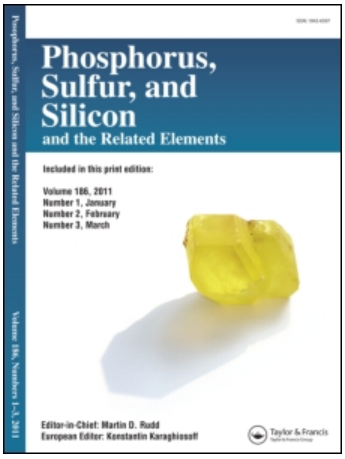

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THE SYNTHESIS AND BIOLOGICAL ACTIVITY OF *S*-ALKYL PHOSPHOHOMOCYSTEINE SULFOXIMIDES

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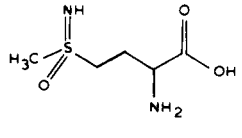
The phosphonate analogues of methionine and buthionine sulfoximide were synthesized by the action of NaN_3 on the corresponding sulfoxides. Analogues **3** and **4** were prepared to determine the effect of altering the carboxyl portion of known inhibitors in the glutamate synthase cycle. Results of biological testing revealed that **3** and **4** did not posses the potency found with buthionine and methionine sulfoximide in cellular systems. However **3** was found to be an effective and irreversible inhibitor of glutamine synthetase.

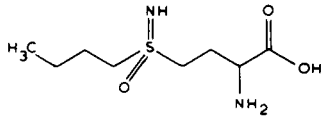
Key words: Sulfoximide synthesis; phosphonate isosteres; glutamine synthetase inhibition; γ-glutamylcysteine synthetase inhibition; amino acid; phosphomethionine sulfoximide; phosphobuthionine sulfoximide.

INTRODUCTION

Both methionine sulfoximide **1** and buthionine sulfoximide (*S*-*n*-butylhomocysteine sulfoximide) **2** have been studied extensively as enzyme inactivators most notably by the Meister group.[2] The chemistry of these inhibitors has recently been reviewed by Silverman[3] and they are described as mechanism-based transition state analogues. Noteworthy biological and pharmacological applications are, in the case of methionine sulfoximide **1**, the inhibition of glutamine synthetase, and in the case of buthionine sulfoximide **2**, potent and specific inhibition of γ-glutamylcysteine synthetase which has found effective applications in cancer chemotherapy research.[4,5]



1

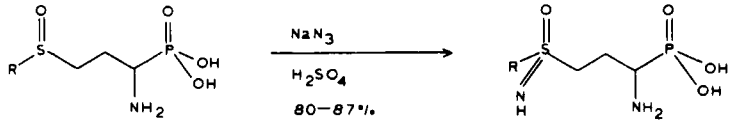


2

Reported surveyed structural-activity relationships of glutamic acid derived transition state enzyme inhibitors have not demonstrated the importance of the carboxy function located at the primary end of the glutamic acid template. With this in mind we have synthesized analogues of *S*-alkyl homocysteine sulfoximides, substituting the carboxyl group with a phosphonate. The synthesis of these materials and the preliminary results of biological testing are reported here.

RESULTS AND DISCUSSION

Bentley reported the synthesis of alkyl homocysteine sulfoximides in 27–28% yields by adding 1.2 equivalents of sodium azide in small portions to a mixture of alkyl homocysteine sulfoxide, sulfuric acid, and dry chloroform, followed by product isolation on an ion exchange resin.[6] This procedure was later modified by Griffith using 4.7 equivalents of sodium azide to one equivalent alkyl homocysteine to yield the sulfoximide directly in 50–75% yield without the preformation of the sulfoxide.[7] Applying the latter procedure with *S*-alkyl phosphohomocysteines resulted in mixtures of sulfoxide and and sulfoximide. Although the Bentley procedure worked well, we found that the most convenient preparation of **3** and **4** follows closely to that reported by Johnson describing the synthesis of *S*-alkyl-*S*-aryl sulfoximides.[8] Thus **3** and **4** were obtained by adding 98% sulfuric acid dropwise to a well stirred suspension of *S*-alkyl phosphohomocysteine sulfoxide, **5** and **6**, and one to two equivalents sodium azide in dry chloroform kept below 10°C. After addition of the sulfuric acid the mixture was maintained at 50° for 12–18 hours (Scheme 1). The products in both cases were essentially pure as indicated by ^{1}H NMR. However elemental analysis indicated the presence of NH_3 remaining from ammonium hydroxide elution of the ion exchange resin. The desired compounds **3** and **4**, were obtained in 80–87% yield without further purification. Analytically pure samples were prepared directly by one of two methods: (A) slow elution of a Dowex resin with 2N NH_4OH resolving pure **3** or **4**; (B) the formation of the amino oxo sulfonium picrates **7** and **8** from the crude isolated reaction products (Scheme 2). The salts (**7** and **8**) were readily recrystallized from water and the sulfoximides regenerated in analytical purity. Removal of residual NH_3 by other methods, i.e. reflux with co-solvent, solvent coevaporation, vacuum drying at elevated temperatures, was



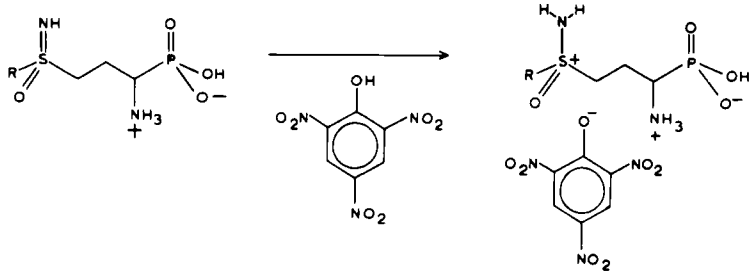
5 R: Methyl

6 R: n-Butyl

3 R: Methyl

4 R: n-Butyl

SCHEME 1



3 R = CH_3 7

4 R = n-Butyl 8

SCHEME 2

not as effective. The presence of sulfoximide was demonstrated by ^1H NMR. Addition of deuterium chloride to a solution of **3** or **4** in D_2O caused the signals from protons α to sulfur to shift downfield by about 0.6 ppm. The same phenomena was not observed performing the same experiment with a sample of phosphomethionine sulfone.

The synthesis of *S*-alkyl phosphohomocysteine sulfoximides proceeded in better yields than reported for the synthesis for the carbon analogues, **1** and **2**, using techniques similar to those reported in the literature. The use of picric acid salts for purification of sulfoximides was a novel application of an old idea and works well in the case of the materials studied here.

BIOLOGICAL ACTIVITY

Preliminary studies carried out with compounds **3** and **4** on murine tumor cells in tissue culture resulted in no effect on the level of cellular glutathione (no observed -γ-glutamylcysteine synthetase inhibition whereas compounds **1** and **2** under the same conditions show significant depletion of glutathione levels).[9]

In greenhouse studies the methionine sulfoximide analogue, **3**, showed no herbicidal effects. Thus, by inference, may not significantly inhibit plant glutamine synthetase. Replacement of a carboxyl group by a phosphono group alters the activity of these *S*-alkyl homocysteine sulfoximide analogues. Since the phosphorylated sulfoximide fragment is considered to be a transition state mimic of an enzymatic reaction,[3] it can be assumed that the amino acid portion influences membrane transport and/or conformation within the active site.

Analysis of the behavior of **3** in an cell free enzymatic system resulted in the discovery of its properties as an irreversible inhibitor.

The Km(obs) for glutamic acid (NH4Cl) was found to be 5.0×10^{-3} M. Literature Km values range from 2.4 to 6.7 mM depending upon the source of the enzyme and assay conditions.[12] Methionine sulfoximide (**1**) is a known inhibitor of glutamine synthetase and was reported to exhibit competitive inhibition initially followed by gradual irreversible (or tight binding) inhibition.[13] In the case of the pea enzyme, **1** was reported to have an initial Ki of 1.6×10^{-4} M

(pKi 3.80). The present investigation yielded an initial Ki of 7.0×10^{-5} M (pKi 4.15). The phosphonate analogue of methionie sulfoximide, i.e., **3**, tested in our system, exhibited a similar initial competitive binding phase followed by gradual tight-binding. An estimate of Ki = 1.2×10^{-3} M was made for analogue **3** using a standard double reciprocal plot. The relative rates of enzyme inactivation due to tight binding for **1** and **3** can be expressed as the ratio $k_1/k_3 = 4.8$, where k_1 and k_3 are the calculated inactivation rate constants for **1** and **3** respectively. Thus, analogue **3** was demonstrated to be an inhibitor of glutamine synthetase, with initial reversible binding somewhat less tight than methionine sulfoximide (**1**) but with a subsequent tight-binding rate similar in nature to **1**. This observation is consistent with the structural similarities between **1** and **3**, i.e., carboxylate (1) vs. phosphonate (3).

A number of glutamine synthetase inhibitors are known. It has recently become possible to use computer-assisted methodology to construct a predictive hypothetical active site lattice (HASL) from inhibitor binding data.[14] Using the Ki data for 16 inhibitors[15] to construct such a lattice at a resolution of 2.5 angstroms, predicted pKi values obtained for **1** and **3** were 4.09 and 2.64, respectively. These predictions compare favorably with the pKi's observed in this study. The HASL model of the glutamine synthetase active site is expected to serve as a modelling stratagem for the design of more effective inhibitors.

EXPERIMENTAL

The sulfoxides **5** and **6** were prepared by known methods.[10] (3-Amino-3-phosphonopropyl)alkyl sulfoximides **3** and **4**: To a stirring suspension of (3-amino-3-phosphonopropyl)alkyl sulfoxide (**5** or **6**, 40 mmol) and sodium azide (60–80 mmol) in 120 mL of dry chloroform cooled to 4° was added concentrated sulfuric acid (22.6 mL) dropwise at a rate sufficient to maintain the temperature below 10° (30–50 min.). The three phase mixture was then warmed to 50° and vigorously stirred at that temperature for 12–18 hours. After cooling to room temperature, 100 mL of cold water was poured into the reaction vessel with rapid stirring. The aqueous phase was separated and the chloroform layer washed with 3 × 20 mL of H_2O. The combined washings and aqueous extract were loaded onto an ion exchange column (94 g of Dowex 50W–X8 in the H^+ form) and the column washed with one liter of distilled and deionized water. The product, **3** or **4**, was then eluted from the column with 2N NH_4OH. Those fractions which were ninhydrin positive were concentrated to dryness by vacuum, and the residue dissolved in H_2O:CH_3OH (1:1) and reconcentrated to drive off excess NH_3 to obtain **3** and **4** as white solids pure by ^1H NMR.

Analytically pure samples of **3** and **4** were obtained by following the synthetic procedure with modicfiations **A** and/or **B**.

Modification **A**: The sulfoximide **3** and **4** can be eluted from the ion exchange column avoiding NH_3 contamination by slowing the rate of elution so that a zone of pure sulfoximide forms. The fraction containing pure product eluted with an observed drop in pH (from 6 to 4 by pH paper). As the eluant pH return to neutral the fraction was cut and pure sulfoximide obtained by vacuum concentration of the fraction.

Modification **B**: Alternatively the products can be further purified by picrate formation. For example, to a solution of **4** (3.85 g, 17.8 mmol) in 75 mL of H_2O was added a methanolic solution of picric acid (12.2 g, 53.4 mmol, 250 mL CH_3OH). A precipitate formed almost instantly. The mixture was then heated on a steam bath for twenty minutes and allowed to stand at room temperature overnight. The precipitate was filtered off, washed with 20 mL of CH_3OH, 3 × 20 mL of diethyl ether and dried to yield **8** (6.69 g, 16 mmol, 84% yield). The picrate **7** required the following procedure: to a solution of the sulfoximide, **3**, (5.0 g, 20.6 mmol) in 48 mL of H_2O:CH_3OH (1:1) was added a solution of picric acid (9.44 g, 41.2 mmol, 90 mL CH_3OH). Water (10 mL) was then added to the point of turbidity. The mixture was then heated on a steam bath until homogeneous at which point a precipitate was

observed to form. After standing 24 hours at room temperature **7** was filtered off and recrystallized from 200 mL of hot H_2O to yield 7.23 g, 14.8 mmol, 72% yield.

Regeneration of the sulfoximides from their picrate salts was accomplished by stirring the salts in 2% HCl (12 mmol picrate, 100 mL 2% HCl). The insoluble picric acid was filtered off. The filtrate was extracted with 5 × 30 mL benzene and the aqueous phase concentrated to dryness by vacuum. The residue was dissolved in 50% ethanol (24 mL for **3**, 44 ml for **4**) and the solution cooled to 0°. Propylene oxide (6.87 g, 111 mmol) was added dropwise with stirring. After standing for 24 hours the precipitate was fitlered off, washed with absolute ethanol and dried to give pure sulfoximide. The mother liquid was concentrated to dryness and the residue triturated with absolute ethanol to yield a second crop of pure product.

Glutamine synthetase activity was determined using a modification of the coupled assay system.[11] Assay solutions consisted of 0.10 M Tris/HCl buffer at pH 7.8 containing 20 mM MgSO4, 60 mM KCl, 10 mM NH4Cl, 0.83 mM phosphenolpyruvate, 0.67–2.0 mM glutamic acid, 0–0.44 mM analogue **3** (or 0.042 mM methionine sulfoximide), 2 units of glutamine synthetase (*E. coli*, Sigma Chemical Co.), 14 units of pyruvate kinase and 20 units of lactate dehydrogenase (rabbit muscle, Sigma Chemical Co.), with sufficient NADH added to provide a UV (340 nm) absorbance of 2–3 in 3 mL. Reactions were carried out at 30°C and initiated with the addition of ATP (3.3 Mm final concentration) in a total assay volume of 3.00 mL. The rate of glutamine synthesis was monitored by UV at 340 nm.

Analytical Data NMR data were obtained on a Varian XL-200 spectrometer.

Structure 3, D_2O, δ ppm vs. DSS = 0, 2.35 (broad m, 2H); 3.19 (s, 3H); 3.42 (m, 1H); 3.57 (m, 2H): Elemental analysis, calculated 22.22%C, 6.06% H, 12.96% N, Found 21.81%, 6.14%, 12.72%. M.P. 219°C with decomposition.

Structure 4, D_2O, δ ppm vs. DSS = 0, 1.0 (t, 3H); 1.55 (hex, 2H); 1.8 (pent, 2H); 2.37 (m, 2H); 3.07 (t, 2H); 3.17 (m, 2H); 3.48 (dt, 1H): Elemental analysis, calculated %C = 32.55, %H = 7.42, %N = 10.85; Found 32.24, 7.61, 10.89: M.P. 190–192°C with decomposition and foaming.

Structure 7, D_2O, δ ppm vs. DSS = 0, 2.40 (broad m, 2H); 3.52 (m, 1H); 3.63 (s, 3H); 4.03 (m, 2H); 8.98 (s, 2H). Elemental analysis, calculated 26.97%C, 3.62% H, 15.73% N; found 26.26%, 3.6%, 15.49%. M.P. 208–210 with decomposition.

Structure 8, D_2O, δ ppm vs. DSS = 0, 0.952 (t, 3H); 1.51 (hex, 2H); 1.89 (pent., 2H); 2.37 (m, 2H); 3.46 (dt 1H); 3.64 (m, 2H); 3.90 (m, 2H); 8.96 (s, 2H): Elemental analysis, calculated %C = 32.04, %H = 4.55, %N = 14.37; found 31.79, 4.55, 14.27: sample does not melt sharply.

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