This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2001)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2001 Term

Instructors:
GARRY R. BUETTNER, Ph.D.
LARRY W. OBERLEY, Ph.D.

with guest lectures from:
Drs. Freya Q . Schafer, Douglas R. Spitz, and Frederick E. Domann

The Fine Print:

Because this is a paper written by a beginning student as an assignment, there are no guarantees that everything is absolutely correct and accurate.

In view of the possibility of human error or changes in our knowledge due to continued research, neither the author nor The University of Iowa nor any other party who has been involved in the preparation or publication of this work warrants that the information contained herein is in every respect accurate or complete, and they are not responsible for any errors or omissions or for the results obtained from the use of such information. Readers are encouraged to confirm the information contained herein with other sources.

Buthionine sulfoximine: Glutathione depleter

by

Iman Ahmad

B-180 Medical Laboratories
Free Radical and Radiation Biology Program
The University of Iowa
Iowa City, IA 52242-1181

For 77:222, Spring 2001

2. April 2001

Abbreviations

ATP, adenosine triphosphate
Å, angstrom
BSO, buthionine sulfoximine
Cys, cysteine
GSH, glutathione
γ-Glu, γ-glutamyl
Gly, glycine
HPLC, high performance liquid chromatography

Table of contents

Abstract

Although cultured cells from individuals with inborn errors of glutathione (GSH) metabolism provide some useful information, *in vivo* studies are necessary to study the effects of GSH deficiency, gain information for therapy, and understand more clearly the function of GSH. Various non-specific oxidizing compounds, increased hypoxia and radiation have been used to produce oxidative stress and they also deplete GSH. However, these compounds are non-specific for GSH. A satisfactory approach to produce GSH deficiency is by inhibiting γ-glutamylcysteine synthetase enzyme "the rate limiting step of GSH synthesis". L-buthionine sulfoximine (BSO) is a potent and specific inhibitor of γ-glutamylcysteine synthetase. BSO administration to animals or incorporation into tissue culture media inhibits GSH biosynthesis and causes depletion of cellular GSH level. This paper provides a brief review of BSO diastereomers, mechanism of action, metabolism, and toxicity.

Introduction-an overview

Glutathione (L-glutamyl-L-cysteine-glycine) is found in almost all cells in relatively high concentrations (1 to 10 mM) [1,2]. GSH has a number of cellular functions. It is an effective

intracellular reductant. It functions in catalysis, metabolism, transport and in the protection of cells against foreign compounds, free radicals and reactive oxygen compounds. GSH is an active participant in reactions that destroy H_2O_2 and organic peroxides. GSH is synthesized intracellularly by concecutive actions of γ-glutamylcysteine synthetase (reaction 1) and GSH synthetase (reaction 2) [3]:

L-glutamate + L-cysteine + ATP ⟷ L-γ-glutamyl-L-cysteine + ADP + Pi (1)

L-γ-glutamyl-L-cysteine + glycine + ATP ⟷ glutathione + ADP + Pi (2)

The synthesis of GSH is limited by the availability of substrates; cysteine is usually the limiting substrate. γ-Glutamylcysteine synthetase is non-allosterically, feedback inhibited by GSH (Ki about 1.5 mM) [4]. Thus, under physiological conditions, γ-glutamylcysteine synthetase is probably not operating at its maximal rate. A schematic diagram of metabolism and transport of GSH is shown in Figure 1 [2].

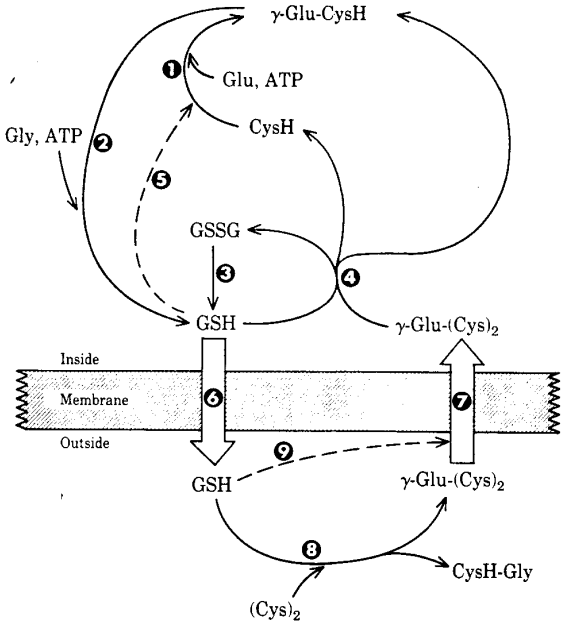


Figure 1. Metabolism and transport of GSH and γ-glutamylcysteine by the kidney. **1,** synthesis of γ-glutamylcysteine from glutamate and cysteine by γ-glutamylcysteine synthetase; **2**, synthesis of GSH from γ-glutamylcysteine and glycine by GSH synthetase; **3**, reduction of GSSG by GSSG reductase; **4**, reduction of γ-glutamylcysteine by transhydrogenation with GSH; **5**, feedback inhibition of γ-glutamylcysteine synthetase by intracellular GSH; **6**, transport of GSH out of the cell; **7**, transport of γ-glutamylcysteine into the cell; **8**, formation of γ-glutamylcysteine from GSH and cysteine by γ-glutamyl transpeptidase; **9**, inhibition of transport of γ-glutamylcysteine by high levels of extracellular GSH [2].

It is of importance in the characterization of the glutathione status of tissues and cells to determine the concentration of GSH as well as the capacity of the system to synthesize GSH. Much useful information about the function of GSH has been derived from studies in which the cellular level of GSH is markedly decreased. A satisfactory approach to the production of GSH deficiency consists of inhibiting γ-glutamylcysteine synthetase. Such inhibition can be produced by BSO.

Mechanism of action

Methionine sulfoximine is an inhibitor of both glutamine synthetase and γ-glutamylcysteine synthetase [11]. Thus, mice treated with methionine sulfoximine experience a decrease in both glutamine and GSH levels, and convulsions [11,12]. To achieve better specificity a series of methionine sulfoximine analogs was synthesized and evaluated *in vitro* and *in vivo* as inhibitors of glutamine synthetase and γ-glutamylcysteine synthetase; BSO was found to be a specific inhibitor of γ-glutamylcysteine synthetase [8]. γ-Glutamylcysteine synthetase catalyzes the reaction of L-glutamate with MgATP to form γ-glutamyl phosphate as an enzyme bound intermediate [11,12]. Reaction of γ-glutamyl phosphate with the α-amino group of L-cysteine completes the catalytic cycle. The γ-glutamylcysteine synthetase also catalyzes an analogous reaction, in which ATP phosphates the sulfoximine nitrogen of L-methionine-S-sulfoximine; the products, MgADP and methionine sulfoximine phosphate, are tightly but non-covalently bound in the active site of the enzyme [9,11]. The enzyme remains inhibited until these products dissociate; so there is no reaction with cysteine to complete a catalytic cycle. It seems that methionine sulfoximine bound to the enzyme as a substrate analog or as a transition state analog in which its structure resembles the tetrahedral adduct formed by addition of γ-carbonyl of γ-glutamyl phosphate to the α-amino group of cysteine.

Buthionine sulfoximine closely resembles the structure of the γ-glutamyl phosphate cysteine adduct [10,11], and is a specific potent inhibitor of γ-glutamylcysteine synthetase. The inhibition of γ-glutamylcysteine synthetase by BSO follows pseudo first-order kinetics (Figure 2) [11].

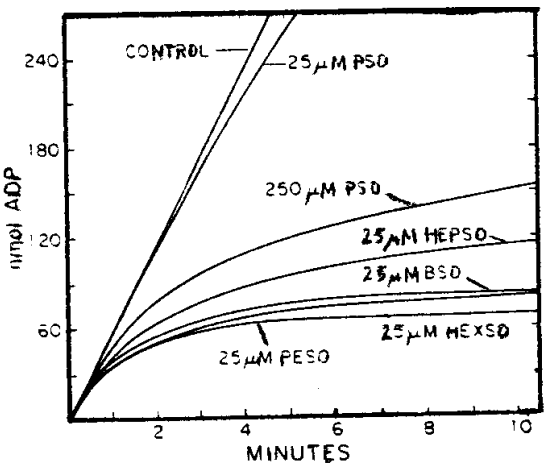


Figure 2. Inhibition of γ-glutamylcysteine synthetase by S-(n-alkyl)-homocysteine sulfoximines. In the experiment represented by the line labeled 'control' no inhibitor was added. In the other experiments the following sulfoximines were present at the final concentrations indicated: DL-prothionine-SR-sulfoximine (PSO); DL-buthionine-SR-sulfoximine (BSO); DL-pentathionine –SR-sulfoximine (PESO); DL-hexathionine-SR-sulfoximine (HEXSO); and DL-heptathionine-SR-sulfoximine (HEPSO) [11].

This indicates that enzyme inhibition is mediated by the initial formation of an enzyme-BSO complex [11]. Such inhibitors do not bind covalently to the enzyme. It is noted that tightly bound non-covalent inhibitors are pharmacologically advantageous in that their use is not accompanied by the risk of supplying or producing electrophiles *in vivo* [11]. In contrast to methionine sulfoximine, BSO does not inhibit glutamyl synthetase and thus does not cause convulsions and death. Also, BSO is not an inhibitor of GSH synthetase, or γ-glutamyl transpeptidase. BSO is not catabolized *in vivo* and it is metabolized primarily by acylation of the α-amino nitrogen. Methionine sulfoximine is more extensively catabolized. It is a substrate of γ-cystathionase, glutamine transaminase and L-amino acid oxidase and is metabolized by these and other enzymes to yield methane sulfinic acid, methane sulfinamide, and vinyl glyoxylate in addition to other products [11]. *In vitro* and *in vivo* studies with higher homologs of BSO containing S-n-alkyl groups of 5, 6, and 7 carbons, increased the

effectiveness of the compounds as inhibitors of γ-glutamylcysteine synthetase. As shown in Figure 2, pentathinine sulfoximine is tightly more effective inhibitor than BSO whereas the 6-carbon analog, hexathionine sulfoximine is approximately equivalent to BSO. Heptathionine sulfoximine is distinctly less effective, but is at least 10 times more inhibitory than prothionine sulfoximine, the 3-carbon analog [11,12]. In conclusion, BSO is the best inhibitor of GSH.

Diastereomers of L-(SR)-BSO

Studies reported to date have been carried out using either DL-(SR)-BSO or L-(SR)-BSO mixtures of four and two isomers respectively [11,12]. The diastereomers of BSO are partially separated by several of the ion exchange or reverse-phase chromatographic procedures. An excellent separation of L-(S)-BSO and L-(R)-BSO was achieved by using D-proline and cupric acetate as buffer in reverse-phase HPLC (Figure 3). As shown, the diastereomers of D-(SR)-BSO elute together in a third peak that is well separated from the L-isomers.

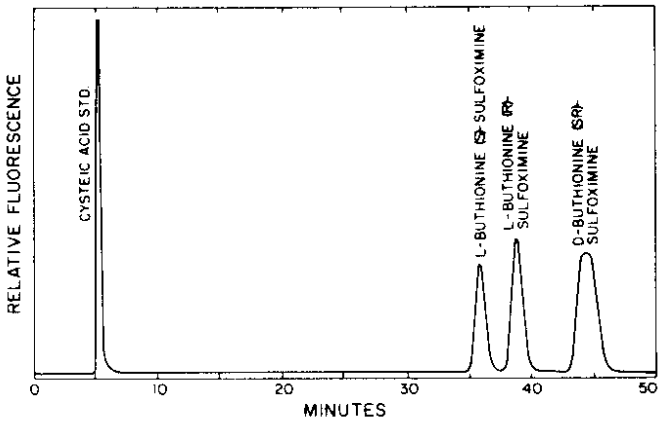


Figure 3. Analytical separation of BSO enantiomers and diastereomers. The compounds indicated were separated by reverse-phase HPLC using 17mM D-proline and 8 mM cupric acetate as buffer; detection was by post column (C-18 silica column) derivatization with O-phthalaldehyde [11].

In additional studies, it has been shown the ability of the resolved L-(SR)-BSO diastereomers to inhibit γ-glutamylcysteine synthetase was examined. Only L- (S)- BSO is phosphorylated by the

enzyme; only that isomer causes essentially irreversible inhibition of the enzyme. At a concentration of 10 μM, L-(S)-BSO caused complete inhibition within 10 min; with concentrations greater than 100 μM, inhibition was complete within seconds. As shown in Figure 4, there is a linear relationship between the amount of L-(S)-BSO added to the initial reaction mixture and the extent of inhibition observed after dilution.

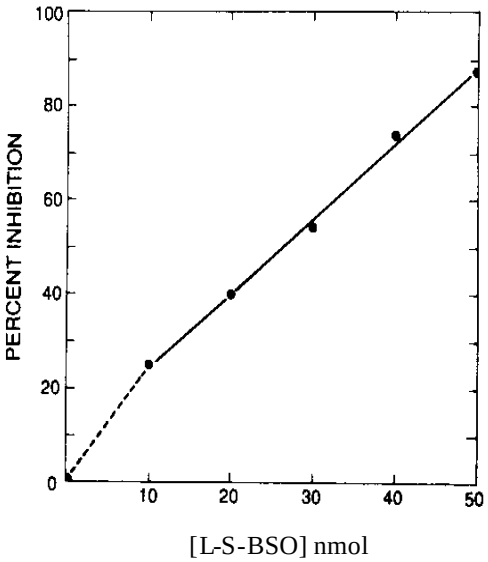
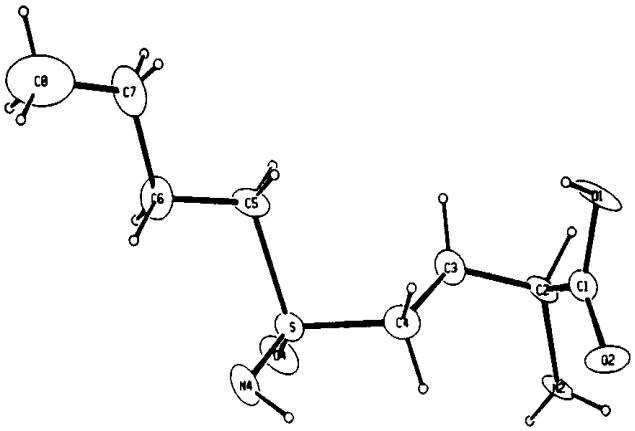


Figure 4. γ-Glutamylcysteine synthetase inhibition as a function of the amount of L-(S)-BSO present in the incubation. γ-Glutamylcysteine synthetase (6 units, corresponding to about 55 pmol of enzyme) was incubated with ATP, Mg^{2+}, and the indicated concentration of L-(S)-BSO for 30 min. A small aliquot of the reaction mixture was then assayed for residual γ-glutamylcysteine synthetase activity, and the percent inhibition was calculated with reference to similar incubations containing no L-(S)-BSO [12].

Inhibition was also seen with highly purified L-(R)-BSO. In contrast to the results with L-(S)-BSO, the extent of inhibition did not increase with time and significant inhibition required high concentrations of L-(R)-BSO [12]. Kinetic studies established that L-(R)-BSO is a reversible inhibitor that binds competitively with respect to L-glutamate; its Ki is 0-11 mM (the K_M for L-glutamate is 1.7 mM) [12]. The absolute configuration of the resolved isomers was determined by x-ray diffraction (Figure 5) [12].

A)



B)

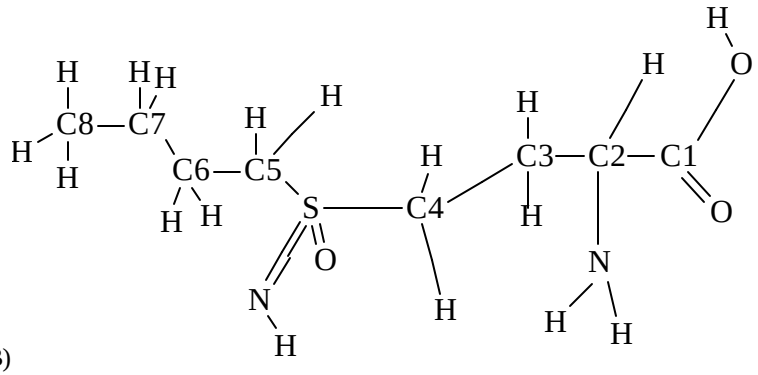


Figure 5. Structure of L-(R)-BSO as determined by x-ray diffraction (A), and the representative chemical configuration (B). Measured bond lengths and angles are all within expected ranges. Both C-S bonds are 1.8 Å in length; the N-S and O-S bonds are 1.48 and 1.55 Å, respectively. The geometry around the sulfoximine sulfur is very close to the regular tetrahedron [12].

This structure was solved based on the known L configuration of the α-amino group. The S-N-H bond angle is 117.8°; note that the geometry around the sulfoximide sulfur is very close to that of a regular tetrahedron [12].

Toxicity of BSO

The toxicity of higher analogs of BSO, *e.g.* hexathionine and heptathionine is an unexpected and unexplained observation. BSO has been given in single doses of 32 mmol/kg [8], and in multiple

doses totaling 72 mmol/kg in 27 h without apparent toxicity [11]. Mice have consumed water containing 20 mM BSO for up to 45 days without toxicity (a dose of 4 to 8 mmol/kg per day) [13]. The toxicity of higher analogs observed may be due to non-specific membrane damage or to the limited solubility of these compounds at physiologic pH [11].

Summary

BSO is a potent and specific inhibitor of γ-glutamylcysteine synthetase. γ-Glutamylcysteine synthetase is the enzyme that catalyzes the first reaction of GSH biosynthesis. Earlier studies established that BSO is initially bound as a transition state analog and that the enzyme then catalyzes the ATP and Mg^{2+} dependent phosphorylation of one or both diastereomer of L-(SR)-BSO. The phosphorylated inhibitor is tightly but non-covalently bound to the enzyme. Studies reported to date have shown that only L-(S)-BSO is phosphorylated by the enzyme. Furthermore, they showed that only that isomer causes irreversible inhibition of the enzyme. BSO has been shown to have considerable utility in the biological role of GSH. Since GSH biosynthesis is strongly inhibited in animals as well as in cultured cells exposed to BSO, treatment with BSO causes tissues or cells with moderate to high rates of GSH turnover to be depleted of GSH.

References

1) Meister A, Anderson ME. (1983) Glutathione. *Annl Rev Biochem.* **52**:711-760.

2) Anderson ME, Meister A. (1983) Transport and direct utilization of gamma-glutamylcyst(e)ine for glutathione synthesis. *Proc Natl Acad Sci.* USA. **80**:707-711.

3) Anderson ME. (1997) Glutathione and glutathione delivery compounds. *Adv Pharmacol*. **38**:65-78.

4) Richman PG, Meister A. (1973) Regulation of γ-glutamylcysteine synthetase by allosteric feedback inhibition by glutathione. *J Biol Chem*. **250**:1422-1426.

5) Meister A. (1983) Selective modification of glutathione metabolism. *Sci*. **220**:472-477.

6) Dethmers JK, Meister A. (1981) Glutathione export by human lymphoid cells: depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation. *Proc Natl Acad Sci.* USA. **78**:7492-7496.

7) Biaglow JE, Varnes ME, Clark EP, Epp ER. (1983) The role of thiols in cellular response to radiation and drugs. *Rad Research.* **95**:437-455.

8) Griffith OW, Meister A. (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). *J Biol Chem*. **254**:7558-7560.

9) Richman PG, Orlowski M, Meister A. (1973) Inhibition of gamma-glutamylcysteine synthetase by L-methionine-S-sulfoximine. *J Biol Chem.* **248**:6684-6690.

10) Griffith OW, Anderson ME, Meister A. (1979) Inhibition of glutathione biosynthesis by prothionine sulfoximine (S-n-propyl homocysteine sulfoximine), a selective inhibitor of gamma-glutamylcysteine synthetase. *J Biol Chem*. **254**:1205-1210.

11) Griffith OW. (1982) Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. *J Biol Chem.* **257**:13704-13712.

12) Campbell EB, Hayward ML, Griffith OW. (1991) Analytical and preparative separation of the diastereomers of L-buthionine (SR)-sulfoximine, a potent inhibitor of glutathione biosynthesis. *Anal Biochem.* **194**:268-277.

13) Griffith OW. Meister A. (1979) Glutathione: interorgan translocation, turnover, and metabolism. *Proc Natl Acad Sci.* USA. **76**:5606-5610.