# Phenylalkyl Isothiocyanate–Cysteine Conjugates as Glutathione S-Transferase Stimulating Agents†

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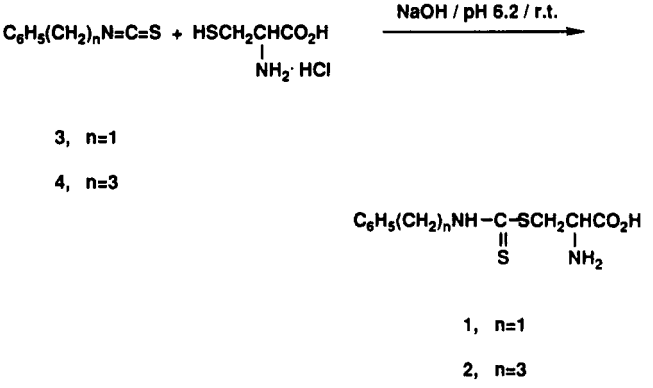
To develop analogues of phenylalkyl isothiocyanate with less toxicity and better biological activity, two water-soluble phenylalkyl isothiocyanate–cysteine conjugates, *S*-[*N*-benzyl(thiocarbamoyl)]-L-cysteine (**1**) and *S*-[*N*-(3-phenylpropyl)(thiocarbamoyl)]-L-cysteine (**2**), were synthesized. The induction of increased activity of the detoxifying enzyme glutathione *S*-transferase by the conjugates and their parent compounds was determined and compared in several tissues of A/J mice. The biological evaluation revealed that the conjugates as GST enzyme inducers appeared to be less toxic and even more potent than the parent compounds in the mouse bladder. Compounds **1** was much more active than **2** in all the tissues examined, while their parent compounds showed an inverse order of activity. Thus, an increase in the alkyl chain length of the parent isothiocyanates or a decrease in the alkyl length of the conjugates could result in higher enzyme-inducing activity in the same compound series. Since a number of nitrosamines have been identified as prime bladder carcinogens and phenylalkyl isothiocyanates have been reported to inhibit a wide range of carcinogenic nitrosamines, the corresponding conjugates may serve as prodrugs to protect against nitrosamine-induced urinary bladder carcinogenesis once they are delivered to the target organ.

Epidemiological studies have shown that consumption of cruciferous vegetables is associated with a reduced risk of human cancer.[1] Isothiocyanate glucosinolates occur widely in these vegetables.[2] The inhibition of carcinogenesis by intact glucosinolates has been reported.[3,4] Glucotropaeolin, the glucosinolate of benzyl isothiocyanate, was found to inhibit both BP-induced pulmonary neoplasia in mice and DMBA-induced mammary tumorigenesis in rats.[4] Glucosinolates can be hydrolyzed enzymatically by myrosinase during processing and digestion of foods to yield the corresponding isothiocyanates.[5] Natural isothiocyanates have been shown to inhibit a wide range of carcinogens including polycyclic aromatic hydrocarbons and nitrosamines in several different animal models.[3,6-8] Recently, Morse and co-workers have synthesized and evaluated a series of phenylalkyl isothiocyanates as the inhibitors of nitrosamine-induced lung tumorigenesis.[9-11]

Although isothiocyanates are effective inhibitors of carcinogenesis, controlling factors such as solubility, toxicity, specificity, and potency limit most of them from becoming clinically useful cancer chemopreventive agents. While isothiocyanates have been considered to be fairly toxic compounds when administered, the water-soluble glucosinolates show promise as less toxic proinhibitors.[12] However, limited structural type of isothiocyanates from plant glucosinolates and low overall yield for the multistep synthesis of glucosinolates[13] make it difficult to study a wide range of isothiocyanate analogues in large quantities. To overcome this limitation, this research focuses on developing a novel type of isothiocyanate analogues, isothiocyanate–cysteine conjugates, as potential chemopreventive agents that can be synthesized in less steps with high yields and may offer better biological activity and less systemic toxicity.

Glutathione *S*-transferase is one of the major detoxifying enzyme systems. GST catalyzes the conjugation of a wide variety of electrophiles including the reactive ultimate carcinogenic forms of chemical carcinogens with glutathione to form less toxic, water-soluble substances that can be readily excreted.[2,14] Thus, induction of GST activity by anticarcinogenic compounds is believed to be a major mechanism for carcinogen detoxification.[15,16] Certain compounds and cruciferous vegetables that induce GST activity may inhibit chemical carcinogenesis in the same tissue.[2,6,17-19] A positive correlation between inhibition of aromatic hydrocarbon induced neoplasia in mice and en-

**Scheme I**

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† Abbreviations: GST, glutathione *S*-transferase; BP, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; and CDNB, 1-chloro-2,4-dinitrobenzene.

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Table I. The Effects of Phenylalkyl Isothiocyanate Compounds (1–4) on the Activity of Glutathione S-Transferase in Target Tissues of A/J Mice

| | | liver | | small bowel mucosa | | forestomach | | lung | | large bowel mucosa | | bladder | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| compounds | total dose,[a] mmol | GST spec act.[b] | ratio[c] | GST spec act.[b] | ratio[c] | GST spec act.[b] | ratio[c] | GST spec act.[b] | ratio[c] | GST spec act.[b] | ratio[c] | GST spec act.[b] | ratio[c] |
| control | | 1.28 ± 0.10 | | 0.55 ± 0.04 | | 0.83 ± 0.04 | | 0.24 ± 0.06 | | 0.34 ± 0.11 | | | |
| 1 | 0.10 | 1.63 ± 0.33 | 1.27 | 1.58 ± 0.23[d] | 2.87 | 1.52 ± 0.27[e] | 1.83 | 0.35 ± 0.03[e] | 1.46 | 0.41 ± 0.05 | 1.21 | | |
| 2 | 0.10 | 1.35 ± 0.17 | 1.05 | 0.69 ± 0.09 | 1.25 | 0.92 ± 0.12 | 1.11 | 0.29 ± 0.04 | 1.21 | 0.38 ± 0.06 | 1.12 | | |
| control | | 1.14 ± 0.55 | | 0.50 ± 0.12 | | 0.93 ± 0.09 | | 0.22 ± 0.04 | | 0.30 ± 0.02 | | 5.08 ± 2.50 | |
| cysteine | 0.04 | 0.91 ± 0.14 | 0.80 | 0.36 ± 0.07 | 0.72 | 0.78 ± 0.08 | 0.84 | 0.25 ± 0.03 | 1.14 | 0.29 ± 0.07 | 0.97 | 5.51 ± 1.04 | 1.08 |
| 1 | 0.04 | 1.02 ± 0.11 | 0.89 | 0.58 ± 0.06 | 1.16 | 0.81 ± 0.07 | 0.87 | 0.33 ± 0.06[f] | 1.50 | 0.31 ± 0.06 | 1.03 | 6.35 ± 0.97 | 1.25 |
| 2 | 0.04 | 1.20 ± 0.33 | 1.05 | 0.49 ± 0.14 | 0.98 | 0.86 ± 0.23 | 0.92 | 0.31 ± 0.07[f] | 1.41 | 0.26 ± 0.07 | 0.87 | 6.16 ± 0.60 | 1.21 |
| 3 | 0.04 | 1.01 ± 0.15 | 0.89 | 1.17 ± 0.36[g] | 2.35 | 1.59 ± 0.15[g] | 1.71 | 0.36 ± 0.05[g] | 1.64 | 0.30 ± 0.07 | 1.00 | 5.30 ± 1.03 | 1.04 |
| 4 | 0.04 | 1.20 ± 0.20 | 1.05 | 1.37 ± 0.08[g] | 2.74 | 0.98 ± 0.10 | 1.05 | 0.42 ± 0.07[g] | 1.91 | 0.37 ± 0.07 | 1.23 | 5.87 ± 0.69 | 1.16 |

[a] The compounds were dissolved in 0.3 mL of cottonseed oil and given in three separate doses once every 2 days. The control mice were given 0.3 mL of cotton seed oil only. [b] μmol/min per mg of protein. [c] Test/control. [d] All $P$ values were obtained by the Student $t$ test ($n = 4$), $P < 0.005$. [e] $P < 0.05$, $n = 4$. [f] All $P$ values were obtained by the Student $t$ test ($n = 5$), $P < 0.005$. [g] $P < 0.05$, $n = 5$.

hancement of GST activity by several classes of inhibitors has been observed.[17-19] The capacity to enhance GST activity can be used as a parameter for the evaluation of inhibitors of chemical carcinogenesis. Thus, the ability to induce increased GST activity by the cysteine conjugates and their parent isothiocyanates was investigated.

## Results

**Chemistry.** The synthetic approach to the phenylalkyl isothiocyanate conjugates *S*-[*N*-benzyl(thiocarbamoyl)]-L-cysteine (1) and *S*-[*N*-(3-phenylpropyl)(thiocarbamoyl)]-L-cysteine (2) was based on the procedure of Brusewitz and co-workers[20] as shown in Scheme I. Commercially available benzyl isothiocyanate (3) and 3-phenylpropyl isothiocyanate (4) were allowed to react with L-cysteine hydrochloride at pH 6.2 to afford the corresponding cysteine conjugates 1 and 2 with high yields. The products were separated and purified by recrystallization. The structures were characterized on the basis of elemental analysis and spectroscopic methods (NMR and MS). Compound 2 has not yet been reported in the literature.

**Biological Evaluation.** The induction of increased GST activity by isothiocyanates and their conjugates was tested in the liver, lung, forestomach, bladder, and large and small intestinal mucosa of female A/J mice (Table I). The GST-specific activities for these compounds were determined under first-order kinetics.[21] All samples were administered to the mice by gavage. The control mice were given cottonseed oil only. The parent isothiocyanates 3 and 4 were too toxic to be tested at the dose level of 0.1 mmol. They were retested at the dose of 0.04 mmol. In the parent isothiocyanate series, compound 4 appeared to be more active than 3 in the mouse liver, bladder, lung, and large and small intestinal mucosa. However, an inverse order for the corresponding cysteine conjugates was observed. At 0.1 mmol the cysteine conjugate 1 was more active than conjugate 2 in all the mouse tissues. With compound 1 at the 0.1 mmol dose, the GST activity in the cytosol of small intestinal mucosa was increased to almost 3 times the control level. In the cytosols of lung and forestomach, the GST enzyme activity was significantly higher than that of the control, ranging from 1.46 to 1.83 times higher. A greater than 20% increase of enzyme activity was observed in the liver and colon. In addition, the differences in the effects on enzyme activity at the two dose levels of 0.1 and 0.04 mmol indicated that at the higher dose 1 appeared to be more active. Compound 2 was not as active as 1 in the induction of GST activity. Only a small increase (10–25% higher than the control) of the GST activity was observed in the mouse tissues examined. Comparison of conjugates with their corresponding parent compounds indicated that conjugate 1 exhibited higher enzyme-inducing activity at 0.1 mmol in most of the tissues. At the dose level of 0.04 mmol compound 1 was more active than 3 only in the bladder, but less active than 3 in the forestomach and small bowel. Compound 2 showed activity similar to that of the parent compound 4 in the liver and forestomach, but appeared to be less active in the lung, colon, and small bowel mucosa.

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However, compound 2 was a little more active than 4 in the bladder. Most of the compounds exhibited high activity in the small bowel mucosa.

## Discussion

The rationale behind the use of isothiocyanate conjugates as less toxic anticarcinogenic agents is based on the physical properties of the conjugates. The bulkiness and hydrophilic character of the cysteine group should decrease the absorption of the isothiocyanate conjugates in the upper gastrointestinal tract after oral administration, so that the systemic toxicity of isothiocyanate can be reduced. Our present studies have indicated that the cysteine conjugates 1 and 2 are much less toxic than the corresponding parent compounds 3 and 4. Compounds 1 and 2 were found to induce increased GST activity in the target organs of mice with no apparent toxic effects observed at the 0.1 mmol dose level. Parent compounds 3 and 4, on the other hand, were too toxic to be evaluated at the same dose that resulted in the death of mice. Similar toxicity of phenylalkyl isothiocyanate in mice aslo has been reported by Morse and co-workers.[8] Bruggeman and co-workers have compared the cytotoxicity of benzyl isothiocyanate with those of its cysteine and glutathione conjugates in RL-4 rat hepatocytes.[22] They found that benzyl isothiocyanate is more toxic than its conjugates and addition of excess L-cysteine or glutathione can lower or abolish the cytotoxicity.

Structure–activity relationship studies on the inhibition of nitrosamine-induced lung neoplasia in mice by a series of homologous isothiocyanates have indicated a general trend of increased inhibitory activity of these isothiocyanates with increase alkyl chain length up to six carbons.[9-11] Our study on phenylalkyl isothiocyanates and their conjugates toward induction of GST activity shows that compound 4 with a three-carbon alkyl side chain is more active than the one-carbon chain homologue 3, which is consistent with the above observation. However, in the cysteine conjugate series, short-chain conjugate 1 appears to be much more active than long-chain conjugate 2. With the above observation, we will proceed with further structure–activity relationship studies on a series of conjugates with different chain length by investigating the optimal side-chain length required for the maximal GST-inducing activity and the influence of different chain length on the activity and toxicity.

The mechanisms by which anticarcinogenic isothiocyanates protect against polycyclic hydrocarbon and nitrosamine carcinogens are not completely understood. Isothiocyanates may affect a number of different enzyme systems. There is some evidence that isothiocyanates may inhibit the monooxygenases such as cytochrome P-450 isozymes responsible for oxidative activation of carcinogens.[3,8,23] Enhancement of the activity of nonoxidative detoxifying enzymes such as GST may also contribute to protection by these compounds against carcinogenicity and toxicity. Induction of increased GST activity by isothiocyanates has been observed in laboratory animal tissues including liver, lung, esophagus, forestomach, colon, small intestine, and urinary bladder.[17,23,24] A number of phenylalkyl isothiocyanates have also been found to inhibit tumorigenesis induced by nitrosamines and hydrocarbons in these tissues.[6,7,9-11,17] These results suggest that enhancement of GST activity by isothiocyanate conjugates may be associated with their inhibition of carcinogenesis in that tissue.

Our present study shows that conjugates 1 and 2 are more active than the parent compounds 3 and 4 in the mouse bladder. It is known that when benzyl isothiocyanate is administered orally to rats, the principal urinary metabolite is *N*-acetylcysteine conjugate, which may be formed through the catabolism of the corresponding glutathione conjugate.[20] Human urine has also been shown to contain the corresponding cysteine conjugate after the ingestion of benzyl isothiocyanate.[25] The distribution and metabolism of phenethyl isothiocyanate in mice have been determined by Eklind and co-workers.[26] Only a small amount of the compound was excreted within 8 h of dosing. Approximately 50% of the total dose was excreted within 24 h and 80% of the total dose was excreted in 72 h. It is possible that these conjugates may revert readily to their parent isothiocyanates under physiological condition in aqueous media, as it has been reported for the glutathione conjugate of benzyl isothiocyanate.[22] The reversibility of 1 and 2 may be a desirable property. The liberated isothiocyanates are known GST inducers and the cysteine component is a precursor for the biosynthesis of GSH.[27] The elevation of GST activity and increased tissue GSH level together form a powerful detoxifying system against carcinogens. Since a number of nitrosamines have been identified as prime bladder carcinogens[28,29] and phenylalkyl isothiocyanates have been reported to inhibit a wide range of carcinogenic nitrosamines by blocking metabolic activation of these carcinogens or by stimulating detoxifying enzymes,[3,8-11] their corresponding conjugates may be considered as the prodrugs to protect against nitrosamine-induced urinary bladder carcinogenesis once they are delivered to the target organ and regenerate free phenylalkyl isothiocyanates. Furthermore, these water-soluble conjugates are expected to have better efficiency than their parent compounds because of their inhibited absorption and degradation in the upper gastrointestinal tract.

In summary, the present results indicate that the isothiocyanate conjugates can be synthesized in one step with high yields. They are less toxic and capable of inducing increased activity of the detoxifying enzyme GST when given orally. These preliminary studies should provide the

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clues for further development of more potent and less toxic analogues or prodrugs of phenylalkyl isothiocyanates. Since an increase of GST activity is associated with anticarcinogenic potential, these types of conjugates show promise to become useful chemopreventive agents.

## Experimental Section

Melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Elemental analysis was performed by the Galbraith Laboratories, Inc. (Knoxville, TN). Ultraviolet (UV) spectra were determined on a Beckman DU-65 spectrophotometer. High-resolution fast atom bombardment mass spectra (FAB-MS) were obtained on a VG 7070E-HF mass spectrometer. Nuclear magnetic resonance (NMR) spectra ($\delta$ in ppm, $J$ in Hz) were recorded on a Nicolet NT-300 NMR spectrometer. Tetramethylsilane (TMS) was used as the internal reference ($\delta$ 0.00) for $^1$H NMR spectra measured in DMSO-$d_6$. The signal multiplicities were described using the following abbreviations: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. L-Cysteine hydrochloride monohydrate and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO). Benzyl isothiocyanate and 3-phenylpropyl isothiocyanate were obtained from Fairfield Chemical Co. (Blythewood, SC). 1-Chloro-2,4-dinitrobenzene was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade or purer and purchased from Aldrich unless noted otherwise.

**Synthesis of *S*-[*N*-Benzyl(thiocarbamoyl)]-L-cysteine (1).** Benzyl isothiocyanate (2.98 g, 20 mmol) in aqueous 80% (v/v) ethanol (50 mL) was added to a solution of L-cysteine hydrochloride monohydrate (3.51 g, 20 mmol) in water (20 mL), followed by the dropwise addition of 1 N aqueous NaOH to raise the pH to 6.2. The mixture was allowed to react for 2 days at room temperature. After the TLC analysis indicated that the reaction was completed, the precipitate was filtered, washed with water (2 × 3 mL) and ethanol (2 × 5 mL), and dried in vacuo. The crude product was recrystallized in water to afford compound 1 as white powder (5.02 g, 93% yield): mp 191–193 °C dec (lit.[20] 190–194 °C); $[\alpha]_D^{25} = -35.0°$ (c 0.5, 1 M HCl) [lit.[20] $[\alpha]_D^{20} = -31.9°$ (c 0.5, 1 M HCl)]; HRMS (FAB) calcd for $C_{11}H_{15}N_2O_2S_2$ ($MH^+$) $m/z$ 271.0575, found 271.0589; $^1$H NMR (DMSO-$d_6$) $\delta$ 3.36 (dd, 1 H, J = 14.2, 6.2, $H_\beta$ of the cysteine), 3.59 (dd, 1 H, $J$ = 6.2, 4.7, $H_\alpha$ of the cysteine), 3.71 (dd, 1 H, $J$ = 14.2, 4.7, $H_\beta$ of the cysteine), 4.83 (s, 2 H, $PhCH_2N$), 7.51 (m, 5 H, $C_6H_5$), 7.70–9.30 (br s, 3 H, exchangeable with $D_2O$, NH's). Anal. ($C_{11}H_{14}N_2O_2S_2$) C, H, N.

**Synthesis of *S*-[*N*-(3-Phenylpropyl)(thiocarbamoyl)]-L-cysteine (2).** This compound was prepared by the reaction of L-cysteine hydrochloride monohydrate (3.51 g, 20 mmol) with 3-phenylpropyl isothiocyanate (3.54 g, 20 mmol), following the same procedure as used for compound 1. The crude product was purified by recrystallization in water to yield compound 2 as white powder (5.70 g, 95% yield): mp 224–225 °C dec; HRMS (FAB) calcd for $C_{13}H_{19}N_2O_2S_2$ ($MH^+$) $m/z$ 299.0888, found 299.0877; $^1$H NMR (DMSO-$d_6$) $\delta$ 1.87 (5 lines, 2 H, $J$ = 7.5, $PhCH_2CH_2CH_2N$), 2.59 (t, 2 H, $J$ = 7.5, $PhCH_2CH_2CH_2N$), 3.34 (dd, 1 H, $J$ = 14.3, 6.3, $H_\beta$ of the cysteine), 3.54 (t, 2 H, $J$ = 7.5, $PhCH_2CH_2CH_2N$), 3.56 (dd, 1 H, $J$ = 6.3, 4.8, $H_\alpha$ of the cysteine), 3.68 (dd, 1 H, $J$ = 14.3, 4.8, $H_\beta$ of the cysteine), 7.22 (m, 5 H, $C_6H_5$), 7.90–9.20 (br s, 3 H, exchangeable with $D_2O$, NH's). Anal. ($C_{13}H_{18}N_2O_2S_2$) C, H, N.

**Enzyme Assay.** Female A/J mice obtained from National Institutes of Health (7 weeks old) were fed a semipurified diet from ICN Nutritional Biochemicals (Cleveland, OH) for 1 week. One week after the start of the semipurified diet they were divided into control and experimental groups with four mice per group. Animals were housed in temperature-controlled animal quarters with a 12/12 h light/dark cycle. Water was given ad libitum.

The procedures for the preparation of cytosolic fractions were essentially those described previously.[30] Briefly, the experimental groups were given by gavage the test compounds suspended in 0.3 mL of cottonseed oil once every 2 days for a total of three doses. The control groups were given cottonseed oil alone. Each sample represents one individual animal. Twenty-four hours after the last administration, the mice were killed by cervical dislocation. The liver, lung, forestomach, and the mucosa of the proximal third of the small intestine and the entire length of the large intestine including the caecum were removed. The tissues were homogenized in cold 1.15% KCl (pH 7.4). The homogenates were centrifuged at 9000$g$ for 20 min and the supernatants were centrifuged at 100000$g$ for 1 h. The cytosolic fractions were kept frozen at −80 °C until use. The protein concentrations of these samples were determined by the method of Lowry and coworkers.[31]

The activity of cytosolic GST was assayed according to the method of Habig and co-workers using 1-chloro-2,4-dinitrobenzene as the substrate.[32] The complete assay solution contained, in a total volume of 2.0 mL, 0.1 M phosphate buffer (pH 6.5), 5 mM glutathione, 1 mM CDNB, and 20 μL of the cytosol. The reaction was monitored at 340 nm in a Beckman Model DU65 UV spectrophotometer. Assays were performed at 30 °C. Complete assay mixture without the cytosolic enzyme was used as the reference blank. Data were analyzed by Student's $t$ test and $P$ values were obtained in comparison to the control.

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