A Study of the Reaction of Calicheamicin γ_1 with Glutathione in the Presence of Double-Stranded DNA

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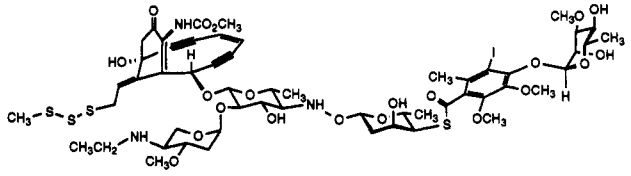
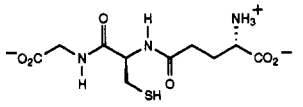
Abstract: The reaction of calicheamicin γ_1 (**1**) with glutathione (GSH) has been studied in the presence of double-stranded DNA and is shown to produce initially all four products arising from S–S bond exchange between the calicheamicin γ_1 trisulfide group and the thiol function of glutathione. The calicheamicin–glutathione disulfide **6** is formed as the major product of the reaction, while the dihydrothiophene derivative **3**, the thiosulfenic acid derivative **4**, and the calicheamicin–glutathione trisulfide **5** are formed as comparatively minor products. The product distribution is highly time dependent for products **4**–**6** undergo further transformation, ultimately producing **3**. The rates of reaction of products **4** and **5** with GSH are roughly comparable to that of **1**, while the calicheamicin–glutathione disulfide **6** forms **3** at a rate approximately 2 orders of magnitude slower than the rate of formation of **3** from **1**. The kinetics of cleavage of double-stranded DNA by **1** and GSH, determined by polyacrylamide gel electrophoretic (PAGE) analysis is found to parallel the kinetics of formation of **3** and is characterized by a two-stage process. Both stages of DNA cleavage proceed with identical sequence specificity. The rates of DNA cleavage by **1** and **6** respond in different fashion to variations in the concentration of DNA. The rate of DNA cleavage by **1** is essentially independent of the concentration of DNA, while the rate of DNA cleavage by **6** is inversely proportional to the concentration of DNA. The data support the hypothesis that **1** undergoes thiol activation as a DNA-bound species, while **6** is activated free in solution. These findings suggest that, under physiologically relevant conditions, the major DNA-damage pathway arising from the reaction of **1** and GSH involves the following sequence: **1** binds to double-stranded DNA; DNA-bound **1** reacts with GSH to form **6**, DNA-bound **6** dissociates and reacts with free GSH to form **A** and then **2**; the product(s) of the latter reaction (likely **2**) bind to DNA; DNA-bound **2** rearranges to the biradical **B**, which then abstracts hydrogen atoms from the ribose backbone of DNA. New and existing data pertaining to the potential role of DNA as a catalyst in the thiol activation reaction and to the potential participation of the carbohydrate amino group of **1** in that reaction is evaluated. It is determined that while there is evidence to support the hypothesis that the carbohydrate amino group of **1** participates in the thiol activation of **1** in organic solvents, no conclusions may be drawn at this time concerning its role in the corresponding reaction in water in the presence of DNA. Similarly, it is concluded that there is presently insufficient data to determine if DNA functions as a formal catalyst for the thiol activation of **1** in water.

The potent natural antitumor agent calicheamicin γ_1 (**1**) and the structurally related esperamicins have been shown to initiate the cleavage of duplex DNA upon incubation with thiols.[1] This activity was rationalized early on as the result of the generalized mechanism of Scheme 1, where thiol-induced cleavage of the allyl trisulfide group of **1** produces the thiol (or thiolate) **A**, which then cyclizes to form the dihydrothiophene derivative **2** and, subsequently, the biradical **B**.[2,3] Much evidence now supports this mechanism, to include the characterization of the products of quenching of the biradical **B** with hydrogen or deuterium (e.g., **3**, Chart 1)[2] and the observation at low temperature of the intermediate **2**.[4]

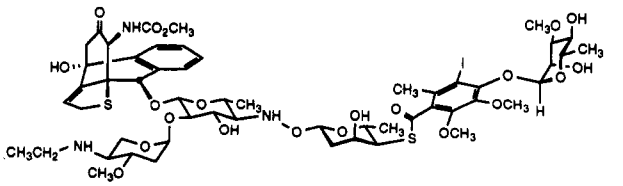
Chart 1



Calicheamicin γ_1 (1)



Glutathione (GSH)



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Evans and Saville have presented evidence showing that, in the base-catalyzed reaction of a thiol with a symmetrical trisulfide,

* Abstract published in *Advance ACS Abstracts,* January 1, 1994.

(1) Calicheamicin: (a) Zein, N.; Sinha, A. M.; McGahren, W. J.; Ellestad, G. A. *Science* **1988**, *240*, 1198. (b) Zein, N.; Poncin, M.; Nilakantan, R.; Ellestad, G. A. *Science* **1989**, *244*, 697. Esperamicin: (c) Long, B. H.; Golik, J.; Forenza, S.; Ward, B.; Rehfuss, R.; Dabrowiak, J. C.; Catino, J. J.; Musial, S. T.; Brookshire, K. W.; Doyle, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2. (d) Sugiura, Y.; Uesawa, Y.; Takahashi, Y.; Kuwahara, J.; Golik, J.; Doyle, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 7672.

(2) (a) Golik, J.; Clardy, J.; Dubay, G.; Groenewold, G.; Kawaguchi, H.; Konishi, M.; Krishnan, B.; Ohkuma, H.; Saitoh, K.; Doyle, T. *J. Am. Chem. Soc.* **1987**, *109*, 3461. (b) Golik, J.; Clardy, J.; Dubay, G.; Groenewold, G.; Kawaguchi, H.; Konishi, M.; Krishnan, B.; Ohkuma, H.; Saitoh, K.; Doyle, T. *J. Am. Chem. Soc.* **1987**, *109*, 3462. (c) Lee, M. D.; Dunne, T. S.; Siegel, M. M.; Chang, C. C.; Morton, G. O.; Borders, D. B. *J. Am. Chem. Soc.* **1987**, *109*, 3464. (d) Lee, M. D.; Dunne, T. S.; Chang, C. C.; Ellestad, G. A.; Siegel, M. M.; Morton, G. O.; McGahren, W. J.; Borders, D. B. *J. Am. Chem. Soc.* **1987**, *109*, 3466. (e) Lee, M. D.; Dunne, T. S.; Chang, C. C.; Siegel, M. M.; Morton, G. O.; Ellestad, G. A.; McGahren, W. J.; Borders, D. B. *J. Am. Chem. Soc.* **1992**, *114*, 985.

(3) Numbers are used to label species which have been observed directly, capital letters to indicate intermediates which have been proposed but not observed directly.

(4) De Voss, J. J.; Hangeland, J. J.; Townsend, C. A. *J. Am. Chem. Soc.* **1990**, *112*, 4554.

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Scheme 1

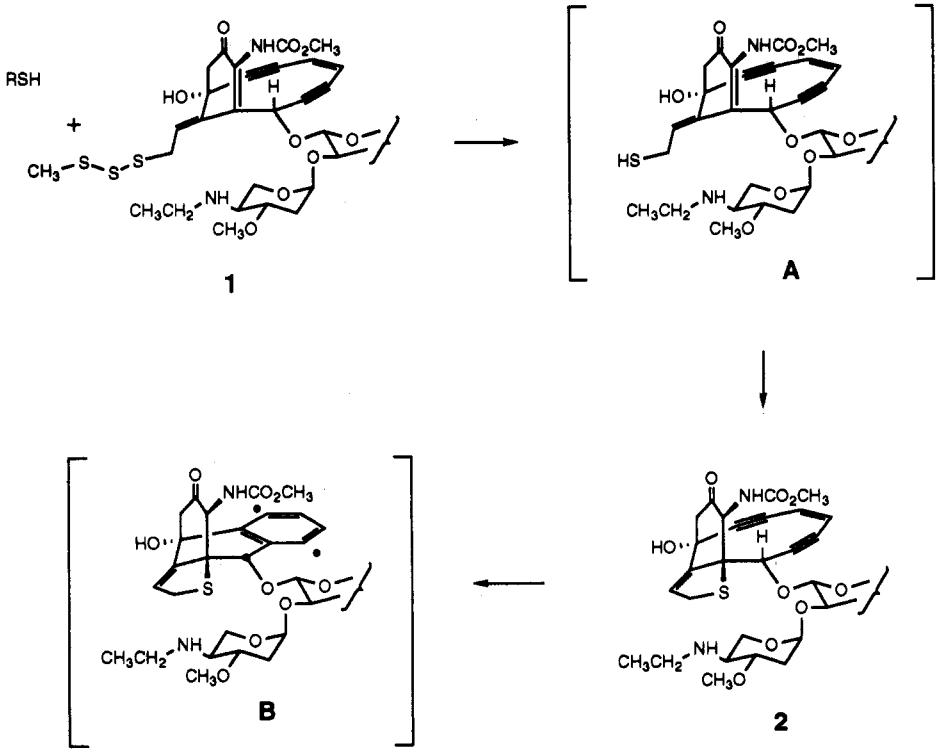
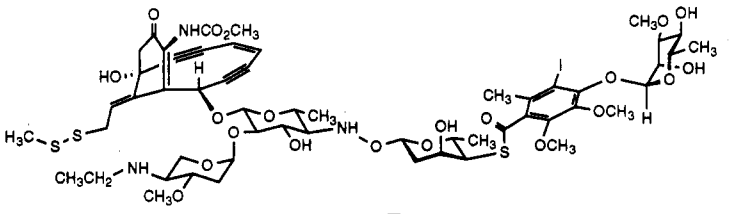
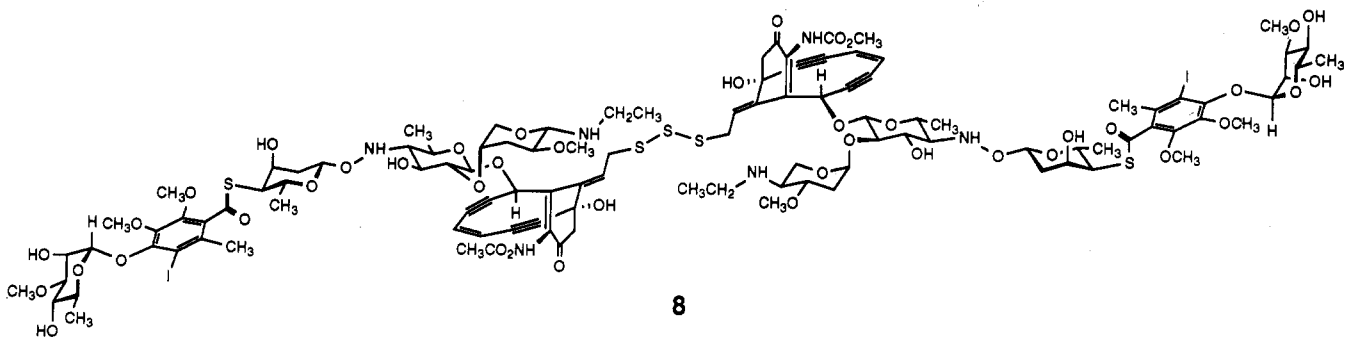


Chart 2





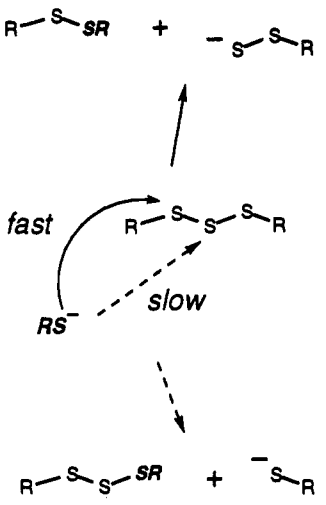
attack on the terminal sulfur atoms is kinetically preferred over attack on the central sulfur atom (Scheme 2).[5] This preference was attributed to a leaving-group effect, where the presumed greater stability of the thiosulfenate anion versus the thiolate anion directs the course of reaction. Nucleophilic attack of thiols upon the unsymmetrical allyl trisulfide functional group of **1** is potentially more complex; in theory, four discrete products can be formed by single-step processes involving S–S bond cleavage and concomitant S–S bond formation, as illustrated in Scheme 3 for the specific case of the reaction of **1** with glutathione (GSH). The direct products of this reaction (**A**, **4**–**6**, Scheme 1 and 3) are not expected to be stable under the reaction conditions but are anticipated to undergo further transformations of one or more steps to produce ultimately the dihydrothiophene derivative **3** via the common final sequence **A** → **2** → **B** → **3** (Scheme 4). The putative intermediate **A** has never been observed; available evidence suggests that its cyclization to **2** is quite rapid, even at low temperature.[4] De Voss, Hangeland, and Townsend have measured the rate of cyclization of **2** to **B** at −10 °C, from which data **2** may be estimated to have a half-life of ~20 s at 23 °C.[4] It is generally assumed that the quenching of biradicals such as **B** by hydrogen atom transfer is exceedingly rapid.[6]

Ellestad et al. have studied the reaction of **1** with methanethiol in organic solvents and have noted that the product distribution is solvent dependent. The reaction of **1** with methanethiol in methanol is reported to form the dihydrothiophene derivative **3**, while in acetonitrile the methyl disulfide derivative **7** (Chart 2) is produced. The methyl disulfide derivative **7** is reported to be much less reactive toward thiols than **1**, permitting its isolation

(5) Evans, M. B.; Saville, B. *Proc. Chem. Soc.* **1962**, 18.

(6) Lockhart, T. P.; Mallon, C. B.; Bergman, R. G. *J. Am. Chem. Soc.* **1980**, *102*, 5976.

Scheme 2



from the reaction medium. In addition to the product **7**, the dimeric calicheamicin trisulfide **8** (Chart 2) was reported to be formed in the early stages of reactions conducted in acetonitrile.[7] The formation of **8** almost certainly involves the thiosulfenic acid derivative **4** as an intermediate which, in turn, most likely arises by nucleophilic attack of methanethiolate on the methyl-terminal sulfur atom. In a study of the reaction of **1** with methyl thioglycolate in methanol containing triethylamine, Townsend and co-workers report that the initial stages of the reaction, as monitored by variable-temperature ^{1}H NMR spectroscopy, are complex, with signals for several methylthio-containing compounds visible.[4] Previous work clearly establishes a rich and potentially complex chemistry of **1** in its reactions with thiols in organic solvents. In conjunction with a parallel series of investigations of the natural antitumor agent neocarzinostatin, we have undertaken a study of the reaction of **1** with glutathione (GSH), the most abundant nonprotein thiol in eukaryotic cells and a putative cofactor in activation processes occurring in vivo.[8] In order to more closely approximate physiologically relevant conditions, our studies were conducted in water and in the presence of double-stranded calf thymus DNA. Our goal was to learn the detailed pathway(s) by which **1** and glutathione cleave DNA and, in particular, to probe the possibility that DNA may play a role in the activation process. It is shown that the reaction of **1** with GSH in the presence of DNA is indeed complex and involves, at a minimum, four distinct pathways of activation that proceed at different rates. These pathways are correlated with the observed kinetics of the cleavage of DNA by **1** and glutathione. A detailed investigation of the role of DNA in two of the primary activation processes is presented. It is shown that one of these likely proceeds as a ternary complex of drug, thiol, and DNA, while the other occurs in a simple bimolecular reaction of DNA-free drug and thiol. Finally, evidence concerning the potential role of DNA as a catalyst in the thiol activation reaction and regarding the participation of the carbohydrate amino group of **1** in that process is critically evaluated.

Reaction of Calicheamicin γ_1 with Glutathione

The reaction of calicheamicin γ_1 (**1**, 5.0×10^{-5} M) with glutathione (GSH, 1.0×10^{-3} M) was conducted in aqueous solution buffered to pH 7.5 (3.0×10^{-2} M Tris-HCl) at 23 °C in the presence of double-stranded calf thymus DNA (5.0×10^{-3} M base pairs, [base pairs]/[drug] = 100) and was monitored by reverse-phase high-performance liquid chromatography (rp-HPLC). These parameters define our standard reaction conditions and were employed throughout this work, unless otherwise specified. In the early stages of the reaction ($t \leq 15$ min) four product peaks are apparent, as well as unreacted calicheamicin γ_1 (**1**; see Figure 1). In the following paragraphs evidence is presented to support the hypothesis that these four products correspond to the four products arising from S–S bond exchange between the calicheamicin γ_1 trisulfide group and the thiol function of glutathione, as shown in Scheme 3. These products are identified as the dihydrothiophene derivative **3**, the thiosulfenic acid derivative **4**, the calicheamicin–glutathione trisulfide **5**, and the calicheamicin–glutathione disulfide **6**. While compounds **4–6** are likely direct products of the reaction of GSH with **1**, the dihydrothiophene derivative **3** presumably arises from the direct product **A** by the sequence **A** → **2** → **B** → **3**, as previously proposed.[2] As suggested in the introduction above, none of the products **4–6** are found to be stable to the reaction conditions; each is transformed, ultimately, to the product **3**, albeit by different pathways and at different rates, as discussed below.

Product Identification

Because experiments were conducted with microgram quantities of calicheamicin γ_1, standard spectroscopic (e.g., ^{1}H NMR) methods of product characterization were not possible. Nevertheless, product structures were assigned with confidence, on the basis of the following analysis. Of the four products, three provide UV absorption spectra that are virtually identical to that of **1**, while the fourth exhibits a substantially different UV spectrum (Figure 2). It is this fourth product to which all others converge; thus, on mechanistic grounds alone it must be assigned as the dihydrothiophene derivative **3** (see Scheme 4). This assignment was confirmed by comparison of the product with an authentic sample of **3**; the two compounds were found to be identical in terms of HPLC retention time (coinjection), UV absorption spectra, and FAB mass spectroscopy (nitrobenzyl alcohol matrix, calcd for $[M + H]^+$ 1292.1; found 1292).

The similarity of the UV absorption spectra of the three remaining products with that of **1** suggests a common chromophore and thus supports the assignment of these products as structures **4–6**. The most predominant (and most stable) of these was isolated by rp-HPLC and was analyzed by electrospray mass spectrometry after lyophilization. The mass of this product corresponded to that calculated for the glutathione–calicheamicin disulfide derivative **6** (calcd for $[M + H]^+$ 1595.6; found 1596). Consistent with the latter structural assignment, this substance was found to be substantially more polar (rp-HPLC) than **1** or **3** (see Figure 1). Also consistent with the structure **6**, it was found that resubjection of this product to the standard reaction conditions led to the clean formation of the dihydrothiophene derivative **3**, albeit ~2 orders of magnitude more slowly than the direct formation of **3** from **1** (vide infra). Product **6** was also found to cleave DNA in the presence of GSH, as described below in detail.

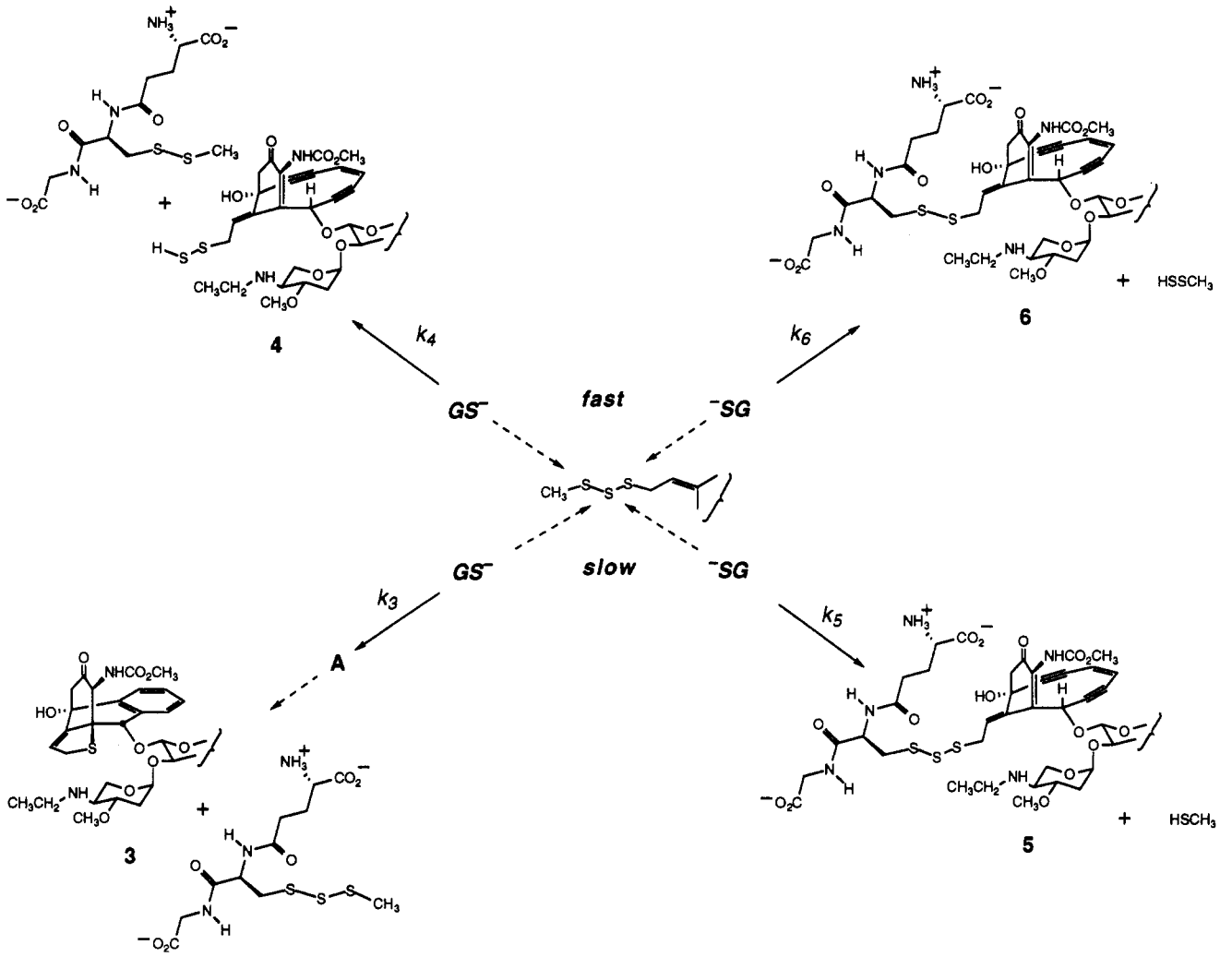
The remaining two products are considerably less stable to the reaction conditions than is **6**. Least prevalent of the four products is a compound barely resolvable by rp-HPLC from the glutathione–calicheamicin disulfide **6**. Given its polarity, and the apparent reactivity of this species (it appears only transiently within the first several minutes of the reaction) and, as mentioned, the near identity of its UV absorption spectrum with that of **1** and **6**, it is proposed that this product is the glutathione–calicheamicin trisulfide **5**. We were unsuccessful in attempts to isolate this substance for further confirmation of the assignment, which must therefore be regarded as tentative.

The remaining, unidentified component is perhaps most interesting. Careful monitoring of the early stages of the reaction shows that this component, only slightly more polar than calicheamicin γ_1 itself, is a significant product, secondary only

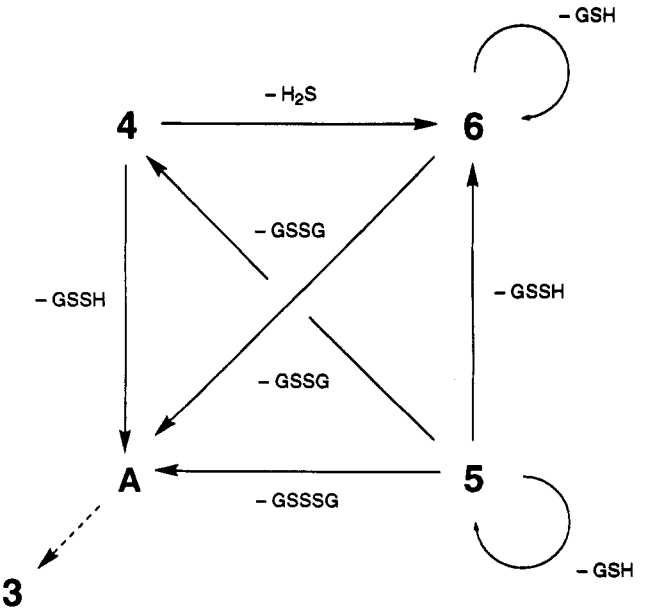
(7) Ellestad, G. A.; Hamann, P. R.; Zein, N.; Morton, G. O.; Sigel, M. M.; Pastel, M.; Borders, D. B.; McGahren, W. J. *Tetrahedron Lett.* **1989**, *30*, 3033.

(8) Meister, A.; Anderson, M. E. *Annu. Rev. Biochem.* **1983**, *52*, 711.

Scheme 3



Scheme 4



to the glutathione disulfide **6**. Like the trisulfide **5**, this product is found to be highly reactive, persists only within the first several minutes of the reaction, and affords UV absorption data very similar to that of **1** and **6**. HPLC fractions containing this product were frozen directly upon elution, without concentration (attempts to obtain mass spectral data on lyophilized samples were not successful); subsequent analysis of these fractions by electrospray mass spectrometry showed the product to be consistent in formulation with its assignment as the thiosulfenic acid derivative **4** (calcd for $[M + H]^+$ 1322.2; found 1324). The observed mass value lies within the error limits of the instrument employed, determined using related compounds for reference (e.g., for **1**, calcd for $[M + H]^+$ 1368.3; found 1369 and 1370 in two separate runs). In further confirmation of the assignment, resubjection of isolated **4** to the standard reaction conditions was found to lead to the rapid formation of the disulfide **6** and the dihydrothiophene derivative **3**. This product profile, the exclusive formation of **3** and **6** by separate reaction paths (the observed rate of appearance of **3** in the latter reaction is consistent only with its direct formation from **4** and not from the secondary transformation of **6** identified above; see below), uniquely identifies the starting material as **4**, as diagrammed within Scheme 4.[9] Like the product **6**, **4** is found to cleave double-stranded DNA in the presence of GSH, as described in detail below. The relative stability of product **4** as compared with **A**, the presumed precursor to **2** and **3**, is noteworthy. It is clear that internal Michael addition within **4** to form a cyclic disulfide derivative analogous to **2** is not a viable reaction pathway, perhaps due to repulsion of the adjacent sulfur lone pair orbitals in the hypothetical transition state for this cyclization.[10]

(9) As mentioned in the introduction, the thiosulfenic acid derivative **4** is practically an obligate intermediate in the formation of the dimeric calicheamicin trisulfate **8** and so may be regarded tentatively as precedented.[7] That we do not detect the dimeric trisulfide **8** observed by Ellestad et al. in our experiments may be attributable to any one of several differences in reaction conditions: a dilution factor (concentrations were not specified in the work of Ellestad et al.), the use of water as solvent rath er than acetonitrile, or the presence of DNA in our experiments.

(10) Houk, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1987**, *109*, 6825 and references therein.

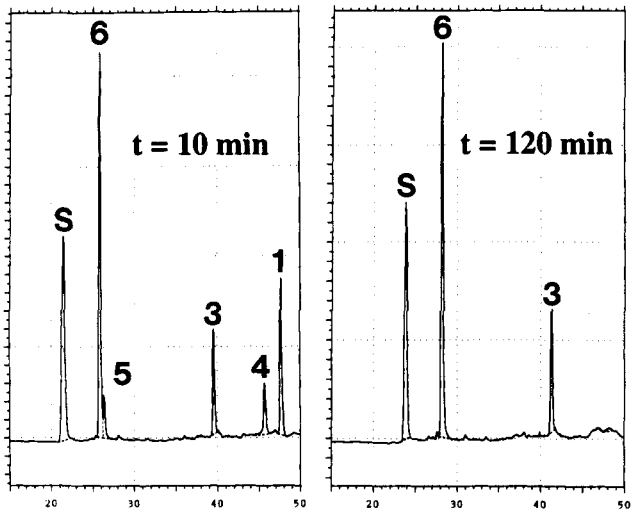


Figure 1. Reverse-phase HPLC traces of the reaction of **1** (5.0×10^{-5} M) with GSH (1.0×10^{-3} M) in the presence of DNA (5.0×10^{-3} M bp) at 10 and 120 min. The products are identified in their order of elution as **S**, 2,5-dimethoxybenzyl alcohol (internal HPLC standard); the calicheamicin–glutathione disulfide **6**; the calicheamicin–glutathione trisulfide **5**; the dihydrothiophene derivative **3**; the thiosulfenic acid derivative **4**; calicheamicin γ_1 (**1**).

Kinetics of Primary and Secondary Activation Processes with Glutathione

Despite the complexity of the reaction of **1** and GSH in the presence of DNA, the kinetics of disappearance of **1** follows simple pseudo-first-order behavior (rp-HPLC determination, $k_\psi = 7.9 \times 10^{-4}$ s^{-1}, 5.0×10^{-4} M GSH; $k_\psi = 1.8 \times 10^{-3}$ s^{-1}, 1.0×10^{-3} M GSH; second-order rate constants 1.6 and 1.8 M^{-1} s^{-1}, respectively, Figure 3). This observation is consistent with the mechanism of Scheme 3, where the partitoning of **1** among the various pathways of reaction with GSH is rate-determining; k_ψ then represents the sum of k_3, k_4, k_5, and k_6, as defined within Scheme 3. Products **4** and **5** grow and decay within the first several minutes of the reaction, partitioning between products **3** and **6** in one or more steps. Because of the complexity this brings to the kinetic analysis, accurate values of k_3, k_4, k_5, and k_6 have not been determined; however, on the basis of the rate of appearance of products **3**, **4**, **5**, and **6** at the onset of the reaction, these rate constants may be rank-ordered as follows: $k_6 > k_4 > k_3 > k_5$ (see Figure 4). The ratio k_6:k_5 (the fastest and slowest steps, respectively) is estimated to lie between 6 and 10.

As illustrated within Figures 1 and 4, the complexity of the early stages of the reaction quickly diminishes; within 2 h only two products remain: the dihydrothiophene derivative **3** (13%) and the disulfide **6** (54%). As discussed above, this is due to the fact that products **4** and **5** react at a rate that is comparable to or greater than that of **1**. For example, resubjection of isolated **4** to the standard reaction conditions leads to its rapid consumption ($t_{1/2} \sim 3$ min, cf. $t_{1/2} \sim 6$ min for **1** under identical conditions) with the formation of **6** and **3** (32 and 21% yield, respectively). The data show that attack of glutathione upon the allyl-terminal sulfur atom of **4** (with concomitant expulsion of hydrogen sulfide) is slightly faster than attack on the less-substituted sulfur atom of **4** and expulsion of **A**. Although we were unable to isolate **5** for study, on the basis of HPLC analysis, it would appear that **5** is consumed at a rate comparable to that of **1** and **4** (Figure 4).

Following its initial stages (>1 h), the reaction may be described by a single process, the transformation of **6** to **3**. This transformation is found to be slower than $k_3 - k_6$ by at least 2 orders of magnitude under our standard reaction conditions. In order to conveniently measure the rate of formation of **3** from **6**, it was necessary to increase the concentration of glutathione by 10-fold over the corresponding reaction conducted with **1**. Under otherwise identical conditions, **6** was observed to undergo clean transformation to **3** with pseudo-first-order kinetics ($k = 5.0$ 10^{-5} s^{-1}, 1.0×10^{-2} M GSH; second-order rate constant 5.0×10^{-3}

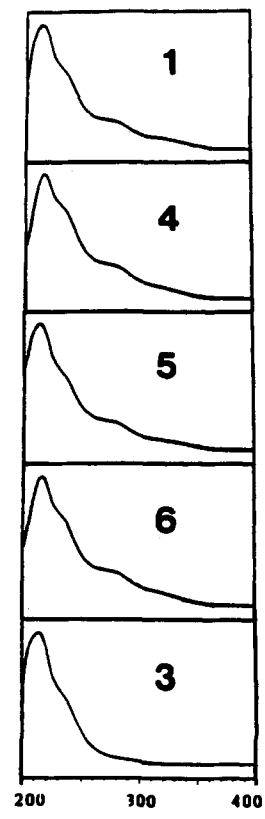


Figure 2. UV absorption spectra (200–400 nm) of **1** and products **3–6**.

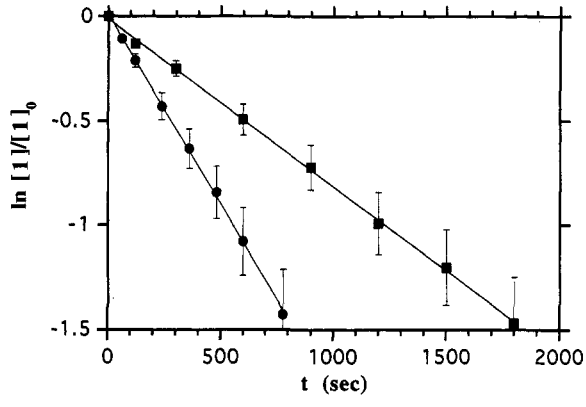


Figure 3. Logarithmic plots of the reaction of **1** (5.0×10^{-5} M) with GSH in the presence of DNA (5.0×10^{-3} M bp), as monitored by HPLC: (■) GSH = 5.0×10^{-4} M ($k = 1.6$ M^{-1} s^{-1}); (●) GSH = 1.0×10^{-3} M ($k = 1.8$ M^{-1} s^{-1}).

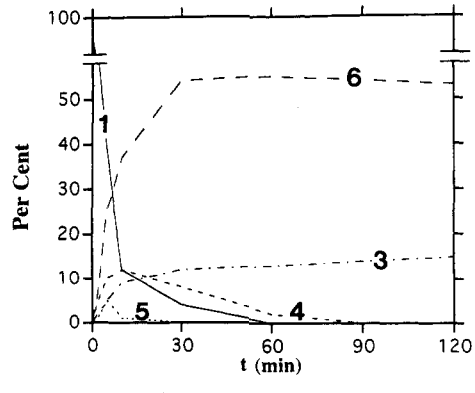


Figure 4. Time profile of the reaction of **1** (5.0×10^{-5} M) with GSH (1.0×10^{-3} M) in the presence of DNA (5.0×10^{-3} M bp), as monitored by rp-HPLC.

M^{-1} s^{-1}).[11] Comparison of the second-order rate constants for the reaction of **1** (cumulative pathways) and **6** with GSH shows that the former is more rapid by a factor of ~340. This result is perhaps not surprising given prior work showing that disulfides are generally less reactive than trisulfides toward thiol exchange;[7] however, as will be shown, in the present case this simple analysis is insufficient. The fact that glutathione bears a net negative charge may slow its reaction with **6** relative to **1**, given the potential for charge repulsion in the transition state. This factor becomes of even greater significance should these disulfide exchanges occur when **1** or **6** is bound to the polyanionic DNA helix. As will be demonstrated below, the reaction of **1** and GSH does, in fact, occur as a ternary complex with DNA, while the reaction of **6** and GSH does not.

The complexity of the reaction of **1** with GSH in the presence of DNA, as determined by the analysis of products derived from **1**, raises several questions about the DNA cleavage chemistry and, more generally, about the activity of **1** in vivo. Given the fact that four discrete species are produced in the activation process, it is reasonable to ask if each of these is capable of cleaving DNA and, if so, to determine the efficiency, rate, and sequence specificity of each respective cleavage reaction. If, as anticipated, each species cleaves DNA at a rate that parallels the rate of its transformation to **3** (determined above), then the consequences for in vivo activity may be significant, notwithstanding questions of sequence specificity. Specifically, the data suggest that DNA cleavage by **1** and GSH will follow roughly bimodal kinetics, exhibiting an initially rapid but minor burst of cleavage followed by the major damage process arising from the disulfide **6** and proceeding approximately 2 orders of magnitude more slowly than the first stage of cleavage. If this prediction is correct, and valid in vivo, then the different time scales of these distinct activation reactions may well produce different biological responses. We have begun to address these questions by studying both the kinetics and sequence specificity of DNA cleavage by **1**, **4**, and **6** in the presence of GSH, as reported below.

DNA Cleavage Experiments

DNA cleavage experiments were conducted under the standard reaction conditions defined above (**1**, 5.0×10^{-5} M; GSH, 1.0×10^{-3} M; calf thymus DNA, 5.0×10^{-3} M bp (base pairs); Tris-HCl buffer (pH 7.5), 3.0×10^{-2} M) with the inclusion of trace quantities of the 5′-^{32}P-labeled synthetic 35-mer duplex DNA: 5′-^{32}P-GCAAAGCACGCTGATCCTCTTGCTGCAA-CGTTGAC-3′. This sequence was anticipated to contain one strong cleavage site on the labeled strand (5′-TCCT-3′) on the basis of previous studies of the sequence specificity of DNA cleavage by **1**.[1a,12] Quantitative analysis of DNA cleavage by **1** and GSH was achieved by polyacrylamide gel electrophoresis (PAGE) of aliquots taken periodically throughout the course of a given reaction.[13] Figure 5 displays cleavage data from two such experiments as well as a control reaction in which GSH was omitted (lane 2). Lanes 3–9 of the gel illustrate the time course of a reacton containing 1.0×10^{-3} M GSH; lanes 10–12 show an identical reaction but with additional GSH incorporated after 60 min (final concentration 1.0×10^{-2} M). As anticipated, all observed DNA cleavage was restricted to the single site 5′-TCCT-3′.[12] The reaction employing 1×10^{-3} M glutathione (lanes 3–9) displayed a monotonic increase in DNA cleavage over a reaction period of 5–1200 min with a cleavage efficiency of approximately 35% at 1200 min. Comparison of this reaction with lanes 10, 11, or 12 shows that even after 1200 min, maximal DNA cleavage has not been attained at the lower concentration of thiol. Complete and highly efficient (~90%) DNA cleavage is observed upon incubation of **1** with the 10-fold higher concentration of GSH (1.0×10^{-2} M, lanes 10–12). Kinetics experiments described above show that any cleavage observed after 60 min must be attributable entirely to the glutathione–calicheamicin disulfide **6**, thereby confirming the conjecture that the bulk of DNA cleavage by **1** arises from the disulfide **6**. It is also notable that the sequence specificity of cleavage does not vary with time (Figure 5, lanes 3–9, 10–12), suggesting that **1** and **6** cleave the same site within this synthetic 35-mer, a conclusion verified below.

In order to verify conclusions concerning the role of the calicheamicin–glutathione disulfide **6** in DNA cleavage, we have conducted DNA cleavage experiments with pure **6**, isolated by preparative rp-HPLC. Figure 6 displays data from three separate cleavage experiments in addition to a control reaction lacking GSH (lane 2). Lane 3 provides for comparison a cleavage reaction employing **1** and GSH (1.0×10^{-3} M) that was quenched after 1 h. Lane 4 illustrates an identical reaction employing **6** in lieu of **1** and lanes 5–10 show the time course of the reaction of **6** with a 10-fold higher concentration of GSH (1.0×10^{-2} M). It is apparent from the gel data that **6** does indeed produce cleavage within this synthetic 35-mer, at the same site as **1**, albeit at a much slower rate. The maximal cleavage efficiency attained with **6** is calculated to be approximately 90%. Comparison of the cleavage intensities of experiments employing **1** and **6** (lanes 3 and 4–12, respectively) supports the earlier conclusion that the reaction of **1** with GSH produces an initial minor burst of DNA cleavage followed by the much slower and major cleavage reaction arising from **6**.

Similar conclusions have been drawn from DNA cleavage experiments conducted with pure **4**. Figure 7 displays data from a cleavage experiment following the time course of the reaction of **4** with GSH (1×10^{-3} M, lanes 3–9), from an identical experiment but with a 10-fold elevation in the concentration of GSH after 60 min (to 1.0×10^{-2} M, lanes 10–12), and, for comparison, from a reaction employing **1** and GSH (1.0×10^{-3} M). The data shows that **1** and **4** cleave the same site within this synthetic 35-mer. Cleavage by **4** also exhibits bimodal kinetics, with an initial burst occurring within the first 20 min of the reaction followed by a much slower process, presumably mediated by the disulfide **6**. Roughly 40% of DNA cleavage by **4** occurs within the first 20 min of reaction, a result consistent with HPLC analysis of the reaction of **4** with GSH (1.0×10^{-3} M), where ≥95% of **4** was consumed within 20 min, affording **3** (21%) and **6** (32%). DNA damage subsequent to this point (ca. 60% of total) must then arise from the disulfide **6**. As with **6** above, maximal DNA cleavage by **4** (~40% efficiency) occurs only at the higher concentration of GSH. The fact that the maximum cleavage efficiency with **4** is less than that observed with **1** and **6** is thought to be due to the difficulty in purification and manipulation of this highly reactive intermediate rather than to an inherent reactivity difference.

Taken together, the DNA cleavage data support the picture which arose from HPLC analysis of the reaction products derived from **1**. The reaction of **1** with GSH in the presence of DNA proceeds via four competing pathways, three of which are rapid and one of which is ~2 orders of magnitude slower under the standard conditions. DNA cleavage follows an essentially bimodal course initiated with a rapid burst followed by a much slower but major cleavage pathway involving the disulfide **6**. Overall, the

(11) Measurement of this rate constant is complicated slightly by the fact that the ultimate product of the reaction, the dihydrothiophene derivative **3**, is also not stable to the reaction conditions and decays on a time scale approximately 1 order of magnitude more slowly than the rate of consum ption of **6**. The instability of **3** has been documented previously[2e] and accounts for the slow but steady decline in material balance that we observe during the course of the reaction.

(12) It has been shown that damage of the labeled strand within this 4-base pair site occurs by abstraction of the 5′-pro-*S* hydrogen atom of the cleaved cytidine residue by C4 of the biradical **B**. (a) Zein, N.; McGahren, W. J.; Morton, G. O.; Ashcroft, J.; Ellestad, G. A. *J. Am. Chem. Soc.* **1989**, *111*, 6888. (b) De Voss, J. J.; Townsend, C. A.; Ding, W.; Morton, G. O.; Ellestad, G. A.; Zein, N.; Tabor, A. B.; Schreiber, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 9669. (c) Hangeland, J. J.; De Voss, J. J.; Heath, J. A.; Townsend, C. A. *J. Am. Chem. Soc.* **1992**, *114*, 9200.

(13) Calf thymus DNA was used as a carrier due to the prohibitive expense of experiments employing pure 35-mer duplex DNA. As a consequence, kinetics measurements were obtained using DNA of a heterogeneous sequence. Any influence this may have upon the rate of cleavage of the labeled 35-mer duplex DNA is considered to be minor in comparison to the large differences in the rate of DNA cleavage exhibited by **1** and **6**.

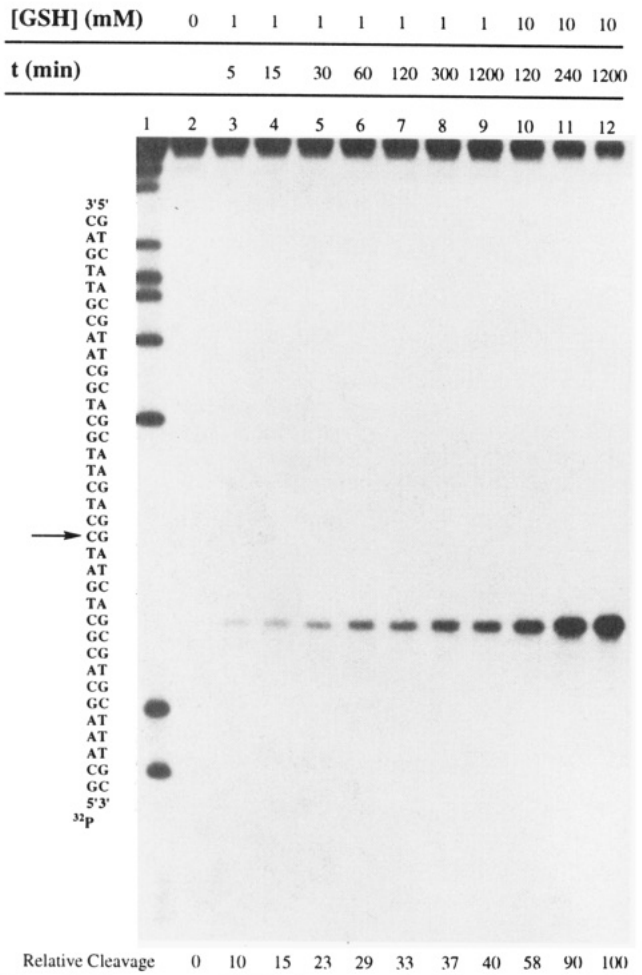


Figure 5. Time course of DNA cleavage produced by the reaction of **1** with GSH: lane 1, Maxam–Gilbert G/A sequencing reaction;[35] lane 2 (control), **1** (5.0×10^{-5} M), calf thymus DNA (5.0×10^{-3} M bp) no GSH, t = 60 min; lanes 3–9, **1** (5.0×10^{-5} M), calf thymus DNA (5.0×10^{-3} M bp) GSH (1.0×10^{-3} M), t = 5, 15, 30, 60, 120, 300, and 1200 min, respectively; lanes 10–12, **1** (5.0×10^{-5} M), calf thymus DNA (5.0×10^{-3} M bp), GSH (1.0×10^{-3} M for the first 60 min, 1.0×10^{-2} M thereafter), t = 120, 240, and 1200 min, respectively. "Relative cleavage" is defined as the percent of DNA cleavage relative to the lane of highest intensity (assigned a value of 100).

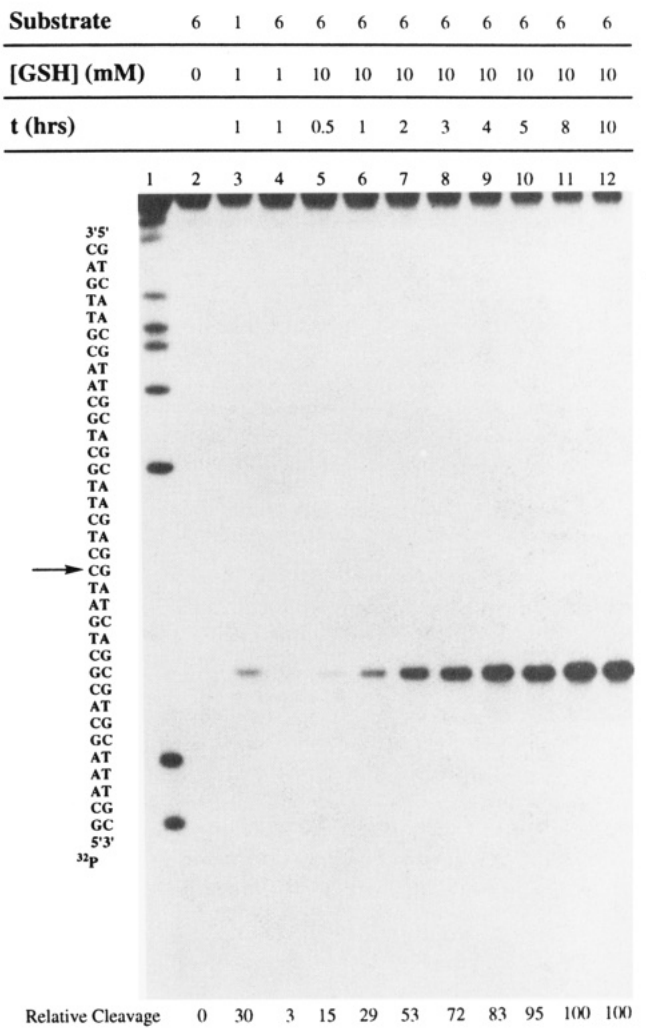


Figure 6. Time course of DNA cleavage produced by the reaction of the calicheamicin–glutathione disulfide **6** with GSH: lane 1, Maxam–Gilbert G/A sequencing reaction;[35] lane 2 (control), **6** (5.0×10^{-5} M), calf thymus DNA (5.0×10^{-3} M bp), no GSH, t = 60 min; lane 3, **1** (5.0×10^{-5} M), calf thymus DNA (5.0×10^{-3} M bp), GSH (1.0×10^{-3} M), t = 60 min; lane 4, **6** (5.0×10^{-5} M), calf thymus DNA (5.0×10^{-3} M bp), GSH (1.0×10^{-3} M), t = 60 min; lanes 5-12, **6** (5.0×10^{-5} M), calf thymus DNA (5.0×10^{-3} M bp), GSH (1.0×10^{-2} M), t = 0.5, 1, 2, 3, 4, 5, 8, 10 h, respectively.

efficiency of DNA cleavage is high; the amount of cleavage arising directly from the reaction of **1** and GSH (**1** → **A** → **2** → **B**) is conservatively estimated to be less than 25%; fully 60% of the cleavage arises indirectly via the disulfide **6**.

Both **1** and **6** cleave the same site within the synthetic 35-mer duplex DNA described. In order to explore more thoroughly the sequence specificity of DNA cleavage by **1** and **6**, we have examined cleavage by these agents within a 167-base pair DNA restriction fragment that contains several cleavage sites (Figure 8). Lanes 3–5 display DNA cleavage by **1** and GSH under varying conditions of time and GSH concentration, while lane 6 displays cleavage arising from **6** and GSH. These data virtually replicate observations with the synthetic 35-mer duplex DNA described above (Figures 5 and 6), where the initial, minor DNA cleavage process, emanating directly from **1** and GSH, occurs on a time scale of minutes, while the major DNA cleavage process, arising from **6** and GSH, occurs on a time scale of several hours. Comparison of the histograms of lanes 3 and 6 determined by phosphorimaging shows that **1** and **6** display identical sequence specificity of cleavage within this 167-base pair restriction fragment.

That **1** and **6** display identical sequence specificity of cleavage is perhaps not surprising, given that all cleavage arises ultimately from the biradical **B** and that each of these intermediates must pass through **A**, **2**, and **B** in the formation of the final product **3**. The critical issues here concern the site of activation and the dynamics of rearrangement versus equilibration among DNA binding sites for each species in the pathway. Scenarios may be envisioned where the observed cleavage specificity is kinetically determined,[14] e.g., if **2** were formed as a DNA-bound intermediate and the rate of its cycloaromatization were rapid relative to its rate of equilibration among DNA binding sites. Existing data suggests that this is not likely to be the case. Townsend et al. calculate a half-life of ~20 s for the intermediate **2** (the last common intermediate arising from **1**, **4**, and **6** prior to the formation of the biradical **B**) at 23 °C.[4] Consideration of the rates of equilibration of a representative sample of nonintercalative minor groove binding drugs between DNA binding sites (Table 1)[15] suggests that it is likely that **2** would have more than sufficient time to equilibrate among DNA binding sites prior to the cycloaromatization reaction that produces **B**. This hypothesis is further supported by the recent work of Walker, Murnick, and

(14) For an example of kinetic selectivity in DNA damage, see: Baker, B. F.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 2700.

(15) (a) Klevit, R. E.; Wemmer, D. E.; Reid, B. R. *Biochemistry* **1986**, *25*, 3296. (b) Leupin, W.; Chazin, W. J.; Hyberts, S.; Denny, W. A.; Wüthrich, K. *Biochemistry* **1986**, *25*, 5902. (c) Lee, M.; Chang, K.; Hartley, J. A.; Pon, R. T.; Krowicki, K.; Lown, J. W. *Biochemistry* **1988**, *27*, 445. (d) Lee, M.; Shea, R. G.; Hartley, J. A.; Kissinger, K.; Pon, R. T.; Vesnaver, G.; Breslauer, K. J.; Dabrowiak, J. C.; Lown, J. W. *J. Am. Chem. Soc.* **1989**, *111*, 345.

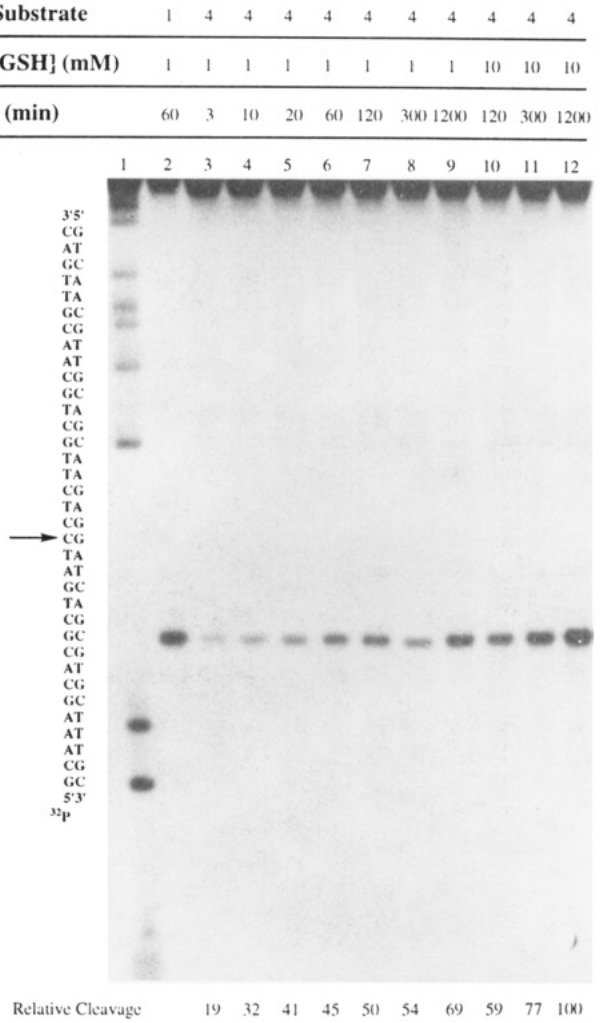


Figure 7. Time course of DNA cleavage produced by the reaction of the thiosulfenic acid derivative **4** with GSH: lane 1, Maxam–Gilbert G/A sequencing reaction;[35] lane 2, **1** (5.0×10^{-5} M), calf thymus DNA (5.0×10^{-3} M bp), GSH (1.0×10^{-3} M), $t = 60$ min; lanes 3–9, **4** (5.0×10^{-5} M), calf thymus DNA (5.0×10^{-3} M bp), GSH (1.0×10^{-3} M), $t = 3$, 10, 20, 60, 120, 300, 1200 min, respectively; lanes 10–12, **4** (5.0×10^{-5} M), calf thymus DNA (5.0×10^{-3} M bp), GSH (1.0×10^{-3} M for the first 60 min, 1.0×10^{-2} M thereafter), t = 120, 300, and 1200 min, respectively.

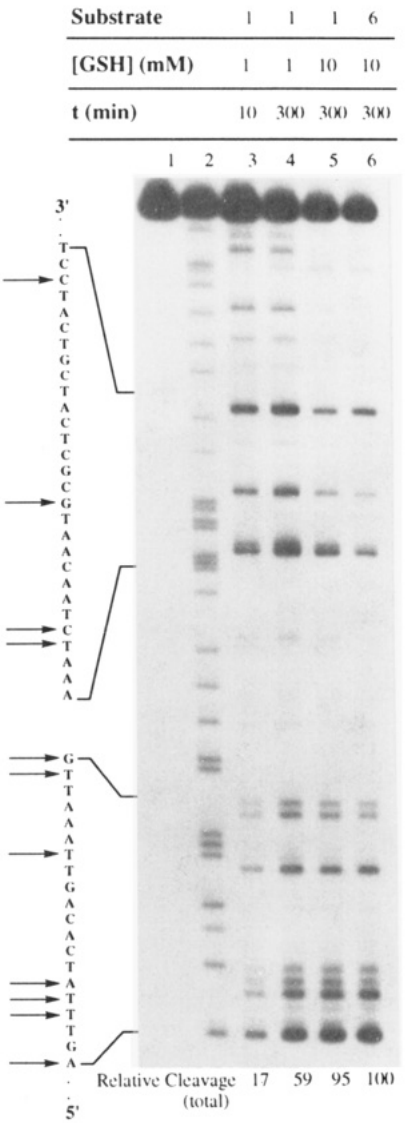


Figure 8. DNA cleavage of a 5′-^{32}P-labeled 167-base pair restriction fragment of pBR322 (Eco RI-Rsa I digest) produced by the reaction of **1** or **6** with GSH: lane 1, calf thymus DNA (1.0×10^{-3} M bp) alone; lane 2, cleavage products of an adenine-specific sequencing reaction;[36] lane 3, **1** (5×10^{-6} M), calf thymus DNA (1.0×10^{-3} M bp), GSH (1.0×10^{-3} M), $t = 10$ min; lane 4, **1** (5×10^{-6} M), calf thymus DNA (1.0×10^{-3} M bp), GSH (1.0×10^{-3} M), $t = 300$ min; lane 5, **1** (5×10^{-6} M), calf thymus DNA (1.0×10^{-3} M bp), GSH (1.0×10^{-3} M for the first 10 min, 1.0×10^{-2} M thereafter, $t = 300$ min); lane 6, **6** (5×10^{-6} M), calf thymus DNA (1.0×10^{-3} M bp), GSH (1.0×10^{-2} M), $t = 300$ min.

Kahne, wherein the rate of dissociation of **1** bound to an 8-mer DNA duplex was estimated to be 3.1 ± 1.1 s^{-1} at 25 °C.[16] The proposal that double-helical DNA may catalyze the rearrangement of **2** to **B**[4] seems unlikely in view of the nature of the unimolecular rearrangement involved, and experimental data contradicting this hypothesis have been reported.[17] The almost certain rapid quenching of the biradical **B** (relative to its equilibration among DNA binding sites) then forces the conclusion that **2** is the sequence-determing species in DNA cleavage, as previously proposed.[4] The fact that **1** and **6** display identical DNA cleavage specificity supports this hypothesis. Experiments described below will show that thiol activation of **6** occurs free in solution, thus generating **A** and, presumably, **2** prior to DNA binding, whereas **1** undergoes thiol activation while bound to DNA. Thus, the product of thiol activation (**2**, formed by rapid cyclization of **A**) functions equivalently in DNA cleavage whether generated bound to DNA or free in water. Again, the implication is that **2** is the sequence-determining species in DNA cleavage.

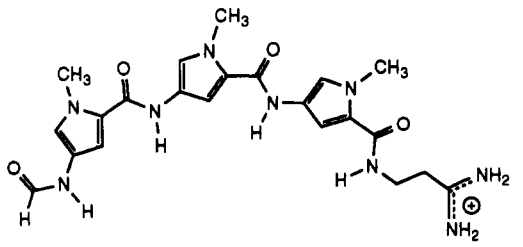
(16) Walker, S.; Murnick, J.; Kahne, D. *J. Am. Chem. Soc.* **1993**, *115*, 7954.

(17) Walker, S.; Landovitz, R.; Ding, W.; Ellestad, G. A.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4608.

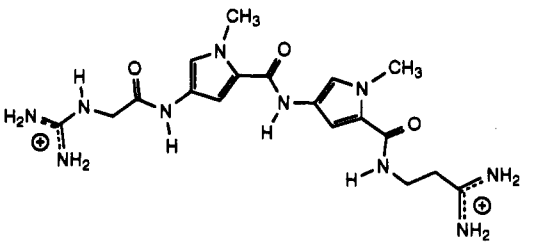
Role of DNA in Thiol Activation of Calicheamicin γ_1

A natural question arises as to the role of DNA in the chemistry of activation of **1** by glutathione. Does the reaction occur as a ternary complex of **1**, GSH, and DNA, or is **1** activated free in solution with subsequent binding of the reaction product(s) to DNA? What is the role of DNA in the primary and secondary activation steps? We have been able to address these questions, in part, by a kinetic analysis of the reaction of **1** and of **6** with GSH as a function of the concentration of DNA. The method pertains to the specific situation where drug is primarily bound to double-helical DNA. By then increasing the concentration of DNA in the medium, the concentration of bound drug is changed negligibly, while the concentration of free drug decreases markedly. If bound drug reacts faster than free drug, then the rate of DNA cleavage will be unaffected with increasing concentrations of DNA. The same result will be obtained in the unlikely situation that bound and free drug react at identical rates. If, however, free drug reacts appreciably faster than bound

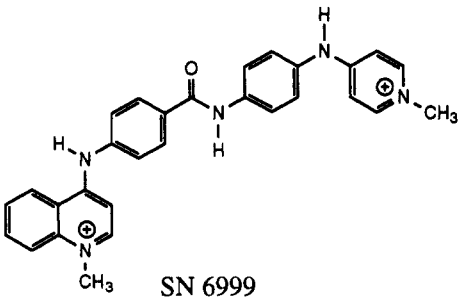
Table 1. Rates and Equilibration of Representative DNA-Binding Drugs[a]



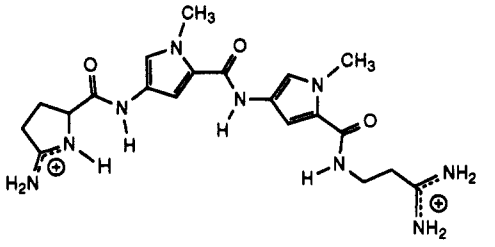
Distamycin A



Lexitropsin



SN 6999



(4*S*)-(+)-Anthelvencin A

drug	half-life (s)	*T* (°C)	ref
distamycin A	0.173	27	15a
SN 6999	0.007	16	15b
lexitropsin	0.016	21	15c
(4*S*)-(+)-anthelvencin A	0.019	21	15d

[a] Based on a two-site binding model of drug within a self-complementary DNA duplex. Exchange rates were determined by dynamic ^{1}H NMR spectroscopy. Search limited to nonintercalative minor-groove DNA-binding drugs.

drug, then the rate of DNA cleavage will decrease as the concentration of DNA is increased. The latter is precisely the situation observed with **6**. Figure 9 displays the time course of DNA cleavage by **6** and GSH (1.0×10^{-2} M) as analyzed by PAGE at four different concentrations of DNA spanning 2 orders of magnitude (4.0×10^{-5} M to 5.0×10^{-3} M).[13] In each experiment, the concentration of **6** was varied so as to maintain a constant ratio of drug to DNA ([**6**]:[DNA] = 1:100). Analysis of the data of Figure 9 clearly indicates that double-stranded DNA serves to inhibit the cleavage reaction, supporting the idea that thiol activation of **6** occurs free in solution with subsequent binding of the thiol-activated product (**A** or, more likely **2**) to DNA. These results have been confirmed by HPLC analysis of the reaction of **6** and GSH (1×10^{-2} M) with varying concentrations of calf thymus DNA (4.5×10^{-3} and 0.9×10^{-4} M bp, Figure 10). The rate of reaction of **6** is observed to increase by a factor of 4.8 upon dilution of the concentration of DNA by a factor of 5 ($k = 5.0 \times 10^{-3}$ and 2.4×10^{-2} M^{-1} s^{-1}, respectively), again demonstrating the inhibitory properties of DNA upon the thiol activation reaction of **6** and, consequently, upon DNA cleavage.

Conceptually, the same experiment may be conducted with **1**, but analysis of the outcome of this experiment is complicated by the fact that **1** is rapidly transformed to **6**. In order to dissect the component of DNA cleavage attributable to **1** alone, DNA cleavage experiments were conducted with **1** and **6** in parallel with analysis (PAGE) restricted to the very early stages of the reaction ($t \leq 15$ min), where cleavage by **1** dominates. An upper bound on the cleavage due to **6** as a secondary process emanating from **1** may then be roughly approximated and corrected for by quantitation of the cleavage intensity of the appropriate experiment conducted with pure **6**. As is evident from inspection of the data of Figures 9, 11, and 12, the DNA cleavage reactions mediated by **1** and **6** display very different kinetic behavior as a function of the concentration of DNA. Whereas DNA cleavage by **6** is strongly inhibited by DNA, DNA cleavage by **1** is essentially unaffected by variations in the concentration of DNA. Discounting the unlikely possibility that bound and unbound forms of **1** react with GSH at equivalent rates, the data show that the reaction of **1** with GSH proceeds as a termolecular event, while the reaction of **6** with GSH is a simple bimolecular process. This is an entirely reasonable outcome given the likelihood for charge repulsion in the reaction of **6** with GSH proximal to the polyanionic DNA helix. This significance of this observation is that the products of thiol activation of **1** function equivalently in the cleavage of DNA in terms of efficiency and specificity whether generated in DNA-bound form (from **1**) or free in solution (from **6**). As discussed above, this suggests that thiol-activated **1** (**A** or, more likely **2**) is sufficiently long-lived to equilibrate among DNA binding sites or, equivalently, is the sequence-determining species in DNA cleavage by **1**. A similar conclusion has been reached regarding the cumulene product of thiol activation of neocarzinostatin chromophore.[18]

While the activation of **1** with GSH may be said to occur via DNA-bound **1** at millimolar concentrations of DNA, it may not be concluded that the absolute rate of reaction of DNA-bound **1** with GSH is faster than the corresponding reaction of unbound **1** with GSH. The high affinity of **1** for DNA ($K_B > 10^6$ M^{-1}; see below) could easily offset, through a concentration effect, a disfavorable rate of reaction of bound **1** versus free **1** with GSH. This issue is of importance because it deals with the fundamental question of DNA catalysis in the thiol activation of **1**.

The possibility that DNA may catalyze the reaction of **1** with GSH is, in theory, trivially addressed. All that is necessary is to measure and compare the rates of reaction of **1** with GSH in the presence and absence of DNA. Unfortunately, our efforts to conduct this seemingly trivial experiment have been wholly unsuccessful due to the complete insolubility of **1** in aqueous media or in mixtures of water and organic solvents in the absence

(18) Myers, A. G.; Cohen, S. B.; Kwon, B. M. *J. Am. Chem. Soc.*, in press.

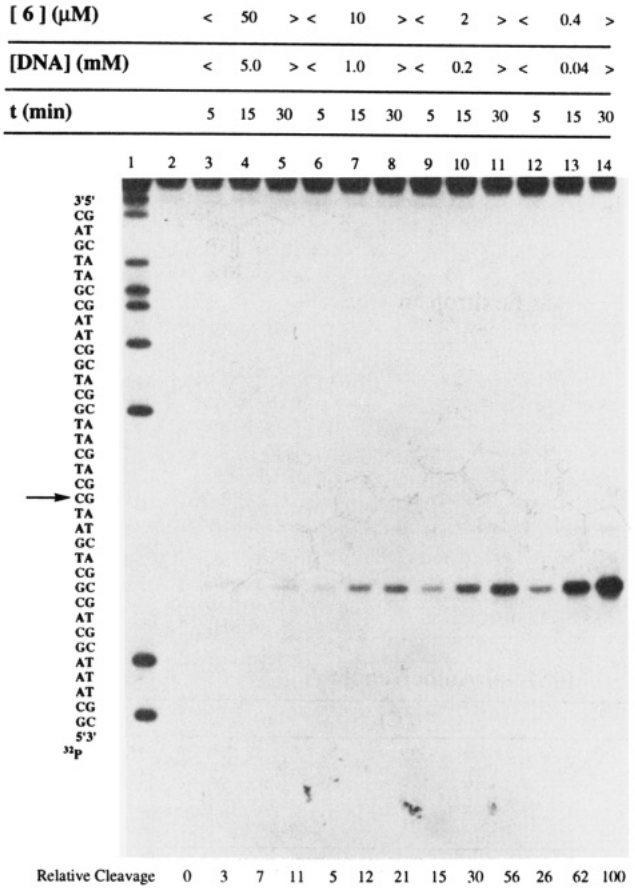


Figure 9. Kinetics of DNA cleavage produced by the reaction of the calicheamicin–glutathione disulfide **6** with GSH at varying concentrations of DNA: lane 1, Maxam–Gilbert G/A sequencing reaction;[35] lane 2 (control), **6** (5×10^{-5} M), DNA (5×10^{-3} M bp), no GSH, t = 60 min; lanes 3–5, **6** (5×10^{-5} M), DNA (5×10^{-3} M bp), GSH (1×10^{-2} M), t = 5, 15, 30 min, respectively; lanes 6–8, **6** (1×10^{-5} M), DNA (1×10^{-3} M bp), GSH (1×10^{-2} M), t = 5, 15, 30 min, respectively; lanes 9–11, **6** (2×10^{-6} M), DNA (2×10^{-4} M bp), GSH (1×10^{-2} M), t = 5, 15, 30 min, respectively; lanes 12–14, **6** (4×10^{-7} M), DNA (4×10^{-5} M bp), GSH (1×10^{-2} M), t = 5, 15, 30 min, respectively.

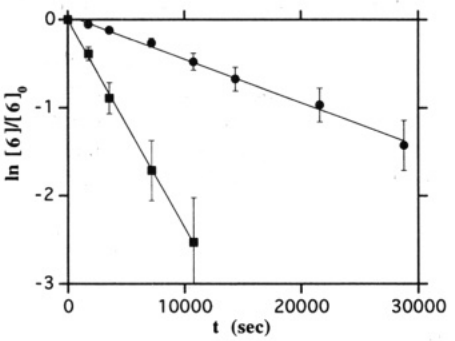


Figure 10. Logarithmic plots of the rate of disappearance of the calicheamicin–glutathione disulfide **6** in its reaction with GSH (1×10^{-2} M) in the presence of DNA, as monitored by rp-HPLC: (●) **6** (4.5×10^{-5} M), DNA (4.5×10^{-3} M bp) ($k = 5.0 \times 10^{-3}$ M^{-1} s^{-1}); (■) **6** (9×10^{-6} M) , DNA (9×10^{-4} M bp) ($k = 2.4 \times 10^{-2}$ M^{-1} s^{-1}).

of DNA.[19] We have carefully studied the solubility properties of **1** in aqueous media by light-scattering analysis with a submicron particle analyzer. For example, addition of a solution of **1** in DMSO (1×10^{-3} M) to 19 volumes of the aqueous buffer system of our standard reaction conditions, modified by the incorporation of potassium dimethylphosphate (1×10^{-2} M) in lieu of DNA to maintain constant ionic strength, is found to produce a

(19) We thank Professor Craig Townsend of The Johns Hopkins University for bringing to our attention the solubility problems encountered with **1** in aqueous solution in the absence of DNA, a fact we did not fully appreciate at the outset of our studies.

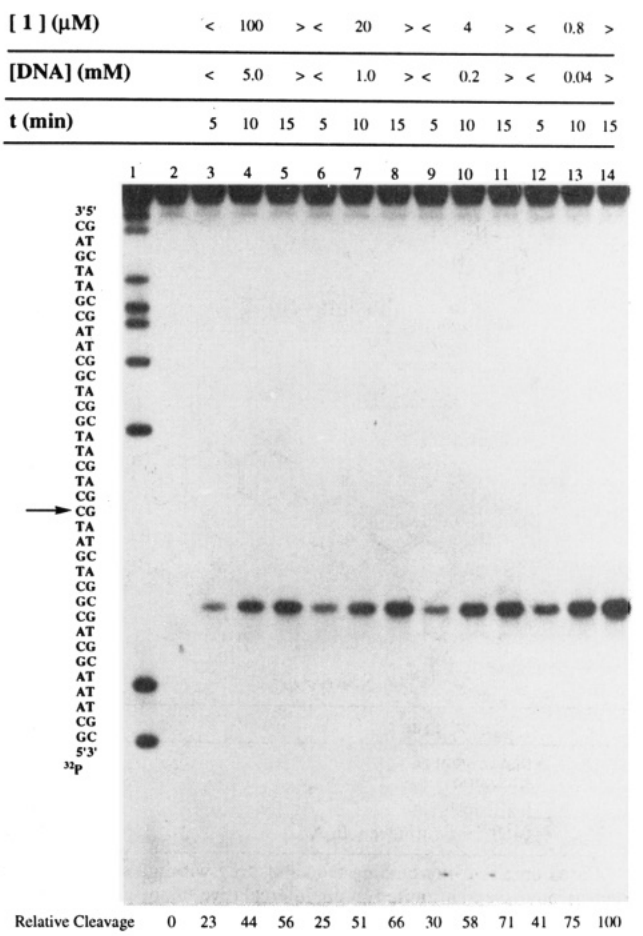


Figure 11. Kinetics of DNA cleavage produced by the reaction of calicheamicin γ_1 (**1**) with GSH at varying concentrations of DNA: lane 1, Maxam–Gilbert G/A sequencing reaction;[35] lane 2 (control), **1** (1×10^{-4} M), DNA (5×10^{-3} M bp), no GSH, t = 60 min; lanes 3–5, **1** (1×10^{-4} M), DNA (5×10^{-3} M bp), GSH (1×10^{-3} M), t = 5, 10, 15 min, respectively; lanes 6–8, **1** (2×10^{-5} M), DNA (1×10^{-3} M bp), GSH (1×10^{-3} M), t = 5, 10, 15 min, respectively; lanes 9–11, **1** (4×10^{-6} M), DNA (2×10^{-4} M bp), GSH (1×10^{-3} M), t = 5, 10, 15 min, respectively; lanes 12–14, **1** (8×10^{-7} M), DNA (4×10^{-5} M bp), GSH (1×10^{-3} M), t = 5, 10, 15 min, respectively.

particulate suspension of mean particle diameter 5–10 μm. The larger particles can be precipitated from solution with a benchtop centrifuge (~80% precipitation by HPLC analysis; see Experimental Section), affording a supernatant suspension of mean particle diameter 0.5 μm. Repetition of this experiment with successive dilution of **1** while maintaining a constant DMSO:buffer ratio (1:19) leads to suspensions of progressively smaller mean particle diameter, but in no case was a homogeneous aqueous solution of **1** obtained. By this method, we estimate that the maximum solubility of **1** in 1:19 DMSO:Tris buffer (3×10^{-2} M, pH 7.5) is 1×10^{-8} M. At this dilution, the particle density was sufficient to produce scattered light distinguishable from background, from which data a mean particle diameter of ~0.5 μm was determined. Accurate quantitation of particulate suspensions of lesser particle density is not possible with our instrumentation; thus, 1×10^{-8} M represents an upper limit for the solubility of **1** in the medium described.

Recently, the question of DNA participation in thiol activation of **1** was investigated by measuring the rates of reaction of **1** with aminoethanethiol and with GSH[20] in the presence and absence of DNA in the medium 30% methanol–aqueous Tris buffer (3

(20) Experiments investigating the reaction of **1** with GSH in the presence of DNA, the analysis of the products of the latter reaction by rp-HPLC, kinetics monitoring of that reaction by rp-HPLC, and the identification of the calicheamicin–glutathione disulfide **6** as the primary product of that reaction, were initially conducted in our laboratories, and this information was shared with Professor Craig Townsend of The Johns Hopkins University.

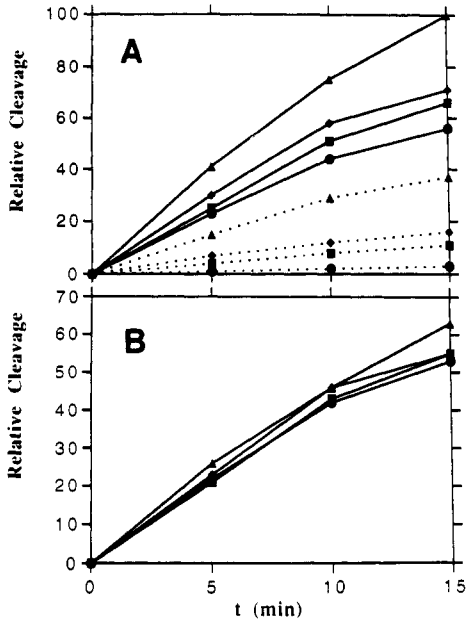


Figure 12. (A) Plot of relative cleavage intensities from Figure 10 (solid lines): (●) lanes 3–5; (■) lanes 6–8 (◆), lanes 9–11; (▼) lanes 12–14. Dashed lines represent cleavage intensities from a parallel experiment using **6** in lieu of **1** (gel not shown due to the weak cleavage intensities observed). (B) "Corrected" rates of DNA cleavage by **1** over the varying concentrations of DNA from Figure 10. The corrected values were obtained by subtracting the cleavage intensities obtained from the reaction of **6** from the cleavage intensities obtained from the reaction of **1**.

$\times 10^{-2}$ M, pH 7.5). It was found that thiol activation of **1** in the presence of DNA is slightly slower than reactions conducted in its absence.[21] We have studied the solubility of **1** in the medium employed in the latter studies and find that **1** forms particulate suspensions in that medium as well. Addition of a homogeneous solution of **1** in methanol (4×10^{-3} M) to 79 volumes of 30% methanol–aqueous Tris buffer (3×10^{-2} M, pH 7.5, 50 mM NaCl) produced a particulate suspension with particles of 2.5-μm maximum diameter. Centrifugation (16000*g*, 30 min) removed the larger particles (30% precipitation), affording a supernatant suspension of mean particle diameter 0.5 μm. Assuming a density of ~0.8 g/mL, these particles contain $\sim 3 \times 10^7$ molecules of **1**, of which <1% reside at the particle surface. The rate of the heterogeneous reaction of GSH with particulate **1** is potentially quite different from the rate of the hypothetical, homogeneous bimolecular reaction of GSH with an isolated molecule of **1** in aqueous solution. The statement "any possibility that the drug is not fully dissolved in the absence of DNA would only serve to increase the relative rate of solution reaction" is unfounded.[22] In studies of the rate of nucleophilic addition of GSH to DNA-bound and to free neocarzinostatin chromophore, we have found that the termolecular activation process is, in fact, slower than the bimolecular reaction by a factor of ~15 (the termolecular reaction dominates nevertheless, due to the high affinity of neocarzinostatin chromophore for DNA).[18] Although a similar conclusion concerning the reaction of **1** with GSH is perhaps not unreasonable, it is not supported by the data available at present.

Solubility studies of **1** in water in the presence and absence of DNA provide an estimate of the binding affinity of **1** for double-stranded DNA. Addition of a solution of **1** in DMSO (1×10^{-3} M) to 19 volumes of the aqueous buffer system of our standard reaction conditions containing double-stranded calf thymus DNA (5.0×10^{-3} M bp) produced a homogeneous solution, as determined by light-scattering analysis. It may therefore be concluded that the concentration of free **1** in this solution is <1.0 $\times 10^{-8}$ M (a higher concentration would produce a particulate suspension). Thus, the concentration of bound **1** is $\sim 5.0 \times 10^{-5}$ M, the concentration of DNA not bound to **1** is $\sim 5.0 \times 10^{-3}$ M ([base pairs]/[drug] = 100), and the binding constant of **1** and double-stranded DNA, K_B,[23] can be estimated as follows:

$$K_B = \frac{[\mathbf{1}\cdot\mathrm{DNA}]}{[\mathbf{1}_{free}][\mathrm{DNA}]} \cong \frac{5.0 \times 10^{-5}\ \mathrm{M}}{(<1.0 \times 10^{-8}\ \mathrm{M})(5 \times 10^{-3}\ \mathrm{M})} > 1 \times 10^{6}\ \mathrm{M}^{-1}$$

It follows that the ratio of free to bound **1** is $<2 \times 10^{-4}$ under our standard reaction conditions. If the rate of activation of **1** by GSH is a composite of free and bound terms, $k_{free}[\mathbf{1}_{free}][\mathrm{GSH}] + k_{bound}[\mathbf{1}\cdot\mathrm{DNA}][\mathrm{GSH}]$, then k_{free} must be on the order of 10^4 faster than k_{bound} if it is to figure significantly in the reaction. This is inconsistent with the DNA dependence of DNA cleavage by **1** determined above, where it was shown that the rate of DNA cleavage is essentially independent of the concentration of DNA in the regime of >90% bound **1**. If k_{free} were 4 orders of magnitude larger than k_{bound}, then the rate of DNA cleavage would be extraordinarily sensitive to small variations in the concentration of DNA in this range.

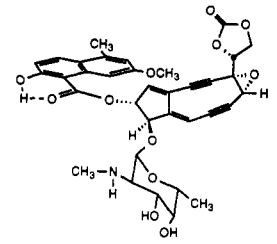
Role of the Amino Group in Thiol Activation of Calicheamicin γ_1

There is strong evidence to support the hypothesis that the carbohydrate amino group of **1** facilitates thiol activation of **1** in organic solvents. Ellestad et al. report that derivatives of **1** lacking the amino group are unreactive toward thiols in organic solvents in the absence of added triethylamine, whereas **1** itself requires no added amine for thiol activation.[1b] Kahne et al. find that the dihydrothiophene derivative **3** has a well-defined solution conformation in which the carbohydrate amino group is proximal to the thiophene ring, consistent with the idea that this group may play a role in the activation of **1**.[24] Although there is no reason, a priori, why it should necessarily be the case, it is nevertheless interesting to note that similar observations have been reported concerning the carbohydrate amino group of the structurally unrelated antitumor antibiotic neocarzinostatin chromophore (**9** Chart 3). Synthetic analogs of **9** lacking the amino group are found to be completely unreactive toward thiols in 9:1 tetrahydrofuran:acetic acid in the absence of added triethylamine, whereas **9** itself reacts rapidly with methyl thioglycolate at −70 °C in the same medium.[25] In a recent X-ray crystallographic study of the neocarzinostatin protein–chromophore complex, the carbohydrate amino group of **9** was found to be oriented directly above C12, the site of nucleophilic thiol addition, at a distance of ~5 Å, or approximately the van der Waals diameter of a sulfur atom.[26]

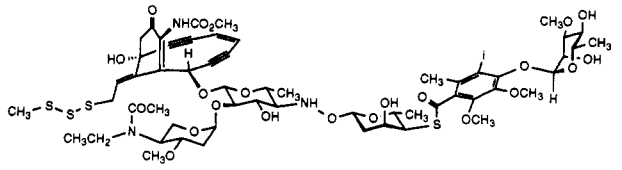
Within the context of this study of the mechanistic details of calicheamicin activation, it is worthwhile and important to question whether the carbohydrate amino group of **1** participates in the thiol activation process when **1** is bound to DNA, since this would appear to be most relevant to events occurring in vivo, on the basis of experiments described above. It is useful to define precisely the mechanisms for participation of the carbohydrate amino group in the thiol activation chemistry. Scheme 5 depicts two limiting mechanisms, 5a and 5b. Mechanism 5a involves deprotonation of the neutral thiol by the neutral amino group with concomitant attack of the developing thiolate anion upon the trisulfide (illustrated for the specific case of the generation

(21) Chatterjee, M.; Cramer, K. D.; Townsend, C. A. *J. Am. Chem. Soc.* **1993**, *115*, 3374.
(22) See footnote 13 in ref 21.
(23) Previously, K_B for a TCCT site within a synthetic double-stranded DNA dodecamer was determined to be ~ 1–3×10^{-8} M: Drak, J.; Iwasawa, N.; Danishefsky, S.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7464.
(24) Walker, S.; Valentine, K. G.; Kahne, D. *J. Am. Chem. Soc.* **1990**, *112*, 6428.
(25) Myers, A. G.; Harrington, P. M.; Kwon, B. M. *J. Am. Chem. Soc.* **1992**, *114*, 1086.
(26) Kim, K.; Rees, D. C.; Myers, A. G. *Science*, in press.

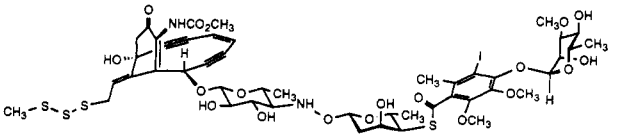
Chart 3



9



10



11

of the intermediate **A**, the mechanism holds for the three alternative modes of trisulfide cleavage as well). Mechanism 5b represents the alternative extreme, involving a thiolate–ammonium ion pair, formed in a rapid preequilibrium step, with rate-determining attack of thiolate upon the trisulfide group. While conceptually distinct, it is important to recognize that these two mechanisms are kinetically equivalent and that both fall within the definition of general-base "catalysis" (the word catalysis is used somewhat loosely here due to to the intramolecular nature of the reaction).[27] Thus, the protonation state of the amino group does not, per se, support or rule our mechanisms involving the participation of this group in the thiol activation step.[28]

In a recent study comparing the rates of thiol activation of **1** in the presence and absence of DNA, it was concluded that DNA conferred no kinetic advantage in thiol activation, rather that the rate of activation was slightly attenuated in the presence of DNA. It was also concluded from this result that the amino sugar is not a general-base catalyst in the reaction. Our finding that **1** is insoluble in 30% methanol–aqueous Tris buffer (3×10^{-2} M, pH 7.5), the medium utilized in these studies, calls into question the validity of these conclusions. It should also be noted that, even were it true that the rate of thiol activation of **1** is slower in the presence of DNA, this would not mitigate against participation of the amino group in the reaction. In order to determine if the amino group offers a kinetic advantage in the thiol activation step, it is necessary to have a reference state, a compound lacking the amino group. The "kinetic advantage" is then defined relative to some standard, and the validity of any conclusions arising from such a comparison is intimately connected to the "accuracy" of the model compound chosen.

In prior work, Cramer and Townsend attempt just such a comparison, using the *N*-acylated calicheamicin derivative **10** and the *des*amino sugar derivative **11** as reference compounds (Chart 3).[28] Unfortunately, these experiments, which compare the rates of reaction of **1**, **10**, and **11** with the nonbiological thiol aminoethanethiol, were also conducted in the medium 30% methanol–Tris buffer (30 mM, pH 7.4, 50 mM NaCl), which was shown above to lead to precipitation of **1**. The more hydrophobic calicheamicin derivatives **10** and **11** are expected to be even less soluble in this medium. In addition, kinetics measurements were determined by a continuous UV assay without characterization of the reaction products, e.g., by rp-HPLC analysis.[20] Such an analysis obfuscates the complex trisulfide interchange chemistry which dominates calicheamicin activation, as shown above, because the glutathione disulfide **6**, the glutathione trisulfide **5**, the thiosulfenic acid **4**, and **1** are virtually indistinguishable by UV spectroscopy (Figure 2), and it is expected that the same would be true of the corresponding derivatives of aminoethanethiol.[29] Furthermore, the use of aminoethanethiol (net positive charge at physiological pH) as the activating thiol bears little resemblance to potential in vivo activation factors such as GSH (net negative charge at physiological pH) and further complicates interpretation of the data by the presence of an additional amino group.

In summary, the question of amino participation in the thiol activation of **1** in water, whether **1** is free or DNA bound, remains an open issue. While compelling data exist to support the idea that the carbohydrate amino group of **1** (and of **9**) facilitates the thiol activation of this substrate in organic solvents, no meaningful conclusions concerning the corresponding experiments in aqueous media can be reached at this time.

Conclusions

The reaction of calicheamicin γ_1 (**1**) with glutathione (GSH), the most prevalent thiol in mammalian cells[8] and a putative cofactor in the activation of **1** in vivo, has been studied in water in the presence of double-stranded DNA and is shown to produce each of the four products of S–S bond exchange between the thiol function of GSH and the trisulfide group of **1** (**3**–**6**, Scheme 3). The major reaction pathway produces the calicheamicin–glutathione disulfide **6**, while the dihydrothiophene derivative **3**, the thiosulfenic acid derivative **4**, and the calicheamicin–glutathione trisulfide **5** are formed in relatively minor competing processes. Products **4**–**6** react further, each converging upon the product **3**, albeit by different reaction paths and at different rates. Thus, the most direct pathway for biradical formation from **1** (Scheme 1) is, in fact, a minor process under conditions mimicking a physiological setting. The major reaction product (**6**) forms **3** at a rate approximately 2 orders of magnitude slower than the rate of formation of **3** from **1**, **4**, or **5**. The kinetics of appearance of **3** then displays a bimodal profile with an initial rapid burst as **1**, **4**, and **5** react, followed by a much slower period as **6** is transformed into **3**. This bimodal kinetic profile is reflected in the kinetics of cleavage of double-stranded DNA by **1** and GSH, as anticipated if the putative biradical precursor to **3** (**B**) were to initiate the DNA cleavage reaction.

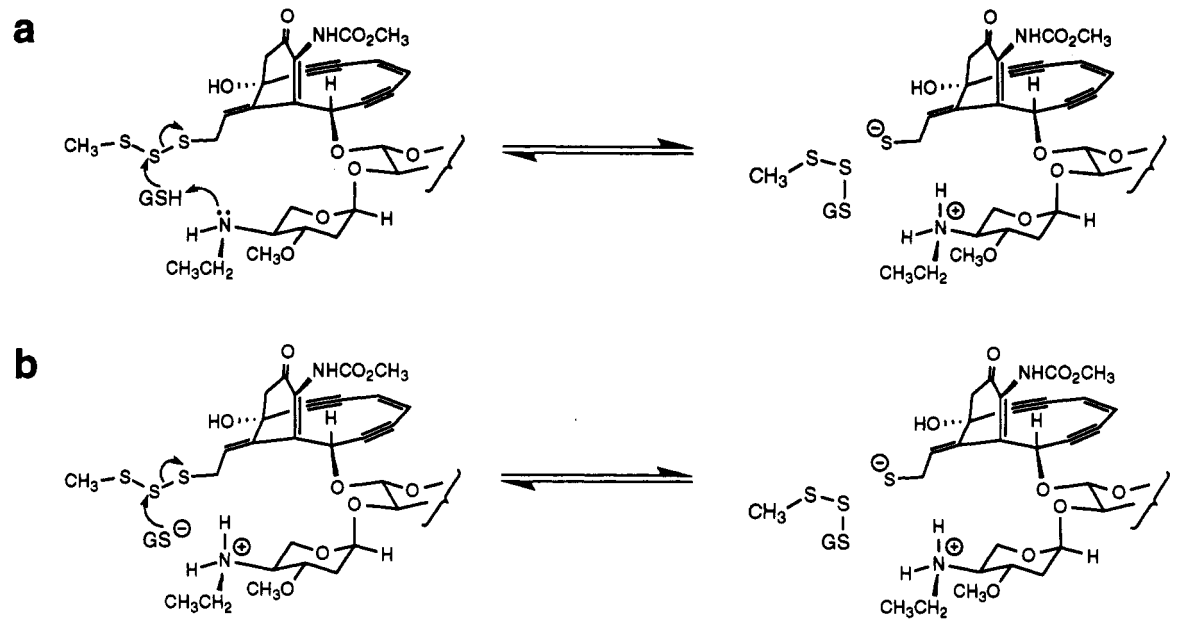
It is found that the rate of DNA cleavage by **1** is essentially independent of the concentration of DNA, whereas the rate of DNA cleavage by **6** slows markedly with increasing concentrations of DNA. Similarly, the rate of reaction of **6** with GSH in the presence of double-stranded DNA is inversely proportional to the concentration of DNA and parallels the observed rate of DNA cleavage by **6** and GSH. The most reasonable interpretation of these observations is that **1** undergoes thiol activation as a DNA-bound species, while **6** is preferentially activated free in

(27) Jencks, W. P. *Catalysis in Chemistry and Enzymology*, Dover ed.; Dover Publications, Inc.: New York, 1969; p 184.

(28) This view stands in contrast to prior arguments, where it was concluded that because "the carbohydrate ethyl ammonium group [of **1**] has a pK_a comparable to that of a thiol ... at physiological pH (6.8–7.2) it may not function as a general base." See: Cramer, K. D.; Townsend, C. A. *Tetrahedron Lett.* **1991**, *32*, 4635.

(29) In preliminary studies, we have found that aminoethanethiol reacts more rapidly (2–3-fold) with **1** in the presence of DNA than does GSH. Due to the rapidity of transformations with aminoethanethiol versus GSH, we were unable to detect the thiosulfenic acid derivative **4** or the aminoethanethiol–calicheamicin trisulfide in the reaction of **1** with aminoethanethiol; however, we did observe the aminoethanethiol–calicheamicin disulfide as an intermediate, and in contrast to the observations of Townsend et al.,[21] we find no significant distinction in the UV absorption characteristics of this intermediate versus **1**. The reaction of the aminoethanethiol–calicheamicin disulfide with aminoethanethiol in the presence of DNA (5×10^{-3} M bp) is found to be 1–2 orders of magnitude more rapid than the reaction of **6** with GSH under identical conditions.

Scheme 5



solution. Because **1** and **6** display identical sequence specificity and efficiency in DNA cleavage, one implication of these findings is that the products of thiol activation of **1** (**A** → **2**) function equivalently in the cleavage of DNA whether generated free in water or bound to DNA. This supports the idea that the sequence specificity of DNA cleavage by **1** is determined by a species formed postactivation, most probably the intermediate **2**.

The concentration of double-stranded DNA in a eukaryotic cell nucleus is estimated to range from 4 mM to ~0.5 M,[30] while the concentration of nuclear GSH in cultured rat hepatocytes has been determined to be ~20 mM, some 4-fold higher than cytosolic GSH in the same cells.[31] Our experiments suggest that the following would pertain to the hypothetical activation of **1** by GSH in a eukaryotic cell nucleus: (1) the reaction of **1** with GSH occurs as a ternary complex of **1**, GSH, and DNA and produces **6** as the major product; (2) **6** must dissociate from the DNA helix prior to reacting with free GSH; (3) the products of the latter reaction, **A** → **2**, then bind to DNA and, subsequent to formation of the biradical **B**, induce DNA damage; (4) DNA cleavage will follow a bimodal kinetic profile where the initial cleavage event will occur with a half-life on the order of a few minutes and the second, major stage of the cleavage will occur with a half-life of several hours, depending critically upon the exact concentration of nuclear DNA. It is conceivable that the differing time scales of this dual-stage cleavage process will produce different biological responses.

Given that the reaction of **1** with GSH occurs via DNA-bound **1** in the presence of millimolar concentrations of DNA, the question then arises as to the role of DNA in this reaction. We were unable to answer the simplest question, does DNA accelerate (catalyze) the thiol activation step? due to the complete insolubility of **1** in aqueous media in the absence of DNA. Previous claims in this regard are called into question for the same reason.[21] The fact that the thiol activation reaction is indiscriminate, producing all possible S–S exchange products, demonstrates that the DNA-mediated process lacks one feature that typifies many protein-catalyzed or enzymatic processes, that of selectivity. The finding that the products of thiol activation of **1** (**A** → **2**) function equivalently in DNA cleavage whether generated free in solution or bound to DNA in some ways diminishes the importance of the DNA catalysis question because it suggests that the sequence specificity of DNA cleavage by **1** is determined after thiol activation. This hypothesis is supported by earlier estimates of the half-life of the activated intermediate **2**[4] vis-à-vis representative rates of binding and debinding of related small molecules to and from DNA (Table 1)[15] and has been previously proposed by others.[4] The possibility that the sequence specificity of DNA cleavage by **1** is kinetically determined, reflecting DNA sequences that catalyze the activation of **1** (or the cyclization of **2**) as opposed to most-favored binding sites of the activated product **2**, appears unlikely.

Critical evaluation of existing data concerning the possible role of the carbohydrate amino group of **1** in the thiol activation step has shown that prior conclusions discounting participation of the amino group in aqueous media are invalid. While there is good evidence for participation of the amino group in thiol activation reactions conducted in organic media,[1b,25] no meaningful conclusions concerning the corresponding experiments conducted in water may be reached at this time. It is pointed out that mechanisms involving a thiolate–ammonium ion pair are kinetically equivalent to mechanisms involving the neutral thiol–amine couple and thus that the protonation state of the amino group, per se, does not support or rule out mechanisms involving participation of this group in the thiol activation step.[28] NMR studies in organic solvents suggest that the calicheamicin oligosaccharide is highly preorganized and adopts a conformation in which the amino group is proximal to the allyl trisulfide functional group,[24] an observation consistent with its demonstrated participatory role in thiol activation reactions conducted in organic media. Though it is reasonable to speculate that such would be the case in water and in the presence of DNA, this issue remains an open question at present.

Experimental Section

General. Calicheamicin γ_1 (**1**) was generously supplied by Dr. George Ellestad of the American Cyanamid Co., Lederle Laboratories. The drug was stored as a dry powder at –80 °C and was weighed with a Mettler microbalance. All manipulations of the drug were conducted with extreme caution due to its potential human toxicity. An authentic sample of the dihydrothiophene derivative **3** was kindly provided by Professor Daniel Kahne of Princeton University. All reaction solutions were prepared with ultrapure water, obtained from a Millipore Milli-Q Plus water purification system. "Double-stranded calf thymus DNA" refers to sonicated, phenol-extracted calf thymus DNA (Pharmacia) of approximately 90% double strand content, analyzed as follows. Calf thymus DNA was dissolved in sufficient aqueous sodium phosphate buffer (10 mM, pH 7.2) to prepare a solution 1 mM bp in DNA. A 50-μL aliquot of this solution was injected onto a Waters 600E HPLC system

(30) Suciu, D. *J. Theor. Biol.* **1986**, *117*, 587.
(31) Bellomo, G.; Vairetti, M.; Stivala, L.; Mirabelli, F.; Richelmi, P.; Orrenius, S. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4412.

configured with a Bio-Rad Econo Pac hydroxylapatite cartridge (5 mL) with the following profile of elution (1 mL/min) with aqueous solutions A (10 mM sodium phosphate, pH 7.2) and B (400 mM sodium phosphate, pH 6.8), respectively: 0–2 min, 100:0 v/v A:B; 2–30 min, linear gradient from 100:0 to 20:80 v/v A:B; 31–60 min, isocratic elution with 20:80 v/v A:B. Peaks were detected by ultraviolet absorption at 220, 230, 240, and 250 nm with a Waters 994 programmable photodiode detector. Aqueous solutions of DNA were adjusted to pH 7.5 by the addition of Tris base (Fisher). Aqueous solutions of glutathione (GSH, Sigma, 10 or 100 mM, pH adjusted to 7.5 with Tris base) were prepared just prior to use; control experiments established that these solutions were stable toward air oxidation during the time of their use.[32] A standard buffer solution (100 mM, pH 7.5) was prepared from Tris base (Fisher) and 1.00 M aqueous hydrochloric acid solution. All pH measurements were determined with a Beckman *ϕ40* digital pH meter equipped with a MI-410 micro-pH electrode (Microelectrodes, Inc.). Unless otherwise specified, all reactions were conducted with Tris-HCl buffer solutions (30 mM, pH 7.5) and were quenched by the transfer of a specified reaction volume to an Eppendorf tube containing aqueous ammonium acetate buffer solution (10 μL, 1 M, pH 5.5) followed by rapid freezing by immersion of the tube in liquid nitrogen. Electrospray mass spectra were obtained on a Vestec 201 electrospray mass spectrometer with an extended mass range of 0–2000 amu; meter voltage = 2000 V; electrospray current = 0.2 mamp; flow = 4 μL/min; solvent matrix 50:47:3 CH_3OH:H_2O:CH_3CO_2H. The spray chamber was maintained at 50 °C.

Reaction of 1 with GSH, HPLC Analysis. Reactions were performed at 23 °C in 1.5-mL Eppendorf tubes containing a total reaction volume of 1.0 mL. In a typical reaction, a 50-μL aliquot of a freshly prepared solution of **1** (1.0 mM) in DMSO containing 2,5-dimethoxybenzyl alcohol (5.0 mM) as an internal standard was combined with a freshly prepared solution of double-stranded calf thymus DNA (500 μL, 10 mM bp) in water, Tris-HCl aqueous buffer solution (300 μL, 100 mM, pH 7.5), and water (50 μL). An initial ratio of **1** to 2,5-dimethoxybenzyl alcohol (internal standard) was established by HPLC analysis (50-μL injection volume) employing a Waters 600E HPLC system equipped with a Beckman Ultrasphere ODS (C_{18}, 5 μm) rp-HPLC column, 4.6 × 250 mm, flow = 0.40 mL/min with the following step gradient of acetonitrile and aqueous ammonium acetate buffer solution (10 mM, pH 6.0), respectively: 0–5 min, 27:73 v/v; 6–20 min, 40:60 v/v; 21–60 min, 60:40 v/v. Peaks were detected by ultraviolet absorption at 220, 230, 240, and 250 nm with a Waters 994 programmable photodiode detector. The reaction was initiated by the addition of an aqueous solution of glutathione (100 μL, 10 mM), thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.05 mM; GSH, 1.0 mM; double-stranded calf thymus DNA, 5.0 mM bp; Tris-HCl buffer, 30 mM. The reaction was monitored periodically by quenching 100-μL aliquots of the reaction solution (see General) with subsequent HPLC analysis, as described. Representative retention times are as follows: **1**, 48.4 min; **3**, 39.6 min; **4**, 45.7 min; **5**, 26.5 min; **6**, 25.8 min (see Figure 1). Ultraviolet absorption spectra for each component are depicted in Figure 2. The identity of component **3** was established by coinjection with an authentic sample of **3** (~0.05 mM in methanol), kindly provided by Professor Daniel Kahne of Princeton University.

Isolation of 6. The reaction of **1** with GSH was conducted as previously described, albeit with a 5-fold increase in scale, and was quenched after 1 h at 23 °C by the addition of aqueous ammonium acetate buffer solution (500 μL, pH 5.5) and freezing (liquid nitrogen). The quenched reaction mixture was thawed and, in 11 separate 500-μL injections, was loaded onto a Beckman Ultrasphere ODS (C_{18}, 5 μm) rp-HPLC column, 10 × 250 mm, as part of a Waters 600E HPLC system, flow = 2.00 mL/min, with the following step gradient of acetonitrile and aqueous ammonium acetate buffer solution (10 mM, pH 6.0), respectively: 0–15 min, 27:73 v/v; 15–30 min, 40:60 v/v; 31–60 min, 60:40 v/v. Fractions containing **6** (retention time 26 min) were collected and pooled; the combined fractions were concentrated by lyophilization. The disulfide **6** was obtained as an off-white solid (~45% yield, as determined by integration against the internal standard in HPLC analysis). Electrospray mass spectrometry: calcd for $[M + H]^+$ 1596.6; found 1596.

Isolation of 4. A 100-μL aliquot of a freshly prepared solution of **1** (2.5 mM) in DMSO containing 2,5-dimethoxybenzyl alcohol (10 mM) as an internal standard was combined with a freshly prepared solution of double-stranded calf thymus DNA (500 μL, 10 mM bp) in water and Tris-HCl aqueous buffer solution (300 μL, 100 mM, pH 7.5). The reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (100 μL, 20 mM) and was quenched after 5 min by the addition of aqueous ammonium acetate buffer solution (100 μL, 2 M, pH 5.5) and freezing (liquid nitrogen). The thiosulfenic acid derivative **4** was purified by rp-HPLC (retention time 46 min) as described for **6**; the pooled fractions containing **4** were treated with aqueous ammonium acetate butter solution (50 μL, 2 M, pH 5.0) and were concentrated to a volume of ~1 mL at 0 °C and 0.01 Torr. The resulting solution, estimated to be approximately 0.1 mM in **4** by ultraviolet absorption at 215 nm (assuming an extinction coefficient of 75 000 at 215 nm,[33] ~12% yield), was stored frozen at −80 °C. Compound **4** is found to decompose upon lyophilization but exhibits moderate stability when stored frozen in solution. Electrospray mass spectrometry: calcd for $[M + H]^+$ 1322.2; found 1324.

Reaction of 6 with GSH, HPLC Analysis. An aqueous stock solution of the disulfide **6** (0.09 mM), 2,5-dimethoxybenzyl alcohol (internal standard, 0.45 mM), and double-stranded calf thymus DNA (9.0 mM bp, drug:DNA = 1:100) was prepared by combining a methanolic solution of **6** (50 μL, 1.0 mM, based on an assumed extinction coefficient of 75 000 at 215 nm)[33] and 2,5-dimethoxybenzyl alcohol (5.0 mM) with an aqueous solution of double-stranded calf thymus DNA (500 μL, 10 mM bp) . The ratio of **6** to internal standard at time 0 was determined by HPLC analysis of the stock solution. This stock solution was diluted 2-fold and 10-fold, respectively, for parallel reactions with GSH (10 mM) at a constant ratio of drug to DNA = 1:100. Thus, 350 μL of the stock solution was combined with aqueous Tris-HCl buffer solution (210 μL, 100 mM, pH 7.5) and water (70 μL), and the reaction was initiated at 23 °C by the addition of an aqueous solution of GSH (70 μL, 100 mM, pH 7.5). The concentrations of solution components at the onset of this reaction were as follows: **6**, 0.045 mM; GSH, 10.0 mM; DNA, 4.5 mM; Tris HCl buffer, 30.0 mM. In a parallel incubation, 70 μL of the stock solution was combined with aqueous Tris-HCl buffer solution (210 μL, 100 mM, pH 7.5) and water (350 μL), and the reaction was initiated at 23 °C by the addition of an aqueous solution of GSH (70 μL, 100 mM, pH 7.5). The concentrations of solution components at the onset of this reaction were as follows: **6**, 0.009 mM; GSH, 10.0 mM; DNA, 0.9 mM bp; Tris-HCl buffer, 30.0 mM. In each of the parallel incubations at times of 0.5, 1, 2, 3, 4, 6, and 8 h, an aliquot (100 μL) was withdrawn, quenched, and analyzed by HPLC, as described above.

Reaction of 4 with GSH, HPLC Analysis. The frozen solution of purified **4** (see Isolation of 4) was thawed, and its pH was adjusted to 7.5 by the addition of Tris base (~0.01 mmol). An aliquot of the latter solution (250 μL, ca. 0.1 mM **4**) was withdrawn and combined with an aqueous solution of 2,5-dimethoxybenzyl alcohol (10 μL, 10 mM, internal standard), double-stranded calf thymus DNA (1.60 mg, 0.0025 mmol), aqueous Tris-HCl buffer solution (100 μL, 100 mM, pH 7.5), and water (140 μL). The mixture was vortexed thoroughly, and an aliquot (50 μL) of the resulting solution was withdrawn and analyzed by HPLC to establish an initial ratio of **4** to internal standard. The reaction was initiated at 23 °C by the addition of an aqueous solution of GSH (50 μL, 10 mM, pH 7.5). The concentrations of solution components at the onset of the reaction were as follows: **4**, 0.045 mM; GSH, 1.0 mM; DNA, 4.5 mM bp; Tris-HCl buffer, 40 mM. The reaction was monitored by withdrawing aliquots (100 μL) of the reaction mixture at times of 3, 20, and 60 min followed by quenching and HPLC analysis, as described above.

Preparation of ^{32}P-labeled 35-Base Pair Duplex DNA. The single-stranded 35-base DNA oligomer 5′-GCAAAGCACGCTGATCCTCT-TGCTGCAACGTTGAC-3′ and its complementary sequence were synthesized on an Applied Biosystems DNA synthesizer (1.0 μmol scale each) using standard phosphoramidite methodology.[34] Removal of protective groups was achieved by the incubation of each protected synthetic oligomer with concentrated aqueous ammonium hydroxide

(32) An aqueous solution of glutathione (10 mL, 10 mM, pH 7.5) was prepared by dissolving glutathione (0.1 mmol) in Tris-HCl aqueous buffer solution (10 mL, 30 mM, pH 7.5), and the pH was adjusted to 7.5 by the addition of Tris base. A portion (1 mL) of the resulting solution was transferred to a 1.5-mL Eppendorf tube and was monitored by HPLC analysis by withdrawing 50-μL aliquots at various intervals (Waters 600E HPLC system equipped with a Beckman Ultrasphere ODS (C_{18}, 5 μm) rp-HPLC column, 4.6 × 250 mm, flow = 0.40 mL/min with isocratic elution of aqueous potassium phosphate buffer solution (100 mM, pH 4.0)). Peaks were detected by ultraviolet absorption at 220 nm with a Waters 994 programmable photodiode detector. After 24 h, glutathione (retention time 8 min) remained predominantly (~90%) in its reduced form; About 10% of the glutathione disulfide (retention time 12 min) had formed. In an analogous fashion, an aqueous solution of glutathione (1.0 mM) was prepared and monitored by HPLC. After 24 h, glutathione remained predominantly (~90%) in its reduced form.

(33) The UV absorption spectrum of calicheamicin γ_1 is dominated by the thiobenzoate chromophore in the range about 215 nm (ϵ (215 nm) ~ 75 000 M^{-1} cm^{-1}). It is assumed that compounds **1**, **4**, and **6** have nearly the same extinction coefficient at this wavelength. Lee, M. D.; Manning, J. K.; Williams, D. R.; Kuck, N. A.; Testa, R. T.; Borders, D. B. *J. Antibiot.* **1989**, *42*, 1070.

(34) Gait, M. J. *Oligonucleotide Synthesis: A Practical Approach*, Oxford University Press: New York, 1984.

solution (1 mL) for 12 h at 55 °C. Each product was dissolved in formamide loading buffer solution (50 μL),[35] and the resulting solution was applied to the top of a 15% denaturing polyacrylamide gel, 1.5-mm thickness, for purification by electrophoresis. The bands containing the DNA oligomers were located by UV shadow and were excised from the gel. The oligomers were isolated by the crush and soak method[35] followed by dialysis against ultrapure water (2 days) and lyophilization. The single-stranded oligomer 5′-GCAAAGCACGCTGATCCTCTTGCT-GCAACGTTGAC-3′ (50 pmol) was 5′-end-labeled with [γ-^{32}P]ATP (NEN, ≥5000 Ci/mmol) and polynucleotide kinase (Boehringer Mannheim) using standard procedures.[35] The labeled single-stranded oligomer was purified over a 15% denaturing polyacrylamide gel, 0.4-mm thickness, and the band containing the oligomer was located by autoradiography. The band was excised from the gel, was crushed thoroughly and, after combination with aqueous Nonidet P-40 detergent solution (Sigma, 350 μL, 0.05%), was vortexed for 30 min at 23 °C. The resulting suspension was filtered through a Centrex filter (0.45 μm), and the filtrate was extracted twice with 1:1 v/v phenol:chloroform (300 μL). The labeled product was precipitated by the addition of aqueous sodium acetate buffer solution (100 μL, 0.3 M, pH 5.3) and ethanol (900 μL), followed by centrifugation at 2 °C (16000*g*, 20 min), and was then washed with aqueous ethanol (1 mL, 70%). The purified labeled fragment was dissolved in an aqueous solution of Tris-acetate buffer (25 μL, 50 mM, pH 7.4) and sodium chloride (100 mM), and the complementary synthetic single-stranded DNA oligomer (50 pmol) was added. The mixture was annealed by heating at 90 °C for 5 min with subsequent slow cooling to 23 °C (maintained at 23 °C for 12 h) to form the labeled duplex DNA.

Preparation of ^{32}P-labeled 167-Base Pair Restriction Fragment. Plasmid pBR322 (40 μL, 0.25 μg/μL, Boehringer Mannheim) was linearized by digestion with Eco RI (Boehringer Mannheim) according to the manufacturer's specifications. Following removal of the 5′-phosphate groups with alkaline phosphatase (Boehringer Mannheim), the DNA was 5′-end-labeled with [γ-^{32}P]ATP (NEN, ≥5000 Ci/mmol) and polynucleotide kinase (Boehringer Mannheim) using standard procedures.[35] The 5′-labeled product was digested with Rsa I (Boehringer Mannheim) according to the manufacturer's specifications, and the 167-base pair fragment was purified over a 8% nondenaturing polyacrylamide gel, 0.8-mm thickness. The band containing the 167-base pair fragment was located by autoradiography and was excised from the gel. The gel slice was crushed thoroughly and, after combination with aqueous Nonidet P-40 detergent solution (Sigma, 350 μL, 0.05%), was vortexed for 1 h at 23 °C. The resulting suspension was filtered through a Centrex filter (0.45 μm), and the filtrate was extracted twice with 1:1 v/v phenol: chloroform (300 μL). The labeled product was precipitated by the addition of aqueous sodium acetate buffer solution (100 μL, 0.3 M, pH 5.3) and ethanol (900 μL), followed by centrifugation at 2 °C (16000*g*, 20 min), and then was washed with aqueous ethanol (1 mL, 70%). The purified labeled fragment was stored frozen in Tris-HCl aqueous buffer solution (10 mM, pH 7.4) with EDTA (1 mM).

Analysis of DNA Cleavage Products, General. The products from a given DNA cleavage reaction were precipitated by the addition of aqueous sodium acetate buffer solution (50 μL, 0.3 M, pH 5.3) and ethanol (300 μL) followed by centrifugation at 2 °C (16000*g*, 20 min). The resulting product pellet was washed with aqueous ethanol (1 mL, 70%) and was dried on a Savant rotary speed-vac. The dried pellet was dissolved in formamide loading buffer (8 μL),[35] and the resulting solution was transferred to a 1.5-mL Eppendorf tube. After assaying for radioactivity with a Beckman LS 6000SC scintillation counter, the solution was diluted with additional formamide loading buffer so as to produce a radiation density of 3000 cpm/μL. After heating at 85 °C for 5 min to induce denaturation, the solution (5 μL) was analyzed by gel electrophoresis. Cleavage products from the 5′-labeled 35-mer were loaded onto a 20% denaturing polyacrylamide gel (42 × 34 cm × 0.4 mm thickness) and were separated by electrophoresis in 1 × TBE buffer at 1800 V for 10 min and then at 1200 V until the bromophenol blue dye had migrated to ~5 cm from the bottom of the gel. Cleavage products from the 5′-labeled 167-base pair restriction fragment were loaded onto a 8% denaturing polyacrylamide gel (42 × 34 cm × 0.4 mm thickness) and were separated by electrophoresis in 1 × TBE buffer at 2000 V for 15 min and then at 1500 V until the bromophenol blue dye and migrated off the gel.[35] The gel was exposed to a storage phospor plate, and the DNA cleavage products were quantified with a molecular Dynamics 400 S PhosphorImager.

(35) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.
(36) Iverson, B. L.; Dervan, P. B. *Nucleic Acids Res.* **1987**, *15*, 7823.

Reaction of 1 with GSH, DNA Cleavage Analysis. Reactions were performed at 23 °C in 1.5-mL Eppendorf tubes containing a total reaction volume of ≤300 μL. A 30-μL aliquot of a freshly prepared solution of **1** (0.55 mM) in methanol was combined with a solution of double-stranded calf thymus DNA (165 μL, 10 mM bp) in water, Tris-HCl aqueous buffer solution (90 μL, 100 mM, pH 7.5), water (15 μL), and labeled duplex DNA (~10^6 cpm). A 30-μL aliquot of the resulting solution was removed and was held at 23 °C for 60 min as a control (Figure 5, lane 2). The reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (30 μL, 10 mM, pH 7.5) to the remaining solution, thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.05 mM; GSH, 1.0 mM; double-stranded calf thymus DNA, 5.0 mM bp; Tris-HCl buffer, 30 mM. At times of 5, 15, 30, 60, 120, 300, and 1200 min, 20-μL aliquots of the reaction solution were quenched and analyzed subsequently by gel electrophoresis (lanes 3–9, respectively). At a reaction time of 60 min, 54 μL of the reaction mixture was transferred to a fresh 1.5-mL Eppendorf tube and was treated with an aqueous solution of glutathione (5.4 μL, 100 mM, pH 7.5, [GSH] = 10 mM). At times of 120, 240, and 1200 min, 20-μL aliquots (lanes 10–12, respectively) of this reaction solution were quenched and analyzed by gel electrophoresis, as described above.

Reaction of 6 with GSH, DNA Cleavage Analysis. DNA cleavage reactions with **6** and GSH (Figure 6, lanes 5–12) were performed at 23 °C in 1.5-mL Eppendorf tubes containing a total reaction volume of ≤200 μL. A freshly prepared solution of **6** in methanol (20 μL, 0.55 mM) was combined with a solution of double-stranded calf thymus DNA (110 μL, 10 mM bp) in water, Tris-HCl aqueous buffer solution (90 μL, 100 mM, pH 7.5), and labeled duplex (~10^6 cpm). A 20-μL aliquot of the resulting solution was removed and held at 23 °C for 60 min as a control (Figure 6, lane 2). The reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (20 μL, 100 mM, pH 7.5) to the remaining solution, thus producing the following concentrations of solution components at the onset of the reaction: **6**, 0.05 mM; GSH, 10 mM; double-stranded calf thymus DNA, 5.0 mM bp; Tris-HCl buffer, 30 mM. At times of 0.5, 1, 2, 3, 4, 5, 8, and 10 h (lanes 5–12, respectively), 20-μL aliquots of the reaction solution were quenched and analyzed by gel electrophoresis, as described above. The DNA cleavage reaction with **6** and GSH (1.0 mM, lane 4) was performed at 23 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 50 μL. A freshly prepared solution of **6** in methanol (5 μL, 0.5 mM) was combined with a solution of double-stranded calf thymus DNA (25 μL, 10 mM bp) in water, Tris-HCl aqueous buffer solution (15 μL, 100 mM, pH 7.5), and labeled duplex (~10^5 cpm). The reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (5 μL, 10 mM, pH 7.5) to the remaining solution, thus producing the following concentrations of solution components at the onset of the reaction: **6**, 0.05 mM; GSH, 1.0 mM; double-stranded calf thymus DNA, 5.0 mM bp; Tris-HCl buffer, 30 mM. At a reaction time of 60 min the reaction solution was quenched and analyzed by gel electrophoresis, as described above. The DNA cleavage reaction with **1** and GSH (1.0 mM, lane 3) was performed at 23 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 50 μL. A freshly prepared solution of **1** in methanol (5 μL, 0.5 mM) was combined with a solution of double-stranded calf thymus DNA (25 μL, 10 mM bp) in water, Tris-HCl aqueous buffer solution (15 μL, 100 mM, pH 7.5), and labeled duplex (~10^5 cpm). The reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (5 μL, 10 mM, pH 7.5) to the remaining solution, thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.05 mM; GSH, 1.0 mM; double-stranded calf thymus DNA, 5.0 mM bp; Tris-HCl buffer, 30 mM. At a reaction time of 60 min the reaction solution was quenched and analyzed by gel electrophoresis, as described above.

Reaction of 4 with GSH, DNA Cleavage Analysis. The DNA cleavage reaction with **4** and GSH was performed at 23 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 400 μL. The frozen solution of purified **4** (see Isolation of **4**) was thawed, and its pH was adjusted to 7.5 by the addition of Tris base (~0.01 mmol). An aliquot of the latter solution (200 μL, ca. 0.1 mM **4**) was withdrawn and combined with an aqueous Tris-HCl buffer solution (80 μL, 100 mM, pH 7.5), double-stranded calf thymus DNA (1.30 mg, 0.0020 mmol), and water (80 μL). After thorough vortexing, the reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (40 μL, 10 mM, pH 7.5), producing the following concentrations of solution components at the onset of the reaction: **4**, 0.05 mM; GSH, 1.0 mM; double-stranded calf thymus DNA, 5.0 mM bp; Tris-HCl buffer, 40 mM. At times of 3, 10, 20, 60, 120, 300, and 1200 min (Figure 7, lanes 3–9, respectively), 30-μl aliquots of the reaction solution were quenched and analyzed by gel

electrophoresis, as described above. At a reaction time of 60 min, 54 μL of the reaction mixture was transferred to a fresh 1.5-mL Eppendorf tube and was treated with an aqueous solution of glutathione (5.4 μL, 100 mM, pH 7.5, [GSH] = 10 mM). At times of 120, 240, and 1200 min, 20-μL aliquots (lanes 10–12, respectively) of this reaction solution were quenched and analyzed by gel electrophoresis, as described above. The DNA cleavage reaction with **1** and GSH (1.0 mM, lane 2) was performed at 23 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 50 μL. A freshly prepared solution of **1** in methanol (5 μL, 0.5 mM) was combined with a solution of double-stranded calf thymus DNA (25 μL, 10 mM bp) in water, Tris-HCl aqueous buffer solution (15 μL, 100 mM, pH 7.5), and labeled duplex (~10^5 cpm). The reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (5 μL, 10 mM, pH 7.5) to the remaining solution, thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.05 mM; GSH, 1.0 mM; double-stranded calf thymus DNA, 5.0 mM bp; Tris-HCl buffer, 30 mM. At a reaction time of 60 min, the reaction solution was quenched and analyzed by gel electrophoresis, as described above.

Cleavage of 167-Base Pair Restriction Fragment by 1 or 6. The reaction of **1** with GSH was performed at 23 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 150 μL. A solution of **1** (45 μL, 20 μM, recovered by HPLC, concentration determined by UV at 215 nm[33]) was combined with a solution of double-stranded calf thymus DNA (15 μL, 10 mM bp) in water, Tris-HCl aqueous buffer solution (45 μL, 100 mM, pH 7.5), water (30 μL), and labeled restriction fragment (~1.5 × 10^5 cpm). The reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (15 μL, 10 mM, pH 7.5), thus producing the following concentrations of solution components at the onset of the reaction: **1**, 5 μM; GSH, 1.0 mM; double-stranded DNA, 1.0 mM bp: Tris-HCl buffer, 30 mM. At a reaction time of 10 min, the reaction solution was partitioned into three equal portions of 50 μL. One 50-μL aliquot of the reaction solution was quenched at 10-min time. A second 50-μL aliquot was treated with an aqueous solution of glutathione (5 μL, 100 mM, pH 7.5, [GSH] = 10 mM). At a reaction time of 300 min, the two remaining reaction solutions were quenched. The reaction of **6** with GSH was performed at 23 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 50 μL. A solution of **6** (15 μl, 20 μM, recovered by HPLC, concentration determined by UV at 215 nm[33]) was combined with a solution of double-stranded calf thymus DNA (5 μL, 10 mM bp) in water, Tris-HCl aqueous buffer solution (15 μL, 100 mM, pH 7.5), water (10 μL), and labeled restriction fragment (~5 × 10^4 cpm). The reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (5 μL, 100 mM, pH 7.5), thus producing the following concentrations of solution components at the onset of the reaction: **6**, 5 μM; GSH, 10.0 mM; double-stranded DNA, 1.0 mM bp; Tris-HCl buffer, 30 mM. At a reaction time of 300 min, the reaction was quenched. The reaction solutions were analyzed subsequently by gel electrophoresis, as described above.

Reaction of 6 with GSH at Varying Concentrations of DNA. An aqueous solution of the disulfide **6** (0.09 mM) and double-stranded calf thymus DNA (9.0 mM, drug:DNA = 1:100) was prepared by combining a methanolic solution of **6** (20 μL, 1.0 mM, based on an assumed extinction coefficient of 75 000 at 215 nm)[33] with an aqueous solution of double-stranded calf thymus DNA (200 μL, 10 mM bp). A 50-μL aliquot of this solution was diluted to a volume of 250 μL by the addition of water (200 μL), affording a second, more dilute solution containing **6** (0.018 mM) and calf thymus DNA (1.8 mM bp). A 50-μL aliquot of this second solution was diluted with water (200 μL) to afford a third solution containing **6** (3.6 μM) and calf thymus DNA (360 μM bp). A fourth solution containing **6** (0.72 μM) and DNA (72 μM bp) was prepared in the same manner. Four parallel reactions of **6** with GSH (10 mM) at a constant ratio of drug to DNA (1:100, respectively) were performed on a reaction volume of 100 μL each by combining 55 μL of each respective solution of **6** and DNA with aqueous Tris-HCl buffer solution (30 μL, 100 mM, pH 7.5), water (5 μL), and labeled duplex (~10^5 cpm). The reactions were initiated at 23 °C by the addition of an aqueous solution of glutathione (10 μL, 100 mM, pH 7.5). The concentrations of **6** and calf thymus DNA, respectively, at the onset of these reactions were, in order of decreasing concentrations, as follows: (1) **6**, 0.05 mM; DNA, 5.0 mM; (2) **6**, 0.01 mM; DNA, 1.0 mM; (3) **6**, 2 μM; DNA, 200 μM; (4) **6**, 0.4 μM; DNA, 40 μM; each reaction solution also contained Tris-HCl buffer (30 mM, pH 7.5) and GSH (10 mM). At times of 5, 15, and 30 min, a 30-μL aliquot for each reaction solution was quenched and analyzed by gel electrophoresis, as described above.

Reaction of 1 with GSH at Varying Concentrations of DNA. An aqueous solution of **1** (0.18 mM) and double-stranded calf thymus DNA (9.0 mM, drug:DNA = 1:50) was prepared by combining a methanolic solution of **1** (20 μL, 2.0 mM) with an aqueous solution of double-stranded calf thymus DNA (200 μL, 10 mM bp). A 50-μL aliquot of this solution was diluted to a volume of 250 μL by the addition of water (200 μL), affording a second, more dilute solution containing **1** (0.036 mM) and double-stranded calf thymus DNA (1.8 mM bp). A 50-μL aliquot of this second solution was diluted with water (200 μL) to afford a third solution containing **1** (7.2 μM) and calf thymus DNA (360 μM bp). A fourth solution containing **1** (1.4 μM) and DNA (72 μM bp) was prepared in the same manner. Four parallel reactions of **1** with GSH (1.0 mM) at a constant ratio of drug to DNA (1:50, respectively) were performed on a total reaction volume of 100 μL by combining 55 μL of each solution of **1** and DNA with aqueous Tris-HCl buffer solution (30 μL, 100 mM, pH 7.5), water (5 μL), and labeled duplex (~10^5 cpm). The reactions were initiated at 23 °C by the addition of an aqueous solution of glutathione (10 μL, 10 mM, pH 7.5). The concentrations of **1** and calf thymus DNA, respectively, at the onset of these reactions were, in order of decreasing concentrations, as follows: (1) **1**, 0.10 mM; DNA, 5.0 mM; (2) **1**, 0.02 mM; DNA, 1.0 mM; (3) **1**, 4 μM; DNA, 200 μM; (4) **1**, 0.8 μM; DNA, 40 μM; each reaction solution also contained Tris-HCl buffer (30 mM, pH 7.5) and GSH (1.0 mM). At reaction times of 5, 10, and 15 min, a 30-μL aliquot from each reaction solution was quenched and analyzed by gel electrophoresis, as described above. Four parallel reactions employing **6** in lieu of **1** (drug:DNA = 1:50) were performed in an identical fashion in parallel in order to place an upper bound on DNA cleavage attributable to **6** as a secondary process emanating from **1**.

Light-Scattering Analysis. The solubility of **1** in aqueous solution in the absence of DNA was analyzed using a Malvern System 4700-C submicron particle analyzer employing a Spectra Physics Series 2000 argon laser (488 nm). The detector was set at an angle of 90° to the laser beam. To examine the solubility of **1** under our standard reaction conditions (employing potassium dimethyl phosphate in lieu of DNA to maintain constant ionic strength), a solution of **1** (50 μL, 1.0 mM) in DMSO containing 2,5-dimethoxybenzyl alcohol (5.0 mM) was combined in a 1.5-mL Eppendorf tube with an aqueous solution of potassium dimethyl phosphate (500 μL, 20 mM), aqueous Tris-HCl buffer solution (300 μL, 100 mM, pH 7.5), and water (150 μL), affording a total volume of 1.00 mL. The final concentrations of solution components are calculated as follows: **1**, 0.05 mM (maximum, if **1** were completely dissolved); potassium dimethyl phosphate, 10.0 mM; Tris-HCl buffer, 30 mM. A 50-μL aliquot of the resulting aqueous suspension was analyzed by rp-HPLC, as previously described, to establish an initial ratio of **1** to internal standard. The fine suspensions produced upon addition of solutions of **1** in DMSO to water are found to analyze as homogeneous solutions by HPLC, presumably due to the rapid dissolution of **1** in the HPLC eluent system. The aqueous suspension of **1** was transferred to a 5-mL Pyrex test tube (for placement in the sample holder) and was examined by light-scattering analysis. The Malvern System reported an average particle diameter of 5–10 μM. A 750-μL aliquot of the latter aqueous suspension was transferred into a fresh 1.5-mL Eppendorf tube and was centrifuged at 16000*g* for 30 min (23 °C). A portion of the supernatant (ca. 500 μL) was carefully withdrawn and was examined by light-scattering analysis; the Malvern System reported an average particle diameter of ~0.5 μm. Analysis of the supernatant by rp-HPLC showed that it contained approximately 20% of **1** originally added. Two controls were also examined in parallel by light-scattering analysis. Control solution 1 was prepared by a combining a solution of **1** (50 μL, 1.0 mM) in DMSO containing 2,5-dimethoxybenzyl alcohol (5.0 mM) with DMSO (950 μL) to afford a solution of **1** (0.05 mM) in DMSO. Control solution 2 was prepared by combining DMSO (50 μL) with an aqueous solution of potassium dimethyl phosphate (500 μL, 20 mM), aqueous Tris-HCl buffer solution (300 μL, 100 mM, pH 7.5), and water (150 μL). The control solutions were found not to scatter light. To examine the solubility of **1** at lower concentrations (≤1 μM), three solutions of **1** (20 μM, 2 μM, and 0.2 μM) in DMSO were prepared. Aliquots (50 μL) of each of the solutions were combined in 1.5 mL Eppendorf tubes with an aqueous solution of potassium dimethyl phosphate (500 μL, 20 mM), aqueous Tris-HCl buffer solution (300 μL, 100 mM, pH 7.5), and water (150 μL) to afford solutions nominally containing 10^{-6}, 10^{-7}, and 10^{-8} M **1**, respectively (maximum, if **1** were completely dissolved). These solutions were found to scatter light distinguishable from the control solutions. Solutions of lesser particle density did not scatter light distinguishable from background. To examine the solubility of **1** under the conditions of Cramer and Townsend,[28] a solution of **1** (12.5 μL, 4.0 mM) in methanol containing 2,5-dimethoxybenzyl alcohol (20 mM, internal HPLC standard) was combined in a 1.5-mL Eppendorf tube with a solution of 70:30 aqueous Tris-HCl buffer (30 mM, pH 7.4, 50 mM NaCl):methanol (987

μL) to afford a solution of **1** at a nominal concentration of 0.05 mM (maximum, if **1** were completely dissolved). A 50-μL aliquot of the resulting suspension was analyzed by rp-HPLC before light-scattering analysis to establish an initial ratio of **1** to internal standard. The suspension of **1** was transferred to a 5-mL Pyrex test tube and was examined by light-scattering analysis. The Malvern System reported an average particle diameter of 2.6 μm. After light-scattering analysis, 250-μL aliquots of the suspension were transferred to each of three fresh 1.5-mL Eppendorf tubes (750 μL total) and were centrifuged at 16000*g* for 30 min (23 °C). A portion of the supernatant was carefully withdrawn from each tube (200 μL). The supernatant solutions were combined (600 μL), and the resulting solution was examined by light-scattering analysis. The Malvern System reported an average particle diameter of 0.54 μm. Analysis of the supernatant solution by rp-HPLC showed that it contained approximately 70% of **1** originally added.[37] Two controls were also examined in parallel by light-scattering analysis. Control solution 1 was prepared by combining a solution of **1** (12.5 μL, 4.0 mM) in methanol containing 2,5-dimethoxybenzyl alcohol (20 mM) with methanol (987 μL) to afford a solution of **1** (0.05 mM) in methanol. Control solution 2 was prepared by combining methanol (12.5 μL) with a solution of 70:30 aqueous Tris-HCl buffer (30 mM, pH 7.4, 50 mM NaCl):methanol (987 μL). The control solutions were found not to scatter light.

(37) The intensity of scattered light is roughly proportional to the surface area of the particle or equivalently, to the square of the particle radius. Although the large particles (removable by centrifugation) represent only 30% of total calicheamicin, these particles account for the majority of light scattering in the sample.

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