was washed with CH_2Cl_2, allowing CySSPe to be obtained as a white solid. The structure of CySSPe was confirmed by comparing NMR data with a published result.[19] ^{1}H NMR (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). HR-ESI-MS: m/z $[M + H]^+$ at 194.0308 (calcd 194.0309), $[M - H]^-$ at 192.0166 (calcd 192.0153).

DTNB Assay of Extracellular Thiols in Cell Culture Medium. Extracellular thiols were assayed by a slightly modified method described by Biaglow et al.[20] RAW 264.7 cells (ATCC, 5 × 10^4 cells per well) were cultured in 200 μL of Dulbecco's modified Eagle's medium (DMEM) in a 96-well plate for 24 h. The medium was decanted and washed with Dulbecco's phosphate buffered saline (DPBS, Invitrogen, fortified with 10 mM glucose), then a 100 μL sample serially diluted in DPBS buffer was added to each well, and an equal volume of 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in DPBS buffer was added. Absorbance at 412 nm was semicontinuously measured to record kinetics of extracellular thiol formation (via measuring 5-thio-2-nitrobenzoic acid (TNB) anion formation) over 3 h. In another experiment, cells were treated with compounds in DPBS buffer, and at predetermined incubation times, 100 μL of DPBS medium was removed and mixed with an equal volume of 1 mM DTNB solution in DPBS buffer to measure thiol concentration. A series of cysteine solutions were prepared to establish a standard curve for calculation of thiol concentration in the samples.

HPLC Analysis of Extracellular Thiols by DTNB Derivatization. To identify the extracellular thiols produced by treated cells, at different time intervals, 100 μL of DPBS medium was removed and mixed with an equal volume of 1 mM DTNB solution in DPBS and

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incubated for 5 min. After centrifuging, the clarified sample was subjected to HPLC analysis on a Jupiter 4 μm Proteo 90 Å, 250 × 10 mm HPLC column (Phenomenex, CA) to resolve the TNB-SS-thiol disulfide conjugates using a gradient of solvent A (water with 0.1% trifluoroacetic acid) in solvent B (80% acetonitrile in water with 0.085% trifluoroacetic acid). Solvent B was started at 5%, then increased to 30% in 9 min, increased further to 80% in 7 min, and then kept at 80% for 5 min before decreasing to 5% in 4 min, and held there for 10 min. Flow rate was 0.25 mL/min, and detection at 326 nm.[21]

Glutaredoxin (Grx) Assay. The enzymatic reduction of CySSR by glutaredoxin (human glutaredoxin, American Diagnostica, Stamford, CT) was measured by a spectrophotometric assay. An assay mixture containing 0.15 mM CySSR, 0.5 mM GSH, 0.2 mM NADPH, and 2 U/mL glutathione reductase (GR) was incubated in the absence or presence of 40 μM Grx in 100 mM Tris-HCl, pH = 7.5. The kinetics of the reaction was semicontinuously monitored by NADPH oxidation at 340 nm.

Quinone Reductase (QR) Induction Assay. A bioassay based on cultured murine hepatoma cells (Hepa 1c1c7) was used to assess QR induction essentially as described earlier.[22] Hepa 1c1c7 cells were grown for 24 h in 96-well plates (5×10^3 cells per well) in 200 μL of minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37 °C in 5% CO_2 in air. The medium was replaced with fresh MEM medium containing test compounds, and the cells were incubated for 48 h. A standard assay cocktail was prepared, and QR activity was determined by measuring absorbance of the reduced tetrazolium dye over a 10 min period at 490 nm. 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.

LPS-Induced Nitric Oxide (NO) Assay. Mouse macrophage cells (RAW 264.7) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 15% FBS at 37 °C in 5% CO_2 in a 96-well plate (5×10^4 cells per well) for 24 h. The medium was then replaced with fresh medium containing test compounds and 1 μg/mL lipopolysaccharide (LPS). The plate was incubated for another 24 h. A 100 μL sample was removed 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 absorbance was measured at 542 nm. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 550 nm.[23]

RESULTS AND DISCUSSION

Chemical Synthesis of CySSR. Non-1-propenyl cysteine mixed disulfide conjugates CySSR (R = methyl, ethyl, propyl, and allyl) were synthesized by the reaction of dithiophosphoric-alk(en)yl disulfide with cysteine (Scheme 1A).[11] However this method cannot be used to prepare the 1-propenyl conjugate CySSPe, because dithiophosphoric-1-propenyl disulfide failed to react with cysteine to generate the corresponding 1-propenyl conjugate CySSPe. This result indicated a different synthetic strategy was required for the preparation of 1-propenyl disulfide conjugate.

In this study, the reaction of a sulfenyl halide with trityl 1-propenyl sulfide (TrSPe) was deployed to prepare CySSPe (Scheme 1B). This strategy was simpler than the previously reported method for CySSPe synthesis.[19] Trityl (*E*/*Z*)-1-propenyl sulfide (TrSPe, *E*:*Z* ≈ 6:1 estimated from ^{1}H NMR analysis) was synthesized by isomerization of trityl allyl sulfide (TrSAll) using *tert*-BuOK in a mixed solvent of *tert*-BuOH and THF.[19] Boc-L-cystine reacts with bromine in CH_2Cl_2 to form *N*-Boc-L-cysteine sulfenyl bromide, which quickly reacts with (*E*/*Z*)-TrSPe to form *S*-1-propenylmercapto-*N*-Boc-L-cysteine (Boc-CySSPe) with an isolable yield of ~20%. Removing Boc group by TFA generated the final product *S*-1-propenylmercapto-L-cysteine (CySSPe). Scheme 1C shows a proposed reaction mechanism for the formation of Boc-CySSPe, involving the formation of a sulfonium bromide intermediate of which selective C−S bond cleavage is mediated by the trityl group because trityl carbocation is stable whereas 1-propenyl carbocation is unstable.[24,25] For all synthesized CySSR conjugates (R = methyl, ethyl, propyl, allyl, and 1-propenyl), > 98% purity was obtained based on ^{1}H NMR analysis.

CySSR Induces Phase II Enzyme Induction and Inhibits LPS-Induced NO Formation in Vitro. Figure 2A shows the QR inducing activities of CySSR (R = methyl, ethyl, propyl, 1-propenyl, and allyl) in Hepa 1c1c7 cells, with an order of potency CySSPe > CySSA > CySSP ≈ CySSE ≈ CySSM. CySSPe was the most potent QR inducer with a CD value ~25 μM, which was more potent than other CySSR species including the most intensively studied water-soluble *Allium* organosulfur compound CySSA (CD value of CySSA ≈ 125 μM), as well as CySSM, CySSE, CySSP with CD values 246−439 μM.

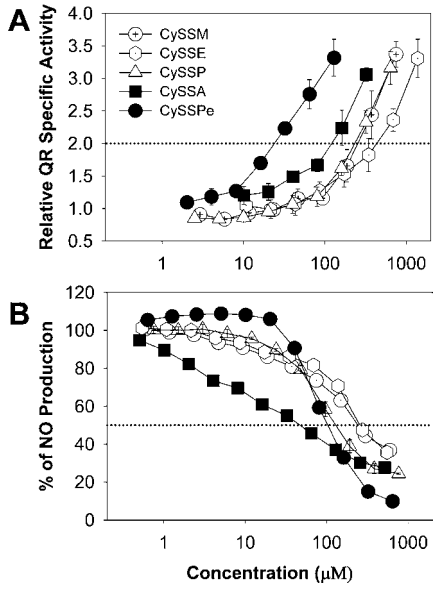

Figure 2. CySSR induce QR activity and inhibit LPS-induced NO production in vitro. (A) QR inducing assay of CySSR (R = methyl, ethyl, propyl, 1-propenyl, and allyl) in Hepa 1c1c7 cells. (B) LPS-induced NO production assay of CySSR in RAW 264.7 cells. Results are expressed as mean ± SD from three independent experiments.

The structures of CySSR compounds differ in the existence and position of a double bond in the R group. CySSPe, which has a double bond adjacent to the disulfide bond, showed the most potent QR inducing activity. CySSA, which contains a double bond one carbon atom away from the disulfide bond, was the second most potent. CySSM, CySSE and CySSP, which are devoid of double bond in the R group, were the least potent compounds and exhibited similar potency for QR induction. The existence of an electron-rich double bond and the distance of the double bond to the disulfide bond in the structure of CySSR might be correlated with the chemical reactivity of the disulfide bond (such as thiol−disulfide exchange reaction) due to the inductive effect. These structure−activity relationships suggest a model where the CySSR may exert their phase II enzyme inducing effects through a direct interaction with

cysteine residues of Keap1 protein.[26] Previous research has shown that a common mechanistic feature of phase II enzyme inducers is that they directly modify active cysteine residues of Keap1 protein, leading to dissociation of Nrf2 protein from Keap1 and activation of Nrf2 signaling.[26] In support of this hypothesis, CySSA has been shown to covalently modify protein thiols.[12,15,16] Further studies are required to investigate whether CySSR activates Nrf2 signaling through a direct interaction with cysteine residues of Keap1 protein. The effects of CySSR on phase I enzymes (P450 enzymes) are not studied here, however, previous studies have shown that CySSA inhibits P450 2E1 in animals.[27,28]

For LPS-induced NO production, CySSPe was a weaker NO inhibitory agent ($IC_{50} \approx 120\ \mu M$) compared with CySSA ($IC_{50} \approx 36\ \mu M$) in LPS-activated RAW 264.7 cells. The potency of CySSPe was similar to the other CySSR species with saturated R groups (R = methyl, ethyl and allyl, $IC_{50} \approx 130-260\ \mu M$).[11] These results indicate that CySSR compounds may induce QR and inhibit LPS-induced NO via different mechanisms; more studies are required to characterize the potential anti-inflammatory effects of CySSR compounds.

An order of potency for *Allium* TS of 1-Pe TS > allicin > PrSS(O)Pr > EtSS(O)Et > MeSS(O)Me was observed for QR induction in Hepa 1c1c7 cells (ref 18 and unpublished results of Qin Ren and Kirk Parkin), which was similar to the order of potency of CySSR for QR induction. 1-Propenyl TS (PeSS-(O)Pr, PrSS(O)Pe, PeSS(O)Me) showed the most potent QR inducing activity with CD values ~10 μM,[18] which was more potent than allicin (AllSS(O)All, CD = 28 μM) and other TS species including PrSS(O)Pr (CD = 38 μM), EtSS(O)Et (CD = 60 μM), and MeSS(O)Me (CD = 100 μM) (unpublished results, Qin Ren and Kirk Parkin). For NO inhibition, allicin is the only TS species which has been studied (IC_{50} = 15−20 μM), which was more potent than CySSA (IC_{50} = 36 μM).[29] Since 1 molar TS can react with 2 molar cysteine to generate 2 molar CySSR, thus the potency of CySSR and TS for phase II enzyme induction and anti-inflammation are well correlated. These data support that the CySSR conjugates may be the bioactive species responsible for the health benefits of *Allium* TS.

Disulfide Bond Is Essential for the Biological Activities of CySSR. The biological activities of CySSR and CySR were compared to investigate the roles of the disulfide (−SS−) bond. Figure 3 shows that CySR (R = methyl, ethyl, and allyl) was inactive to induce QR or inhibit LPS-induced NO production in vitro. In the tested dose range, CySSR dose-dependently induced QR activity in Hepa 1c1c7 cells (Figure 3A) and inhibited NO formation in LPS-activated RAW 264.7 cells (Figure 3B). In contrast, CySR showed no such effects at doses as high as 1 mM, which is consistent with a previous study.[30] CySSA has been shown to have more potent antiproliferative activity than CySA.[13] These results demonstrate the importance of the disulfide bond for the biological activities of CySSR. The disulfide bond may not be the only required structural moiety because the CySSR analogue cystine (Cy-SS-Cy) or disulfides RSSR (diallyl disulfide, dipropyl disulfide) does not have QR inducing or NO inhibitory effects in vitro (data not shown, see ref 30). Diallyl disulfide has been shown to have potent phase II enzyme inducing activities in animals,[31] suggesting a potential metabolic activation of this compound in vivo.

Coaddition of GSH and NAC, Not ASC, Suppresses the Biological Effects of CySSR. Many cancer chemopreventive agents have been shown to exert their effects through induction

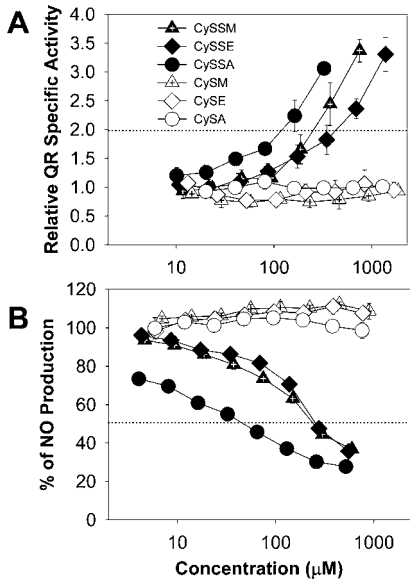

Figure 3. Compared with CySSR, CySR is inactive to induce QR or inhibit LPS-induced NO production. QR (A) and NO (B) assay of CySSR and CySR (R = methyl, ethyl, and allyl). Results are expressed as mean ± SD from three independent experiments.

of oxidative stress via generation of ROS or depletion of intracellular GSH, leading to modulation of redox sensitive signaling pathways.[32] For the organosulfur compounds, the diallyl trisulfide (DATS)-induced Nrf2 activation has been shown to be suppressed by cellular antioxidants such as GSH, *N*-acetylcysteine (NAC, a GSH boosting agent), and microsomal epoxide hydrolase,[33] supporting a potential redox-related mechanism involved in the phase II enzyme induction of DATS.

Here we investigate whether the biological activities of CySSR are mediated by a redox-related mechanism, using cellular antioxidants GSH, NAC, and ascorbic acid (ASC). Using CySSA and CySSPe as examples, we found that GSH and NAC dose-dependently suppressed multiple biological effects of CySSA and CySSPe (Figure 4). Treatments of 162 μM CySSA or 32.4 μM CySSPe alone caused ~2-fold increase of QR activity after 48 h treatment in Hepa 1c1c7 cells, while coaddition of GSH dose-dependently inhibited CySSA- or CySSPe-induced QR induction. With coaddition of 1 mM GSH, the CySSA- or CySSPe-induced QR induction was almost abolished (Figure 4A,B). For LPS-induced NO production in RAW 264.7 cells, treatment with 32.4 μM CySSA or 162 μM CySSPe inhibited 50−60% of NO production, and the inhibitory effects were also dose-dependently reversed by coaddition of GSH (Figure 4C,D). For cell proliferation, treatment with 647.7 μM CySSA or 259 μM CySSPe caused severe cell death after 48 h treatment in Hepa 1c1c7 cells, while coaddition of GSH dose-dependently "rescued" the cells from the cytotoxicity of CySSA and CySSPe (Figure 4E,F). NAC also showed a similar inhibitory effect on the biological activities of CySSA and CySSPe (data not shown). GSH or NAC treatments alone had no effect on QR induction, NO inhibition, or cellular proliferation in the tested dose range (data not shown). Incubation of GSH or NAC with CySSA or CySSPe showed no chemical reaction (or degradation of CySSA or CySSPe, data not shown). In contrast to GSH and NAC, coaddition of other cellular antioxidants

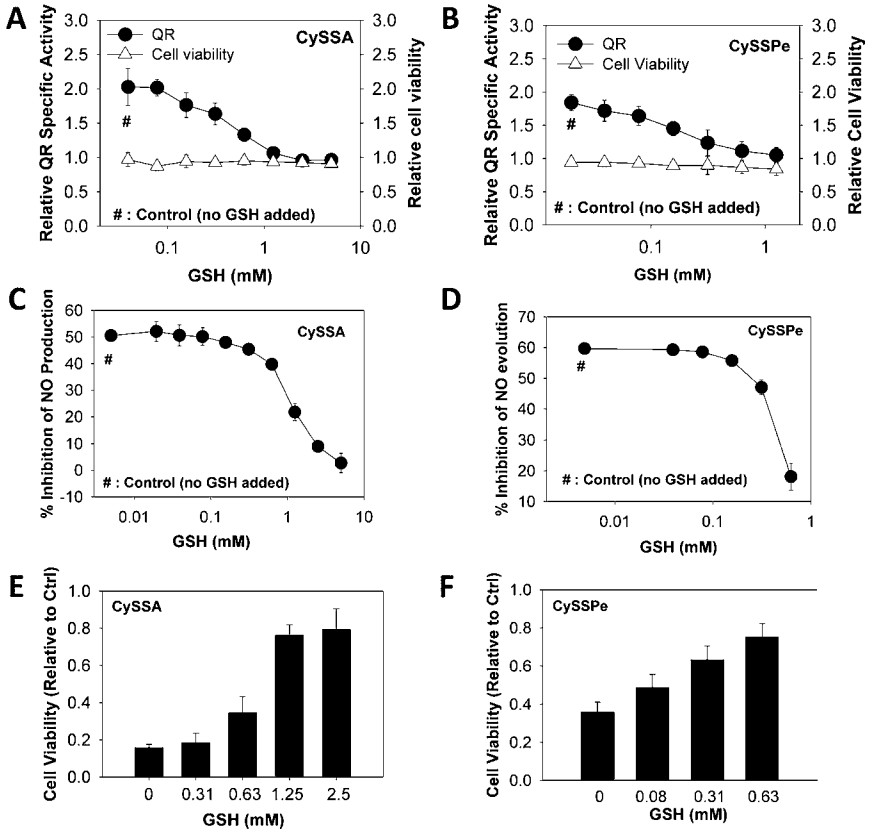

Figure 4. Coaddition of GSH suppressed multiple biological activities of CySSA and CySSPe, including QR induction, inhibition of LPS-induced NO production, and inhibition of cell proliferation. (A, B) Hepa 1c1c7 cells were treated with 162 μM CySSA (A) or 32.4 μM CySSPe (B), with or without varied doses of GSH. After 48 h treatment, QR activity and cell viability were measured. (C, D) LPS-stimulated RAW 264.7 cells were treated with 32.4 μM CySSA (C) or 162 μM CySSPe (D), with or without coaddition of varied doses of GSH. After 24 h treatment, NO production in the medium and cell viability were measured. (E, F) Hepa 1c1c7 cells were treated with 647.7 μM CySSA (E) or 259 μM CySSPe (F), with or without coaddition of varied doses of GSH. After 48 h treatment, cell viability was measured. Results are expressed as mean ± SD from three independent experiments.

such as ASC had no effect to suppress the biological activities of CySSA and CySSPe (data not shown).

Together, these results support a model that CySSR exerts its biological activities via induction of cellular redox stress, which activates redox signaling pathways and causes beneficial effects such as phase II enzyme induction and anti-inflammation. Induction of redox stress has been demonstrated to be a general mechanistic feature of many chemopreventive compounds.[32] In addition, our results seem to suggest a specific thiol-dependent redox mechanism of CySSR, rather than a general redox mechanism since only GSH and NAC instead of other cellular antioxidants such as ASC suppressed the effects of CySSR. ASC decreases ROS level and has been shown to inhibit the biological activity of ROS-generating compound such as 3,3′-diindolylmethane.[34] Both GSH and NAC boost the level of intracellular GSH, thus it is likely that the selective inhibitory effects of GSH and NAC on the biological activities of CySSR were due to an intracellular GSH-dependent "detoxification" process. Addition of GSH or NAC to cells increased the level of intracellular GSH, favoring the GSH-dependent metabolism of CySSR to generate less-active metabolites. Therefore next we studied the metabolism of CySSR in vitro, and tested whether GSH is involved in the metabolism of CySSR in cell cultures.

CySSR Is Metabolized To Form Cysteine and RSH in Extracellular Medium in Vitro. RAW 264.7 macrophage cells exposed to CySSR (R = methyl, ethyl, propyl, 1-propenyl, and allyl) in DPBS buffer led to a dose- and time-dependent increase of extracellular thiols, as measured by a semi-continuous DTNB assay (Figure 5A). The control cells treated with DTNB alone showed almost no thiol increase, indicating the produced thiols are induced by and/or sourced from CySSR compounds. Glucose was found to be required for this process, as the production of extracellular thiols was not observed without addition of glucose in DPBS buffer (data not shown). The experiment was done in DPBS buffer instead of complete cell culture medium for easy manipulation of the extracellular environment.

HPLC was employed to identify the produced thiol species after DTNB derivatization by analyzing the formed TNB-SS-thiol conjugates. For each CySSR compound, four peaks were observed on HPLC: besides DTNB (peak 3) and TNB (peak 2), the other two peaks were found to be TNB-SS-cysteine (peak 1) and TNB-SS-R (peaks 4−8) that were confirmed by coelution with standards (Figure 5B). These results indicate that the evolved extracellular thiols from CySSR species include cysteine and RSH (other thiol species could also be in the extracellular medium), which are reducing products of CySSR. This result supports a model where CySSR undergoes a cellular reduction to generate cysteine and RSH which remain an end-up in the extracellular culture medium. A similar phenomenon of cellular disulfide reduction to form extracellular thiol species has been described in a previous study.[20]

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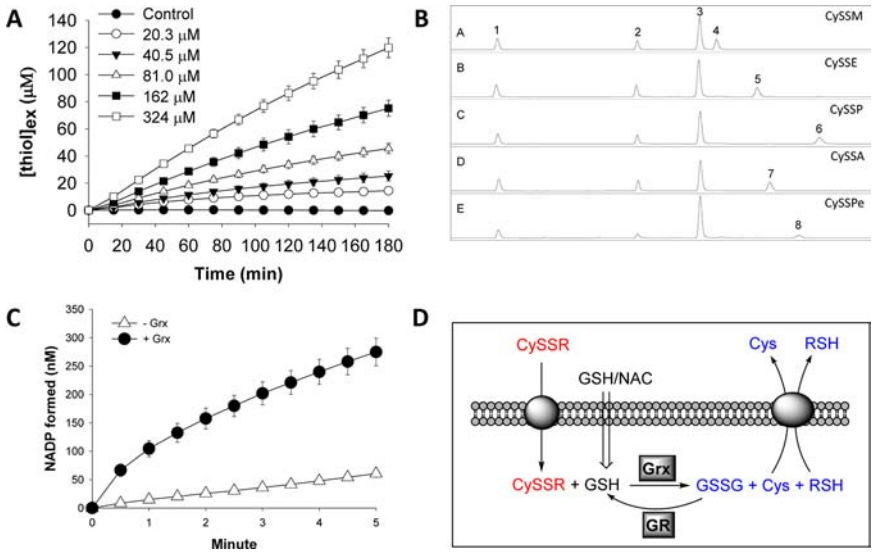

Figure 5. CySSR was metabolized to form cysteine and RSH in the extracellular medium, via a likely mechanism involving the metabolism by glutaredoxin (Grx). (A) Treatment with CySSA in DPBS buffer (fortified with 10 mM glucose) caused a time- and dose-dependent increase of thiols in medium of RAW 264.7 cells, measured by a DTNB assay. (B) HPLC analysis of cell medium of CySSR-treated RAW 264.7 cells after DTNB assay, demonstrating the formed thiols are cysteine and RSH derived from CySSR. The RAW 264.7 cells were treated with 62.5 μg/mL CySSR and 0.5 mM DTNB in 10 mM glucose-fortified DPBS buffer for 3 h at room temperature; then 100 μL of medium was taken out, centrifuged, and subjected to HPLC analysis, detection at 326 nm. Panel from top to bottom: (A) CySSM, (B) CySSE, (C) CySSP, (D) CySSA, and (E) CySSPe. Peak 1: TNB-SS-cysteine. Peak 2: TNB. Peak 3: DTNB. Peak 4: TNB-SS-Me. Peak 5: TNB-SS-Et. Peak 6: TNB-SS-Pr. Peak 7: TNB-SS-All. Peak 8: TNB-SS-1-Pe. (C) Glutaredoxin (Grx) enzymatic assay of CySSA in 100 mM Tris-HCl (pH = 7.5). (D) Proposed mechanism for the redox-related cellular responses of CySSR.

CySSR could be reduced to form cysteine and RSH via multiple mechanisms. In the present study, we found that CySSR is a substrate of glutaredoxin (Grx). Figure 5C shows the enzymatic assay of Grx, using a standard Grx assay system which contains Grx, GSH reductase (GR), GSH, NADPH, and CySSR.[35] Addition of Grx caused a ~5-fold increase in initial reaction velocity of CySSR reduction compared with the non-Grx control, supporting that CySSR is a substrate of Grx. To further study whether Grx was involved in cellular reduction of CySSR in RAW 264.7 cells as shown in Figure 5A,B, cells were treated with CySSR together with $CdCl_2$, a chemical inhibitor of Grx.[36] Coaddition of 2.5−10 μM $CdCl_2$ inhibited 30−40% of CySSA-induced extracellular thiol production after 1 h treatment in RAW 264.7 cells (data not shown). MTT assay of the cells after the treatment showed no decrease of cell viability, indicating the observed inhibitory effects were not due to loss in cell number (data not shown). Together, these results imply that CySSR could be reduced by a Grx-dependent process to form cysteine and RSH in vitro, and the produced cysteine and RSH are then secreted to extracellular medium, most likely via multiple drug resistance protein (MRP) (proposed model shown in Figure 5D). Other cellular reducing enzymes, such as thioredoxin reductase (TRxR), thioredoxin (Trx), and GR, could also be involved in the cellular reduction of CySSR. The Grx-mediated disulfide bond reduction requires NADPH, which might be derived from glucose metabolism through glucose-6-phosphate dehydrogenase (G6PDH). This would explain the requirement of glucose in the DPBS medium for extracellular thiol production of CySSR. The Grx-dependent reduction of CySSR may at least partially explain the selective inhibitory effects of GSH and NAC on the biological activities of CySSR. Coaddition of GSH or NAC, but not other types of cellular antioxidants such as ASC, increases the level of intracellular GSH, favoring the Grx-dependent metabolism of CySSR since Grx has a high K_m value for GSH.[37] This process leads to removal of CySSR from intracellular space, and the formed cysteine and RSH are inactive for QR induction and NO inhibition (data not shown), leading to decreased biological effects of CySSR species.

Interestingly, *S*-alk(en)ylmercaptoglutathione (GSSR, R = methyl, ethyl, propyl, 1-propenyl, and allyl), which showed similar effects of CySSR to induce QR and inhibit LPS-induced NO formation,[11] did not induce extracellular thiol production (data not shown). This result indicates GSSR might not enter intracellular space, because if GSSR pass cell membrane, GSSR would be intracellularly reduced to form RSH, which would be secreted into extracellular medium. Multidrug resistance protein transporters (MRP) function to export glutathione *S*-conjugates, GSH, and GSSG from cells;[38] importation of these chemotypes and the similarly structured GSSR species into cells would pose a futile cycle. It would be expected that GSSR would remain in extracellular space until further metabolized.

Together, in the present study we report the chemical synthesis, biological activities and redox-related mechanisms of CySSR compounds. The 1-propenyl conjugate CySSPe showed the most potent phase II enzyme inducing activities, which is well correlated with the potent effects of the *Allium* 1-propenyl TS. CySSR compounds were metabolized to form cysteine and RSH, which were secreted into extracellular medium, via a process which could involve Grx-dependent enzymatic activity. This GSH/Grx-dependent metabolism could explain the selective inhibitory effects of GSH and NAC on the biological activities of CySSR.

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Notes

The authors declare no competing financial interest.

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