Notes

Synthesis and pH-Dependent Stability of Purine-6-sulfenic Acid, a Putative Reactive Metabolite of 6-Thiopurine

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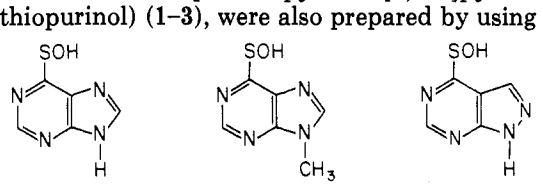
Previous studies have shown that 6-thiopurine is metabolically activated by hepatic cytochrome P-450 to an intermediate capable of binding to proteins by a mixed disulfide linkage. The identity of the active metabolite was postulated to be purine-6-sulfenic acid. In the present report, we describe the synthesis of the sulfenic acid derivatives of 6-thiopurine and two structurally similar compounds, 9-methyl-6-thiopurine and 4-mercapto-1*H*-pyrazolo[3,4-*d*]-pyrimidine. The unusual pH-dependent stability profiles of these compounds in buffered aqueous media are presented and explained on the basis of a disproportionation mechanism of sulfenic acid decomposition. Studies with radiolabeled purine-6-sulfenic acid demonstrate that this species binds directly to hepatic microsomal protein. These results support the proposed involvement of purine-6-sulfenic acid in the metabolic activation and tissue binding of 6-thiopurine.

6-Thiopurine is an antineoplastic agent that is used for the treatment of leukemias.[1] The drug is also immunosuppressive and is used, as the prodrug azathioprine, for organ transplantation.[2] We have recently demonstrated that 6-thiopurine is metabolically activated by the cytochrome P-450 system of mammalian liver to a reactive metabolite which subsequently binds to tissue protein.[3] We have proposed that the reactive intermediate is purine-6-sulfenic acid (1) and that the binding of this metabolite is largely through formation of mixed disulfides with protein thiols. The covalent binding of purine-6-sulfenic acid to liver proteins may explain the hepatotoxic effects associated with the use of 6-thiopurine.[4] However, the enzymatic oxidation of thiol compounds to sulfenic acids has been difficult to prove, since these metabolites are notoriously unstable and reactive. To date, only a few sulfenic acids have been prepared chemically.[5] Recently, evidence was presented for the involvement of sulfenic acids in the oxidation of thiols.[6]

In order to further study the metabolic activation of 6-thiopurine, we have synthesized purine-6-sulfenic acid via chemical oxidation of the parent thiol. Sulfenic acids of two structurally related compounds, 9-methyl-6-thiopurine and 4-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidine (allothiopurinol) (1–3), were also prepared by using this



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procedure. In this paper, we describe the synthesis and isolation of these sulfenic acids and present the unusual pH-dependent stabilities of these compounds in buffered aqueous media. We also report that chemically synthesized [8-^{14}C]purine-6-sulfenic acid binds directly to hepatic microsomal protein in vitro.

Results and Discussion

Chemistry. The sulfenic acids (1–3) were synthesized by reaction of the parent thiol with 1 equiv of *m*-chloroperoxybenzoic acid. The sulfenic acids were purified as the silver salts after Pal et al.,[7] who isolated and characterized 1-methyluracil-4-sulfenic acid as its silver salt. The free sulfenic acids could not be isolated, but their properties in solution were examined after regeneration of the acid in solution by acidification of the silver salt.

That we are observing the sulfenic acid of 6-thiopurine is supported by the following evidence. Synthesis of 1 was performed via oxidation with 1 equiv of *m*-chloroperoxybenzoic acid. The addition of an 8% solution (w/v) of $FeCl_3$ in methanol to a solution of the product in methanol yielded a soluble blue complex, indicative of a sulfur monoxide,[8] a tautomeric form of purine-6-sulfenic acid. As shown in Figure 1, the UV spectrum of 1 in aqueous solution revealed an absorption maximum between 355 and 360 nm, depending on the solution pH. The bathochromic shift in maximum UV absorption observed upon oxidation of the parent thiol to the sulfenic acid was comparable to that previously reported for conversion of 1-methyl-4-thiouracil to its sulfenic acid.[7] The observed UV spectrum was not that of other possible reaction products, i.e., purine, hypoxanthine, 6-thiopurine, 6-thiopurine disulfide, purine-6-sulfinic acid, or purine-6-sulfonic acid.[9] The decomposition of the putative sulfenic acid in solution was consistent with the disproportionation mechanism shown in Scheme I.[10,11] Thus, the sulfenic acid (1) decomposes in base via the thiosulfinate (2) to 6-thiopurine (4) and purine-6-sulfinic acid (3) only (Figure 1). Similarly, under

(1) Paterson, A. R. P.; Tidd, D. M. In "Antineoplastic and Immunosuppressive Agents"; Sartorelli, A. C.; Johns, D. G., Eds.; Springer-Verlag: Berlin, 1975; pp 384–403.
(2) Bach, J.-F. In "The Mode of Action of Immunosuppressive Agents", Elsevier: New York, 1975; pp 93–172.
(3) Hyslop, R. M.; Jardine, I. *J. Pharmacol. Exp. Ther.* **1981**, *218*, 621 and 629.
(4) Zimmerman, H. J. *Drugs* **1978**, *16*, 25.
(5) For recent reviews of sulfenic acid chemistry, see: (a) Hogg, D. R. *Compr. Org. Chem.* **1979**, *3*, 261. (b) Kice, J. L. *Adv. Phys. Org. Chem.* **1980**, *17*, 65.
(6) Davis, F. A.; Billmers, R. L. *J. Am. Chem. Soc.* **1981**, *103*, 7016.
(7) Pal, B. C.; Uziel, M.; Doherty, D. G.; Cohn, W. E. *J. Am. Chem. Soc.* **1969**, *91*, 3634.
(8) Walter, W.; Randau, G. *Ann. Chem.* **1969**, *722*, 52.
(9) Doerr, I. L.; Wempen, I.; Clarke, D. A.; Fox, J. J. *J. Am. Chem. Soc.* **1961**, *26*, 3401.
(10) Block, E.; O'Connor, J. *J. Am. Chem. Soc.* **1974**, *96*, 3921 and 3929.
(11) Davis, F. A.; Billmers, J. L. *J. Am. Chem. Soc.* **1981**, *103*, 7016.

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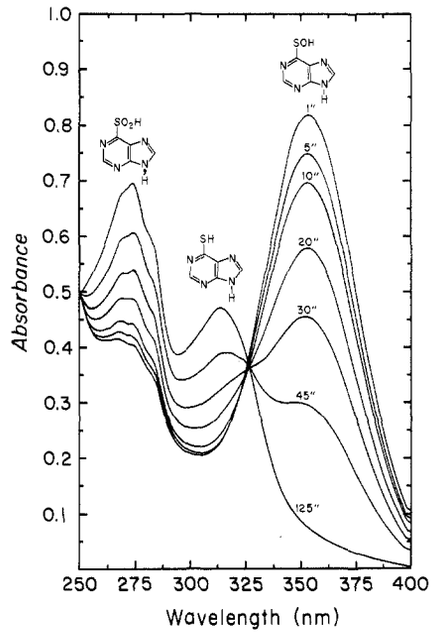
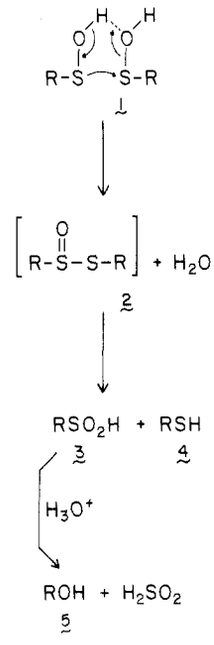


Figure 1. UV absorption spectra obtained during decomposition of purine-6-sulfenic acid into purine-6-sulfinic acid and 6-thiopurine. Individual scans were recorded in 0.1 M Tris-HCl buffer (pH 8.0) at 1, 8, 10, 20, 30, 45, and 125 min after acidification of the silver salt of purine-6-sulfenic acid.

Scheme I



acidic conditions the products of purine-6-sulfenic acid breakdown were 6-thiopurine and hypoxanthine. The latter product is generated by rapid acid-catalyzed decomposition of purine-6-sulfinic acid.[9] Finally, the thiopurine oxidation product reacted readily with sulfhydryl groups, as would be expected of a sulfenic acid. Thus, the addition of 1 mM 2-mercaptoethanol to a buffered solution of the reaction product resulted in the almost immediate disappearance of the 355–360-nm UV absorption peak. Finally, when the putative sulfenic acid was prepared from 6-thio[8-^{14}C]purine and added to a buffered solution containing rat liver microsomes, radioactivity was immediately bound to microsomal protein via a disulfide bond. Such binding was demonstrated by release of 6-thio[^{14}C]purine from the protein by reaction with dithiothreitol or glutathione (see below).

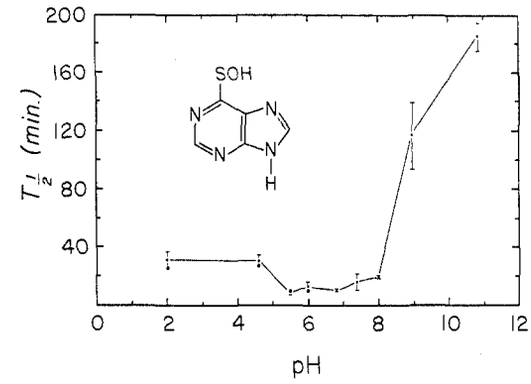


Figure 2. Effect of pH on the apparent half-life ($T_{1/2}$) of purine-6-sulfenic acid in aqueous solution. $T_{1/2}$ determinations were performed in 0.1 M buffers at pH 2.0 (HCl–KCl), 4.6 (sodium acetate), 5.5 (sodium citrate), 6.0 (sodium phosphate), 6.8 (sodium phosphate), 7.4 (sodium phosphate), 8.0 (Tris-HCl), 9.0 (Tris-HCl), and 10.9 (glycine–sodium hydroxide). Values represent means plus or minus standard deviations of three separate determinations. See text for further details.

The existence of a characteristic UV spectrum for 1 distinct from those of the known decomposition products, hypoxanthine and purine-6-sulfinic acid, enabled us to observe the decomposition of 1 in aqueous solution. Since the protonation or deprotonation of 1 should strongly influence the rate of formation of thiosulfinate (2 in Scheme I), the rate of decomposition of 1 in solution should be pH dependent. This possibility was examined by using chemically synthesized sulfenic acids. With the present methodology, we were unable to determine the true initial concentration of sulfenic acid in solution. However, it was observed that the time-dependent decrease in absorbance at the sulfenic acid UV λ_{max} followed a log-linear relationship. Further, the slope of this line was highly reproducible when a constant initial absorbance value was used. For example, the results shown in Figure 2 were obtained by rapidly diluting freshly generated sulfenic acid in a quartz cuvette until an absorbance reading of 0.5–0.6 unit was obtained. The sulfenic acid was then allowed to decompose at room temperature. The resulting log absorbance vs. time data were subjected to linear regression analyses, and apparent half-lives ($T_{1/2}$) were calculated from the best-fit line. Linear correlation coefficients (r) were always greater than 0.95. The unusual half-life vs. pH profile of 1 is shown in Figure 2. The apparent decomposition $T_{1/2}$s were minimal (10–20 min) in the intermediate pH range (pH 5.5–8.0); however, $T_{1/2}$ increased at both more basic and more acidic pHs. Thus, the apparent $T_{1/2}$ of 1 was 31.4 ± 5.6 min (mean ± SD, n = 3) at pH 2 and 185.3 ± 9.8 min at pH 10.9. As stated previously, the apparent decomposition $T_{1/2}$s of 1 were calculated from the decrease in UV absorbance with time by using a constant initial absorbance (i.e., concentration). When apparent $T_{1/2}$ vs. pH experiments were performed at different starting concentrations of 1, qualitatively similar results to those shown in Figure 2 were obtained. However, the apparent $T_{1/2}$ of 1 at any pH varied inversely with the starting concentration of 1 in the cuvette. An example of the concentration dependence of the apparent $T_{1/2}$ of 1 is shown in Table I. This observation is consistent with the bimolecular decomposition mechanism depicted in Scheme I. The kinetics of this reaction would be second order in nature; therefore, the rate of sulfenic acid decomposition should be highly concentration dependent, as we have observed.

A comparison of the stabilities of sulfenic acids, 1–3 at selected pH values is provided in Table II. The sulfenic

Table I

initial absorbance (OD 360 nm)	apparent $T_{1/2}$,[a] min
0.34	18.2
0.66	14.0
1.10	8.0
1.20	5.4
1.40	4.2
1.46	3.7

[a] Concentration dependence of apparent half-life ($T_{1/2}$) measurement for sulfenic acid 1. $T_{1/2}$ determinations were performed in 0.1 M sodium phosphate buffer, pH 7.4. Sulfenic acid 1 was diluted to different initial concentrations in the quartz cuvette based on the initial optical density reading recorded at 360 nm (OD 360 nm). Absorbance at 360 nm vs. time was then recorded from this point, and the apparent decomposition $T_{1/2}$ was calculated as described under Results.

Table II

pH	apparent $T_{1/2}$,[a] min		
	1	2	3
4.6	30.9 ± 3.6	119.4 ± 22.1	>240
7.4	16.3 ± 5.6	38.3 ± 6	>240
9.0	116.6 ± 22.9	>180	>240

[a] Apparent half-lives ($T_{1/2}$) of sulfenic acids 1–3 in buffered aqueous solutions at acidic, neutral, and basic pH. $T_{1/2}$ determinations were performed in 0.1 M buffers at pH 4.6 (sodium acetate), 7.4 (sodium phosphate), and 9.0 (Tris-HCl). Values represent means plus or minus standard deviations of three separate determinations.

acid **2** (UV λ_{max} 355–360 nm) possessed a pH-dependent stability profile qualitatively similar to that observed with **1**; however, the stability of **2** was greater than that of **1** throughout the pH range tested. Sulfenic acid **3** was markedly more stable (apparent $T_{1/2} \gg 4$ h) than either **1** or **2** over the pH range 4.6–9.0. Thus, the steric and electronic characteristics of the adjacent aromatic ring play determinant roles in the relative stabilities of these sulfenic acids.

The stabilization of sulfenic acids by aromatic solvents has been reported previously,[12,13] and we have observed a similar phenomenon for **1**. For example, the apparent $T_{1/2}$ of **1** in pH 8.0 buffer was 19.6 ± 1.1 min; the presence of 0.62 M pyridine in this buffer increased the $T_{1/2}$ to 32.2 ± 4.1 min. Similar results were observed at pH 4.6, where the apparent $T_{1/2}$ of **1** increased from 30.9 ± 3.6 to 106.6 ± 11.0 min in the presence of 0.62 M phenol. The pHs of the buffered solutions were unchanged by the addition of the aromatic solvent.

The effect of pH on the stabilities of the sulfenic acids in solution may be rationalized from the mechanism of decomposition illustrated in Scheme I. The increased stability at basic pH is the result of conversion of the sulfenic acid to the conjugate base (RSO^-). At acidic pH it could be expected that the highly electrophilic protonated sulfenic acid ($RSOH_2^+$) would facilitate decomposition. However, electrostatic repulsion between similarly charged $RSOH_2^+$ species inhibits the decomposition reaction sufficiently to cause an increase in stability at more acidic pH.

Protein Binding Studies. We have previously reported that hepatic microsomal cytochrome P-450 converts 6-thiopurine to a reactive metabolite capable of binding to microsomal protein via a mixed disulfide bond.[3] This reaction was shown to be dependent on both oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The proposed mechanism of 6-thiopurine activation involves oxidation of the parent molecule to purine-6-sulfenic acid (**1**), which then reacts with protein thiol groups. In order to further substantiate this hypothesis, we have tested the reactivity of chemically generated [8-^{14}C]purine-6-sulfenic acid with hepatic microsomal protein. Freshly prepared [8-^{14}C]purine-6-sulfenic acid was added to rat liver microsomes suspended in 0.4 M potassium phosphate buffer, pH 7.4 (for details of this procedure, see Experimental Section). A similar quantity of 6-thio[8-^{14}C]purine was added to a second microsomal suspension under identical conditions to serve as a control. The reaction was peformed at room temperature in the absence of NADPH. After 2 min, the microsomal protein was exhaustively extracted with organic solvents to remove unbound radioactivity, and the protein-bound radioactivity was determined by liquid scintillation counting. Reaction of **1** with hepatic microsomal protein resulted in the binding of 1.76 ± 0.12 nmol/mg of protein, vs. only 0.14 ± 0.00 nmol/mg of protein in the control flask containing unoxidized 6-thiopurine. Thus, the binding of **1** to microsomal protein was 13-fold higher than that of 6-thiopurine. Furthermore, nearly 90% of the bound radioactivity could be released as 6-thio[^{14}C]purine by incubation of the radiolabeled microsomes with 2 mM glutathione or 2 mM dithiothreitol for 20 min at 37 °C, which indicated that binding occurred via a disulfide bond. The level of 6-thio[^{14}C]purine bound per milligram of microsomal protein in these experiments with chemically generated **1** was remarkably similar to that measured following addition of the parent thiol to an NADPH-fortified microsomal activating system.[3] Taken together, these data raise the possibility that a similar limited number of sulfenic acid binding sites, presumably protein thiol groups, are accessible to both exogenously added and metabolically generated purine-6-sulfenic acid. In a parallel series of experiments, we investigated the reactivity of **3** (synthesized from allothio[2-^{14}C]purinol) with protein thiol groups of rat hepatic microsomes. The experimental results were markedly different from those obtained with **1**. The level of protein binding following incubation of **3** with hepatic microsomes (0.14 ± 0.02 nmol bound/mg of microsomal protein) was actually less than the level of binding observed with the parent thiol compound (0.29 ± 0.02 nmol bound/mg of microsomal protein). Thus, sulfenic acid **3** was clearly more stable than **1**, both in buffered aqueous solution, where disproportionation is the principal decomposition mechanism, and in solution with biological protein, where sulfenic acid decomposition proceeds via two competing pathways, disproportionation and disulfide bond formation with protein sulfhydryl groups. Moreover, the binding data for **3** suggests that the NADPH-dependent oxidation of allothio[2-^{14}C]purinol to the sulfenic acid metabolite (**3**) by hepatic microsomes in vitro would not lead to the generation of covalently bound radioactivity. This suggestion was subsequently confirmed by incubation of allothio[2-^{14}C]purinol with rat liver microsomes in the presence or absence of NADPH. After exhaustive organic solvent extraction of microsomal protein, only background levels of radioactivity remained bound to tissue biomolecules (data not shown). In contrast, under identical incubation conditions, the NADPH-dependent metabolism of 6-thio[8-^{14}C]purine resulted in significant binding of radioactivity to microsomal protein.[3] We have

(12) Shelton, J. R.; Davis, K. E. *Int. J. Sulfur Chem.* **1973**, *8*, 205.
(13) Davis, F. A.; Jenkins, R. H., Jr.; Rizvi, S. Q. A.; Yocklovick, S. G. *J. Org. Chem.* **1981**, *46*, 3467.

recently obtained preliminary UV spectral evidence for the oxidation of allothiopurinol to 3 by hepatic microsomes in the presence of NADPH (unpublished observations). Our results to date suggest that sulfenic acid formation may be a common pathway of heteroaromatic thiol oxidation mediated by the microsomal cytochrome P-450 system. We have also demonstrated that marked differences exist between the chemical reactivities of purine-6-sulfenic acid and the sulfenic acids of other, closely related, heterocyclic compounds. Therefore, the biological consequences of oxidative metabolism of these thiol compounds to sulfenic acids may vary markedly, depending on the reactivity of the particular sulfenic acid generated. Further experimentation will be required to establish the apparent correlation between the stabilities in aqueous media of chemically synthesized sulfenic acids of 6-thiopurine and analogous thiol-containing heterocyclic compounds and the capacities of chemically or enzymatically generated sulfenic acids to covalently react with tissue proteins in biological systems.

Conclusions

These studies with chemically generated purine-6-sulfenic acid support the proposed involvement of this reactive species in the oxidative metabolic activation and tissue binding of 6-thiopurine. The availability of synthetic purine-6-sulfenic acids will permit further investigations of the role of oxidative metabolism and tissue binding in the immunosuppressive and hepatotoxic effects of 6-thiopurine.

Experimental Section

Ultraviolet spectra were determined with a Cary Model 219 UV/VIS spectrophotometer (Varian Instruments).

Purine-6-sulfenic Acid (1). For the synthesis of 1, a solution of 0.2 mmol of 6-thiopurine in 32 mL of methanol was placed in an ice bath and thoroughly deoxygenated with a continuous flow of pure nitrogen gas. A nitrogen atmosphere was maintained throughout the synthetic procedure. A solution containing 0.2 mmol of *m*-chloroperoxybenzoic acid in 8 mL of methanol was added dropwise over a 5-min period with continuous stirring. The reaction mixture was stirred for an additional 4 min. At this time, 20 mL of 5 N NH_4OH containing 0.4 mmol of silver acetate was slowly added with vigorous stirring. The reaction was continued for an additional 0.5 h. The organic solvent was removed by rotary evaporation, and the remaining solution was placed on ice and left undisturbed for 1 h to allow precipitation of the silver salt of 1.[7] The silver salt was then pelleted by centrifugation at 4 °C, and the resulting supernatant was discarded. The pellet was washed by vortexing with 30 mL of ice-cold distilled water and reprecipitated by centrifugation. The salt was washed two additional times with water and then once with 10 mL of cold methanol. The free sulfenic acid 1 was regenerated from the purified silver salt by careful acidification of an aqueous suspension of the salt to pH 2 with 0.05 N HCl. After centrifugation to remove the insoluble AgCl, the concentrated sulfenic acid solution was diluted in various buffered aqueous solutions for UV spectroscopy. The overall yield of the reaction as determined with ^{14}C-labeled 6-thiopurine was 20–25%.

The synthesis of 2 and 3 were carried out in a similar fashion, except that the starting concentrations of the parent thiols in methanol were reduced because of decreased solubility.

Protein Binding. Studies of the binding of sulfenic acids 1 and 3 to rat liver microsomal protein were performed with 6-thio[8-^{14}C]purine and allothio[2-^{14}C]purinol (radiochemical purities, 99%) supplied by Research Products International Corp. (Elk Grove, IL). The synthesis of radiolabeled 1 was performed by a modification of the procedure described above. Fifty micromoles of 6-thio[^{14}C]purine (specific activity, 2 $\mu Ci/\mu mol$) was dissolved in 16 mL of ice-cold, nitrogen-saturated methanol. Four milliliters of methanol containing 50 μmol of *m*-chloroperoxybenzoic acid was added dropwise over 5 min. Subsequently, 100 μmol of silver acetate in 5 mL of 5 N NH_4OH was added to the reaction with vigorous stirring, and the solution was stirred for an additional 20 min. The silver salt of 1 was precipitated as described above and then pelleted by centrifugation. The supernatant was discarded, and the precipitate was washed once with ice-cold methanol, centrifuged, and washed once with 5 mL of cold 1.15% KCl. The silver salt was collected by centrifugation and resuspended in 10 mL of 1.15% KCl. The suspension was transferred to a syringe barrel with a 0.8 $\mu m/0.2$ μm polysulfone membrane filter system (Gelman Sciences, Inc., Ann Arbor, MI) attached in series at the syringe outlet. The suspension was rapidly acidified to pH 2 with 0.1 N HCl and immediately forced through the filter system into 8 mL of rat liver microsomal suspension at room temperature. The microsomes were suspended in 0.4 M sodium phosphate buffer (pH 7.4) at a protein concentration of 15.6 mg/mL. Techniques for microsomal preparation and protein determination have been described previously.[14,15] The pH of the microsomal suspension was decreased to 7.25 by the acidic solution of 1. The solution was stirred for several minutes, and then triplicate 1.0-mL samples were withdrawn and added with vortexing to tubes containing 2.0 mL of ice-cold 10% trichloroacetic acid. The precipitated protein was pelleted by centrifugation and then extracted 3 times with 4 mL of methanol/water, 80:20, containing 20% (w/v) trichloroacetic acid. The resulting pellet was then extracted once with ethanol/ether, 80:20, and centrifuged, and the solvent was discarded. The remaining tissue was then digested with 1.0 mL of 0.5 N NaOH at 60° until solubilization of biological material was complete. The tissue digest was transferred to a glass scintillation vial with 10 mL of RPI 3a70 liquid scintillation cocktail (Research Products International Corp., Elk Grove, IL). Finally, 100 μL of glacial acetic acid was added to each vial, and the samples were assayed by liquid scintillation counting. The counting efficiency was approximately 85%. Additional 1.0-mL samples were drawn from the microsomal suspension and added in triplicate to tubes containing 2.0 mL of either 6 mM glutathione or 6 mM dithiothreitol in 1.15% KCl. These samples were incubated with shaking for 20 min at 37 °C and then prepared for assay of bound radioactivity as described above. As a control, the binding of unoxidized 6-thio[^{14}C]purine to hepatic microsomes was determined by dissolving 50 μmol of 6-thio[^{14}C]purine in 14.5 mL of 1.15% KCl and adding directly to the microsomal suspension. Triplicate samples were then processed for the radioactive binding assay by the procedure detailed above.

The binding of the sulfenic acid derivative of 4-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidine (3) to microsomal protein was examined by methods identical with those developed for [^{14}C]purine-6-sulfenic acid, except that 30 mL of methanol was used to dissolve the allothio[^{14}C]purinol prior to addition of the oxidant.

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Registry No. 1, 86335-59-3; 2, 86335-60-6; 3, 86335-61-7; 4, 50-44-2; 6-mercapto-9-methylpurine, 1006-20-8; 4-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidine, 5334-23-6; *m*-chloroperoxybenzoic acid, 937-14-4.

(14) Stripp, B.; Greene, F. E.; Gillette, J. R. *J. Pharmacol. Exp. Ther.* **1969**, *170*, 347.
(15) Gornall, A. G.; Bardawill, C. J.; David, M. M. *J. Biol. Chem.* **1949**, *177*, 751.