DNA Cleavage by Neocarzinostatin Chromophore. Establishing the Intermediacy of Chromophore-Derived Cumulene and Biradical Species and Their Role in Sequence-Specific Cleavage

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Abstract: Experiments establishing the intermediacy of the cumulene **3** and the biradical **4** in the cleavage of double-stranded DNA by neocarzinostatin chromophore (**1**) and methyl thioglycolate (**2**) are described. It is shown that, in the presence of millimolar concentrations of **2**, ≥95% of DNA cleavage arises via the cumulene **3**; pathways of DNA cleavage not involving **3** are, at best, minor. The following detailed mechanism emerges for the DNA cleavage reaction. The rate-determining step in the damage of DNA by neocarzinostatin chromophore and the thiols glutathione (GSH), cysteine (CySH), or methyl thioglycolate at physiologically relevant concentrations and pH values is shown to be thiol addition to the chromophore. Evidence is presented to support the notion that the addition of thiols to **1**, whether **1** is free in solution or bound to DNA, is an inherently efficient process. The addition of GSH or CySH is shown to proceed via a ternary complex of DNA, thiol, and chromophore. In neither case does DNA accelerate (catalyze) the thiol addition reaction; rather, it is found to induce a modest decrease in the rate of thiol addition versus control solutions lacking DNA. The greater concentration of DNA-bound chromophore versus chromophore free in solution offsets the attenuated rate of thiol addition to the former. The site of activation appears not to be critical, however, because it is likely that the cumulene intermediate produced is sufficiently long-lived to equilibrate among DNA binding sites. In support of this idea, it is shown that the sequence specificity of DNA cleavage by externally generated cumulene is identical to that of the presumptive cumulene formed in situ from **1** and **2** in the presence of DNA. It is proposed that the species that determines the sequence specificity of DNA cleavage is the cumulene intermediate. The experimental evidence suggests that the cumulene intermediate undergoes cycloaromatization while bound to DNA and that the biradical formed in this cycloaromatization reaction is a highly reactive and poorly selective intermediate. The yield-determining step in the production of thiol adducts from **1** is found to be the quenching of the biradical intermediate by hydrogen atom transfer. It is shown that double-stranded calf thymus DNA and the water-soluble 1,4-cyclohexadiene derivative **14** are approximately equally effective in trapping of the biradical intermediate at concentrations of 5 mM (base pairs) and 1 M, respectively, supporting the idea that the biradical is generated as a DNA-bound species. Although the data do not rule out the possibility that DNA may catalyze the cycloaromatization reaction, this proposal is considered to be unlikely. It is shown that thiol activation of **1** in the presence of single-stranded calf thymus DNA or a heterogeneous mixture of cellular RNA, but not bovine serum albumin, likely occurs as a ternary complex with the biopolymer. Furthermore, single-stranded DNA and heterogeneous cellular RNA are shown to serve as effective hydrogen atom donors for quenching of the biradical product of thiol activation, suggesting that biopolymers other than double-stranded DNA are potential targets for neocarzinostatin-induced damage.

Neocarzinostatin is the first of the enediyne antibiotics to be isolated[1] and characterized[2] and differs from other members of the class[3] both in its formulation as a chromoprotein complex and in the detailed mechanism by which the chromophore portion (**1**)[4] is transformed into a carbon-centered biradical.[5] The latter event is believed to underlie the biological activity of this agent, where evidence has accrued to support the notion that double-stranded DNA is an important cellular target, if not the prime site of action.[6] Treatment of double-stranded B-form DNA with neocarzinostatin chromophore (**1**) and 2-mercaptoethanol in aqueous buffer produces both single- and double-stranded DNA damage.[7] Extensive characterization of the DNA-derived reaction products has implicated a mechanism for the damage process involving hydrogen atom abstraction from the ribose backbone of DNA by one or more radical species.[8] In the absence of DNA and in organic solvents, **1** and methyl thioglycolate (**2**) have been shown to form the monoaddition product **5** via the observable cumulene intermediate **3**[5b] and, as inferred on the basis of deuterium labeling, the biradical **4** (Scheme 1).[4c] While this pathway and, in particular, the biradical **4** provide an appealing mechanistic rationale for DNA damage by neocarzinostatin chromophore, these events have not yet been linked unequivocally.

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(1) (a) Shoji, J. *J. Antibiot.* **1961**, *14*, 27. (b) Ishida, N.; Miyazaki, K.; Kumagai, K.; Rikimaru, M. *J. Antibiot.* **1965**, *18*, 68.

(2) (a) Napier, M. A.; Holmquist, B.; Strydom, D. J.; Goldberg, I. H. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 635. (b) Koide, Y.; Ishi, F.; Hasuda, K.; Koyama, Y.; Edo, K.; Katamine, S.; Kitame, F.; Ishida, N. *J. Antibiot.* **1980**, *33*, 342.

(3) Esperamicin: (a) Golik, J.; Clardy, J.; Dubay, G.; Groenewold, G.; Kawaguchi, H.; Konishi, M.; Krishnan, B.; Ohkuma, H.; Saitoh, K.; Doyle, T. W. *J. Am. Chem. Soc.* **1987**, *109*, 3461. (b) Golik, J.; Dubay, G.; Groenewold, G.; Kawaguchi, H.; Konishi, M.; Krishnan, B.; Ohkuma, H.; Saitoh, K.; Doyle, T. W. *J. Am. Chem. Soc.* **1987**, *109*, 3462. Calicheamicin: (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. Dynemicin: (e) Sugiura, Y.; Shiraki, T.; Masataka, K.; Oki, T. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3831. Kedarcidin (a chromoprotein): (f) Zein, N.; Colson, K. L.; Leet, J. E.; Schroeder, D. R.; Solomon, W.; Doyle, T. W.; Casazza, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 2822. C-1027 (a chromoprotein): (g) Minami, Y.; Yoshida, K.; Azuma, R.; Saeki, M.; Otani, T. *Tetrahedron Lett.* **1993**, *34*, 2633. (h) Yoshida, K.; Minami, Y.; Azuma, R.; Saeki, M.; Otani, T. *Tetrahedron Lett.* **1993**, *34*, 2637. For a review of the enediyne antibiotics, see: (i) Nicolaou, K. C.; Dai, W. M. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1387.

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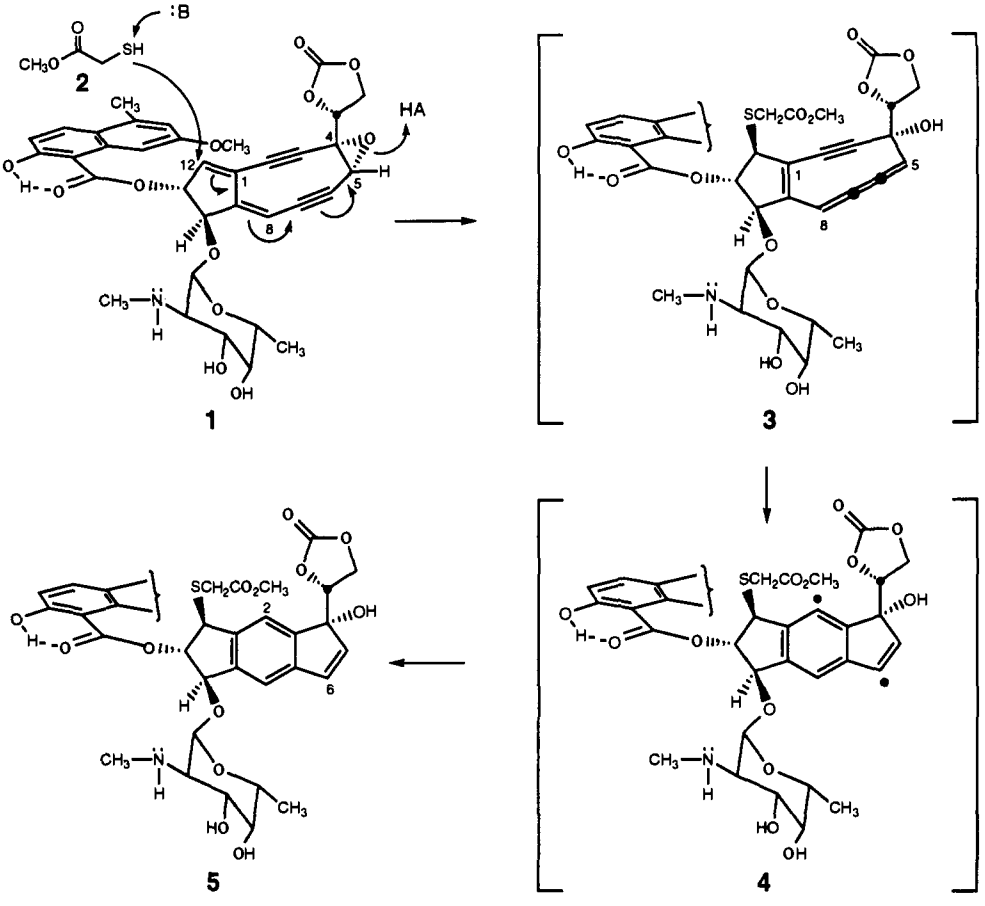
Scheme 1



In this work it is established unequivocally that the cumulene **3** is an intermediate in the cleavage of double-stranded DNA by **1** and **2** in water. Evidence supporting the intermediacy of the biradical **4**, formed by the unimolecular rearrangement of **3**, is also summarized. Given that the pathway **1** + **2** → **3** → **4** → **5** is the chemical sequence by which neocarzinostatin damages DNA in vitro, several questions regarding the details of the molecular mechanism arise. What are the dynamics of the cycloaromatization, DNA-binding, and DNA-damaging steps? Which species determines the sequence-specificity of DNA cleavage? What, if any, is the role of DNA in mediating the transformation of **1** to **5**? More specifically, does DNA function as a formal catalyst for any step in the process? What is the rate-determining step in the DNA cleavage process, and to what extent is this step responsive to changes in parameters of potential physiological significance? Does the chemistry of the biologically relevant thiols glutathione (GSH) and cysteine (CySH) parallel that of methyl thioglycolate (**2**)? These and related questions are addressed below.

(4) Chromophore structure: (a) Edo, K.; Mizugaki, M.; Koide, Y.; Seto, H.; Furihata, K.; Otake, N.; Ishida, N. *Tetrahedron Lett.* **1985**, *26*, 331. Carbohydrate stereochemistry: (b) Edo, K.; Akiyama, Y.; Saito, K.; Mizugaki, M.; Koide, Y.; Ishida, N. *J. Antibiot.* **1986**, *39*, 1615. Chromophore stereochemistry: (c) Myers, A. G.; Proteau, P. J.; Handel, T. M. *J. Am. Chem. Soc.* **1988**, *110*, 7212.

(5) (a) Myers, A. G. *Tetrahedron Lett.* **1987**, *28*, 4493. (b) Myers, A. G.; Proteau, P. J. *J. Am. Chem. Soc.* **1989**, *111*, 1146.

(6) (a) Ono, Y.; Watanabe, Y.; Ishida, N. *Biochim. Biophys. Acta* **1966**, *119*, 46. (b) Beerman, T. A.; Goldberg, I. H.; Poon, R. *Excerpta Medica Internatl Con. Ser.* **1974**, *353*, 347.

(7) (a) Poon, R.; Beerman, T. A.; Goldberg, I. H. *Biochemistry* **1977**, *16*, 486. (b) Hatayama, T.; Goldberg, I. H.; Takeshita, M.; Grollman, A. P. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3603. (c) D'Andrea, A. D.; Haseltine, W. A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3608.

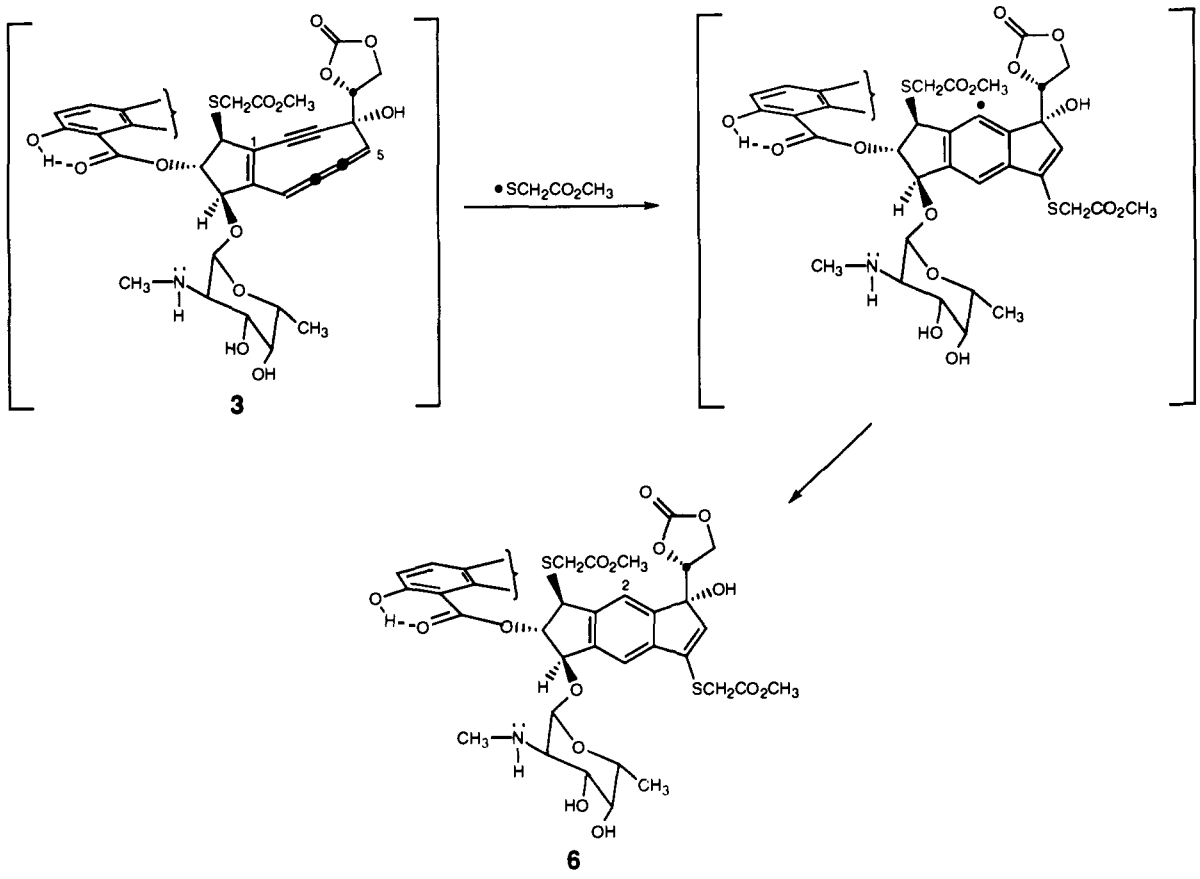
(8) (a) Kappen, L. S.; Goldberg, I. H.; Liesch, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 744. (b) Kappen, L. S.; Goldberg, I. H. *Nucleic Acids Res.* **1985**, *13*, 1637.

Reaction of Neocarzinostatin Chromophore with Thiols in Organic Solvents

The reaction of neocarzinostatin chromophore (**1**, 0.01 M) with methyl thioglycolate (**2**, 0.2 M) in the solvent tetrahydrofuran:acetic acid (9:1; −78 °C → 0 °C) produces the monothiol addition product **5** and the bisthiol addition product **6** in approximately 25% yield each. The formation of **5** is rationalized by the mechanistic pathway previously advanced involving the sequence **1** + **2** → **3** → **4** → **5** (Scheme 1), while the formation of **6** is believed to occur by the mechanism shown in Scheme 2.[4c] Support for the pathway within Scheme 1 is obtained by the direct observation of the proposed cumulene intermediate **3** when the reaction of **1** and **2** is conducted at −70 °C in the probe of an NMR spectrometer. The cumulene is formed in virtually quantitative yield. By conducting the reaction with a lesser concentration of methyl thioglycolate (0.03 M) and by incorporating 1,4-cyclohexadiene (0.2 M), the formation of **6** is suppressed and **3** is observed to undergo first-order decomposition [$k = (1.0 \pm 0.2) \times 10^{-4}$ s^{-1}, −38 °C] to form **5** exclusively, in 68% yield.[5b] Thus, under the conditions described, the rate-determining step for the formation of the adduct **5** is the unimolecular rearrangement of the cumulene **3** to the putative biradical **4** and not the thiol addition reaction that forms **3**. Though the biradical **4** is not observed directly, its intermediacy is supported by the finding that deuterium is incorporated at C2 and C6 of the product **5** when the reaction is conducted in tetrahydrofuran-d_8.[4c]

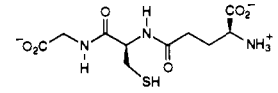
Low-temperature NMR evidence also establishes that the cumulene is an intermediate in the formation of the bisthiol addition product **6**.[4c] The pathway of Scheme 2 is further supported by the fact that the formation of **6** is suppressed in the presence of 1,4-cyclohexadiene and by the observation that deuterium is incorporated at C2 of **6** when the reaction is conducted in tetrahydrofuran-d_8. Because the thiol was not deuterated in

Scheme 2

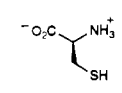


the latter experiment, a pathway involving the polar addition of thiol to the cumulene intermediate may be ruled out. The structures of products **5** and **6** were confirmed by the full range of spectroscopic and analytical methods; these studies led to the determination of all stereochemistry for the product **5** and, by induction, of neocarzinostatin chromophore (**1**).[4c]

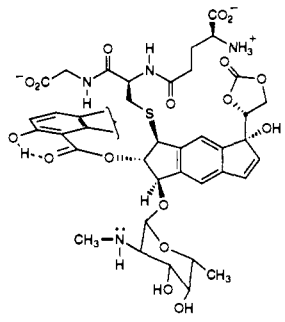
The formation of the adduct **5** from **1** and **2** is not unique to the medium tetrahydrofuran–acetic acid, nor is the thiol addition reaction limited to the thiol methyl thioglycolate (**2**). The reaction of **1** (1×10^{-2} M) with GSH or CySH in 9:1 methanol:water (1×10^{-2} M thiol, 0.3 M 1,4-cyclohexadiene, 0.5 M acetic acid, 0 °C) produces the thiol adduct **7** or **8**, respectively, in approximately 60% yield each. The structures **7** and **8** were fully confirmed by spectroscopic and analytical characterization data. The insolubility of GSH and CySH in tetrahydrofuran–acetic acid or other solvent combinations that are liquids at low temperature has precluded the direct observation by low-temperature NMR spectroscopy of cumulene intermediates from the reactions of **1** with these thiols. The observation that deuterium is incorporated at C2 and C6 of **7** when the thiol addition reaction is conducted in deuterated media supports the intermediacy of biradical intermediates analogous to **4** in the formation of this product as well.[9]



Glutathione (GSH)



Cysteine (CySH)



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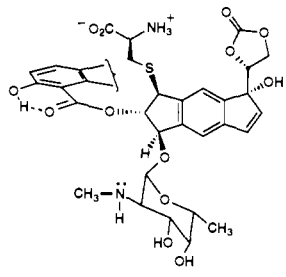

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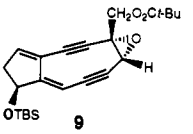
Cumulene Formation by Remote Addition of Thiols—A Rationale for an Unusual Reaction Pathway

Experiments described above demonstrate that S_N'-type epoxide opening by the remote addition of thiols to C12 of **1** in organic solvents is an inherently efficient process. It is shown later that this assertion also extends to reactions conducted in aqueous media. This rather unusual nucleophilic addition reaction and its particular efficiency deserve comment. First, it should be noted that neocarzinostatin chromophore is undoubtedly a highly strained molecule. In addition to the strain attributable to the epoxide ring, recent X-ray crystallographic data indicate that the cyclononadienediyne ring likely contributes to the total strain of **1** as well; the triple bonds are strikingly nonlinear, with a mean

(9) In none of the experiments described did the levels of deuterium incorporated at C2 measurably exceed those at C6; thus, internal hydrogen transfer of the bound thiol α-hydrogen atoms to the C2-centered radical cannot be a very rapid reaction. Consideration of the likely conformation of the biradical, with the thiol group in a pseudoaxial orientation, provides a rationale for the relatively low rate of internal transfer. It would appear that in order for such a reaction to take place, it is necessary to decrease substantially the concentration of potential trapping species by the use of a nondonor solvent. The recent documentation of low levels of internal transfer when glutathione is the activating thiol in an aqueous medium may represent such a case. See: Chin, D.; Goldberg, I. H. *J. Am. Chem. Soc.* **1992**, *114*, 1914.

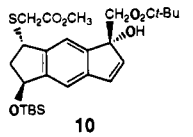
C—C≡C angle of 161.5 ± 1.2°.[10] The strain within **1** is released in a stepwise fashion, such that each of the two steps leading to biradical formation is facilitated. Thus, the strain released upon the opening of the epoxide provides a driving force for the thiol addition reaction, while the strain released upon cycloaromatization of the cumulene **3** drives the formation of the biradical **4**.

It is not immediately obvious why thiol addition occurs at C12 of **1** (formal 1,8-addition) rather than by the more direct 1,2-opening of the epoxide, as is observed upon treatment of **1** with hydrogen chloride or hydrogen bromide.[4a,11] A rationale for this unusual reactivity, in which it is proposed that the carbohydrate amino group participates in the thiol addition reaction, was recently suggested on the basis of experiments conducted with the synthetic chromophore analogue **9**. It was found that **9** is



9

unreactive toward methyl thioglycolate (**2**, 0.3 M) in 9:1 tetrahydrofuran:acetic acid below +60 °C (decomposition ensues at this temperature), whereas **1** reacts rapidly with **2** at −70 °C in the same medium. When triethylamine (0.3 M) was added to the former reaction solution at 23 °C, **9** was observed to form the addition product **10** in modest yield.[12] Parenthetically, it can



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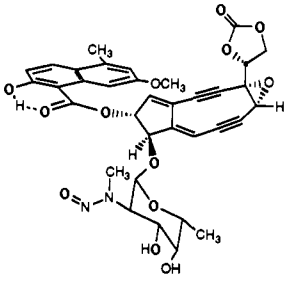
be concluded from this result that the epoxide opening reaction is not restricted to an anti addition mode. The more important conclusion, however, concerns the likely role of the carbohydrate amino group in the thiol addition reaction in organic solvents. This hypothesis is supported by the observation that the *N*-nitroso derivative of neocarzinostatin chromophore (**11**) is also unreactive toward methyl thioglycolate in tetrahydrofuran–acetic acid.[12]



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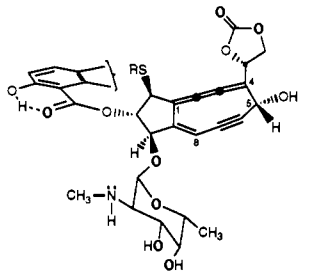
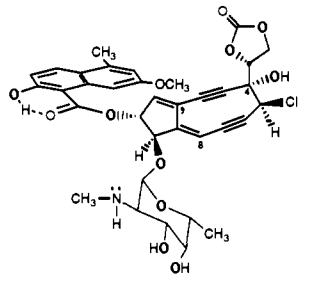
The thiol addition reaction, then, may be viewed as being facilitated by the formation of an amine–thiol hydrogen bond or ion pair, thus positioning the thiol or thiolate directly above C12.[13] In further support of this hypothesis, it was found that in the X-ray crystal structure of the neocarzinostatin protein–chromophore complex, the amino group of **1**, believed to be protonated, is located directly above C12 at a distance of ~5 Å, or approximately the van der Waals diameter of a sulfur atom.[10] At present, the thiol addition reaction is viewed as a "push–pull" mechanism, that is, as potentially involving a degree of concert, where proton-assisted opening of the epoxide accompanies ammonium-directed thiolate addition. Such a mechanism would invoke the formation of a partial positive charge at C5 in the transition state, a suggestion that may explain the observed direction of the epoxide opening. The alternative mode of opening, opening at C4, would produce the cumulene intermediate **12** and would require that C4 become electron deficient in the transition state. The acyloxy substituent adjacent to C4 should disfavor



12

this mode of opening over opening at C5. Alternatively, it may be that **12** is simply enthalpically disfavored relative to **3**. The observation that mineral acids add to **1** in a 1,2-fashion (see structure **13**)[4a,11] is believed to reflect a shift in the mechanistic continuum toward a more S_N1-type nucleophilic addition of halide ion to a more fully formed C5 cation.



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Translation of Thiol Addition Experiments in Organic Media to DNA Cleavage in Aqueous Solution

A prime objective of our work was to establish the relevance of experiments with **1** and thiols in organic media to the observed cleavage of double-stranded DNA by **1** and thiols in water. Existing data support the idea that the biradical **4** of Scheme 1, or the analogous biradical from the addition of a thiol other than methyl thioglycolate, is the species directly responsible for part or all of the observed DNA damage. Detailed characterization of both single- and double-stranded DNA cleavage sites is consistent only with free-radical-mediated chemistry, involving specifically the abstraction of 1′-, 4′-, and 5′-hydrogen atoms from the ribose backbone of DNA.[8b,14] Subsequent trapping of the resultant ribosyl radicals by molecular oxygen leads to irreversible DNA damage.[15]

Early experiments had suggested that the free-radical agent responsible for the initial abstraction event was chromophore-derived. Treatment of 5′-^{3}H-labeled poly(dA-dT) with **1** and dithiothreitol was reported to form a chromophore-derived product with covalent incorporation of tritium, although this product was

(10) Kim, K.-H.; Kwon, B.-M.; Myers, A. G.; Rees, D. C. *Science* **1993**, *262*, 1042.

(11) Edo, K.; Akiyama-Murai, Y.; Saito, K.; Mizugaki, M.; Koide, Y.; Ishida, N. *J. Antibiot.* **1988**, *41*, 1272.

(12) Myers, A. G.; Harrington, P. M.; Kwon, B.-M. *J. Am. Chem. Soc.* **1992**, *114*, 1086.

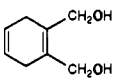
(13) Mechanisms involving a neutral amine–thiol pair or an ammonium–thiolate ion pair are kinetically indistinguishable (Jencks, W. P. In *Catalysis in Chemistry and Enzymology*; Dover, Ed.; Dover Pub. Inc.: New York, 1987; p 182). Thus, the protonation state of the amino group does not, per se, support or rule out mechanisms involving participation of this group in the thiol activation step, contrary to the arguments of Cramer and Townsend (Cramer, K. D.; Townsend, C. A. *Tetrahedron Lett.* **1991**, *32*, 4635).

(14) (a) Kappen, L. S.; Chen, C.; Goldberg, I. H. *Biochemistry* **1988**, *27*, 4331. (b) Sugiyama, H.; Kawabata, H.; Fujiwara, T.; Dannoue, Y.; Saito, I. *J. Am. Chem. Soc.* **1990**, *112*, 5252.

(15) (a) Chin, D.; Carr, S. A.; Goldberg, I. H. *J. Biol. Chem.* **1984**, *259*, 9975. (b) Goldberg, I. H. *Free Rad. Biol. Med.* **1987**, *3*, 41.

not characterized.[8b] More recently, low levels of deuterium transfer from various synthetic oligonucleotides specifcally labeled at 1′ and 5′ sites to C2 and C6, respectively, of the glutathione addition product **7** have been observed,[16] supporting a proposed model for the binding of **4** in double-stranded DNA damage.[17]

The chemistry of neocarzinostatin chromophore (**1**) is decidedly complex, however, and it remained to be established at the outset of our studies the extent to which the mechanism of Scheme 1 was responsible for the damage of DNA. For example, **1** is known to decompose within minutes in water (pH 7.5), in the absence of thiols, to form an ill-characterized product mixture.[18] It has also been shown that **1** is capable of DNA cleavage in the absence of thiols within certain bulged DNA conformations. The latter DNA cleavage reaction proceeds with different sequence specificity than the corresponding thiol-induced reaction.[19] Alternative paths for the reaction of **1** with thiols in water, both in the presence and in the absence of the neocarzinostatin apoprotein, have also been proposed.[20] Furthermore, while the incubation of **1** with methyl thioglycolate in water in the presence of the water-soluble 1,4-cyclohexadiene derivative **14** (conducted in analogy to experiments in organic solvents) does produce the thiol adduct **5** as the major reaction product, it is formed in less than 12% yield (vide infra). It is clear that the potential exists for multiple



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mechanisms to operate in the production of the spectrum of cleavage products observed upon aerobic incubation of DNA with **1** and methyl thioglycolate in water. We describe below a conceptually simple experiment that unequivocally establishes that ≥95% of DNA cleavage observed upon incubation of a synthetic 35-mer oligonucleotide with **1** and **2** in water arises from the cumulene **3** and, likely, the biradical **4**.

Establishing the Intermediacy of a Chromophore-Derived Cumulene in DNA Cleavage by Neocarzinostatin Chromophore and Methyl Thioglycolate

The ability to generate and study the cumulene **3** at low temperatures in organic solvents provides the opportunity to probe directly the role of this intermediate in the DNA cleavage reaction. The specific experiment suggested is to compare the cleavage of double-stranded DNA by the cumulene **3**, generated independently, with cleavage by the incubation of **1** and **2** in the presence of DNA. If **3** is an intermediate leading to all or part of DNA damage by **1** and **2**, then this should be revealed upon comparison of the two cleavage reactions. A caveat in the interpretation of this experiment concerns the possibility that the reaction of **1** and **2** occurs as a ternary complex with DNA such that **3** is generated as a DNA-bound species. In this case, the dynamics of binding of externally generated **3** to DNA relative to the rearrangement of **3** to the biradical **4** becomes important. Should the rearrangement of **3** to **4** be faster or comparable in rate to the binding of **3** to DNA, then the DNA cleavage patterns could differ for the two experiments.

This classic test of a mechanistic proposal—that a postulated intermediate generated by two independent pathways should transform identically under equivalent conditions—proved to be challenging in practice. It was necessary to repeat the experiment several times in order to perfect techniques for the low-temperature manipulation and transfer of the highly reactive cumulene intermediate (see Experimental Section). With practice, highly reproducible results could be obtained. The cumulene **3** (3×10^{-5} M) was prepared in near-quantitative yield at −70 °C, as previously described, and was transferred cold to a parallel series of solutions of double-stranded calf thymus DNA (4×10^{-4} M, base pairs) in aqueous buffer of varying pH (final pH values 6.0, 6.5, 7.5, and 8.3) at 2 °C, each containing trace quantities of the 5′-^{32}P-labeled synthetic 35-mer duplex DNA (5′-^{32}P-GCAAAC-CAGCGTGGACCGCTTGCTGCAACGTGGAC-3′) and sufficient methyl thioglycolate (**2**) to achieve a final concentration of 7.5×10^{-4} M. A parallel series of experiments was conducted using **1** in lieu of **3**; final concentrations of solution components and pH values were otherwise the same. Portions of each reaction solution were quenched after 20 and 60 min, and the quenched aliquots were applied to a 20% denaturing polyacrylamide gel for analysis of the DNA cleavage products. Quantitative analysis of the cleavage bands was obtained by storage phosphor autoradiography.

Consideration of the gel data (Figure 1) provides several insights into the details of DNA cleavage by neocarzinostatin chromophore. First, it should be noted that the cumulene **3** does cleave DNA, and it does so with identical sequence specificity to DNA cleavage arising from **1** and **2**. Second, it can be seen that while DNA cleavage by **3** is almost invariant with pH, the intensity of DNA cleavage from the reaction of **1** and **2** varies markedly with pH. Importantly, the maximal cleavage efficiency with **3** and with **1** and **2** is approximately the same, approaching a value of 25%. One conclusion from these observations is that the cumulene is an obligate intermediate in ≥95% of the observed DNA cleavage by **1** and **2**. While minor reaction paths not involving the cumulene **3** may operate, they must contribute negligibly to DNA cleavage. It may also be concluded that the sequence specificity of DNA cleavage by **1** and **2** is determined by the cumulene **3** or a later intermediate. Because the next species in the reaction pathway is the biradical **4**, and given that the lifetime of this intermediate is likely too brief to permit its equilibration among DNA binding sites, it is reasonable to narrow this conclusion by proposing that the cumulene **3** is the sequence-determining species in DNA cleavage by **1** and **2**.[21]

Analysis of the gel data reveals that, in contrast to reactions conducted in organic solvents, thiol addition is the rate-determining step in DNA cleavage by **1** and **2** in water at pH 7.5 and below. This is shown by comparison of cleavage bands from the cumulene **3**, from the reaction of **1** and **2** (20 min duration), and from the reaction of **1** and **2** (60 min duration) at a given solution pH (e.g., lanes 4 and 8, 20 and 60 min reaction time, respectively, Figure 1). The relative insensitivity of DNA cleavage by **3** toward variations in pH versus the markedly diminished rate of DNA cleavage by **1** and **2** at lower pH values demonstrates that thiol addition is rate-determining at or below pH 7.5 (that the diminished cleavage by **1** and **2** is due to a lower reaction rate and not, e.g., reduced binding affinity at lower pH or an alternative mode of reaction is revealed on examination of the gel data for the extended reaction period of 60 min). The fact that the maximal efficiency of DNA cleavage by **3** and by **1** and **2** is effectively the

(16) (a) Meschwitz, S. M.; Goldberg, I. H. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 3074. (b) Meschwitz, S. M.; Schultz, R. G.; Ashley, G. W.; Goldberg, I. H. *Biochemistry* **1992**, *31*, 9110.

(17) Galat, A.; Goldberg, I. H. *Nucleic Acids Res.* **1990**, *18*, 2093.

(18) Povirk, L. F.; Goldberg, I. H. *Biochemistry* **1980**, *19*, 4773.

(19) Kappen, L. S.; Goldberg, I. H. *Science* **1993**, *261*, 1319.

(20) (a) Tanaka, T.; Fujiwara, K.; Hirami, M. *Tetrahedron Lett.* **1990**, *31*, 5947. (b) Sugiyama, H.; Yamashita, K.; Nishi, M.; Saito, I. *Tetrahedron Lett.* **1992**, *33*, 515.

(21) The rate of equilibration among DNA binding sites for the cumulene and biradical intermediates may be roughly approximated by the rate of reversible association of the chromophore to poly(dA-dT). Free chromophore binds to poly(dA-dT) with a rate constant of 7.8×10^5 M^{-1} s^{-1} at 2 °C. Dissociation of the chromophore from the bound complex occurs more slowly, with a rate of 21 s^{-1}, corresponding to a half-life of ~0.03 s (Dasgupta, D.; Auld, D. S.; Goldberg, I. H. *Biochemistry* **1985**, *24*, 7049). The rate of hydrogen atom transfer from DNA to the DNA-bound biradical may be estimated by the rate of hydrogen atom transfer from methanol to phenyl radical ($k \sim 1.4 \times 10^5$ M^{-1} s^{-1}, 25 °C).[22] If the effective concentration of DNA-bound transferable hydrogen atoms is 1 M, then the rate of quenching of the DNA-bound biradical may be approximated as $k \geq 1.4 \times 10^5$ s^{-1}. This corresponds to a half-life for the bound biradical of $\leq 5 \times 10^{-6}$ s.

(22) Packer, J. E.; House, D. B.; Rasburn, E. J. *J. Chem. Soc. B* **1971**, 1574.

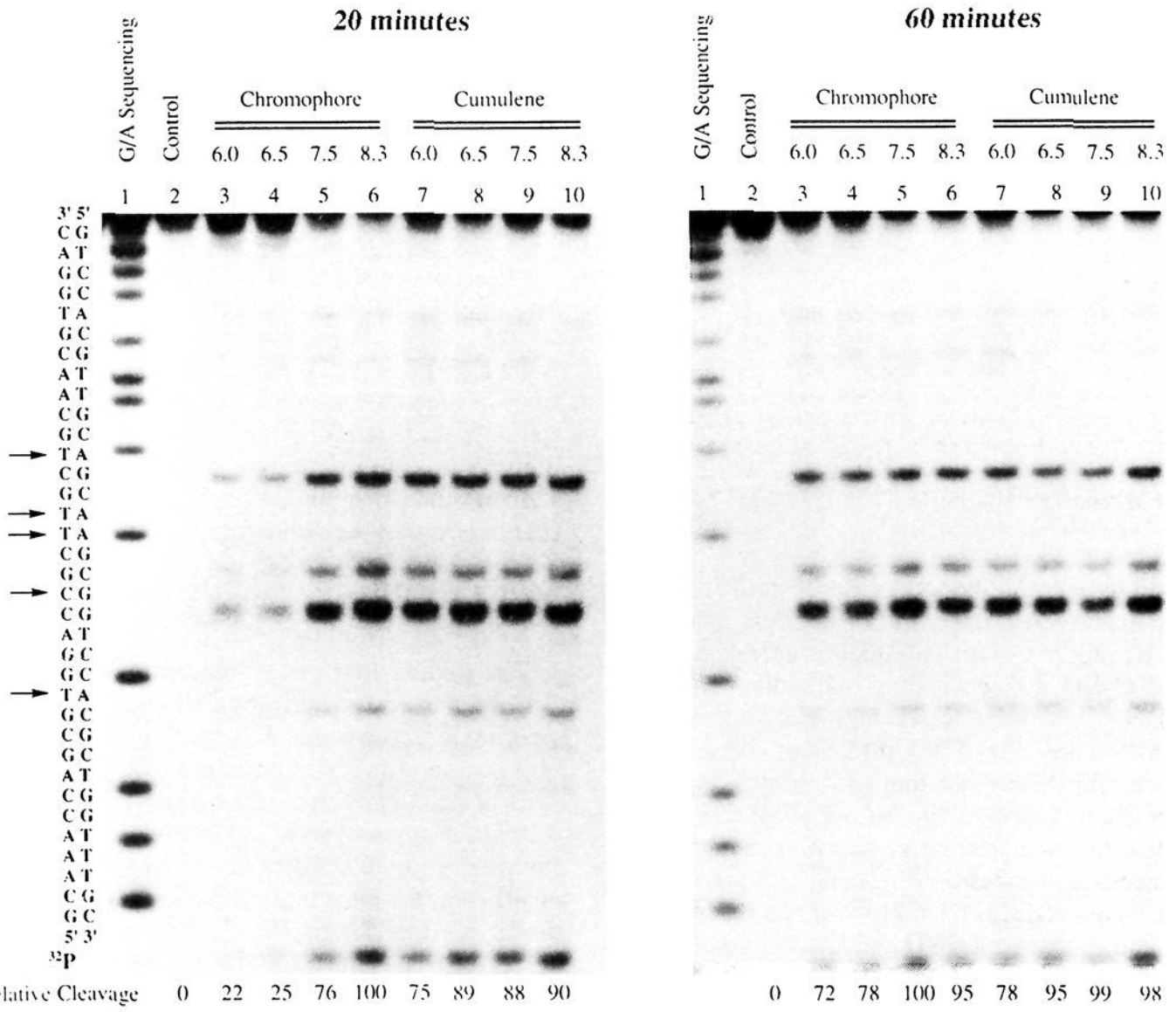

Figure 1. Time course of DNA cleavage by neocarzinostatin chromophore (**1**) and methyl thioglycolate (**2**) and by the cumulene **3**. Reactions were monitored at times of 20 and 60 min. Lane 1, Maxam–Gilbert G/A sequencing reaction;[32] lane 2 (control reaction lacking thiol), **1** (3×10^{-5} M), DNA (4×10^{-4} M, base pairs); lanes 3–6, **1** (3×10^{-5} M), DNA (4×10^{-4} M, base pairs), **2** (7.5×10^{-4} M), pH 6.0, 6.5, 7.5, and 8.3, respectively; lanes 7–10, **3** (3×10^{-5} M), DNA (4×10^{-4} M, base pairs), **2** (7.5×10^{-4} M), pH 6.0, 6.5, 7.5, and 8.3, respectively. "Relative cleavage" is defined as the percent of DNA cleavage relative to the lane of highest cleavage intensity (assigned a value of 100).

same demonstrates that thiol addition is an efficient reaction in the presence of DNA. This conclusion is confirmed in experiments described below.

Thiol Activation of Neocarzinostatin Chromophore (1)—A General and Efficient Process in Aqueous Solution

The efficiency of formation of the thiol adduct **5** from the reaction of **1** (1×10^{-4} M) and **2** (5×10^{-3} M) in aqueous solution (Tris–acetate buffer, 5×10^{-2} M, pH 7.5, 2 °C) containing either double-stranded calf thymus DNA (5×10^{-3} M, base pairs) or a control solution[23] containing equimolar amounts of each of the four nucleosides A, C, G, and T (2.5×10^{-3} M each) and potassium dimethyl phosphate (1×10^{-2} M) was determined quantitatively by reverse-phase high-performance liquid chromatography (rp-HPLC). Both experiments incorporated the water-soluble 1,4-cyclohexadiene derivative **14** (4×10^{-2} M) as a hydrogen atom donor. In each experiment, the adduct **5** was obtained as, by far, the major reaction product; however, the yield of **5** in the presence of DNA was found to be almost twice that obtained in the control solution (23% versus 12%). These results were found to be reproducible through multiple determinations of the data, forcing the conclusion that DNA must somehow play a beneficial role in the formation of **5**. This may be envisioned to occur in any or all of the following steps: (1) thiol addition, (2) cycloaromatization of the cumulene **3** to the biradical **4**, and (3) quenching of the biradical **4**. The ability to generate the cumulene **3** independently provides a means to evaluate specifically the role of DNA in improving the efficiency of the thiol addition step. Parallel incubations of the cumulene **3** (1×10^{-4} M) with double-stranded calf thymus DNA or a control solution lacking DNA (as above) were found to afford the adduct **5** in 26% and 12% yield, respectively. These results virtually replicate those obtained with **1** above. Two conclusions may be drawn from this observation. First, it is clear that the improved efficiency of formation of the adduct **5** in the presence of DNA must be attributable to steps 2 and/or 3 identified above and not the thiol addition step. Second, because the yields of the adduct **5** are virtually identical whether the reaction is conducted with the chromophore (**1**) or with the cumulene **3**, the efficiency of thiol addition to **1** in water must be quite high, both in the presence and in the absence of DNA. The loss in yield, then, must occur either at the stage of cycloaromatization of the cumulene **3** and/or in the trapping of the biradical **4**. Evidence presented below suggests that it is the latter event that is the yield-determining step in adduct formation.

Similar conclusions follow from experiments conducted with the thiols GSH and CySH. Incubation of **1** (1×10^{-4} M) and GSH or CySH (5×10^{-3} M) with increasing concentrations of double-stranded calf thymus DNA (0, 5×10^{-4}, and 5×10^{-3} M, base pairs) leads to a monotonic increase in the yield of adduct formation for both thiols (Table 1). In light of experiments with **1** and **3** just described, it is reasonable to ascribe the improvement in yield in these experiments to the influence of DNA in a step after thiol addition, e.g., in biradical trapping. Competition experiments involving the incubation of **1** with a 1:1 mixture of GSH and CySH (2.5×10^{-3} M each thiol) and varying concentrations of DNA (0, 5×10^{-4}, 5×10^{-3} M, base pairs, entries 7, 10, and 11, respectively, Table 1) suggest that this

(23) Neocarzinostatin chromophore was found to be fully soluble in aqueous solution (solubility $\leq 1 \times 10^{-4}$ M) in the absence of DNA, as determined by light-scattering analysis. This stands in marked contrast to calicheamicin, which is virtually insoluble in aqueous solution in the absence of DNA (Myers, A. G.; Cohen, S. B.; Kwon, B.-M. *J. Am. Chem. Soc.*, in press).

Table 1. Influence of DNA on Yields of Thiol Adducts 7 and 8[a]

entry	[GSH]	[CySH]	[DNA]	[14]	7	8	7 + 8
1	5.0			40	7		7
2	5.0		0.5	40	26		26
3	5.0		5.0	40	44		44
4		5.0		40		8	8
5		5.0	0.5	40		27	27
6		5.0	5.0	40		47	47
7	2.5	2.5		40	5	2	7
8	2.5	2.5		200	17	7	24
9	2.5	2.5		1000	38	13	51
10	2.5	2.5	0.5	40	13	13	26
11	2.5	2.5	5.0	40	18	26	44

[a] Concentrations expressed in units of mM, yields as percent of theoretical maximum. Yields determined by rp-HPLC by integration against an internal standard of 2,5-dimethoxybenzyl alcohol. All reactions employed 1 (1×10^{-4} M) and Tris-HCl buffer (4×10^{-2} M, pH 7.5) and were conducted at 2 °C.

interpretation is correct. While the total yield of thiol adducts is found to be constant at a given concentration of DNA, whether GSH, CySH, or a 1:1 mixture of the two thiols is employed, the ratio of GSH and CySH adducts varies markedly with the concentration of DNA. In order to see how the competition experiments support the idea that DNA influences a step after thiol addition, it is useful to consider that the converse is true, i.e., that DNA somehow improves the efficiency of the thiol addition step relative to some alternative transformation of 1, such as its spontaneous decomposition in water. If the latter were true, comparison of entries 1–6 (Table 1) shows that DNA must facilitate the addition of both GSH and CySH to the same degree. However, comparison of entries 7, 10, and 11 (Table 1) shows that the ratio of GSH and CySH adducts (7 and 8, respectively) decreases with increasing concentrations of DNA, although the total yield of adducts is constant. This is inconsistent with the proposal that DNA facilitates both thiol addition reactions equivalently.

The data are interpreted in the following way. Thiol addition is believed to be inherently efficient for both GSH and CySH in the presence and absence of DNA. The ratio of adducts varies with the concentration of DNA in the medium because the relative rates of thiol addition to DNA-bound and free chromophore are different for GSH and CySH, an assertion which is verified below. The effect of DNA upon the efficiency of adduct formation, then, is exerted after cumulene formation, and, as confirmed in experiments described below, most likely at the stage of biradical trapping.

If the trapping of the biradical is the yield-determining step, then it should be possible to mimic the beneficial effect of DNA in reactions lacking DNA by increasing the concentration of the trapping agent in the medium. This is found to be the case (entries 7, 8, 9, Table 1). Significant enhancements in the yields of thiol adducts are observed upon increasing the concentration of 14 in the medium, supporting the idea that thiol addition is an inherently efficient process; it is the trapping of the biradical that determines the efficiency of adduct formation.

It is particularly revealing that 200–400-fold greater concentrations of 14 are required to mimic the beneficial effect of DNA upon the efficiency of the reaction. The data cannot be rationalized in terms of the C–H bonds strengths (bond dissociation energies, BDEs) of the trapping agents; the allylic C–H bonds of 14 (BDE ~ 73 kcal/mol)[24] are substantially weaker than any ribosyl C–H bond (BDEs estimated to be 85–95 kcal/mol). The only reasonable interpretation of the data is that the trapping of the biradical by DNA is an unimolecular process, while its trapping by 14 is a bimolecular event. In other words, the data suggest that the biradical is generated as a DNA-bound species and that it is trapped at the site of its generation. This

(24) Burkey, T. J.; Majewski, M.; Griller, D. *J. Am. Chem. Soc.* **1986**, *108*, 2218.

Table 2. Second-Order Rate Constants for the Reactions of Neocarzinostatin Chromophore with Glutathione and Cysteine in the Presence and Absence of Double-Stranded DNA[a]

thiol	DNA (mM bp)	k (M^{-1} s^{-1})
GSH	0.0	60 ± 9
GSH	5.0	4.0 ± 0.4
CySH	0.0	30 ± 4
CySH	5.0	6.5 ± 0.5

[a] Reactions monitored by rp-HPLC: 1 (1×10^{-4} M), thiol (5×10^{-4} M), Tris-HCl buffer (4×10^{-2} M, pH 7.5), 2 °C.

is not a surprising result and has been widely conjectured on the basis of previous estimates of the reactivity of biradicals of this type. The data reinforce the idea that the biradical has insufficient lifetime to equilibrate among DNA binding sites[21] and thus supports the assertion above that the cumulene 3 is the species that determines the sequence specificity of DNA cleavage by 1.

Is DNA a Catalyst for the Thiol Activation of Neocarzinostatin Chromophore?

The finding that molar concentrations of the water-soluble 1,4-cyclohexadiene derivative 14 mimic the efficiency of DNA (millimolar, base pairs) in the trapping of chromophore-derived biradical intermediates permits a meaningful comparison of the rates of reaction of neocarzinostatin chromophore with thiols in water in the presence and absence of DNA. Prior to this finding, the yields of thiol adducts in water in the absence of DNA were insufficient for meaningful kinetics measurements. The importance of this comparison is that it allows for the direct determination of the role of DNA as a potential catalyst in the reaction.

Results from competition experiments with 1:1 mixtures of GSH and CySH, described above, suggest that DNA influences the rate of addition of one or both of these thiols versus the corresponding reaction in water without DNA. Kinetics measurements for the reactions of both GSH and CySH (5×10^{-4} M) with 1 (1×10^{-4} M) were obtained by rp-HPLC monitoring of the disappearance of 1 at 2 °C (Tris-HCl buffer, pH 7.5) in the presence of either double-stranded calf thymus DNA (5×10^{-3} M, base pairs) or a control solution containing 14 (1 M), each of the four nucleotides A, C, G, and T (2.5×10^{-3} M each), and potassium dimethyl phosphate (1×10^{-2} M). For both GSH and CySH it is found that the thiol addition reaction is slower in the presence of DNA that in its absence, albeit to different degrees (Table 2). The rate ratio (–DNA/+DNA) is 15 for GSH and 4.6 for CySH. Thus, DNA does not function as a catalyst for either reaction. The rate constants confirm observations from competition experiments employing a 1:1 mixture of the two thiols. The rate ratio (GSH/CySH) in the presence of DNA is calculated to be 0.6 (observed product ratio 0.6), while the rate ratio (GSH/CySH) in the absence of DNA is 2 (observed product ratio 2.6).

The relative concentrations of DNA-bound chromophore and free chromophore in experiments with calf thymus DNA (5×10^{-3} M, base pairs) may be calculated from the established binding constant:[18]

$$[1{\cdot}\mathrm{DNA}]/[1_{\mathrm{free}}] = [\mathrm{DNA}_{\mathrm{free}}]K_{\mathrm{B}} = (\sim 5 \times 10^{-3}\ \mathrm{M})(4 \times 10^{6}\ \mathrm{M}^{-1}) = 2 \times 10^{5}$$

From this ratio it can be determined that the reactions of both GSH and CySH with 1 in the presence of DNA occur primarily as a ternary complex of 1, thiol, and DNA. Although both thiols react more rapidly with free chromophore than with bound chromophore (factor of 5–15), the rate difference is insufficient to offset the concentration effect (factor of 2×10^5). The fact that the smaller, neutral thiol CySH is faster to react with 1 bound to the polyanion DNA than is the larger, negatively-charged

thiol GSH can be rationalized on the basis of steric and electronic factors. It is less obvious why GSH is faster to react with free chromophore than is CySH.

Although our experiments demonstrate that the cumulene intermediate is generated as a DNA-bound species for both GSH and CySH activation, estimates of the lifetime of this intermediate ($t_{1/2} \sim 20$ s at 2 °C)[5b] suggest that it is likely that it is sufficiently long-lived to equilibrate among DNA binding sites.[21] Experiments with the cumulene **3** described above support this notion. The data suggest that proposals involving a kinetic basis for the sequence specificity of DNA cleavage by **1**,[12,25] as opposed to a thermodynamic binding of the cumulene **3**, are unlikely to be correct. While the data do not address the possibility that DNA may catalyze the cycloaromatization of the cumulene **3** to the biradical **4** (presenting another possibility for a kinetic effect in the sequence specificity of DNA cleavage by **1**), this proposal seems unlikely in view of the nature of the unimolecular reaction involved, and evidence refuting a similar proposal[26] concerning the activated intermediate in the cleavage of DNA by calicheamicin has recently been presented.[27]

Biopolymers Other than Double-Stranded DNA as Potential Substrates for Neocarzinostatin Chromophore-Induced Damage

Competition experiments described above employing a 1:1 mixture of GSH and CySH provide a sensitive assay for the participation of double-stranded DNA in both the thiol addition and the biradical trapping steps. The former is manifested in an altered ratio of thiol adducts (glutathione adduct (**7**) is favored in the absence of double-stranded DNA, cysteine adduct (**8**) is favored in the presence of double-stranded DNA), while the latter is manifested in an improved yield of thiol adducts (see Table 1). This simple assay has been used to explore the potential participation of biopolymers other than double-stranded DNA in the thiol activation of **1** and to evaluate the possibility that these biopolymers may serve as potential targets for neocarzinostatin-induced damage.

Figure 2 illustrates the results of competition experiments employing a 1:1 mixture of GSH and CySH (**1**, 1×10^{-4} M; GSH, 2.5×10^{-3} M; CySH, 2.5×10^{-3} M; **14**, 4×10^{-2} M; Tris-HCl buffer, 4×10^{-2} M, pH 7.5) in the presence of the following biopolymers: double-stranded calf thymus DNA (5×10^{-3} M, base pairs, Figure 2b), single-stranded calf thymus DNA (1×10^{-2} M, nucleotide, Figure 2c), a heterogeneous mixture of calf liver cellular RNA (1×10^{-2} M, nucleotide, primarily ribosomal RNA,[28] Figure 2d), tRNA (1×10^{-2} M, nucleotide, heterogeneous anticodon, Figure 2e), and bovine serum albumin (1.5×10^{-5} M, Figure 2f). A control experiment conducted with A, C, G, and T (2.5×10^{-3} M each nucleoside) and potassium dimethyl phosphate (1×10^{-2} M) in lieu of biopolymer is also shown (Figure 2a). Results from these experiments are readily interpreted, as described in the preceding paragraph. Thus, bovine serum albumin (Figure 2f) appears to play no role in either thiol addition to **1** or the trapping of biradical intermediates derived from **1**. In contrast, tRNA (Figure 2e) induces an altered ratio of thiol adducts, but does not substantially improve the efficiency of thiol adduct formation. This suggests that thiol addition to **1** occurs at least partially via tRNA-bound **1** but that tRNA does not serve as a trap for the biradical products of thiol activation and thus is not a target for neocarzinostatin-induced damage. Results from experiments with single-stranded DNA (Figure 2c)[29] and heterogeneous cellular RNA (Figure 2d) show that, like

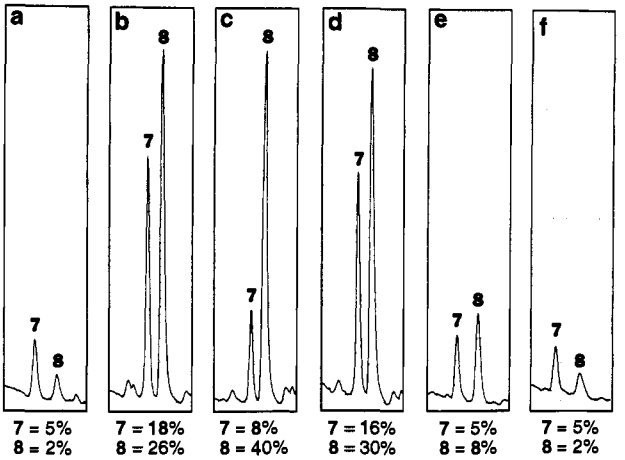

Figure 2. HPLC chromatograms illustrating the distribution of GSH and CySH thiol adducts (**7** and **8**, respectively) arising from competition experiments with **1** and a 1:1 mixture of GSH and CySH in the presence of various biopolymers. All reactions contained **1** (1×10^{-4} M), GSH (2.5×10^{-3} M), CySH (2.5×10^{-3} M), **14** (4×10^{-2} M), Tris-HCl buffer (4×10^{-2} M, pH 7.5, 2 °C). (a) A, C, G, and T (2.5×10^{-3} M each nucleoside) with potassium dimethyl phosphate (1×10^{-2} M). (b) Double-stranded calf thymus DNA (5×10^{-3} M, base pairs). (c) Single-stranded calf thymus DNA (1×10^{-2} M, nucleotide). (d) Heterogeneous mixture of calf liver cellular RNA (1×10^{-2} M, nucleotide). (e) tRNA (1×10^{-2} M, nucleotide, heterogeneous anticodon). (f) Bovine serum albumin (1.5×10^{-5} M).

double-stranded DNA, these biopolymers influence both stages of the thiol adduct formation from **1**, and, as efficient traps for the biradical products of thiol activation, they are potential candidates for neocarzinostatin-induced damage. The fact that the heterogeneous mixture of cellular RNA is apparently an efficient trap for the biradical products of neocarzinostatin chromophore activation, whereas tRNA is not, must be attributable to the presence of binding sites within the conformationally variable cellular RNA that are lacking in the compact, ordered tRNA structure.[30]

These conclusions have, in part, been confirmed by cleavage assays of the appropriate radiolabeled biopolymers. Thus, while no observable cleavage of ^{32}P-labeled tRNA^{Phe} by **1** and GSH is observed (data not shown), ^{32}P-labeled single-stranded DNA is found to be a viable target for cleavage by **1** in the presence of GSH or CySH.[29] Figure 3 displays the results of the time course of reactions of **1** with both a ^{32}P-labeled 35-mer single-stranded DNA oligonucleotide and a ^{32}P-labeled 35-mer double-stranded DNA duplex incubated separately with the thiols GSH and CySH, as determined by gel electrophoresis. The gel data corroborate conclusions derived from the neocarzinostatin chromophore product analysis studies just described (Figure 2). That is, single-stranded DNA is shown to be a target for neocarzinostatin chromophore-induced damage. It should be noted that this finding stands in marked contrast to prior studies.[19,29] It can also be seen that single-stranded DNA favors CySH addition over GSH addition to an even greater extent than does double-stranded DNA, and thus single-stranded DNA must influence the thiol addition step in the reaction as well.

The more-involved experiments necessary to evaluate cleavage (or lack thereof) within the biopolymers bovine serum albumin or the heterogeneous mixture of cellular RNA employed above have not been conducted; conclusions regarding these molecules must, therefore, be regarded as tentative at this time. The

(25) For an example of kinetic selectivity in DNA damage, see: Baker, B. F.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 2700.
(26) De Voss, J. J.; Hangeland, J. J.; Townsend, C. A. *J. Am. Chem. Soc.* **1990**, *112*, 4554.
(27) Walker, S.; Landovitz, R.; Ding, W. D.; Ellestad, G. A.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4608.
(28) Zubay, G. L. *Biochemistry*; The Benjamin/Cummings Publishing Co., Inc.: Menlo Park, CA, 1983; Chapter 21.
(29) As pointed out by a reviewer, it cannot be ruled out at present that double-stranded regions of DNA are formed transiently by association of single-stranded DNA in these experiments and that these regions are the targets of neocarzinostatin chromophore-induced damage.
(30) Saenger, W. *Principles of Nucleic Acid Structure*; Spinger-Verlag: New York, 1984.

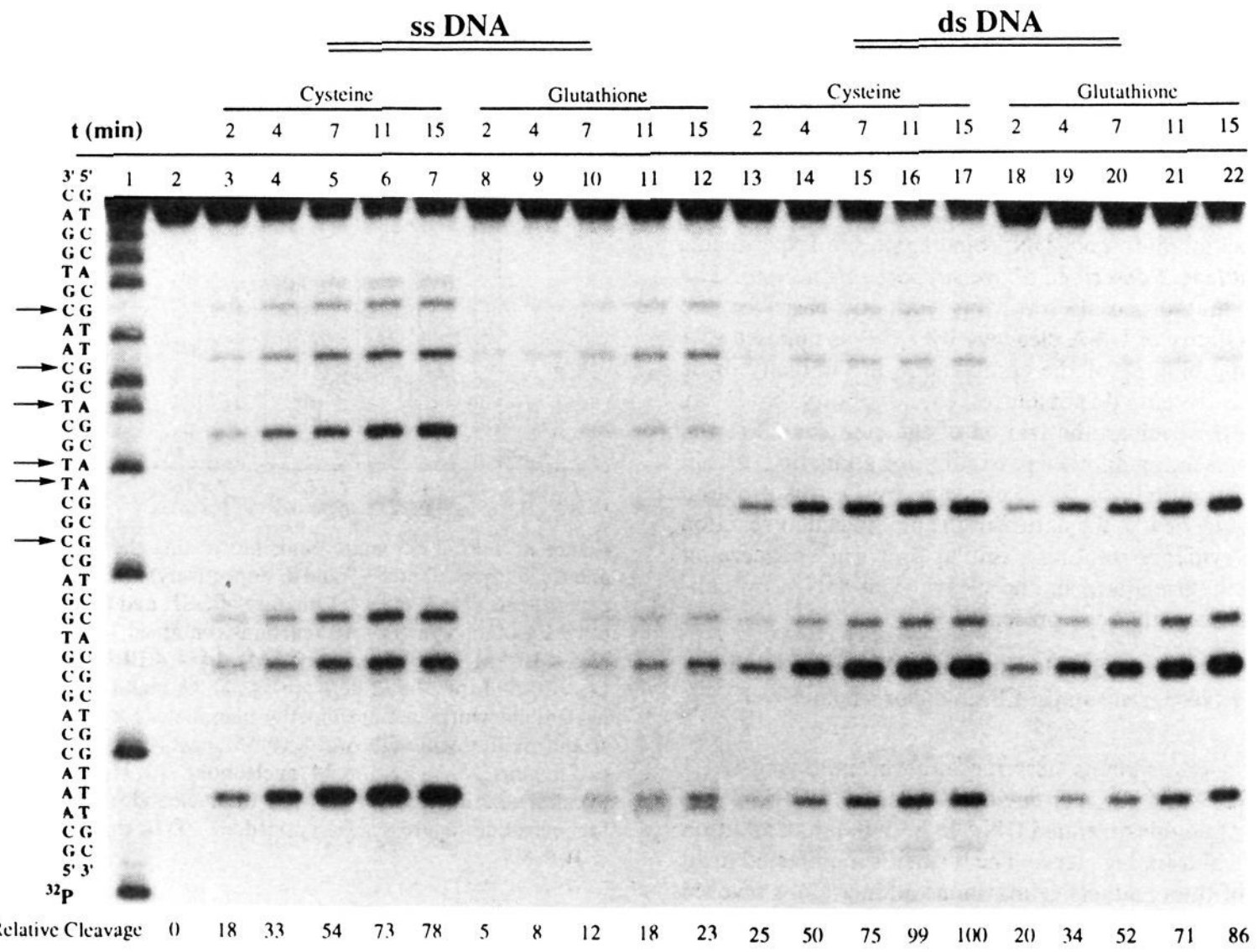

Figure 3. Time course of DNA cleavage by neocarzinostatin chromophore (**1**) with GSH and with CySH in the presence of single- and double-stranded DNA. Lane 1, Maxam–Gilbert G/A sequencing reaction;[32] lane 2 (control reaction lacking thiol), **1** (2×10^{-4} M), double-stranded calf thymus DNA (5×10^{-3} M, base pairs); lanes 3–7, **1** (2×10^{-4} M), single-stranded calf thymus DNA (1×10^{-2} M, nucleotide), CySH (5×10^{-4} M), t = 2, 4, 7, 11, and 15 min, respectively; lanes 8–12, **1** (2×10^{-4} M), single-stranded calf thymus DNA (1×10^{-2} M, nucleotide), GSH (5×10^{-4} M), t = 2, 4, 7, 11, and 15 min, respectively; lanes 13–17, **1** (2×10^{-4} M), double-stranded calf thymus DNA (5×10^{-3} M, base pairs), CySH (5×10^{-4} M), t = 2, 4, 7, 11, and 15 min, respectively; lanes 18–22, **1** (2×10^{-4} M), double-stranded calf thymus DNA (5×10^{-3} M, base pairs), GSH (5×10^{-4} M), t = 2, 4, 7, 11, and 15 min, respectively. "Relative cleavage" is defined as the percent of DNA cleavage relative to the lane of highest cleavage intensity (assigned a value of 100).

available evidence, however, suggests that biopolymers other than double-stranded DNA and, in particular, single-stranded DNA and certain forms of RNA, must be considered as potential targets for neocarzinostatin chromophore-induced damage in vivo.

Experimental Section

General. Neocarzinostatin protein–chromophore complex was generously provided by Kayaku Co., Ltd. and was stored as a dry powder at –80 °C. All manipulations of the drug were conducted in a cold room maintained at 2 °C. Reaction solutions were prepared with ultrapure water, obtained from a Millipore Milli-Q Plus water purification system. "Double-stranded calf thymus DNA" refers to an aqueous solution of 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 of 1 mM (base pairs) in DNA. A 50-μL aliquot of this solution was injected onto a Waters 600E HPLC system configured with a BioRad 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. Single-stranded calf thymus DNA was prepared by heating an aqueous solution of double-stranded calf thymus DNA at 90 °C for 20 min, followed by immediate freezing of the solution by immersion in liquid nitrogen. Analysis of the resulting solution (as described for double-stranded DNA) indicated a single-strand content of approximately 95%; after the solution was allowed to stand at 23 °C for 6 h, no significant change in the single-strand content due to renaturation was observed. Aqueous solutions of DNA were adjusted to pH 7.5 by the addition of Tris base (Fisher). Aqueous solutions of methyl thioglycolate (Aldrich), glutathione (Sigma), and cysteine (Sigma) were prepared just prior to use and were adjusted to pH 7.5 by the addition of Tris base. All pH measurements were determined with a Beckman ϕ40 digital pH meter equipped with a MI-410 micro-pH electrode (Microelectrodes, Inc.).

Neocarzinostatin Chromophore (1). Neocarzinostatin chromophore was extracted from the protein–chromophore complex as follows. Neocarzinostatin powder (0.5 g) was suspended in a solution of acetic acid (0.5 M) in methanol (100 mL, 0 °C), and the resulting suspension was stirred in the dark for 2 h at 0 °C. Solids were removed by centrifugation (1000g) at 2 °C for 5 min; the supernatant was decanted and stored briefly in the dark at 0 °C. The protein pellet was resuspended in fresh cold solvent (100 mL), and the extraction procedure was repeated as described. The supernatants were combined and concentrated to a volume of ~2 mL at 0 °C by rotary evaporation. Purification of the chromophore extract was achieved by chromatography over Sephadex LH-20 resin (Sigma, preswelled in the eluting solvent, column dimensions 2 × 5 cm) eluting with acetic acid:methanol:dichloromethane (2:5:93). The column fractions were assayed for chromophore content by UV and were concentrated in the dark at 0 °C to afford approximately 15 mg of chromophore as a light brown powder. ^{1}H NMR analysis indicated a purity of ~95%. The chromophore was stored at –80 °C as a solution in 0.5 M acetic acid–methanol (8 mM) without observable decomposition.

Preparation of the Cumulene 3. A standard solution of **1** (375 μL, 8 mM) in 0.5 M acetic acid–methanol (see Neocarzinostatin Chromophore) was transferred to a 10-mL side-armed Schlenk flask, and the solvents were removed under vacuum (0.1 mm) in the dark. To deuterate exchangeable protons and thus simplify ^{1}H NMR analysis, the chromophore was dissolved in CD_3OD (1 mL, Cambridge, 100% D) at 0 °C; the resulting solution was held at 0 °C for 10 min and was then concentrated under vacuum (0.1 mm). The deuterated chromophore residue was dissolved in THF-d_8 (675 μL, Cambridge, 100% D) and CD_3CO_2D (65 μL, Cambridge, 100% D). A solution of 1,2-*trans*-dichloroethylene (260

mM, Aldrich) in CD_3CO_2D (500 μL) was prepared for use as an internal standard. A 10-μL aliquot of this solution was added to the Schlenk flask, thus affording a solution of **1** (4 mM) and 1,2-*trans*-dichloroethylene (4 mM) in 9:1 THF-d_8:CD_3CO_2D. A portion of this solution (150 μL) was removed and stored at 0 °C for DNA cleavage reactions with **1** (see DNA Cleavage by **1** and **2** at Varying pH). The remaining solution was transferred to an argon-purged, 5-mm NMR tube sealed with a rubber septum. A ^1H NMR spectrum (500 MHz) was recorded at 210 K, and the sample was removed from the NMR probe and cooled in a dry ice–acetone bath. Neat methyl thioglycolate (5.5 μL, 0.061 mmol, 24 equiv, freshly distilled at 10 mm) was added, affording a thiol concentration of 94 mM. The sample was vortexed briefly for mixing and was quickly inserted into the cold NMR probe, precooled to 200 K. The reaction was monitored by ^1H NMR at 30-min intervals. The transformation of **1** to **3** was readily observed by several characteristic changes in the ^1H NMR spectrum (e.g., the collapse of signals at δ 6.80, 6.12, 5.66, and 4.11, representing H12, H11, H8, and H5 of **1**, respectively, and the increase of new signals at δ 4.20, 5.72, 6.24, and 5.81, assigned as H12, H11, H8, and H5 of **3**, respectively). After ~4 h, complete and quantitative conversion of **1** to **3** was achieved as determined by integration of the signal for the 3′ (napthoate) proton against the internal standard. The cumulene solution was removed from the cold NMR probe and stored in a dry ice–acetone bath in the dark until ready for use, at most 4 h. ^1H NMR (500 MHz, 210 K, 9:1 THF-d_8:CD_3CO_2D): δ 8.04 (d, 1H, J = 9.3 Hz, H4″), 7.58 (s, 1H, H8), 6.96 (d, 1H, J = 9.3 Hz, H3″), 6.80 (s, 1H, H6″), 6.24 (d, 1H, J = 5.1 Hz, H8), 5.81 (d, 1H, J = 5.1 Hz, H5), 5.72 (s, 1H, H11), 5.60 (d, 1H, J = 3.2 Hz, H1′), 4.97 (s, 1H, H10), 4.55 (m, 3H, H13 and H14), 4.20 (s, 1H, H12). The large excess of methyl thioglycolate precludes assignment of the remaining resonances for **3**.

Neocarzinostatin Chromophore–Methyl Thioglycolate Adduct (5). For preparation purposes, the reaction of **1** and **2** was performed in a total volume of 8 mL in a 25-mL round-bottomed flask. A 1-mL aliquot of the standard chromophore solution (8 mM, see Neocarzinostatin Chromophore) in 0.5 M acetic acid–methanol was mixed with a solution of 2,5-dimethoxybenzyl alcohol (300 μL, 100 mM, internal HPLC standard) in methanol, 1,4-cyclohexadiene (240 μL, 2.4 mmol), and 0.5 M acetic acid–methanol (6.45 mL). The resulting solution was cooled to −70 °C, and the reaction was initiated by the addition of neat methyl thioglycolate (10 μL), thus producing the following concentrations of solution components at the onset of the reaction: **1**, 1 mM; **2**, 10 mM; 1,4-cyclohexadiene, 300 mM. After the thiol addition, the solution was transferred to an ice–salt bath at −10 °C, and the reaction mixture was held at −10 °C for 2 h. Volatiles were removed in vacuo (0.1 mm) at 0 °C, and the solid residue was dissolved in sufficient methanol:water (1:1) so as to afford a product solution of ~2 mg/mL. Product **5** was isolated by reverse-phase HPLC as follows. Sample volumes of 0.5 mL were injected onto a Beckman Ultrasphere ODS (C_{18}, 5 μm) rp-HPLC column, 10 × 250 mm, as part of a Waters 600E HPLC system, flow rate 2.00 mL/min, with a linear gradient of methanol:aqueous ammonium acetate buffer (10 mM, pH 4.06): 40:60 v/v methanol:aqueous ammonium acetate buffer (10 mM, pH 4.06) to 70:30 v/v methanol:aqueous ammonium acetate buffer (10 mM, pH 4.06) over a period of 40 min. Peaks were detected by ultraviolet absorption at 220, 230, 240, and 250 nm with a Waters 994 Programmable Photodiode Detector. Fractions containing **5** (retention time, t_R ~ 50 min) were collected and pooled. Methanol was removed at 0 °C (0.1 mmHg) and the remaining aqueous buffer was removed by lyophilization. Product **5** was obtained in approximately 50% yield, as determined by integration against the internal HPLC standard. IR (neat): 3345, 3310, 1807, 1738, 1733, 1644, 1616, 1204, 1174, 1158, 1086, 1030 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 8.01 (d, 1H, J = 9.3 Hz, H4″), 7.77 (s, 1H, H2), 7.50 (d, 1H, J = 2.2 Hz, H8″), 7.23 (s, 1H, H8), 7.02 (d, 1H, J = 9.3 Hz, H3″), 6.94 (d, 1H, J = 5.6 Hz, H6), 6.77 (d, 1H, J = 2.2 Hz, H6″), 6.30 (d, 1H, J = 5.6 Hz, H5), 5.78 (s, 1H, H11), 5.57 (d, 1H, J = 3.2 Hz, H1′), 5.24 (s, 1H, H10), 4.85 (dd, 1H, J = 6.4, 8.5 Hz, H13), 4.63 (s, 1H, H12), 4.44 (t, 1H, J = 8.5 Hz, *anti*-H14), 4.22 (dd, 1H, J = 6.4, 8.5 Hz, *syn*-H14), 4.00 (q, 1H, J = 6.6 Hz, H5′), 3.80 (d, 1H, J = 3.2 Hz, H4′), 3.74 (s, 3H, CO_2CH_3), 3.62 (d, 1H, J = 15.1 Hz, SCH_aH_b), 3.54 (dd, 1H, J = 3.2, 10.5 Hz, H3′), 3.46 (d, 1H, J = 15.1 Hz, SCH_aH_b), 3.28 (s, 3H, Ar-OCH_3), 2.86 (dd, 1H, J = 3.2, 10.5 Hz, H2′), 2.58 (s, 3H, NCH_3), 2.56 (s, 3H, Ar-CH_3), 1.37 (d, 3H, J = 6.6 Hz, CH_3 at C5′). HRFABMS (glycerol matrix): calcd for $[M + H]^+$ 768.2326, found 768.2429.

Neocarzinostatin Chromophore–Glutathione Adduct (7). For preparation purposes, the reaction of **1** and GSH was performed in a total volume of 24 mL in a 100-mL round-bottomed flask. A 3-mL aliquot of the standard chromophore solution (8 mM, see Neocarzinostatin Chromophore) in 0.5 M acetic acid–methanol was mixed with a solution of 2,5-dimethoxybenzyl alcohol (1 mL, 100 mM, internal HPLC standard) in methanol, 1,4-cyclohexadiene (0.72 mL, 7.2 mmol), and 0.5 M acetic acid–methanol (16.9 mL). The resulting solution was cooled to 0 °C, and the reaction was initiated by the addition of an aqueous solution of GSH (2.4 mL, 100 mM), thus producing the following concentrations of solution components at the onset of the reaction: **1**, 1 mM; GSH, 10 mM; 1,4-cyclohexadiene, 300 mM. The reaction mixture was held at 0 °C for 5 h, after which time volatiles were removed in vacuo (0.1 mm) at 0 °C. The crude product was dissolved in sufficient methanol:water (1:1) so as to afford a product solution of ~5 mg/mL. Product **7** was isolated by reverse-phase HPLC as follows. Sample volumes of 0.5 mL were injected onto a Beckman Ultrasphere ODS (C_{18}, 5 μm) rp-HPLC column, 10 × 250 mm, flow rate 2.00 mL/min, with a linear gradient of methanol:aqueous ammonium acetate buffer (10 mM, pH 4.06): 30:70 v/v methanol:aqueous ammonium acetate buffer (10 mM, pH 4.06) to 60:40 v/v methanol:aqueous ammonium acetate buffer (10 mM, pH 4.06) over a period of 40 min. Fractions containing **7** (t_R ~ 45 min) were collected and pooled. Methanol was removed at 0 °C (0.1 mmHg), and the remaining aqueous buffer was removed by lyophilization. Product **7** was obtained in approximately 60% yield, as determined by integration against the internal HPLC standard. IR (neat): 3300 (br), 1793, 1747, 1642, 1556, 1410, 1202, 1089, 1029 cm^{-1}. ^1H NMR (400 MHz, DMF-d^7): δ 8.66 (d, 1H, J = 8.1 Hz, N-H, glycine residue), 8.41 (t, 1H, J = 5.3 Hz, N-H, cysteine residue), 8.04 (d, 1H, J = 9.2 Hz, H4″), 7.73 (s, 1H, H2), 7.58 (s, 1H, H8), 7.39 (d, 1H, J = 2.2 Hz, H8″), 7.17 (d, 1H, J = 9.2 Hz, 3″), 7.03 (d, 1H, J = 5.8 Hz, H6), 6.87 (d, 1H, J = 7.5 Hz, H6″), 6.62 (d, 1H, J = 5.8 Hz, H5), 6.06 (s, 1H, H11), 5.92 (d, 1H, J = 3.3 Hz, H1′), 5.46 (s, 1H, H10), 4.91 (m, 1H, H-g3), 4.78 (dd, 1H, J = 5.5, 8.5 Hz, H 13), 4.69 (t, 1H, J = 8.5 Hz, H14), 4.22 (dd, 1H, J = 2.9, 10.8 Hz, H3′), 4.17 (m, 1H, H-g6), 4.04 (q, 1H, J = 6.6 Hz, H5′), 3.93 (d, 2H, J = 5.3 Hz, H-g1), 3.79 (d, 1H, J = 2.9 Hz, H4′), 3.65 (dd, 1H, J = 3.3, 10.8 Hz, H2′), 3.65 (s, 3H, Ar-OCH_3), 3.51 (dd, 1H, J = 4.7, 12.8 Hz, H-g2), 3.08 (s, 3H, NCH_3), 3.01 (dd, 1H, J = 9.5, 12.8 Hz, H-g2), 2.69 (s, 3H, Ar-CH_3), 2.72 (t, 2H, J = 7.3 Hz, H-g4), 2.26 (q, 2H, J = 5.1 Hz, H-g5), 1.19 (d, 3H, J = 7.3 Hz, CH_3 at C5′). HRFABMS (nitrobenzyl alcohol matrix): calcd for $[M + H]^+$ 969.3076, found 969.3118.

Neocarzinostatin Chromophore–Cysteine Adduct (8). For preparation purposes, the reaction of **1** and CySH was performed in a total volume of 1.20 mL in a 1.5-mL Eppendorf tube. A 150-μL aliquot of the standard chromophore solution (8 mM, see Neocarzinostatin Chromophore) in 0.5 M acetic acid–methanol was mixed with a solution of 2,5-dimethoxybenzyl alcohol (60 μL, 100 mM, internal HPLC standard) in methanol, 1,4-cyclohexadiene (36 μL, 0.36 mmol), and 0.5 M acetic acid–methanol (834 μL). The resulting solution was cooled to 0 °C, and the reaction was initiated by the addition of an aqueous solution of CySH (120 μL, 100 mM), thus producing the following concentrations of solution components at the onset of the reaction: **1**, 1 mM; CySH, 10 mM; 1,4-cyclohexadiene, 300 mM. The reaction mixture was held at 0 °C for 5 h, after which time the reaction mixture was concentrated to dryness on a Savant rotary speed-vac. The crude product was dissolved in methanol–water (0.5 mL, 1:1). Product **8** was isolated by reverse-phase HPLC, as described for **7**. The fraction containing **8** (t_R ~ 50 min) was concentrated at 0 °C (0.1 mmHg) to remove the methanol, and the remaining aqueous buffer was then removed by lyophilization. Product **8** was obtained in approximately 60% yield, as determined by integration against the internal HPLC standard. IR (neat): 3300 (br), 1795, 1747, 1643, 1558, 1409, 1204, 1087, 1029 cm^{-1}. ^1H NMR (500 MHz, CD_3CO_2D): δ 8.07 (d, 1H, J = 9.3 Hz, H4″), 7.81 (s, 1H, H2), 7.55 (s, 1H, H8), 7.50 (s, 1H, H8″), 7.03 (d, 1H, J = 9.3 Hz, H3″), 6.97 (d, 1H, J = 5.5 Hz, H6), 6.77 (s, 1H, H6″), 6.40 (d, 1H, J = 5.5 Hz, H5), 6.20 (s, 1H, H11), 5.78 (d, 1H, J = 3.0 Hz, H1′), 5.40 (s, 1H, H10), 4.83 (m, 1H, cysteine C_α-H), 4.75 (s, 1H, H12), 4.55 (m, 2H, H13, H14), 4.40 (m, 2H, H3′, H14), 3.80 (m, 2H, H4′, H5′), 3.75 (m, 2H, -CH_2S, H2′), 3.60 (m, 1H, -CH_2S), 3.20 (s, 3H, Ar-OCH_3), 3.05 (s, 3H, NCH_3), 2.55 (s, 3H, Ar-CH_3), 1.10 (d, 3H, J = 2.0 Hz, CH_3 at C5′). HRFABMS (nitrobenzyl alcohol matrix): calcd for $[M + H]^+$ 783.2418, found 783.2435.

Preparation of ^{32}P-Labeled 35-Base Pair DNA. The single-stranded 35-base DNA oligomer 5′-GGAAACCAGCGTGGACCGCTTGCT-GCAACGTGGAC-3′ and its complementary sequence were synthesized on an Applied Biosystems DNA Synthesizer (1.0-μmol scale each) using

standard phosphoramidite methodology.[31] Removal of protective groups was achieved by the incubation of each protected synthetic oligomer with concentrated aqueous ammonium hydroxide solution (1 mL) for 12 h at 55 °C. Each product was dissolved in formamide loading buffer solution (50 μL),[32] 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[32] followed by dialysis against ultrapure water (2 days) and lyophilization. The single-stranded oligomer 5′-GGAAACCAGCGTGGACCGCTTGCTGCAACGTGGAC-3′ (50 pmol) was 5′ end-labeled by phosphorylation with [γ-^{32}P]ATP (NEN, ≥5000 Ci/mmol) and polynucleotide kinase (Boehringer Mannheim) using standard procedures.[32] 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, crushed thoroughly, and, after combination with aqueous Nonidet P-40 detergent solution (350 μL, 0.05%, Sigma), vortexed for 30 min at 23 °C. The resulting suspension was filtered through a Centrex filter (0.45 μm). The filtrate was washed twice with phenol (100 μL) and once with chloroform (100 μL), and 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 (16 000*g*, 20 min). The purified labeled fragment was dissolved in an aqueous solution of Tris–acetate buffer (50 μL, 50 mM, pH 7.4) and sodium chloride (100 mM), and the resulting solution was divided into two equal portions. One portion was left in single-stranded form. The other portion was mixed with the complementary synthetic single-stranded DNA oligomer (20 pmol), and 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.

Analysis of DNA Cleavage Products. General. The products from a given DNA cleavage reaction were quenched by the transfer of a 35-μL aliquot of the reaction solution to a fresh 1.5-mL Eppendorf tube containing ethanol (300 μL), aqueous sodium acetate buffer solution (50 μL, 0.3 M, pH 5.3), and aqueous ammonium acetate solution (20 μL, 2 M, pH 5.5), followed by rapid freezing by immersion of the tube in liquid nitrogen. The solution was thawed for analysis. The cleavage products were precipitated by centrifugation at 2 °C (16 000*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), and the resulting solution was transferred to a 1.5-mL Eppendorf tube. After radioactivity was assayed for 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 being heated at 90 °C for 5 min to induce denaturation, the solution (5 μL) was loaded onto a 20% denaturing polyacrylamide gel (42 × 34 cm, 0.4 mm thickness). The products were separated by gel electrophoresis in TBE buffer at 1800 V for the first 10 min and then at 1200 V until such point as the bromophenol blue dye had migrated to ~5 cm from the bottom of the gel.[31] The gel was exposed to a storage phosphor plate, and the DNA cleavage products were quantified with a molecular Dynamics 400 S PhosphorImager.

DNA Cleavage by 1 and 2 at Varying pH (Figure 1). Four reactions were performed in parallel in 1.5-mL Eppendorf tubes containing a total reaction volume of 500 μL each. For each reaction, a 200-μL aliquot of an aqueous solution of double-stranded calf thymus DNA (1.0 mM, base pairs) was combined with an aqueous solution of methyl thioglycolate (50 μL, 7.5 mM), water (146 μL), labeled duplex DNA (~10^6 cpm), and Tris-HCl aqueous buffer solution (100 μL, 200 mM). The pH values of the Tris-HCl buffer solutions were 7.8, 7.9, 8.3, and 9.4, such that the desired reaction pH (6.0, 6.5, 7.5, or 8.3, respectively) would be obtained after addition of a 4-μL aliquot of a solution of the chromophore (4 mM in 9:1 THF-d_8:CD_3CO_2D, see Preparation of the Cumulene **3**). Each reaction was initiated at 2 °C by the addition of a solution of **1** (4 μL, 4 mM) in 9:1 THF-d_8:CD_3CO_2D, thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.03 mM; **2**, 0.75 mM; double-stranded calf thymus DNA, 0.4 mM, base pairs; Tris-HCl buffer, 40 mM. At reaction times of 20 and 60 min, 35-μL aliquots of each reaction solution were quenched and analyzed subsequently by gel electrophoresis. A control reaction lacking thiol was performed in a 1.5-mL Eppendorf tube containing a total reaction volume of 500 μL by combining an aqueous solution of double-stranded calf thymus DNA (200 μL, 1.0 mM, base pairs), water (196 μL), Tris-HCl aqueous buffer solution (100 μL, 200 mM, final reaction pH 7.5), labeled duplex DNA (~10^6 cpm), and a solution of **1** (4 μL, 4 mM in 9:1 THF-d_8:CD_3CO_2D). A 35-μL aliquot of the control reaction was quenched after 60 min reaction time and was analyzed subsequently by gel electrophoresis.

DNA Cleavage by 3 at Varying pH (Figure 1). Four reactions were performed in parallel in 1.5-mL Eppendorf tubes containing a total reaction volume of 500 μL each. For each reaction, a 200-μL aliquot of an aqueous solution of double-stranded calf thymus DNA (1.0 mM, base pairs) was combined with water (196 μL), labeled duplex DNA (~10^6 cpm), and Tris-HCl aqueous buffer solution (100 μL, 200 mM). The pH values of the Tris-HCl buffer solutions were 7.8, 7.9, 8.3, and 9.4, such that the desired reaction pH (6.0, 6.5, 7.5, or 8.3, respectively) would be obtained after addition of a 4-μL aliquot of the cumulene (4 mM in 9:1 THF-d_8:CD_3CO_2D, see Preparation of the Cumulene **3**). Each reaction was initiated at 2 °C by the addition of a solution of **3** (4 μL, ~4 mM, as determined by ^{1}H NMR analysis, integration against the internal standard, maintained at −70 °C) in 9:1 THF-d_8:CD_3CO_2D, thus producing the following concentrations of solution components at the onset of the reaction: **3**, 0.03 mM; **2**, 0.75 mM (methyl thioglycolate is present in the cumulene solution and is diluted by a factor of 125 from 94 to 0.75 mM, equal to the concentration of **2** present in the reactions of **1** and **2**); double-stranded calf thymus DNA, 0.4 mM, base pairs; Tris-HCl buffer, 40 mM. To transfer the cumulene solution, the NMR tube was first placed in a shallow (~5 cm) Dewar containing dry ice–acetone. The NMR tube was scored with a file about 1 cm above the solution level and was carefully broken at that point. Transfer micropipet tips (10-μL capacity) were precooled in liquid nitrogen just before transfer of the cumulene solution. A 4-μL aliquot was then quickly (1–2 s) transferred to the reaction solution, followed by immediate vortexing of the mixture. At reaction times of 20 and 60 min, 35-μL aliquots of each reaction solution were quenched and analyzed subsequently by gel electrophoresis.

Synthesis of the Water-Soluble Radical Trap 14. Diethyl acetylenedicarboxylate (2.7 mL, 17 mmol, 1 equiv) was placed in a 20-mL thick-walled Schlenk tube equipped with a high-vacuum valve and a magnetic stir bar. The flask was cooled in dry ice–acetone, and 1,3-butadiene (10 mL, 140 mmol, 8.2 equiv) was condensed directly into the Schlenk tube. The high-vacuum valve was then sealed, and the reaction tube was immersed in an oil bath (located behind a safety shield) preheated to 65 °C. The solution was stirred at 65 °C for 10 h, at which time the reaction flask was submersed in liquid nitrogen. The high-vacuum valve was opened, and the reaction mixture was allowed to warm slowly to 23 °C. After the unreacted butadiene had evaporated, the product residue was transferred to a pear-shaped flask (100 mL) and was diluted with ethyl ether (50 mL). The resulting solution was cooled to −20 °C and was added via cannula to a solution of lithium aluminum hydride (1.6 g, 30 mmol, 1.8 equiv) in ethyl ether (200 mL) at 0 °C. The resulting gray slurry was stirred at 0 °C for 1 h. Excess hydride was quenched by the sequential addition of powdered potassium carbonate (10 g), methanol (20 mL), and water (10 mL). After quenching, the reaction mixture was diluted with dichloromethane (200 mL), and the resulting suspension was filtered over a coarse sintered-glass funnel. The filtered solid was washed with dichloromethane (100 mL). The combined filtrates were washed twice with a saturated aqueous solution of sodium chloride (200 mL), dried (sodium sulfate), and concentrated. The product was purified by flash column chromatography over silica gel (column diameter, 3 cm; column length, 10 cm), eluting with 97:3 v/v dichloromethane:methanol. The fractions containing **14** were identified by TLC and were pooled and concentrated to afford **14** (1.7 g, 72%) as a clear, colorless oil. For convenience in dispensing and manipulating this viscous product, a standard solution of **14** in water (0.50 M) was prepared. This solution was stored frozen at −20 °C. TLC (10% methanol in methylene chloride), R_f 0.55. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.71 (s, 2H, =C*H*), 4.13 (s, 4H, CH_2), 3.13 (s, 2H, −O*H*), 2.83 (s, 4H, CH_2OH).

Reaction of 1 and 2 in Water in the Presence and Absence of Double-Stranded DNA. Reactions were performed in 1.5-mL Eppendorf tubes containing a total reaction volume of 200 μL. A 100-μL aliquot of an aqueous solution of double-stranded calf thymus DNA (10 mM, base pairs) was combined with an aqueous solution of **2** (10 μL, 100 mM), an aqueous solution of **14** (16 μL, 500 mM), water (24 μL), and an aqueous solution of Tris base (40 μL, 250 mM). The reaction was initiated at 2 °C by the addition of a solution of **1** (4 mM) in 9:1 THF-d_8:CD_3CO_2D, thus producing the following concentrations of solution components

(31) Gait, M. J. *Oligonucleotide Synthesis: A Practical Approach*; IRL Press: New York, 1984.

(32) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd Ed.; Cold Spring Harbor Laboratory Press: New York, 1989.

at the onset of the reaction: **1**, 0.1 mM; **2**, 5.0 mM; **14**, 40 mM; double-stranded calf thymus DNA, 5.0 mM, base pairs; Tris–acetate buffer, 50 mM, pH 7.5. 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 methanol and aqueous ammonium acetate buffer solution (10 mM, pH 4.06), respectively: 0–5 min, 40:60 v/v; 5–15 min, 45:55 v/v; 15–30 min, 60:40 v/v; 30–70 min, 80:20 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 incubated at 2 °C for 1 h and then analyzed by rp-HPLC, eluting at 0.40 mL/min with the following step gradient of methanol and aqueous ammonium acetate buffer (10 mM, pH 4.06), respectively: 0–5 min, 40:60 v/v; 5–15 min, 45:55 v/v; 15–30 min, 55:45 v/v; 30–70 min, 70:30 v/v. A control reaction lacking DNA was performed in an identical manner, using a 100-μL aliquot of an aqueous solution containing each of the four nucleosides A, C, G, and T (5 mM each) and potassium dimethyl phosphate (20 mM) in lieu of the aqueous solution of double-stranded calf thymus DNA. The following concentrations of solution components were thus produced at the onset of the control reaction: **1**, 0.1 mM; **2**, 5.0 mM; **14**, 40 mM; A, C, G, T, 2.5 mM each nucleoside; potassium dimethyl phosphate, 10 mM; Tris–acetate buffer, 50 mM, pH 7.5.

Reaction of 3 in Water in the Presence and Absence of Double-Stranded DNA. Reactions were performed in 1.5-mL Eppendorf tubes containing a total reaction volume of 200 μL. A 100-μL aliquot of an aqueous solution of double-stranded calf thymus DNA (10 mM, base pairs) was combined with an aqueous solution of **2** (10 μL, 53 mM), an aqueous solution of **14** (16 μL, 500 mM), water (24 μL), and an aqueous solution of Tris base (40 μL, 250 mM). The reaction was initiated at 2 °C by the addition of a solution of **3** (4 mM) in 9:1 THF-d_8:CD_3CO_2D; **3** was prepared as described (see Preparation of the Cumulene **3**) with the following modification: for an internal standard, a 10-μL aliquot of a solution of 2,5-dimethoxybenzyl alcohol (650 mM) in CD_3CO_2D was used in lieu of a solution of 1,2-*trans*-dichloroethylene. The solution of **3** was transferred cold as described previously, thus producing the following concentrations of solution components at the onset of the reaction: **3**, 0.1 mM; **2**, 5.0 mM; **14**, 40 mM; double-stranded calf thymus DNA, 5.0 mM, base pairs; Tris–acetate buffer, 50 mM, pH 7.5. The reaction was incubated at 2 °C for 1 h and was then analyzed by rp-HPLC, as described above. A control reaction lacking DNA was performed in an identical manner, using a 100-μL aliquot of an aqueous solution containing each of the four nucleosides A, C, G, and T (5 mM each) and potassium dimethyl phosphate (20 mM) in lieu of the aqueous solution of double-stranded calf thymus DNA. The following concentrations of solution components were thus produced at the onset of the control reaction: **3**, 0.1 mM; **2**, 5.0 mM; **14**, 40 mM; A, C, G, T, 2.5 mM each nucleoside; potassium dimethyl phosphate, 10 mM; Tris–acetate buffer, 50 mM, pH 7.5.

Reactions of 1 with GSH and with CySH in Water in the Presence and Absence of DNA. Reactions were performed in 1.5-mL Eppendorf tubes containing a total reaction volume of 200 μL. A 100-μL aliquot of an aqueous solution of double-stranded calf thymus DNA (10 mM, base pairs) was combined with an aqueous solution of GSH (10 μL, 100 mM, pH 7.5), an aqueous solution of **14** (16 μL, 500 mM), water (24 μL), and aqueous Tris-HCl buffer solution (40 μL, 200 mM, pH 7.5). Following mixing of the aforementioned solution components, a 20-μL aliquot of the standard chromophore solution (8 mM, see Neocarzinostatin Chromophore) in 0.5 M acetic acid–methanol was transferred to a 1.5-mL Eppendorf tube, and the solvent was removed on a Savant rotary speed-vac. The chromophore was dissolved in methanol (50 μL), and the solvent was again removed. The chromophore was dissolved in methanol (80 μL) containing 2,5-dimethoxybenzyl alcohol (8 mM, internal HPLC standard) to afford a solution of **1** (2 mM) in methanol. The reaction was initiated at 2 °C by the addition of the solution of **1** (10 μL, 2 mM) in methanol, thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.1 mM; GSH, 5.0 mM; **14**, 40 mM; double-stranded calf thymus DNA, 5.0 mM, base pairs; Tris-HCl buffer, 40 mM. An initial ratio of **1** to 2,5-dimethoxybenzyl alcohol (internal standard) was established by HPLC as described above. The reaction was incubated at 2 °C for 1 h and then analyzed by rp-HPLC (50-μL injection volume), eluting at 0.40 mL/min with the following step gradient of methanol and aqueous ammonium acetate buffer (10 mM, pH 4.06), respectively; 0–5 min, 40:60 v/v; 5–25 min, 55:45 v/v; 25–70 min, 60:40 v/v. A control reaction lacking DNA was performed in an identical manner, using a 100-μL aliquot of an aqueous solution containing each of the four nucleosides A, C, G, and T (5 mM each) and potassium dimethyl phosphate (20 mM) in lieu of the aqueous solution of double-stranded calf thymus DNA. The following concentrations of solution components were thus produced at the onset of the control reaction: **1**, 0.1 mM; GSH, 5.0 mM; **14**, 40 mM; A, C, G, T, 2.5 mM each nucleoside; potassium dimethyl phosphate, 10 mM; Tris-HCl buffer, 40 mM. The reactions of **1** and CySH were performed and analyzed as described above for reactions of **1** and GSH, employing an aqueous solution of CySH in lieu of the aqueous solution of GSH.

Reactions of 1 and GSH/CySH Employing Varying Concentrations of the Radical Trap 14. Reactions were performed in 1.5-mL Eppendorf tubes containing a total reaction volume of 200 μL. To prepare solutions with varying concentrations of **14**, 16-, 80-, and 400-μL aliquots of an aqueous solution of **14** (500 mM) were transferred to each of three Eppendorf tubes, respectively. The water from these solutions was removed on a Savant rotary speed-vac, leaving a clear oil at the bottom of each tube. Each of the three volumes was brought to 40 μL (±2 μL) by the addition of 39-, 34-, and 10-μL aliquots of water, affording aqueous solutions of **14** at 0.2, 1, and 5 M, respectively. To each of these solutions was added a 100-μL aliquot of an aqueous solution containing each of the four nucleosides A, C, G, and T, (5 mM each) and potassium dimethyl phosphate (20 mM), an aqueous solution of GSH and CySH (10 μL, 1:1 GSH:CySH, 100 mM total thiol, pH 7.5), and aqueous Tris-HCl buffer solution (40 μL, 200 mM, pH 7.5). After thorough vortexing of the resulting mixtures, the reactions were initiated at 2 °C by the addition of a freshly prepared solution of **1** (10 μL, 2 mM) in methanol containing 2,5-dimethoxybenzyl alcohol (8 mM, prepared as described above), thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.1 mM; GSH, 2.5 mM; CySH, 2.5 mM: A, C, G, T, 2.5 mM each nucleoside; potassium dimethyl phosphate, 10 mM; Tris-HCl buffer, 40 mM; **14**, 40, 200, or 1000 mM. The reactions were incubated at 2 °C for 1 h and then analyzed by rp-HPLC as described above.

Kinetics of the Reaction of 1 with GSH and with CySH in the Absence of DNA. Reactions were performed in 1.5-mL Eppendorf tubes containing a total reaction volume of 100 μL. An aqueous solution of **14** (200 μL, 500 mM) was placed in an Eppendorf tube, and the water was removed on a Savant rotary speed-vac, leaving a clear oil at the bottom of the tube. **14** was dissolved into water (5 μL), aqueous Tris-HCl buffer solution (20 μL, 200 mM, pH 7.5), an aqueous solution containing each of the four nucleosides A, C, G, and T (5 mM each) and potassium dimethyl phosphate (50 μL, 20 mM), and an aqueous solution of GSH (5 μL, 10 mM). After thorough vortexing of the resulting mixture, the reaction was initiated at 2 °C by the addition of a freshly prepared solution of **1** (5 μL, 2 mM) in methanol containing 2,5-dimethoxybenzyl alcohol (8 mM, prepared as described above), thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.1 mM; GSH, 0.5 mM; A, C, G, T, 2.5 mM each; potassium dimethyl phosphate, 10 mM; **14**, 1 M; Tris-HCl buffer, 40 mM. An initial ratio of **1** to 2,5-dimethoxybenzyl alcohol (internal standard) was established by rp-HPLC as described above. The reaction was incubated at 2 °C for 30 s, at which time a 50-μL aliquot of the reaction solution was analyzed directly by rp-HPLC (50-μL injection volume, syringe chilled in ice), eluting at 0.40 mL/min with the following step gradient of methanol and aqueous ammonium acetate buffer (10 mM, pH 4.06), respectively: 0–5 min, 45:55 v/v; 5–20 min, 55:45 v/v; 20–30 min, 60:40 v/v; 30–70 min, 80:20 v/v. At least three separate measurements were performed. Three separate measurements with a reaction time of 60 s were also performed. The progress of the reaction was determined by integration of the unreacted chromophore against the internal standard. Pseudo-first-order rate constants were calculated according to $k = -\ln[(1_0/1_t)/t]$; second-order rate constants were obtained by dividing the pseudo-first-order rate constant by the concentration of thiol (5×10^{-4} M). Reactions of **1** with CySH were performed in an identical manner, employing an aqueous solution of CySH in lieu of the aqueous solution of GSH. Monitoring the progress of the reaction by the withdrawl and subsequent quenching (by freezing and/or lowering the pH) of a series of aliquots prior to analysis by rp-HPLC gave unreliable and inconsistent results, presumably to ineffective quenching. Effective quenching is achieved by injection onto the HPLC because GSH and CySH are not retained on the C_{18} column under the initial HPLC mobile-phase condition (45:55 methanol:aqueous ammonium acetate buffer), while **1** is retained virtually indefinitely.

Kinetics of the Reaction of 1 with GSH and with CySH in the Presence of Double-Stranded DNA. Reactions were performed in 1.5-mL Eppendorf tubes containing a total reaction volume of 100 μL. A 50-μL

aliquot of an aqueous solution of double-stranded calf thymus DNA (10 mM, base pairs) was combined with an aqueous solution of GSH (5 μL, 10 mM, pH 7.5), an aqueous solution of **14** (8 μL, 500 mM), water (12 μL), and aqueous Tris-HCl buffer solution (20 μL, 200 mM, pH 7.5). The reaction was initiated at 2 °C by the addition of a freshly prepared solution of **1** (5 μL, 2 mM) in methanol containing 2,5-dimethoxybenzyl alcohol (8 mM, prepared as described above), thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.1 mM; GSH, 0.5 mM; **14**, 40 mM; double-stranded calf thymus DNA, 5.0 mM, base pairs; Tris-HCl buffer, 40 mM. An initial ratio of **1** to 2,5-dimethoxybenzyl alcohol (internal standard) was established by rp-HPLC as described above. The reaction was incubated at 2 °C for 180 s, at which time a 50-μL aliquot of the reaction solution was analyzed directly by rp-HPLC as described above for the control solutions. At least three separate measurements were performed. Three measurements with a reaction time of 360 s were also performed. Second-order rate constants were calculated as described above. Reactions of **1** with CySH were performed in an identical manner, employing an aqueous solution of CySH in lieu of the aqueous solution of GSH.

Reactions of 1 and GSH/CySH in the Presence of Double- and Single-Stranded DNA, Heterogeneous Cellular RNA, tRNA, and Bovine Serum Albumin. Reactions were performed in 1.5-mL Eppendorf tubes containing a total reaction volume of 200 μL. The reaction with double-stranded DNA is illustrative. A 100-μL aliquot of an aqueous solution of double-stranded calf thymus DNA (10 mM, base pairs) was combined with an aqueous solution of GSH and CySH (10 μL, 1:1 GSH:CySH, 100 mM total thiol, pH 7.5), an aqueous solution of **14** (16 μL, 500 mM), water (24 μL), and aqueous Tris-HCl buffer solution (40 μL, 200 mM, pH 7.5). The reaction was initiated at 2 °C by the addition of a freshly prepared solution of **1** (10 μL, 2 mM) in methanol containing 2,5-dimethoxybenzyl alcohol (8 mM, prepared as described above), thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.1 mM; GSH, 2.5 mM; CySH, 2.5 mM; **14**, 40 mM; double-stranded calf thymus DNA, 5.0 mM, base pairs; Tris-HCl buffer, 40 mM. An initial ratio of **1** to 2,5-dimethoxybenzyl alcohol (internal standard) was established by HPLC as described above. The reaction was incubated at 2 °C for 1 h and then analyzed by rp-HPLC (50-μL injection volume) as described above. In an identical manner, reactions containing single-stranded calf thymus DNA, heterogeneous calf liver cellular RNA (Sigma),[28] tRNA (Sigma), or bovine serum albumin (Sigma) were performed in parallel, employing a freshly prepared aqueous solution of single-stranded calf thymus DNA (20 mM, nucleotide), an aqueous solution of calf liver RNA (20 mM, nucleotide), an aqueous solution of tRNA (20 mM, nucleotide, heterogeneous anticodon), or an aqueous solution of bovine serum albumin (2 mg/mL) in lieu of an aqueous solution of double-stranded calf thymus DNA. Reaction solutions were analyzed as described above.

Kinetics of the Reactions of 1 with GSH and with CySH, Single- and Double-Stranded DNA Cleavage Analysis (Figure 3). The reactions of **1** and GSH were performed in 1.5-mL Eppendorf tubes containing a total reaction volume of 200 μL. A 100-μL aliquot of an aqueous solution of either single-stranded calf thymus DNA (20 mM, nucleotide) or double-stranded calf thymus DNA (10 mM, base pair) was combined with labeled single- or double-stranded DNA, respectively (~10^6 cpm), an aqueous solution of GSH (10 μL, 10 mM, pH 7.5), water (40 μL), and Tris-HCl aqueous buffer solution (40 μL, 200 mM, pH 7.5). Following mixing of the aforementioned solution components, a 40-μL aliquot of the standard chromophore solution (8 mM, see Neocarzinostatin Chromophore) in 0.5 M acetic acid–methanol was transferred to a 1.5-mL Eppendorf tube, and the solvent was removed on a Savant rotary speed-vac. The chromophore was dissolved in methanol (100 μL), and the solvent was again removed. The chromophore was dissolved in methanol (80 μL) to afford a solution of **1** (4 mM) in methanol. The reactions were initiated at 2 °C by the addition of **1** (10 μL, 4 mM) in methanol, thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.2 mM; either single-stranded calf thymus DNA, 10 mM nucleotide, or double-stranded calf thymus DNA, 5 mM base pair; GSH, 0.5 mM; Tris-HCl buffer, 40 mM. At times of 2, 4, 7, 11, and 15 min, 35-μL aliquots of the reaction solution were quenched and analyzed subsequently by gel electrophoresis. Reactions with CySH were performed in an identical manner employing an aqueous solution of CySH in lieu of an aqueous solution of GSH.

Preparation of ^{32}P-Labeled tRNA^{Phe}. Yeast tRNA^{Phe} (100 pmol, Sigma) was 5′-end-labeled by dephosphorylation with alkaline phosphatase (Boehringer Mannheim) followed by phosphorylation with [γ-^{32}P]ATP (NEN, ≥5000 Ci/mmol) and polynucleotide kinase (Boehringer Mannheim) using standard procedures.[32] The labeled tRNA was purified over a 10% denaturing polyacrylamide gel, 0.4 mm thickness, and the band containing the tRNA was located by autoradiography. The band was excised from the gel and crushed thoroughly, and, after combination with aqueous Nonidet P-40 detergent solution (350 μL, 0.05%, Sigma), the solution was vortexed for 30 min at 23 °C. The resulting suspension was filtered through a Centrex filter (0.45 μm). The filtrate was washed twice with phenol (100 μL) and once with chloroform (100 μL), and 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 (16 000g, 20 min). The purified labeled tRNA was dissolved in an aqueous solution of EDTA (50 μL, 0.1 mM) and stored at −80 °C.

Reaction of 1 with GSH, tRNA Cleavage Analysis. Reactions were performed in 1.5-mL Eppendorf tubes containing a total reaction volume of 50 μL. A 5-μL aliquot of an aqueous solution of tRNA (1 mM, nucleotide, Sigma) was combined with an aqueous solution of glutathione (5 μL, 20 mM, pH 7.5), aqueous Tris-HCl buffer solution (10 μL, 200 mM, pH 7.5), labeled tRNA (~10^5 cpm), and water (25 μL). Following mixing of the aforementioned solution components, a 10-μL aliquot of the standard chromophore solution (8 mM, see Neocarzinostatin Chromophore) in 0.5 M acetic acid–methanol was transferred to a 1.5-mL Eppendorf tube, and the solvent was removed on a Savant rotary speedvac. To assist removal of remaining acetic acid, the chromophore was dissolved in methanol (50 μL), and the solvent was again removed. The chromophore was dissolved in methanol (80 μL) to afford a solution of **1** (1 mM) in methanol. The reaction was initiated at 2 °C by the addition of **1** (5 μL, 1 mM) in methanol, thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.1 mM; tRNA, 0.1 mM, nucleotide; GSH, 2.0 mM; Tris-HCl buffer, 40 mM. A control reaction lacking GSH was performed in parallel by combining an aqueous solution of tRNA (5 μL, 1 mM, nucleotide), labeled tRNA (~10^5 cpm), aqueous Tris-HCl buffer (10 μL, 200 mM, pH 7.5), water (30 μL), and **1** (5 μL, 1 mM) in methanol. The reactions were held at 2 °C for 60 min, and each reaction solution was divided into two equal portions (25 μL). Each of the four 25-μL portions was transferred to a fresh 1.5-mL Eppendorf tube containing ethanol (300 μL) and aqueous sodium acetate buffer solution (50 μL, 0.3 M, pH 5.3). The cleavage products were precipitated by centrifugation at 2 °C (16 000*g*, 20 min). The resulting product pellet was washed with aqueous ethanol (1 mL, 70%) and dried on a Savant rotary speed-vac. Product pellets from the control reaction and from the cleavage reaction (one pellet from each) were separately dissolved in aqueous aniline acetate buffer solution (20 μL, 1 M, pH 4.4) and incubated at 55 °C for 20 min in the dark. Water (100 μL) was added to each solution, and each solution was dried on a rotary speedvac. To assist removal of remaining aniline, water (100 μL) was added to each residue, and the solutions were dried. Each product pellet was dissolved in formamide loading buffer (8 μL), and the resulting solutions were transferred to 1.5-mL Eppendorf tubes. After radioactivity was assayed for with a Beckman LS 6000SC scintillation counter, the solutions were diluted with additional formamide loading buffer so as to produce a radiation density of 3000 cpm/μL. After being heated at 80 °C for 5 min to induce denaturation, each solution (5 μL) was loaded onto a 15% denaturing polyacrylamide gel (42 × 34 cm, 0.4 mm thickness). The products were separated by gel electrophoresis in TBE buffer at 1800 V for the first 10 min and then at 1200 V until such point as the bromophenol blue dye had migrated to ~10 cm from the bottom of the gel.[31]

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