Reactions of Glutathione with Carcinogenic Esters of *N*-Arylhydroxamic Acids

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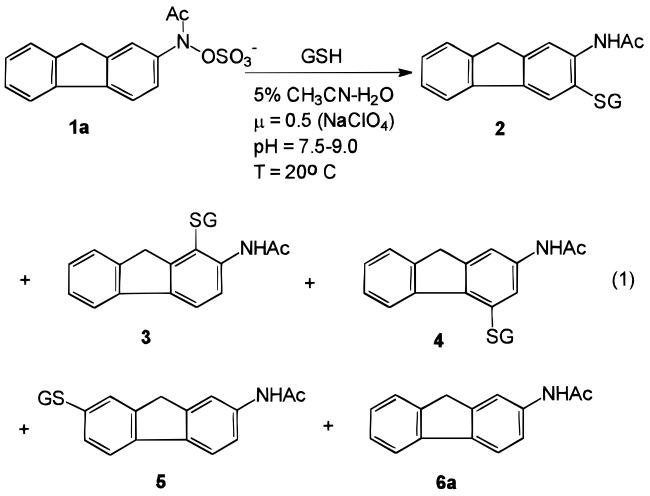
Received October 13, 1995[⊗]

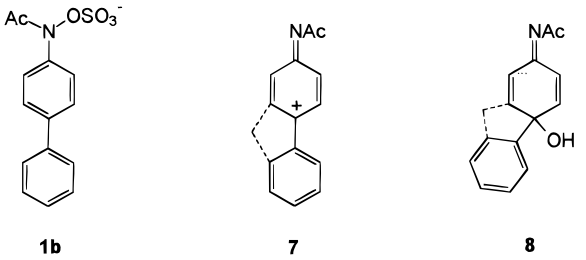
Abstract: The nitrenium ions **7a** and **7b** derived from hydrolysis of *N*-(sulfonatooxy)-*N*-acetyl-2-aminofluorene (**1a**) and *N*-(sulfonatooxy)-*N*-acetyl-4-aminobiphenyl (**1b**) are trapped by glutathione anion (GS^-) with selectivity ratios, k_{gs^-}/k_s, of 8200 ± 600 M^{-1} and 300 ± 15 M^{-1}, respectively. Since k_s is known for both of these ions under our reaction conditions, k_{gs^-} can be calculated. For **7a**, k_{gs^-} is 6.3×10^8 M^{-1} s^{-1}, and for **7b**, k_{gs^-} is 1.8×10^9 M^{-1} s^{-1}. Under physiological conditions (50−100 μM GS^-) neither ion would be efficiently trapped by GS^-. Some of the GSH adducts isolated in this study (**4** from **1a**, **11** and **12** from **1b**) are not derived from nitrenium ion trapping. They arise from GS^- trapping of the quinol imines **8a** and **8b**, which are the initial products of trapping of **7a** and **7b** by H_2O. This reaction is very efficient at physiological GS^- concentrations and could lead to significant GSH depletion *in vivo*. Although it has been known for some time that quinol imines such as **8a** and **8b** are major hydrolysis products of carcinogenic esters of *N*-arylhydroxylamines and *N*-arylhydroxamic acids, no physiological role has been previously suggested for these materials.

Glutathione (GSH) is the major nonprotein thiol present in most animal cells.[1] The concentration of GSH in cells and in intercellular fluids ranges from about 0.1 mM to 10 mM.[1−4] This tripeptide is an important source of reducing equivalents, has a number of regulatory functions, and is also thought to be involved in detoxification of exogenous materials.[2] This detoxification function may depend on the redox properties of GSH or on the high nucleophilicity of the thiol anion of GS^-.[3] Although the nucleophilic reaction of GSH with many electrophiles is catalyzed by glutathione S-transferases, highly reactive substrates such as quinol imines and certain epoxides react rapidly with GSH (as GS^-) under physiological conditions without catalysis.[3−5]

GSH does react with carcinogenic ester metabolites of *N*-arylhydroxylamines or *N*-arylhydroxamic acids such as *N*-(sulfonatooxy)-*N*-acetyl-2-aminofluorene (**1a**) *in vitro* in the absence of glutathione S-transferases (eq 1) to produce both glutathione-carcinogen adducts **2**−**5** and the reduced carcinogen **6a**.[6] There is evidence that similar reactions occur *in vivo*.[7] These reactions have been assumed to occur through a reactive nitrenium ion intermediate, but no evidence to support this supposition has been presented. Both **1a** and *N*-(sulfonatooxy)-*N*-acetyl-4-aminobiphenyl (**1b**) do react with 2′-deoxyguanosine



(d-G), with DNA oligomers, and, by inference, with DNA and RNA, by a nitrenium ion mechanism,[8] but the nature of the carcinogen−GSH reaction and the carcinogen−nucleic acid reaction may be very different. No quantitative data comparing the reactivity of GSH and other nucleophiles with these carcinogens have been presented. Such data could be used to evaluate the effectiveness of GSH in protecting cells from the effects of this class of carcinogens.



This paper presents our results of a mechanistic study of the reaction of GSH with the carcinogens **1a** and **1b**. Both of these

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1996.

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(6) (a) van den Goorbergh, J. A. M.; Meerman, J. H. N.; de Wit, H.; Mulder, G. J. *Carcinogenesis* **1985**, *6*, 1635−1640. (b) Beland, F. A.; Miller, D. W.; Mitchum, R. K. *J. Chem. Soc., Chem. Commun.* **1983**, 30−31.

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(8) (a) Novak, M.; Kennedy, S. A. *J. Am. Chem. Soc.* **1995**, *117*, 574−575. (b) Novak, M.; Kennedy, S. A.; Kolb, B., to be submitted to *J. Am. Chem. Soc.*

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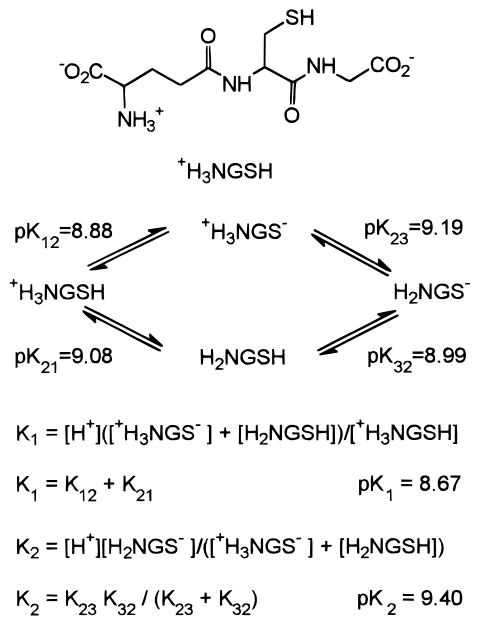
Scheme 1



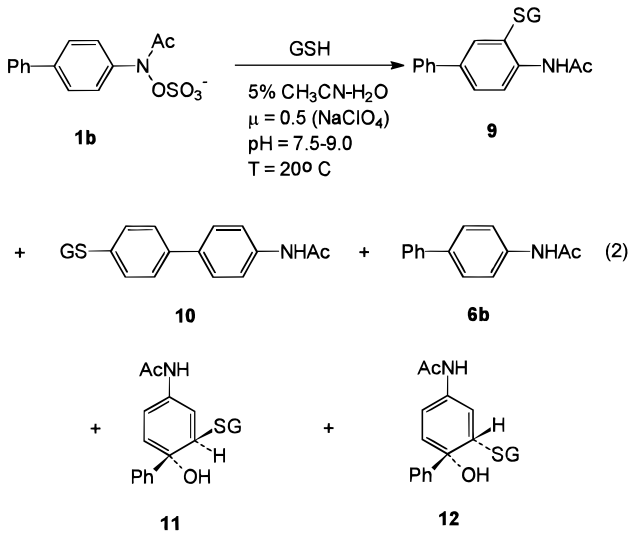
materials react with GSH via two pathways. GS^- does trap the nitrenium ion intermediate **7a** or **7b**, but a second pathway, in which GS^- reacts with the initially formed hydrolysis product **8a** or **8b**, has also been detected. The nitrenium ion trapping is not highly efficient (<50% trapping) under physiological conditions so GSH does not adequately protect against these two carcinogens under conditions prevalent in most cells. However, the second pathway provides a very efficient process for glutathione depletion under physiological conditions. This reaction may be of considerable importance *in vivo*.

Results and Discussion

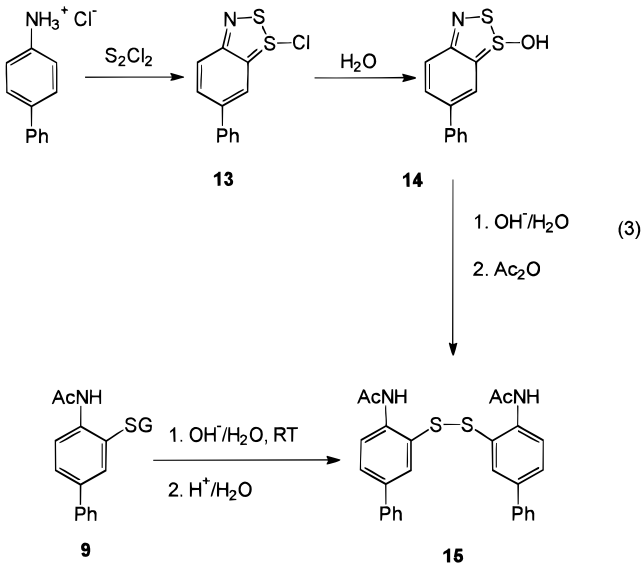
Reaction Mechanism. All experiments were performed in 5 vol % CH_3CN-H_2O, μ = 0.5 ($NaClO_4$), at 20 °C. The conditions were chosen to facilitate comparisons with the reactivities of other nucleophiles with the nitrenium ions **7a** and **7b**.[8−10] GSH (0.5−20 mM) served as its own buffer. Oxidation of GSH to GSSG was minimized by saturating all solutions with N_2. All GSH solutions were prepared fresh daily.

The concentrations of the individual ionized forms of GSH present under our reaction conditions (Scheme 1) are needed for analysis of the reaction data. The system is characterized by four microscopic ionization constants (K_{12}, K_{21}, K_{23}, K_{32}) and two macroscopic constants (K_1, K_2). Ionization constants determined under conditions similar to our own (μ = 0.3, T = 25 °C) are shown in Scheme 1.[11] These constants were used to determine the concentration of the various ionized forms of GSH under our conditions. Confidence in the application of these ionization constants to our system was provided by the excellent agreement (±0.03) between the observed pH values of the GSH buffers (pH 7.5−9.0) and those calculated from the ionization constants. Calculations show that small changes in the microscopic pK_as (±0.1 units) have minimal effects on the calculated concentrations of the various ionized forms shown in Scheme 1. [GS^-], referred to in the text, is the sum of [$^+H_3NGS^-$] and [H_2NGS^-] calculated from the known initial concentrations of GSH and NaOH and the ionization constants of Scheme 1.

Decomposition of **1b** in GSH buffers (3−20 mM $[GSH]_T$): 1.0/1.3 GSH_T/NaOH; pH 8.23, 1.0/1.4 GSH_T/NaOH; pH 8.39, 1.0/1.5 GSH_T/NaOH; pH 8.52) led to the products shown in eq 2. The identity of the adduct **9** was confirmed by the



independent synthesis of its alkaline cleavage product **15** as shown in eq 3. NMR data uniquely established the ring



substitution pattern of **10**. The diastereomers **11** and **12** could not be separated by our procedures, but NMR and MS data leave little doubt about their structures. COSY and NOESY ^{1}H NMR spectra made it possible to uniquely assign most of the ^{1}H NMR peaks for **11** and **12**. In particular, the doublets at 4.06 ppm and 4.09 ppm were assigned to the tertiary proton on the ring carbon bearing the GS-group of **11** and **12**, respectively, based on COSY cross correlations with a vinyl proton at ca. 6.0 ppm and a NOESY cross correlation for one of them (4.09 ppm) with the peak at ca. 7.6 ppm due to the ortho-protons of the aromatic ring. Since GSH is chiral there are four possible diastereomeric products of the type represented by structures **11** and **12**. Only two were detected. The absolute configurations shown for **11** and **12** are, of course, arbitrary, but the NOESY data make it clear that the relative stereochemical configurations shown for the ring carbons in each structure are correct. Integration data show that **11** makes up ca. 55% of the mixture of **11** and **12** isolated from a large scale product

(9) (a) Novak, M.; Kahley, M. J.; Eiger, E.; Helmick, J. S.; Peters, H. E. *J. Am. Chem. Soc*. **1993**, *115*, 9453−9460. (b) Novak, M.; Kahley, M. J.; Lin, J.; Kennedy, S. A.; Swanegan, L. A. *J. Am. Chem. Soc*. **1994**, *116*, 11626−11627.

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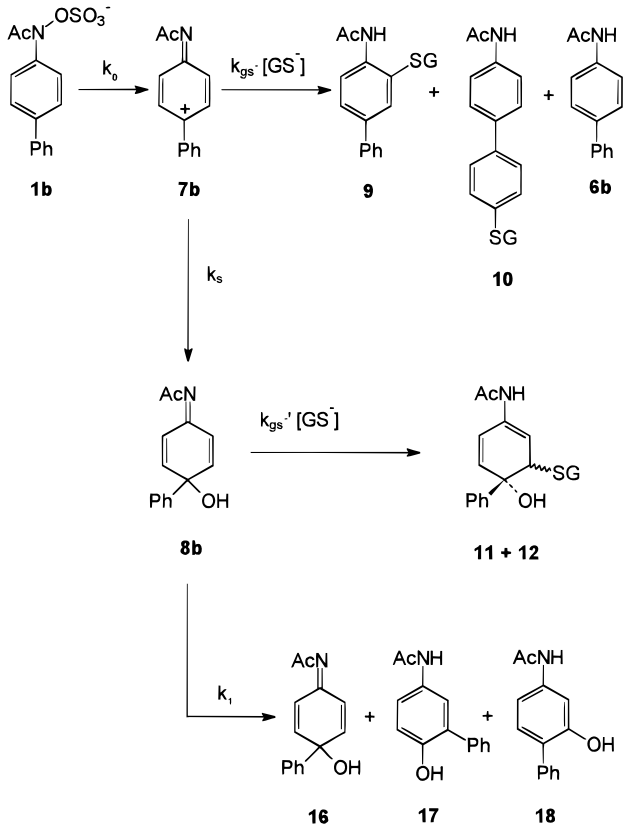
Scheme 2



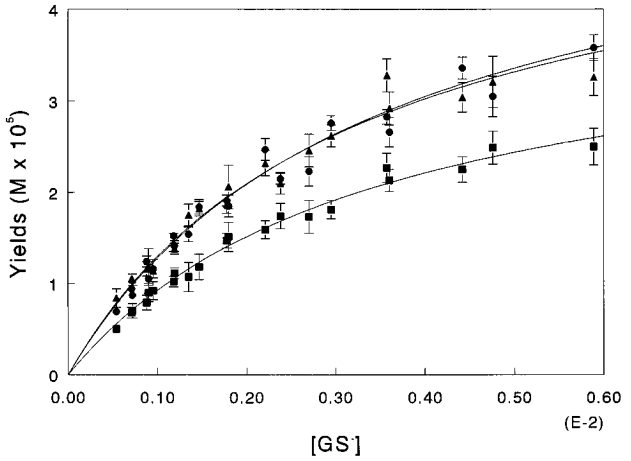


Figure 1. Yields of **6b** (■), **9** (▲), and **10** (●) vs [GS⁻]. Data were taken at pH 8.23, 8.39, and 8.52. Theoretical lines were calculated from eq 4 and k_{gs^-}/k_s data given in the text.

study. Under these conditions the normal hydrolysis products of **1b** (**16**−**18**, Scheme 2)[9a] could not be detected. Product yields were determined from HPLC peak area data. These are summarized in the supporting information for this paper. In determining the yields of **11** and **12** it was assumed that the **11**/**12** product ratio remains constant throughout the buffer concentration and pH range of the study. These compounds coelute under the HPLC conditions used for the quantitative analysis of the reaction mixture. The extinction coefficient of the mixture was obtained from a sample isolated from a large scale product study performed in 3 mM GSH buffer at pH 8.5.

Three of the five products (**6b**, **9**, **10**) increase in concentration with increasing $[GSH]_T$. Figure 1 shows that, within the error limits of the data, the yield of each product depends on [GS⁻] with no apparent differences among data taken at different pH. A similar plot (not shown) of yields vs [GSH] shows three sets

Table 1. Hydrolysis Rate Constants for **1b** and **19** in GSH Buffers at pH 8.52[a]

$[GSH]_T$, mM	$[GS^-]$, mM[b]	$10^4 k_{obs}$		% trapping of the nitrenium ion[f]	
		19	**1b**	**7a**	**7b**
0	0	2.4 ± 0.2[c]	4.0 ± 0.5[c]		
10.0	2.95	2.8 ± 0.2[c]	4.6 ± 0.2[d]	96	47
			4.2 ± 0.1[e]		
20.0	5.89		4.0 ± 0.2[d]		64
			4.0 ± 0.2[e]		

[a] Conditions: 5 vol % CH_3CN-H_2O, μ = 0.5, T = 20 °C. [b] Calculated from $[GSH]_T$, [NaOH] added, and the pK_a data of Scheme 1. [c] Determined by monitoring the disappearance of **1b** or **19** by HPLC. [d] Determined by monitoring the appearance of **10** by HPLC. [e] Determined by monitoring the appearance of **6b** by HPLC. [f] Calculated from the observed k_{gs^-}/k_s and calculated [GS⁻].

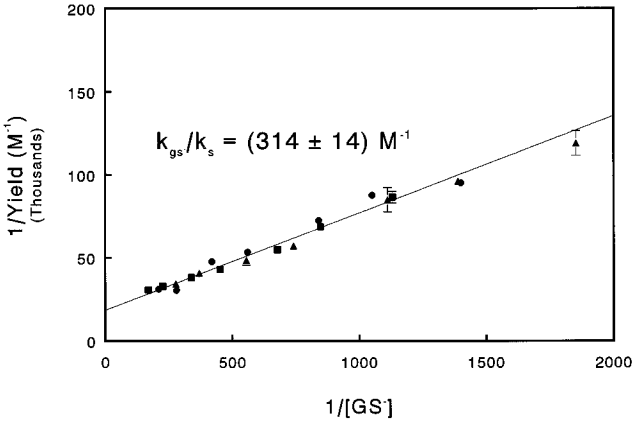


Figure 2. 1/Yield for **9** vs 1/[GS⁻]. Data were taken at pH 8.23 (▲), 8.39 (●), and 8.52 (■). The theoretical line is calculated from a weighted linear least-squares fit to eq 5.

of curves (one for each pH) for each product. The yields of these three products appear to depend only on [GS⁻].

Although product yields definitely depend on $[GSH]_T$, rate constants measured by HPLC methods are independent of $[GSH]_T$. Table 1 shows that even at the highest $[GSH]_T$ and [GS⁻] used in this study, the rate constants for appearance of **6b** and **10** are identical to the rate constants measured for the disappearance of **1b** in the absence of GSH.

If **6b**, **9**, and **10** are formed by competitive trapping of GS⁻ and solvent on the nitrenium ion **7b** formed by rate limiting N−O bond heterolysis ($k_{obs} = k_o$), as in Scheme 2, the yield of each of these products should be governed by eq 4 where $[A]_{max}$ is the maximum yield of a given product obtained at high [GS⁻].

$$[A] = \frac{(k_{gs^-}/k_s)[GS^-][A]_{max}}{1 + (k_{gs^-}/k_s)[GS^-]} \qquad (4)$$

Rearrangement of eq 4 gives eq 5. Figure 2 shows that there is

$$\frac{1}{[A]} = \frac{1}{[A]_{max}} + \frac{1}{[A]_{max}(k_{gs^-}/k_s)[GS^-]} \qquad (5)$$

a linear relationship for 1/[**9**] vs 1/[GS⁻]. The calculated value of k_{gs^-}/k_s is 314 ± 14 M^{-1}. Similar plots for 1/[**6b**] and 1/[**10**] give k_{gs^-}/k_s of 293 ± 10 M^{-1} and 290 ± 14 M^{-1}, respectively. The nearly identical rate constant ratios determined for all three products indicate that they are derived from a common intermediate. The average value of k_{gs^-}/k_s determined from these data is 300 ± 15 M^{-1}. The predicted maximum yields of the trapping products at high [GS⁻] are 27% for **6b**, 36% for **9**, and 37% for **10**. The predicted proportion of products

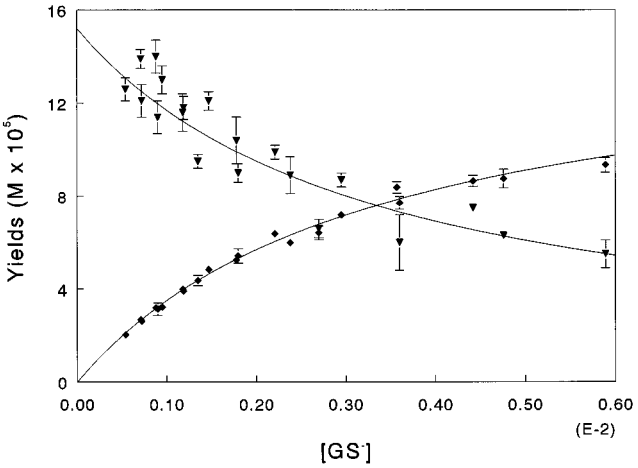


Figure 3. Combined yields of **6b**, **9**, and **10** (◆) and **11** and **12** (▼) vs [GS$^-$]. Theoretical lines were calculated from eq 4 or footnote 12, using the average k_{gs^-}/k_s of 300 M^{-1}, and $[A]_{max} = 1.5 \times 10^{-4}$ M.

obtained by GS$^-$ trapping of **7b** at 5.89 mM GS$^-$ (the highest concentration of GS$^-$ used here) is 64%. At this concentration **6b**, **9**, and **10** account for 63 ± 4% of the observed reaction products.

According to Scheme 2 the products **11** and **12** are formed by diverting **8b** from its normal path to **16**, **17**, and **18** by attack of GS$^-$. Rapid reaction of GS$^-$ with quinone imines and other electron deficient alkenes is well known.[3−5] Figure 3 shows that the yield of **11** and **12** vary with GS$^-$ in the expected manner if they are formed as trapping products of the initial solvent-derived product **8b**.[12] Since even at the lowest [GS$^-$] of 0.54 mM we cannot detect **16**, **17** or **18**, a lower limit of ca. 5×10^4 M^{-1} can be estimated for k_{gs^-}/k_1.[13] Since k_1 has a pH-independent value of 8.2×10^{-5} s^{-1} under these pH conditions,[14] it appears that $k_{gs^-} \geq 4.0$ M^{-1} s^{-1}. This estimate assumes that only GS$^-$ is reactive with **8b**. This is a reasonable assumption,[3,4,5,15] but, unlike the trapping of **7b**, our data do not require that this trapping reaction involves only GS$^-$.

The products of the reaction of **1a** with GSH have been identified previously,[6,7] but the dependence of product yields on $[GSH]_T$ has not been reported. The trapping of **1a** by GSH is significantly more efficient than the corresponding reaction of **1b**. It was necessary to reduce GSH concentration by ca. 1 order of magnitude to observe the dependence of product yields on [GS$^-$]. The decomposition of **1a** occurs too rapidly to allow for convenient monitoring of reaction kinetics, but the pivalic acid ester **19** yields the same GSH adducts under these conditions and decomposes at a significantly slower rate. The data in Table 1 show that the rate constant for the disappearance of **19** is unaffected by GSH under conditions in which >95% trapping of **7a** by GS$^-$ occurs. Figure 4 shows that **2**, **3**, **5**, and **6a** are products of initial trapping of the nitrenium ion. Calculated k_{gs^-}/k_s are 8300 ± 700 M^{-1} for **2**, 8600 ± 800 M^{-1} for **3**, 7400 ± 500 M^{-1} for **5**, and 8600 ± 800 M^{-1} for **6a**. The average value is 8200 ± 600 M^{-1}. The predicted maximum yields of these products at high [GS$^-$] are 35% for **2**, 25% for **3**, 21% for **5**, and 19% for **6a**.

(12) If the formation of **8a** and **8b** is irreversible, and if GS$^-$ quantitatively traps these intermediates, the yield of GS$^-$ trapping products of **8** is equivalent to the yield of **8**, where [I] = initial concentration of the reactant.

$$[\mathbf{8}] = \frac{[I]}{1 + k_{gs^-}/k_s[GS^-]}$$

(13) Extinction coefficients for **16**−**18** indicate that we could have detected a 4% overall yield of these materials equally distributed among the three of them.

(14) Novak, M.; Helmick, J. S.; Oberlies, N.; Rangappa, K. S.; Clark, W. M.; Swenton, J. S. *J. Org. Chem.* **1993**, *58*, 867−878.

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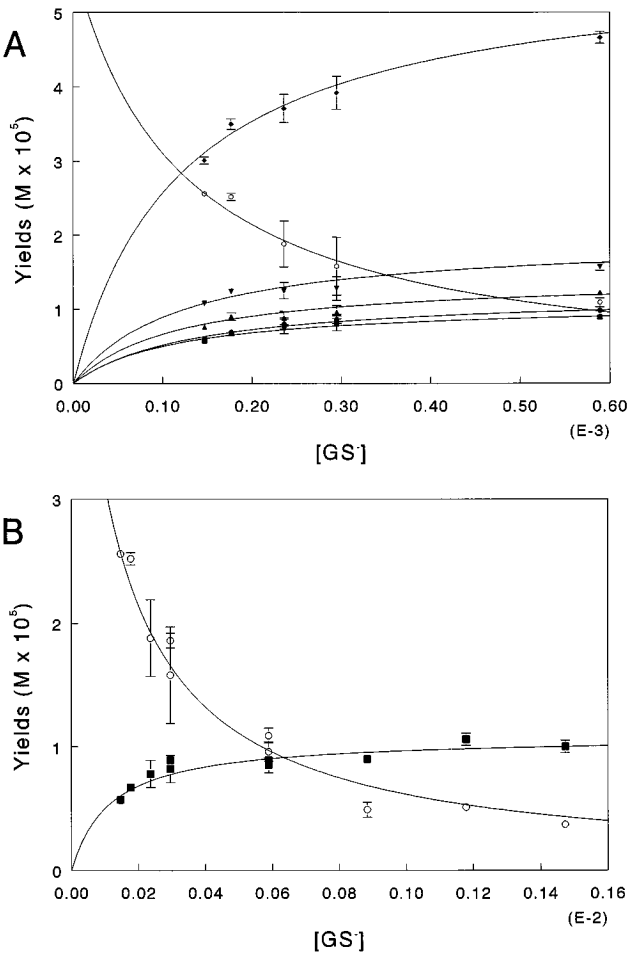
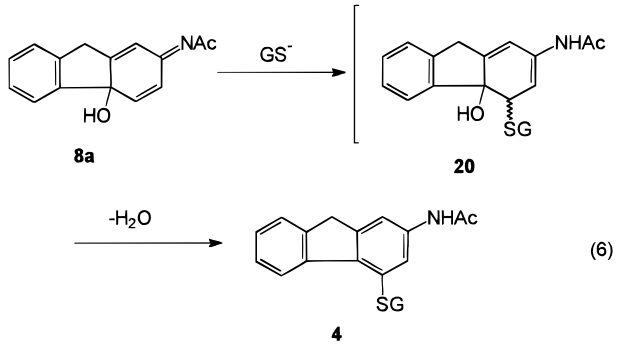


Figure 4. (A) Yields of **2** (▼), **3** (▲), **4** (○), **5** (●), **6a** (■) and the combined yields of **2**, **3**, **5**, and **6a** (◆) vs [GS$^-$] at pH 8.52. Theoretical lines were calculated from eq 4 or footnote 12 and k_{gs^-}/k_s data given in the text. For **4** and the combined yields of **2**, **3**, **5**, and **6a**, the average k_{gs^-}/k_s of 8200 M^{-1} and $[A]_{max} = 5.7 \times 10^{-5}$ M were used. (B) Yields of **4** (○) and **6a** (■) taken over a wider concentration range of [GS$^-$] at pH 8.52. The theoretical lines are identical to those in Figure 4A.

One product (**4**) does not follow the pattern of the others. Figure 4 shows that **4** follows the same pattern observed for **11** and **12**. Its yield is predicted quite well if it is assumed to be produced by GS$^-$ trapping of **8a**.[12] Products similar to **11** and **12** (**20**) were not observed but are likely intermediates on the pathway that produces **4** (eq 6). There may be greater driving



(6)

force for aromatization in **20** provided by ring strain in **20** and the required planarity of the two aromatic rings in **4**.[16] Since the normal hydrolysis product, **21**, observed under these pH

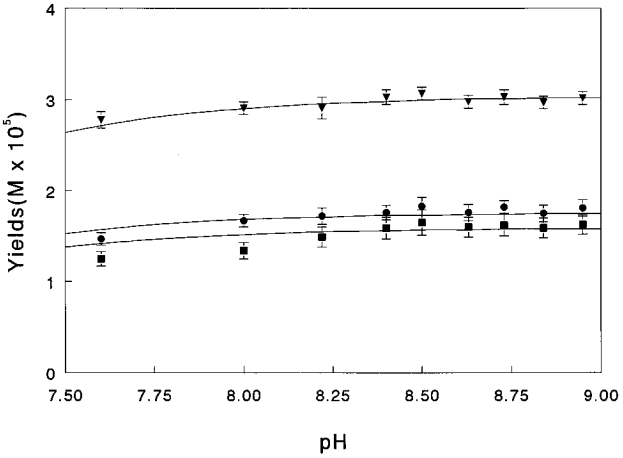
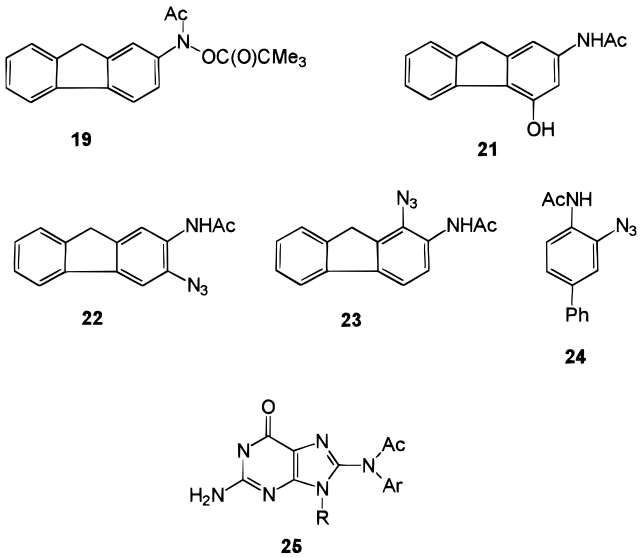


Figure 5. Yields of **2** (▼), **5** (●), and **6a** (■) vs pH in 20 mM $[GSH]_T$ buffers. Theoretical lines were calculated from the average k_{gs^-}/k_s of 8200 M^{-1} and $[GS^-]$ calculated at each pH from the data of Scheme 1.

conditions[17] could not be detected, it is apparent that GS^- trapping of **8a** is very efficient.



Product yields for **2**, **5**, and **6a** were determined as a function of pH in 0.02 M $[GSH]_T$ buffers over the pH range 7.5 to 9.0. Under the conditions used for this analysis **3** and **4** coeluted so they were not monitored. Figure 5 shows that the product yields are generally in good agreement with those predicted from k_{gs^-}/k_s and calculated $[GS^-]$. There are some deviations from the expected results in the more acidic buffers.

GS^- consists of the two ionization states $^+H_3NGS^-$ and H_2NGS^- (Scheme 1). The relative amounts of the two forms change with pH. At pH 7.7 the pK_a data of Scheme 1 predict that H_2NGS^- is ca. 3% of GS^-, while at pH 8.9 H_2NGS^- is calculated to make up ca. 36% of GS^-. Since the proportion of the two ionization states changes continuously throughout the pH range of this study, it is remarkable that the data are in as good agreement with calculated trends as they are. Apparently the reactivity patterns of the two ionization states making up GS^- are not drastically different. No attempt was made to fit our data to a two-state model for GS^- because of the relatively small deviations observed from predicted results calculated assuming a single state.

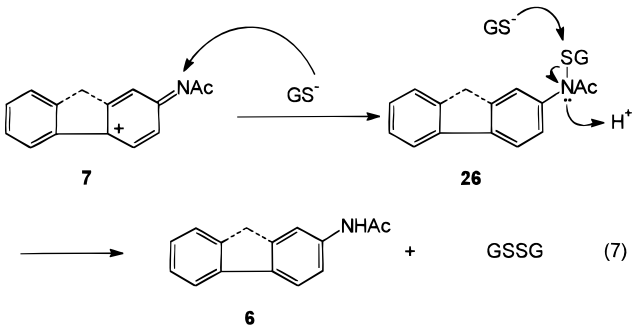
Table 2. Observed and Calculated Rate Constants for Reactions of **7a** and **7b** with Nucleophiles

	ion	
rate constant	**7a**	**7b**
k_s[a,b]	7.7×10^4 s^{-1}	5.9×10^6 s^{-1}
$k_{N_3^-}$[a,b]	4.2×10^9 M^{-1} s^{-1}	5.1×10^9 M^{-1} s^{-1}
	(4.8×10^9 M^{-1} s^{-1})[c]	(6.1×10^9 M^{-1} s^{-1})[c]
$k_{d\text{-}G}$[a,c]	6.2×10^8 M^{-1} s^{-1}	1.7×10^9 M^{-1} s^{-1}
k_{gs^-}[a,c]	6.3×10^8 M^{-1} s^{-1}	1.8×10^9 M^{-1} s^{-1}

[a] Conditions: 5% CH_3CN-H_2O, $\mu = 0.5$, T = 20 °C. [b] Directly measured from **7a** or **7b** generated in laser flash photolysis experiments. See ref 10. [c] Calculated from rate constant ratios determined from product studies and directly measured k_s. See refs 8 or 9, or this work.

Comparison with Other Nucleophiles. Since k_s is known for both **7a** and **7b** under these same reaction conditions from data obtained in laser flash photolysis experiments,[10] it is possible to calculate k_{gs^-} from the experimental rate constant ratios. Table 2 shows a comparison of directly observed or calculated second-order rate constants for reaction of several nucleophiles with **7a** and **7b** under a common set of conditions. Azide ion reacts with both of these nitrenium ions at or very near the diffusion controlled rate.[10] The rate constants for reaction of **7a** and **7b** with GS^- are within 1 order of magnitude of the approximate diffusion controlled limit of 5.0×10^9 M^{-1} s^{-1} and are very similar to the rate constants for reaction of the same two ions with d-G.[8a]

These nucleophiles have very different site selectivity for attack on **7a**, **7b**, and related nitrenium ions. Azide adducts obtained from these two cations are **22**−**24**.[9,10] Attack of N_3^- can also occur at the para-position of the ring proximal to N,[18] but in these two cases such attack does not lead to stable products. Reaction with d-G results in the C-8 adduct **25**.[8] This may not be the initial reaction product. There is evidence that N-7 of d-G initially attacks N of the nitrenium ion to yield a product which undergoes intramolecular rearrangement to produce **25**.[8b,19] GS^- attacks not only the rings of **7a** and **7b** proximal to N to yield **2**, **3** and **9**, but also the distal rings to yield **5** and **10**. The reduction products **6a** and **6b** are thought to arise from the reaction of eq 7.[6a] The intermediate **26** has



not been observed, but similar products are formed during the reaction of d-G and $PhNH_2$ with **7a** and **7b**.[8,19,20] In those cases the reaction products are stable to reduction under the conditions in which they are produced. Some of the differences in site

(16) Although **11** and **12** are stable under neutral or mildly alkaline pH conditions, they do undergo rapid dehydration at pH 2 (Novak, M.; Lin, J., work in progress). The greater driving force for aromatization of **20** may reduce the activation barrier so that dehydration of **20** occurs under the reaction conditions.

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selectivity exhibited here may be rationalized by considering the relative hardness of the attacking nucleophiles. The relatively hard N_3^- attacks the cations at the hard electrophilic sites of highest charge density.[21,22] The softer GS^- attacks these same electrophilic sites, but also attacks softer sites in the distal rings and, apparently, at N. $PhNH_2$ shows behavior similar to that of GS^- except that no attack on the distal rings is observed.[20] $PhNH_2$ also behaves as both a carbon and nitrogen nucleophile.[20] The reaction of d-G appears to be somewhat anamolous because only one site (N) is attacked on **7a** and **7b**. Further studies underway involving the d-G reaction may shed some light on this unusual site selectivity.[8b]

Implications with Respect to Carcinogenesis. Although the reaction of GS^- with **7a** and **7b** is quite rapid, under physiological conditions [GS^-] is too low for effective trapping of these nitrenium ions. GSH concentrations are typically highest in liver tissue where they reach about 5−10 mM.[2,4] The liver is also one of the main target organs of carcinogens such as **1a** and **1b**.[23] Most of the GSH is not present as GS^- under physiological conditions. GSSG concentrations are usually quite low (<1% of $[GSH]_T$) so this oxidized form does not significantly deplete GSH levels.[2] Nevertheless, because of the low acidity of the thiol in GSH, under physiological pH conditions only ca. 1% of GSH_T is present as GS^-. [GS^-] of ca. 50−100 μM will trap only 30−45% of **7a** and only 2−3% of **7b** in an aqueous solution in the absence of other nucleophiles.[24] Since $[GSH]_T$ and [GS^-] are considerably lower in other organs,[2,4] trapping efficiencies will be even lower in those organs. It is clear that GSH cannot provide complete protection against these nitrenium ions *in vivo*. Available data in the literature is in agreement with this conclusion. The binding of **1a** to RNA (2.7 mg/mL) is only reduced by ca. 25% at pH 7.4 when 10 mM GSH is added to the reaction mixture.[6a] Treatment with L-buthionine sulfoximine reduces $[GSH]_T$ from an average of 8.2 mM to 0.6 mM in adult rat heptaocytes, but causes only about a two-fold increase in binding to cellular macromolecules in cells incubated with **6a** which is a precursor to the carcinogen **1a**.[25]

Although the reaction of GS^- with **7a** is very rapid (Table 2), the fact that this reaction occurs at a rate somewhat below the diffusion limit is critical to the inability of GS^- to trap this ion efficiently *in vivo*. If k_{gs^-} for **7a** was at the diffusion limit of ca. 5×10^9 M^{-1} s^{-1}, under physiological conditions prevalent in the liver GS^- would trap ca. 75−85% of **7a** in the absence of other nucleophiles. The effect for **7b** would be considerably less dramatic. If **7b** were trapped by GS^- at the diffusion limit only about 4−8% of the ion would be trapped by GS^- under physiological conditions prevalent in the liver. The significantly lower efficiency of GS^- trapping of **7b** under both actual and diffusion controlled rates is due to its rapid reaction with water (Table 2). No nucleophile can trap **7b** efficiently at concentrations below 1 mM. The inability of GS^- to trap **7b** is less critical than its inability to trap **7a** because under physiological conditions the great majority of **7b** will be trapped by water. That is not the case for **7a**. Because of its relatively slow reaction with water (Table 2), **7a** will efficiently react with nucleophiles present in the 100 μM to 1 mM range if the reaction occurs near the diffusion limited rate.

Because of low concentrations and/or low trapping efficiencies of cellular nucleophiles, a substantial amount of the nitrenium ions **7a** and **7b** generated *in vivo* will be trapped by water to produce **8a** and **8b**. Although it has been known for some time that these types of products are formed during hydrolysis of this class of carcinogens,[9,26] no physiological role for these materials has been proposed or discovered. The results of this study show that GSH is more efficient at trapping **8a** and **8b** than trapping **7a** and **7b**. Since our previous studies have not shown extremely high reactivity of **8a**, **8b**, or related intermediates with other common nucleophiles,[9,14,26] it appears likely that their major fate *in vivo* will be reaction with GSH. Sufficiently high doses of precursors to **8a** and **8b** or related quinol imines could seriously compromise cells by depleting cellular GSH levels. We are unaware of any attempt to verify this experimentally. If GSH depletion can be demonstrated in cell cultures a potentially important physiological role for compounds such as **8a** and **8b** will have to be considered.

Experimental Section

General Procedures. The syntheses of **1a** and **1b** as their K^+ salts have been previously described.[14,27] The pivalic acid ester **19** was synthesized by methods described earlier for similar compounds.[28] General methods for preparation of solutions and analysis of reaction mixtures by HPLC methods have been described.[29] Reverse phase (C_{18}) silica gel 100 was purchased from Fluka, and DEAE Sephadex A-25 was obtained from Pharmacia Biotech.

All GSH solutions were prepared fresh daily. A 0.1 M GSH solution was prepared in N_2-saturated deionized, doubly distilled H_2O. This solution was used to prepare 0.02 M GSH buffers in N_2-saturated solvents using standardized NaOH solutions, reagent grade $NaClO_4 \cdot H_2O$, and purified CH_3CN.[28,29] Dilutions of the original GSH buffers were prepared by dilution with N_2-saturated 0.5 M $NaClO_4$ in 5 vol % CH_3CN-H_2O.

Isolation of Products. Solutions of **1a** and **1b** (0.036 M) were prepared in 5 mL of dry DMF. These solutions were added in 1 mL aliquots at 2 min (**1a**) or 30 min (**1b**) intervals to 200 mL of GSH buffer stirred at 20 °C under a N_2 atmosphere. For **1a**, the reaction was performed in GSH buffers at pH 8.5. For **1b** the products **6b**, **9**, and **10** were isolated from a 50 mM GSH_T buffer at pH 9.0, and the mixture of **11** and **12** was isolated from a 3 mM GSH_T buffer at pH 8.5. Products were isolated using an adaptation of a method described in the literature.[7] After 10 half-lives for the disappearance of **1a** or **1b,** the reaction mixtures were extracted (2×150 mL) with Et_2O. The Et_2O extracts contained **6a** or **6b**, which, after isolation, were identified by comparison with authentic samples.

The isolation and purification of **2-5** was carried out as previously described,[7] except that the final purification was performed on a 1 cm $\times$ 20 cm reverse phase (C_{18}) silica gel 100 column eluted with 1/1 $MeOH/H_2O$ containing 0.02 M AcOH. These products were identified by comparison with previously published NMR data.[7]

For isolation of **9-12**, the aqueous solution which remained after extraction with Et_2O was extracted (8×150 mL) with freshly distilled

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water saturated 1-butanol. The 1-butanol extracts were combined and evaporated on a rotary evaporator at 40 °C. The residue which remained after evaporation was dissolved in a minimum volume of 0.2 M aqueous ammonium formate (pH 7.4). This solution was applied to a 1.5 cm × 25 cm DEAE sephadex A-25 column which had been equilibrated with 0.2 M aqueous ammonium formate. The column was eluted with a stepwise gradient of 0.2 M (50 mL), 0.6 M (350 mL), and 1.0 M (100 mL) aqueous ammonium formate (pH 7.4). Fractions (5 mL) were collected and monitored by UV absorbance at 254 nm. Two main peaks (150−225 mL and 250−350 mL) were pooled. These solutions were extracted with 1-butanol as described above, and the residues which remained after evaporation were dissolved in a minimum volume of 1/1 MeOH/H_2O containing 0.02 M AcOH. These two solutions were separately eluted through a 1 cm × 20 cm reverse phase (C_{18}) silica gel 100 column with the same solvent. In each case one major product was observed by UV analysis of fractions. Fractions containing these products were pooled and lyophilized. The two products **9** (from the 250−350 mL fractions) and **10** (from the 150−225 mL fraction) were identified by analysis of spectral data and (for **9**) comparison of the alkaline cleavage product with an authentic sample.

4-(*N*-Acetylamino)-3-(glutathion-*S*-yl)biphenyl (9): ^{1}H NMR (300 MHz, D_2O) δ 1.94−2.01 (2H, m), 2.22 (3H, s), 2.28−2.44 (2H, m), 3.26 (1H, dd, J = 7.9, 14.6 Hz), 3.43 (1H, dd, J = 4.9, 14.6 Hz), 3.50−3.58 (2H, m), 3.61−3.65 (1H, m), 4.46 (1H, dd, J = 4.9, 7.8 Hz), 7.41−7.46 (1H, m), 7.49−7.54 (3H, m), 7.59 (1H, dd, J = 2.0, 8.3 Hz), 7.66−7.69 (2H, m), 7.79 (1H, d, J = 2.0 Hz); ^{13}C NMR (75.5 MHz, D_2O) δ 25.1 (CH_3), 28.8 (CH_2), 34.1 (CH_2), 45.9 (CH_2), 56.1 (CH), 56.8 (CH), 129.4 (CH), 129.6 (CH), 129.8 (CH), 130.8 (CH), 131.9 (CH), 132.3 (C), 133.5 (CH), 138.5 (C), 141.8 (C), 142.4 (C), 174.0 (C), 176.3 (C), 176.5 (C), 177.3 (C), 178.7 (C); high resolution MS (FAB) *m*/*e* 631.047, $C_{24}H_{26}N_4O_7SK_3^+$ requires 631.043.

4-(*N*-Acetylamino)-4′-(glutathion-*S*-yl)biphenyl (10): ^{1}H NMR (300 MHz, D_2O) δ 2.01−2.08 (2H, m), 2.16 (3H, s), 2.35−2.41 (2H, m) 3.32 (1H, dd, J = 8.2, 14.6 Hz) 3.48 (1H, dd, J = 4.7, 14.6 Hz), 3.50−3.57 (2H, m), 3.64−3.69 (1H, m) 4.55 (1H, dd, J = 4.8, 8.1 Hz) 7.48−7.54 (4 H, m), 7.61−7.68 (4H, m); ^{1}H NMR (300 MHz, DMSO-d_6) (aromatics only) δ 7.42 (2H, d, J = 8.4 Hz), 7.56 (2H, d, J = 8.5 Hz), 7.58 (2H, d, J = 8.9 Hz), 7.65 (2H, d, J = 8.8 Hz); ^{13}C NMR (75.5 MHz, D_2O) δ 25.7 (CH_3), 29.8 (CH_2), 34.2 (CH_2), 37.6 (CH_2), 40.0 (CH_2) 56.0 (CH) 57.1 (CH), 124.5 (CH), 129.9 (CH), 130.0 (CH), 133.7 (CH), 135.4 (C), 138.8 (C), 139.4 (C), 141.2 (C), 173.8 (C), 174.2 (C), 175.4 (C), 177.7 (C), 178.9 (C); high resolution MS (FAB) *m*/*e* 583.129, $C_{24}H_{26}N_4O_7SNa_3^+$ requires 583.121.

Bis[3-[4-(acetylamino)biphenylyl]] Disulfide (15). This material was obtained from the thiazthionium hydrate **14** which was synthesized, in turn, from 4-aminobiphenyl by a known procedure.[30] A 100 mg sample of **14** (0.47 mmol) was slurried with 1−2 mL of H_2O under N_2 at 0 °C while 160 μL of 10 M NaOH was added to the mixture. The mixture was stirred for about 1 h as it was allowed to reach room temperature. Then, a 152 μL (1.6 mmol) aliquot of Ac_2O was added. After a few minutes the reaction mixture was extracted with CH_2Cl_2 (3 × 5 mL). The extracts were combined, and the solvent was evaporated. The residue was applied to a 1 cm × 20 cm silica gel column and eluted with 1/9 EtOAc/CH_2Cl_2. The major band was collected and evaporated to yield **15**: mp 236−237 °C; 1R (KBr) 3200, 1664, 1522, 1378, 1296, 758, 696 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.05 (6H, s), 7.30−7.35 (2H, m), 7.37−7.42 (4H, m) 7.47−7.54 (6H, m), 7.58 (2H, dd, J = 2.0, 8.2 Hz) 7.82 (2H, d, J = 2.0 Hz), 9.77 (2H, s); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 23.1 (CH_3), 126.0 (CH), 126.4 (CH), 126.8 (CH), 127.7 (CH), 127.9 (CH), 129.0 (CH), 131.5 (C), 136.0 (C), 137.9 (C), 138.8 (C), 168.9 (C); high resolution MS *m*/*e* 484.129, $C_{28}H_{24}N_2O_2S_2$ requires 484.128.

Alkaline Cleavage of 9. A 10 mg sample of **9** was dissolved in 5 mL of 0.1 M NaOH. After stirring overnight the solution was neutralized with 0.1 M HCl, and the precipitate was collected by filtration. After purification by TLC on silica gel (2/3 EtOAc/CH_2Cl_2), the ^{1}H and ^{13}C NMR spectra of the cleavage product were found to be identical to that of authentic **15**.

The adducts **11** and **12** would not extract with 1-butanol. The aqueous solution containing these materials was lyophilized after extraction with Et_2O and 1-butanol. The residue was dissolved in a minimum volume of 1/1 MeOH/H_2O containing 0.02 M HOAc and subjected to reverse phase chromatography as described above. The two materials coeluted from the column and no effective method of separation was found. COSY and NOESY cross correlation experiments made it possible to assign most of the ^{1}H NMR peaks to one of the two isomers.

(*E*)-4-(Acetylamino)-2-(glutathion-*S*-yl)-1-phenyl-3,5-cyclohexadien-1-ol (11): ^{1}H NMR (300 MHz, D_2O) δ 2.04 (3H, s), 2.06−2.16 (2H, m), 2.39−2.51 (2H, m), 2.81 (1H, dd, J = 8.9, 14.2 Hz), 3.03 (1H, dd, J = 4.8, 14.2 Hz), 3.68−3.77 (3H, m) 4.06 (1H, d, J = 4.9 Hz), 4.36 (1H, dd, J = 4.8, 8.9 Hz), 5.95 (1H, d, J = 10.1 Hz), 5.99 (1H, m), 6.21 (1H, dd, J = 1.9, 10.0 Hz), 7.35−7.44 (3H, m), 7.57 (2H, t, J = 8.0 Hz).

(*Z*)-4-(Acetylamino)-2-(glutathion-*S*-yl)-1-phenyl-3,5-cyclohexadien-1-ol (12): ^{1}H NMR (300 MHz, D_2O) δ 2.04 (3H, s), 2.06−2.16 (2H, m), 2.39−2.51 (2H, m) 2.80 (1H, dd, J = 8.7, 14.1 Hz), 3.00 (1H, dd, J = 5.3, 14.1 Hz), 3.68−3.77 (3H, m), 4.09 (1H, d, J = 5.3 Hz), 4.52 (1H, dd, J = 5.3, 8.7 Hz), 5.96 (1H, d, J = 10.0 Hz), 5.98 (1H, m), 6.19 (1H, dd, J = 1.9, 10.0 Hz), 7.35−7.44 (3H, m), 7.57 (2H, t, J = 8.0 Hz).

All 44 expected ^{13}C NMR peaks were located: ^{13}C NMR (75.5 MHz, D_2O) δ 25.46 (CH_3), 25.51 (CH_3), 28.97 (CH_2), 29.02 (CH_2), 34.11 (CH_2), 34.17 (CH_2), 34.98 (CH_2), 35.26 (CH_2), 46.08 (CH_2), 46.08 (CH_2), 55.42 (CH), 55.46 (CH), 56.13 (CH), 56.24 (CH), 56.85 (CH), 56.85 (CH), 76.93, (C), 77.22, (C), 115.17 (CH), 115.77 (CH), 126.99 (CH), 127.04 (CH), 128.47 (CH), 128.49 (CH), 130.84 (CH), 130.96 (CH), 131.15 (CH), 131.23 (CH), 134.53 (C), 134.85 (C), 136.31 (CH), 136.58 (CH), 146.13 (C), 146.83 (C), 174.43 (C), 174.56 (C), 174.60 (C), 175.94 (C), 175.98 (C), 176.73 (C), 177.42 (C), 177.54 (C), 177.93 (C), 178.88 (C); high resolution MS (FAB) *m*/*e* 601.141, $C_{24}H_{28}N_4O_8SNa_3^+$ requires 601.132.

Kinetic and Product Studies. The decomposition of **1b** and **19** or the formation of GSH adduct **10** and reduction product **6b** were monitored by HPLC on a μ-Bondapak C-18 column with UV absorbance monitored at 280 nm. The eluent was 7/3 or 8/2 MeOH/H_2O containing 0.02 M 1/1 HOAc/NaOAc. In each case 25 μL of a 0.02 M DMF solution of **1b** or **19** was injected into 5 mL of the GSH buffer (10 mM or 20 mM GSH_T, pH 8.52) or Tris buffer (pH 8.5) which had been incubating at 20 °C for at least 15 min. Aliquots of 20 μL were withdrawn periodically for HPLC analysis. Peak area vs time data were fit to the first-order rate equation by nonlinear least squares procedures.

For product analyses, initial concentrations of **1a** of ca. 5.7×10^{-5} M and **1b** of ca. 1.5×10^{-4} M were obtained in GSH buffers by injection of 25 μL of an appropriate stock solution of **1a** or **1b** in DMF into 5 mL of the buffer. All reactions were allowed to go to completion at 20 °C before analyses were performed by HPLC (triplicate 20 μL injections) on a μ-Bondapak C_{18} column or a Beckman C-8 column. MeOH/H_2O containing 0.02 M HOAc was used at the eluent and UV absorbance was monitored at 280 nm. Products were identified by HPLC comparison to isolated samples. Yields were determined from average peak areas and extinction coefficients obtained from the isolated samples.

Acknowledgment. This work was supported by a grant from the American Cancer Society (CN-23K). The 300 MHz ^{1}H and 75.5 MHz ^{13}C NMR spectra were obtained with equipment funded by NSF Grant No. CHE9012532.

Supporting Information Available: Reaction product yields for the decomposition of **1a** and **1b** in GSH buffers (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

JA9534563

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