Mechanistic Studies of the Natural DNA-Cleaving Agents Neocarzinostatin Chromophore, Calicheamicin γ_1, and Dynemicin A

Thesis by

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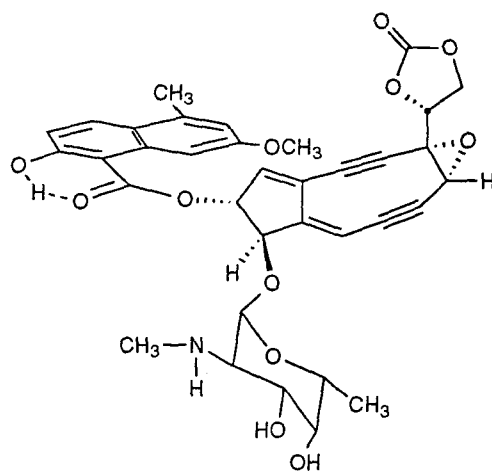
Finally, I would like to thank my parents and family for their support throughout the years.
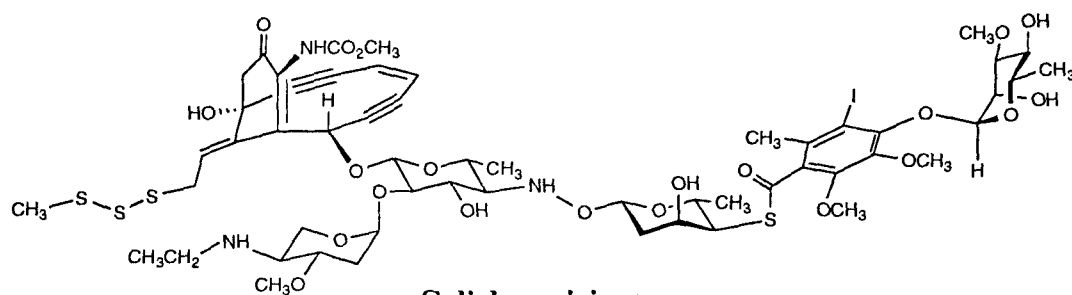
Abstract

Experiments establishing the intermediacy of the cumulene derived from neocarzinostatin chromophore are described. It is shown that $\geq 95\%$ of DNA cleavage arises via the cumulene. The sequence specificity and efficiency of DNA cleavage by externally generated cumulene are identical to that by the cumulene formed in situ, supporting the proposal that the cumulene determines the sequence specificity of DNA cleavage. It is shown that DNA and a water-soluble cyclohexadiene derivative are equally effective in trapping of the biradical intermediate at concentrations of 5 mM and 1 M, respectively, supporting the idea that the biradical must be generated as a DNA-bound species to induce DNA cleavage.

The reaction of calicheamicin γ_1 with glutathione has been studied in the presence of DNA and is shown to produce all four products arising from S–S bond exchange. The calicheamicin-glutathione disulfide is formed as the major product of this reaction, and is shown to be 2-3 orders of magnitude less reactive toward glutathione than is calicheamicin γ_1. The rate of DNA cleavage by calicheamicin γ_1 is essentially independent of the concentration of DNA, while the rate of DNA cleavage by the calicheamicin-glutathione disulfide is inversely proportional to the concentration of DNA. The data support the hypothesis that calicheamicin γ_1 undergoes thiol activation as a DNA-bound species, while the calicheamicin-glutathione disulfide is activated free in solution.
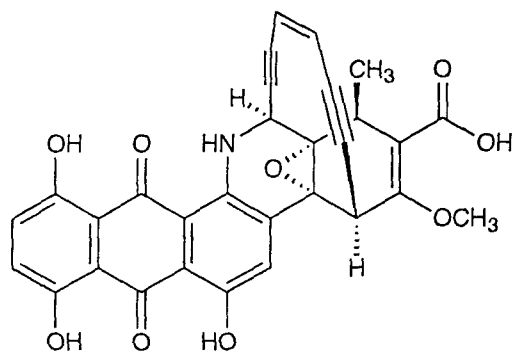
Binding constants of dynemicin A and synthetic analogs to DNA show that the two E-ring hydroxyls of the anthraquinone contribute approximately 2.7 kcal/mol binding energy, and that neutralization of the negatively-charged carboxylate stabilizes the drug-DNA binding complex by ~3 kcal/mol. Dynemicin A and the synthetic analogs display an inverse rate dependence on the concentration of DNA, supporting the proposal that these drugs must dissociate from DNA prior to chemical activation.

Neocarzinostatin Chromophore

Calicheamicin γ₁

Dynemicin A

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List of Abbreviations

AcOH	acetic acid
Å	angstrom
BSA	bovine serum albumin
°C	degrees Celsius
C_6D_6	hexadeuteriobenzene
Ci	curie
cm^{-1}	reciprocal centimeters
CySH	cysteine
δ	chemical shift
d	days
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
equiv	equivalents
FAB	fast atom bombardment
FT	Fourier transform
g	gram
GSH	glutathione
h	hour
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectroscopy
Hz	Hertz
IR	infrared

°K	degrees Kelvin
k	rate constant
$k\psi$	pseudo-first-order rate constant
K_B	equilibrium binding constant
kcal	kilocalories
J	coupling constant
L	liter
ln	natural log function
m	meta
mL	milliliter
μL	microliter
μM	micromolar
mM	millimolar
M	molar
μg	microgram
mg	milligram
μm	micrometer
mm	millimeter
μmol	micromole
mmol	millimole
mol	mole
$[M]^+$	molecular ion
nm	nanometer
NMR	nuclear magnetic resonance
o	ortho
PAGE	polyacrylamide gel electrophoresis
p	para

32p	phosphorous-32
pH	hydrogen ion concentration (log scale)
ppm	parts per million
RNA	ribonucleic acid
s	seconds
$t_{1/2}$	half life
THF	tetrahydrofuran
tRNA	transfer ribonucleic acid
UV	ultraviolet
v/v	volume-to-volume ratio
Z	zusammen

Chapter 1

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

Introduction

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, inferred on the basis of deuterium labeling, the biradical 4 (Scheme 1).[4c] While this pathway and, in particular, the biradical 4 provide an

Scheme 1
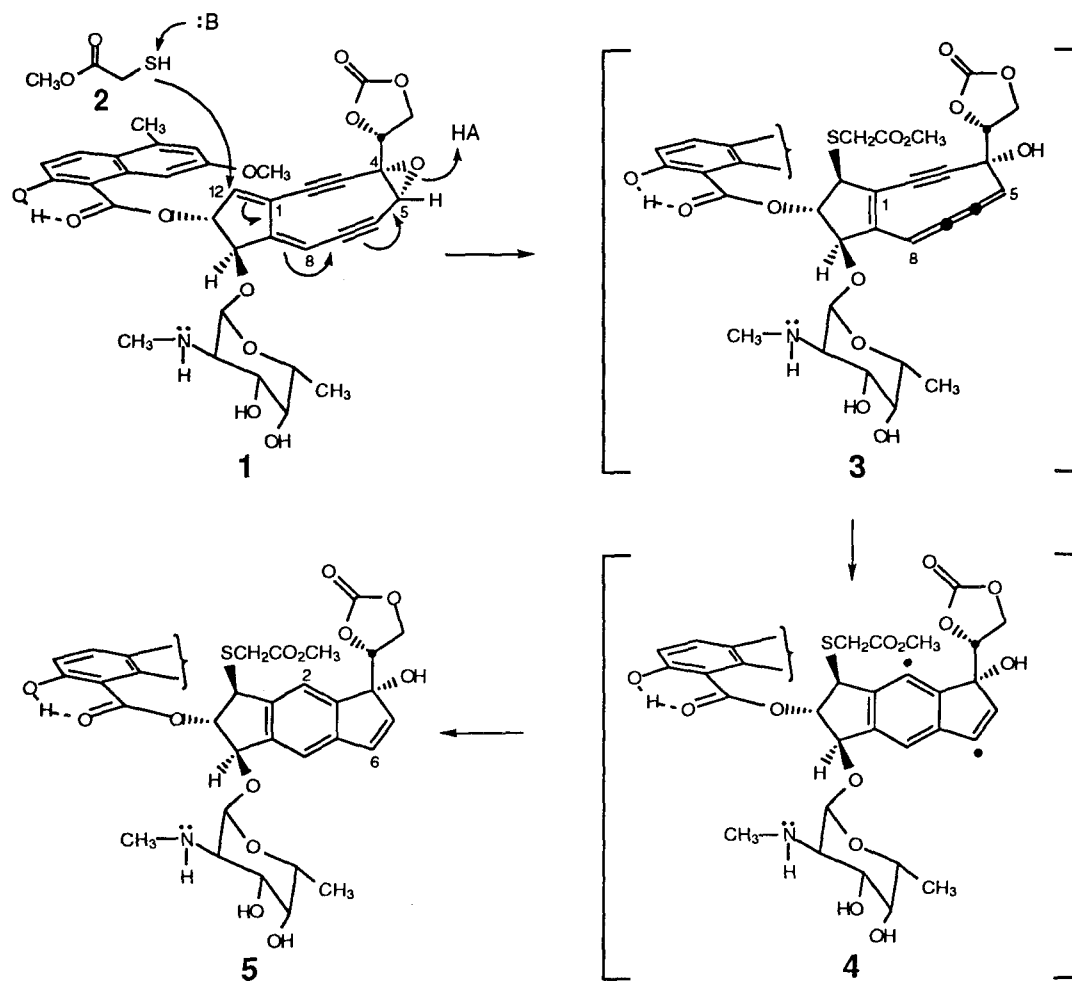
appealing mechanistic rationale for DNA damage by neocarzinostatin chromophore, these events have not as yet been linked unequivocally.

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.

The 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 → 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 the incorporation of 1,4-cyclohexadiene (0.2 M), the formation of **6** is supressed 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]

Scheme 2
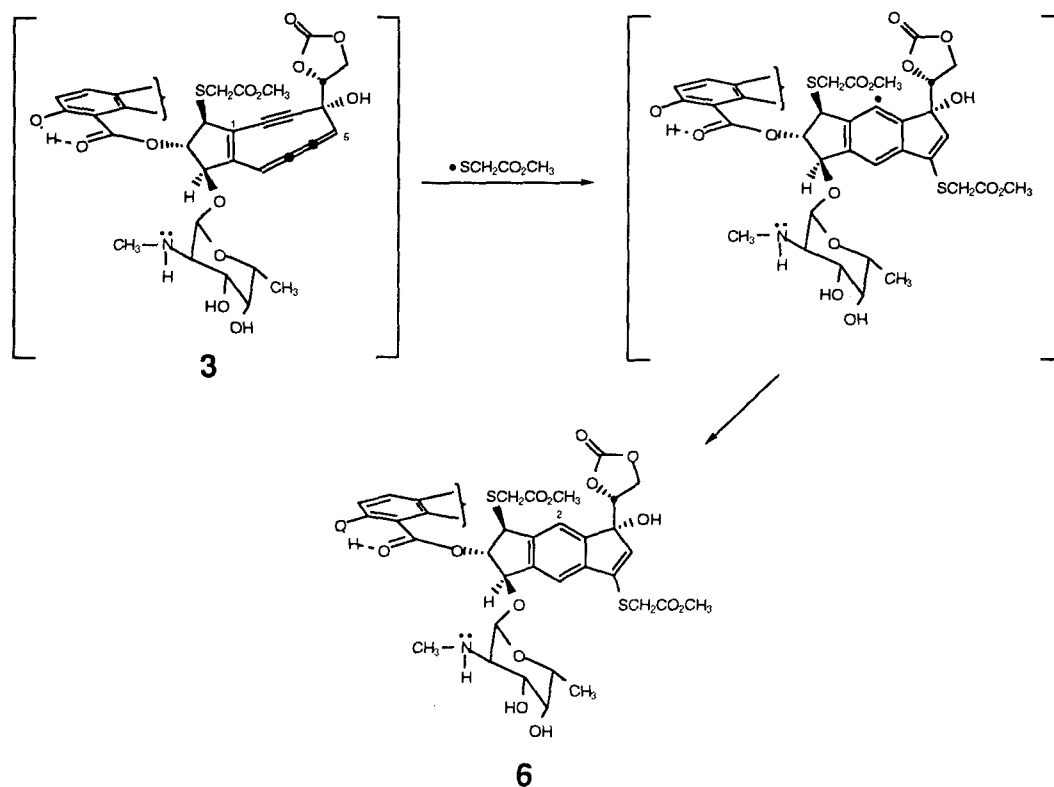
Low-temperature NMR evidence also establishes that the cumulene is an

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 deuteriated in 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^{-3} 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 adducts 7 and 8, respectively, in approximately 60% yield each.
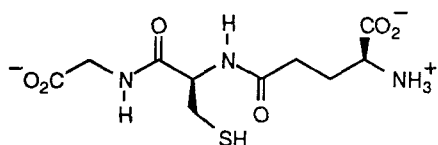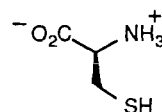
Glutathione (GSH)   Cysteine (CySH)
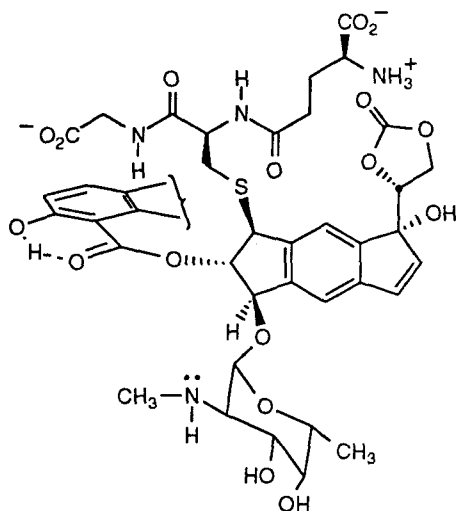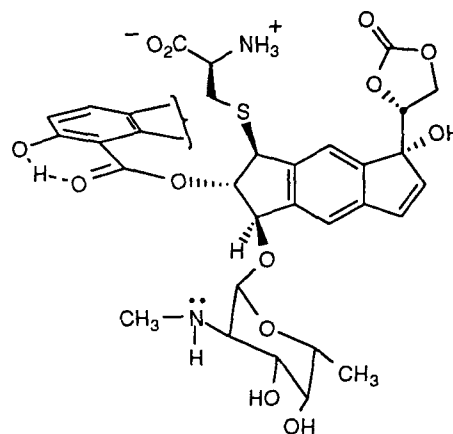
7   8

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 liquid 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 deuteriated media supports the intermediacy of biradical intermediates analogous to **4** in the formation of this product as well.[9]

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 carbon 12 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 indicates that the cyclononadienediyne ring likely contributes to the total strain of **1** as well; the triple bonds are strikingly non-linear, with a mean $C-C{\equiv}C$ angle of $161.5 \pm 1.2°$.[10] The strain within **1** is released in a rather ingeneous, stepwise fashion, such that each of the two steps leading to biradical formation is facilitated. Thus, the strain released upon 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 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

$$CH_2O_2Ct\text{-}Bu$$

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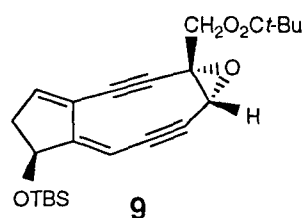
9 is unreactive toward methyl thioglycolate (**2**, 0.3 M) in 9:1 tetrahydrofuran:acetic acid below +60 °C (decomposition ensues at this temperature), whereas **1** reacts rapidly with **2** at −70 °C in the same medium. When triethylamine (0.3 M) was added to the former reaction solution at 23 °C, **9**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

$$SCH_2CO_2CH_3 \quad CH_2O_2Ct\text{-}Bu$$

10
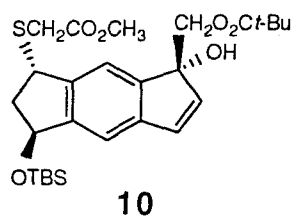
modest yield.[12] Parenthetically, it can be concluded from this result that the epoxide opening reaction is not restricted to an anti addition mode. The more important conclusion, however, concerns the likely role of the carbohydrate amino group in the thiol addition reaction in organic solvents. This hypothesis is supported by the observation that the *N*-nitroso derivative of neocarzinostatin chromophore (**11**) is also
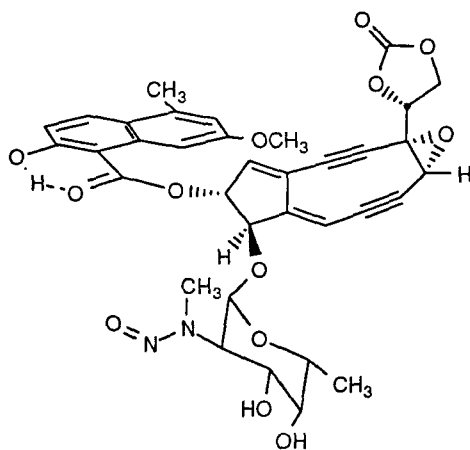
1 1

unreactive toward methyl thioglycolate in tetrahydrofuran-acetic acid.[12] 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, at C4,

would produce the cumulene intermediate **12** and would require that C4 become

12
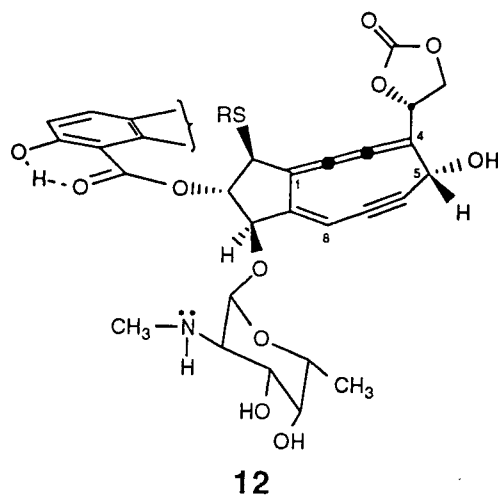
electron deficient in the transition state. The acyloxy substituent adjacent to C4 should

disfavor 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

13
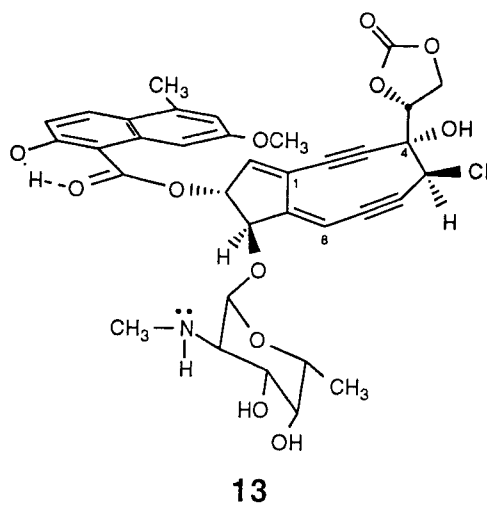
continuum toward a more S_N1-type nucleophilic addition of halide ion to a more fully

formed C5 cation.

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 supports the idea that the biradical **4** of Scheme I, 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 not characterized.[8b] More recently, low levels of deuterium transfer from various synthetic oligonucleotides specifically labeled at 1'- and 5'- sites into 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 I 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 that of the corresponding thiol-induced reaction.[19] Alternative paths for the reaction of **1** with thiols in water, both in the presence and 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
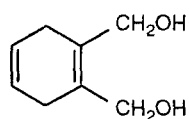
14

less than 12% yield (vide infra). It is clear that the potential exists for multiple 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 olgonucleotide 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 temperature 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, and 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 possiblity 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). 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-GCAAACCAGCGTGGACCGCT TGCTGCAACGTGGAC-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, respectively, 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 with identical sequence specificity as 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]

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;[31] lane 2 (control reaction lacking thiol): **1** (3 x 10^{-5} M), DNA (4 x 10^{-4} M, base pairs); lanes 3-6: **1** (3 x 10^{-5} M), DNA (4 x 10^{-4} M, base pairs), **2** (7.5 x 10^{-4} M), pH 6.0, 6.5, 7.5, and 8.3, respectively; lanes 7-10: **3** (3 x 10^{-5} M), DNA (4 x 10^{-4} M, base pairs), **2** (7.5 x 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).
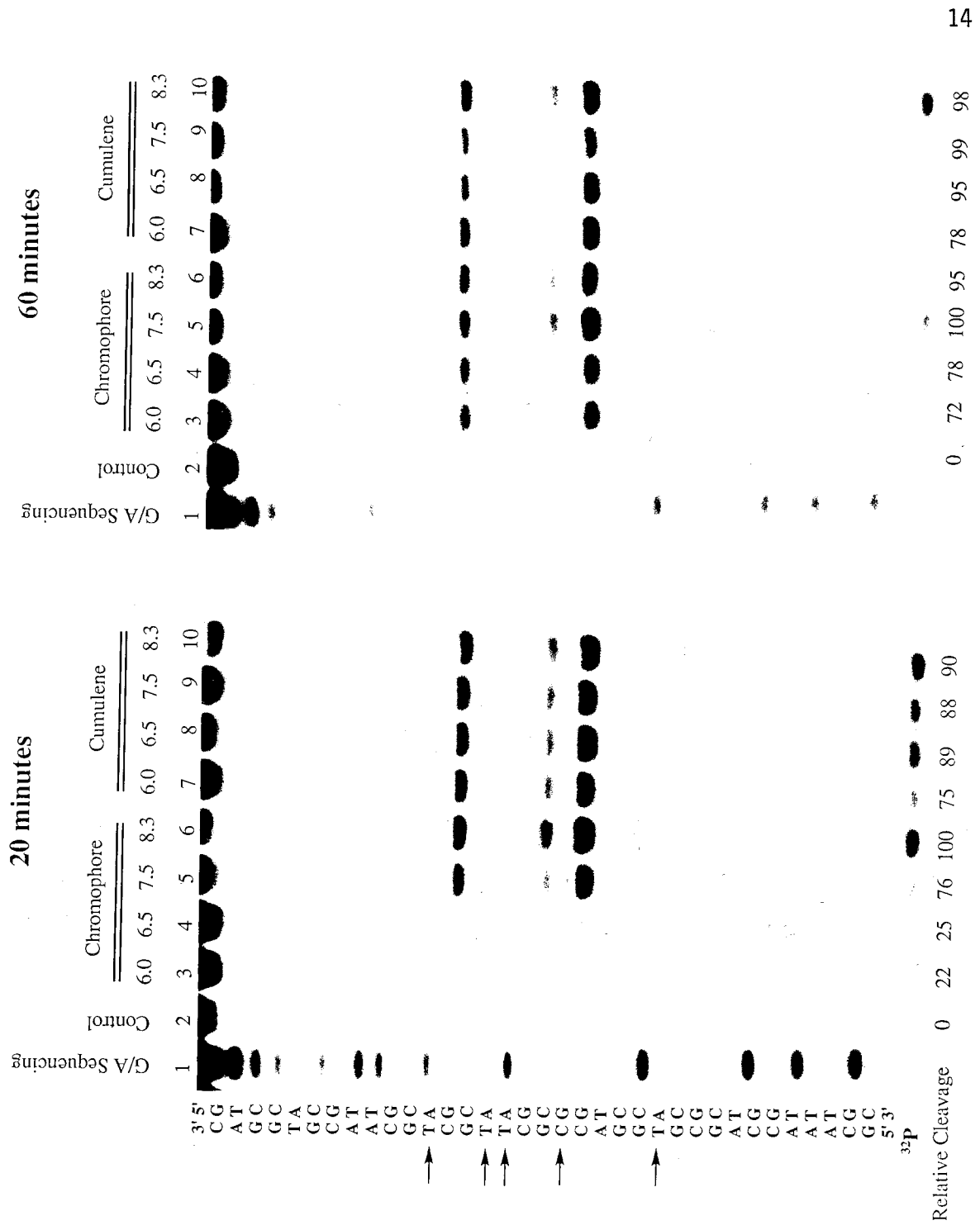
Analysis of the gel data reveals that, in contrast to reactions conducted in organic solvents, thiol addition is the rate-determining step in DNA cleavage by **1** and **2** in water at pH 7.5 and below. This is shown by comparison of cleavage bands from the cumulene **3**, from the reaction of **1** 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 by 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 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/or (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 x 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 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 x 10^{-4} M) and GSH or CySH (5 x 10^{-3} M) with increasing concentrations of double-stranded calf thymus DNA (0, 5 x 10^{-4}, and 5 x 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

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 x 10^{-4} M) and Tris-HCl buffer (4 x 10^{-2} M, pH 7.5) and were conducted at 2 °C.

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 x 10^{-3} M each thiol) and varying concentrations of DNA (0, 5 x 10^{-4}, 5 x 10^{-3} M, base pairs, entries 7, 10, and 11, respectively, Table 1) suggest that this 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 is 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 yield 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 particularily revealing that 200- to 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 bond strengths (bond dissociation energies, BDE's) 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 (BDE's estimated to be 85-95 kcal/mol). The only reasonable interpretation of the data is that the trapping of the biradical by DNA is a unimolecular process, while its trapping by **14** is a bimolecular event. In other words, the data suggests that the biradical is generated as a DNA-bound species, and that it is trapped at the site of its generation. This 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 reinforces 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 nucleosides 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 is 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).

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.

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]

$$[\mathbf{1}\cdot DNA]/[\mathbf{1}_{free}] = [DNA_{free}]K_B = (\sim 5 \times 10^{-3} \text{ M})(4 \times 10^6 \text{ M}^{-1}) = 2 \times 10^5$$

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 suggests 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 does 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 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 x 10^{-4} M; GSH, 2.5 x 10^{-3} M; CySH, 2.5 x 10^{-3} M; **14**, 4 x 10^{-2} M; tris-HCl buffer, 4 x 10^{-2} M, pH 7.5) in the presence of the following biopolymers: double-stranded calf thymus DNA (5 x 10^{-3} M, base pairs, Figure 2b), single-stranded calf thymus DNA (1 x 10^{-2} M, nucleotide, Figure 2c), a heterogeneous mixture of calf liver cellular RNA (1 x 10^{-2} M, nucleotide, primarily ribosomal RNA,[28] Figure 2d), tRNA (1 x 10^{-2} M, nucleotide, heterogeneous anticodon, Figure 2e), and bovine serum albumin (1.5 x 10^{-5} M, Figure 2f). A control experiment conducted with A, C, G, and T (2.5 x 10^{-3} M each nucleoside) and potassium dimethyl phosphate (1 x 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 in 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
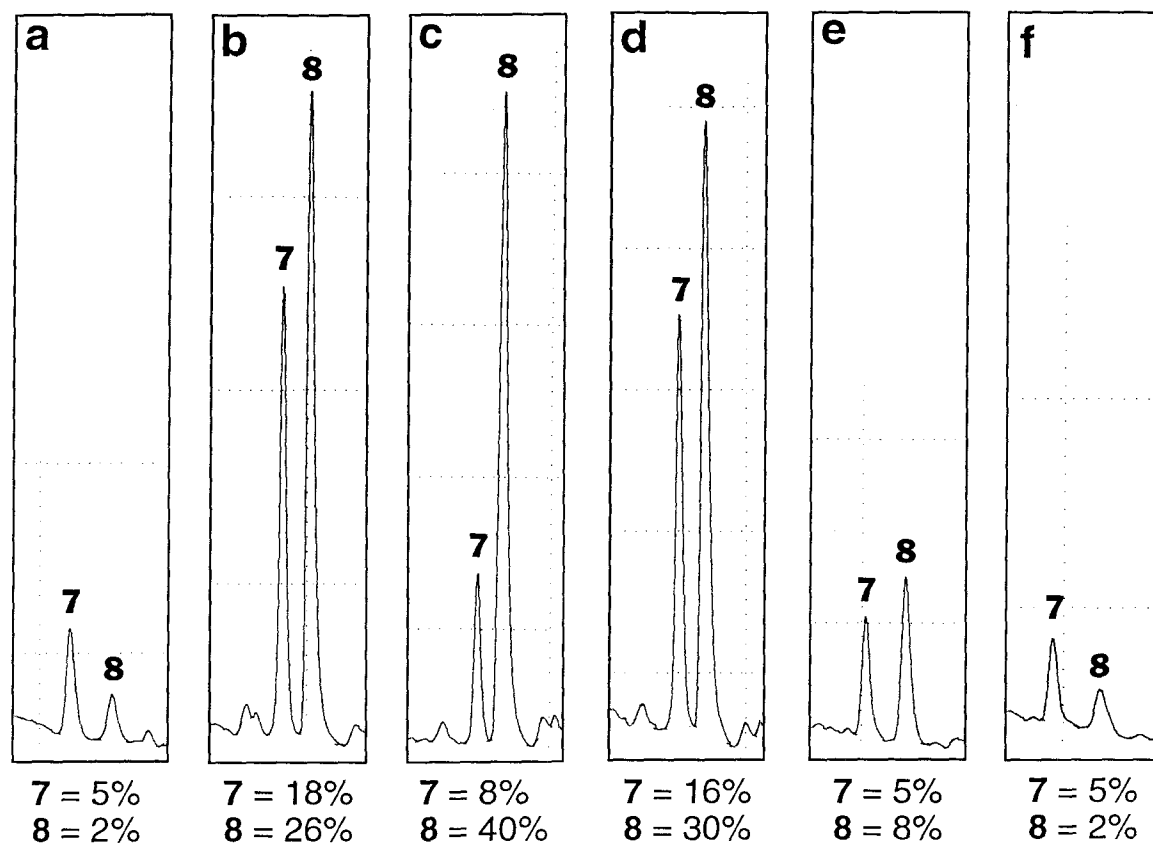
Figure 2. HPLC chromatograms illustrating the distribution of GSH and CySH thiol adducts (**7** and **8**, respectively) arising from competition experiments with **1** and a 1:1 mixture of GSH and CySH in the presence of various biopolymers. All reactions contained: **1** (1×10^{-4} M), GSH (2.5×10^{-3} M), CySH (2.5×10^{-3} M), **14** (4×10^{-2} M), tris-HCl buffer (4×10^{-2} M, pH 7.5, 2 °C). (2a) A, C, G, and T (2.5×10^{-3} M each nucleoside), with potassium dimethyl phosphate (1×10^{-2} M). (2b) Double-stranded calf thymus DNA (5×10^{-3} M, base pairs). (2c) Single-stranded calf thymus DNA (1×10^{-2} M, nucleotide). (2d) Heterogeneous mixture of calf liver cellular RNA (1×10^{-2} M, nucleotide). (2e) tRNA (1×10^{-2} M, nucleotide, heterogeneous anticodon). (2f) Bovine serum albumin (1.5×10^{-5} M).

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) and heterogeneous cellular RNA show that, like 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 conformationaly variable cellular RNA that are lacking in the compact, ordered tRNA structure.[29]

These conclusions have, in part, been confirmed by cleavage assays of the appropriate radio-labeled 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. 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 corroborates conclusions derived from the neocarzinostatin chromophore product analysis studies just described (Figure 2). That is, single-stranded DNA is shown clearly to be a target for neocarzinostatin chromophore-induced damage. It should be noted that this finding stands in marked contrast to prior studies.[19] 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 of) 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 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.

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;[31] lane 2 (control reaction lacking thiol): **1** (2 x 10^{-4} M), double-stranded calf thymus DNA (5 x 10^{-3} M, base pairs); lanes 3-7: **1** (2 x 10^{-4} M), single-stranded calf thymus DNA (1 x 10^{-2} M, nucleotide), CySH (5 x 10^{-4} M), t = 2, 4, 7, 11, and 15 min, respectively. lanes 8-12: **1** (2 x 10^{-4} M), single-stranded calf thymus DNA (1 x 10^{-2} M, nucleotide), GSH (5 x 10^{-4} M), t = 2, 4, 7, 11, and 15 min, respectively. lanes 13-17: **1** (2 x 10^{-4} M), double-stranded calf thymus DNA (5 x 10^{-3} M, base pairs), CySH (5 x 10^{-4} M), t = 2, 4, 7, 11, and 15 min, respectively. lanes 18-22: **1** (2 x 10^{-4} M), double-stranded calf thymus DNA (5 x 10^{-3} M, base pairs), GSH (5 x 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).

ss DNA   ds DNA

Cysteine   Glutathione   Cysteine   Glutathione

t (min) 2 7 11 15 2 4 7 11 15 2 4 7 11 15 2 4 7 11 15

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

3'5'
C G
A T
G C
G C
T A
G C
C G
A T
A T
C G
G C
T A
C G
T A
T A
C G
G C
C G
A T
G C
G C
T A
G C
C G
C G
A T
A T
A T
C G
C G
G C
5'3'
32P

Relative Cleavage 0 18 33 54 73 78 5 8 12 18 23 25 50 75 99 100 20 34 52 71 86

Experimental

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 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 standing 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 (1000 g) at 2 °C for 5 min; the supernatant was decanted and was 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, pre-swelled in the eluting solvent, column dimensions 2 x 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 deuteriate 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 deuteriated 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-transdichloroethylene (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-transdichloroethylene (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 ^{1}H 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 equivalents, 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 to the cold NMR probe, pre-cooled to 200 °K. The reaction was monitored by ^{1}H NMR at 30 min intervals. The transformation of **1** to **3** was readily observed by several characteristic changes in the ^{1}H 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; the increase of new signals at 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. ^{1}H NMR (500 MHz, 210 °K, 9:1 THF-d_8:CD_3CO_2D), δ: 8.04 (d, 1 H, J = 9.3 Hz, H4"), 7.58 (s, 1 H, H8), 6.96 (d, 1 H, J = 9.3 Hz, H3"), 6.80 (s, 1 H, H6"), 6.24 (d, 1 H, J = 5.1 Hz, H8), 5.81 (d, 1 H, J = 5.1 Hz, H5), 5.72 (s, 1 H, H11), 5.60 (d, 1 H, J =

3.2 Hz, H1'), 4.97 (s, 1 H, H10), 4.55 (m, 3 H, H13 and H14), 4.20 (s, 1 H, 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 x 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, ~50 min) were collected and pooled. Methanol

was removed at 0 °C (0.1 mm) 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. ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.01 (d, 1 H, J = 9.3 Hz, H4"), 7.77 (s, 1 H, H2), 7.50 (d, 1 H, J = 2.2 Hz, H8"), 7.23 (s, 1 H, H8), 7.02 (d, 1 H, J = 9.3 Hz, H3"), 6.94 (d, 1 H, J = 5.6 Hz, H6), 6.77 (d, 1 H, J = 2.2 Hz, H6"), 6.30 (d, 1 H, J = 5.6 Hz, H5), 5.78 (s, 1 H, H11) 5.57 (d, 1 H, J = 3.2 Hz, H1'), 5.24 (s, 1 H, H10), 4.85 (dd, 1 H, J = 6.4, 8.5 Hz, H13), 4.63 (s, 1 H, H12), 4.44 (t, 1 H, J = 8.5 Hz, anti-H14), 4.22 (dd, 1 H, J = 6.4, 8.5 Hz, syn-H14), 4.00 (q, 1 H, J = 6.6 Hz, H5'), 3.80 (d, 1 H, J = 3.2 Hz, H4'), 3.74 (s, 3 H, CO$_2$CH$_3$), 3.62 (d, 1 H, J = 15.1 Hz, SCH$_a$H$_b$), 3.54 (dd, 1 H, J = 3.2, 10.5 Hz, H3'), 3.46 (d, 1 H, J = 15.1 Hz, SCH$_a$H$_b$), 3.28 (s, 3 H, Ar-OCH$_3$), 2.86 (dd, 1 H, J = 3.2, 10.5 Hz, H2') 2.58 (s, 3 H, NCH$_3$), 2.56 (s, 3 H, Ar-CH$_3$), 1.37 (d, 3 H, J = 6.6 Hz, CH$_3$ at C5'). IR (neat) : 3345, 3310, 1807, 1738, 1733, 1644, 1616, 1204, 1174, 1158, 1086, 1030 cm^{-1}. 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 x 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 (retention time, ~45 min) were collected and pooled. Methanol was removed at 0 °C (0.1 mm) 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. ^{1}H NMR (400 MHz, DMF-d_7): δ 8.66 (d, 1 H, J = 8.1 Hz, NH, glycine residue), 8.41 (t, 1 H, J = 5.3 Hz, NH, cysteine residue), 8.04 (d, 1 H, J = 9.2 Hz, H4"), 7.73 (s, 1 H, H2), 7.58 (s, 1 H, H8), 7.39 (d, 1 H, J = 2.2 Hz, H8"), 7.17 (d, 1 H, J = 9.2 Hz, H3"), 7.03 (d, 1 H, J = 5.8 Hz, H6), 6.87 (d, 1 H, J = 7.5 Hz, H6"), 6.62 (d, 1 H, J = 5.8 Hz, H5), 6.06 (s, 1 H, H11), 5.92 (d, 1 H, J = 3.3 Hz, H1'), 5.46 (s, 1 H, H10), 4.91 (m, 1 H, H-g3), 4.78 (dd, 1 H, J = 5.5, 8.5 Hz, H13), 4.69 (t, 1 H, J = 8.5 Hz, H14), 4.22 (dd, 1 H, J = 2.9, 10.8 Hz, H3'), 4.17 (m, 1 H, H-g6), 4.04 (q, 1 H, J = 6.6 Hz, H5'), 3.93 (d, 2 H, J = 5.3 Hz, H-g1), 3.79 (d, 1 H, J = 2.9 Hz, H4'), 3.65 (dd, 1 H, J = 3.3, 10.8 Hz, H2'), 3.65 (s, 3 H, Ar-OCH$_3$), 3.51 (dd, 1 H, J = 4.7, 12.8 Hz, H-g2), 3.08 (s, 3 H, NCH$_3$), 3.01 (dd, 1 H, J = 9.5, 12.8 Hz, H-g2), 2.69 (s, 3 H, Ar-CH$_3$), 2.72 (t, 2 H, J = 7.3 Hz, H-g4), 2.26 (q, 2 H, J = 5.1 Hz, H-g5), 1.19 (d, 3 H, J = 7.3 Hz, CH$_3$ at C5'). IR (neat): 3300 (broad), 1793, 1747, 1642, 1556, 1410, 1202, 1089, 1029 cm^{-1}. 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** (retention time, ~50 min) was concentrated at 0 °C (001 mm) 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. ^{1}H NMR (500 MHz, CD$_3$CO$_2$D): δ 8.07 (d, 1 H, J = 9.3 Hz, H4"), 7.81 (s, 1 H, H2), 7.55 (s, 1 H, H8), 7.50 (s, 1 H, H8"), 7.03 (d, 1 H, J = 9.3 Hz, H3"), 6.97 (d, 1 H, J = 5.5 Hz, H6), 6.77 (s, 1 H, H6"), 6.40 (d, 1 H, J = 5.5 Hz, H5), 6.20 (s, 1 H, H11), 5.78 (d, 1 H, J = 3.0 Hz, H1'), 5.40 (s, 1 H, H10), 4.83 (m, 1 H, Cysteine C$_\alpha$-H), 4.75 (s, 1 H, H12), 4.55 (m, 2 H, H13, H14), 4.40 (m, 2 H, H3', H14), 3.80 (m, 2 H, H4', H5'), 3.75 (m, 2 H, -CH$_2$S, H2'), 3.60 (m, 1 H, -CH$_2$S), 3.20 (s, 3 H, Ar-OCH$_3$), 3.05 (s, 3 H, NCH$_3$), 2.55 (s, 3 H, Ar-CH$_3$), 1.10 (d, 3 H, J = 2.0 Hz, CH$_3$ at C5'). IR (neat): 3300 (broad), 1795, 1747, 1643, 1558, 1409, 1204,

1087, 1029 cm^{-1}. 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'-GGAAACCAGCGTGGACCGCTTGCTGCAACGTGGAC-3' and its complementary sequence were synthesized on an Applied Biosystems DNA Synthesizer (1.0 μmole scale each) using standard phosphoramidite methodology.[30] 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)[31] 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[31] followed by dialysis against ultrapure water (2 d) and lyophilization. The single-stranded oligomer 5'-GGAAACCAGCGTGGACCGCT TGCTGCAAC GTGGAC-3' (50 pmole) was 5' end-labeled by phosphorylation with [γ-^{32}P]-ATP (NEN, ≥5000 Ci/mmol) and polynucleotide kinase (Boehringer Mannheim) using standard procedures.[31] The labeled single-stranded oligomer was purified over a 15% denaturing polyacrylamide gel, 0.4 mm thickness, and the band containing the oligomer was located by autoradiography. The band was excised from the gel, was crushed thoroughly and, after combination with aqueous Nonidet P-40 detergent solution (350 μL, 0.05%, Sigma), 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), was washed 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 pmole), 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 assaying for radioactivity with a Beckman LS 6000SC scintillation counter, the solution was diluted with additional formamide loading buffer so as to produce a radiation density of 3000 cpm/μL. After heating at 90 °C for 5 min to induce denaturation, the solution (5 μL) was loaded onto a 20% denaturing polyacrylamide gel (42 x 34 cm, 0.4 mm thickness). The products were separated by gel electrophoresis in 1 x 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 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$_3$CO$_2$D, 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$_3$CO$_2$D, 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$_3$CO$_2$D). 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 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 ^1H 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 mM 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 micro-pipet tips (10-μL capacity) were pre-cooled 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 grey slirry 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), was dried (sodium sulfate), and was 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 $-20\ ^\circ$C. TLC (10% in methanol), R_f 0.55. ^{1}H NMR (400 MHz, CDCl$_3$), δ: 5.71 (s, 2 H, =CH), 4.13 (s, 4 H, CH$_2$), 3.13 (s, 2 H, -OH), 2.83 (s, 4 H, CH$_2$OH).

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$_3$CO$_2$D, thus producing the following concentrations of solution components 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 x 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 was 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$_3$CO$_2$D; **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$_3$CO$_2$D was used in lieu of a solution of 1,2-transdichloroethylene. 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 was 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 were 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: $k = -\ln [(1_o/1_t)/t]$; 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 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 was 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 was 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 ($\sim10^6$ 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 pmole, 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.[31] 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, was crushed thoroughly and, after combination with aqueous Nonidet P-40 detergent solution (350 μL, 0.05%, Sigma), 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), was washed 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 tRNA was dissolved in an aqueous solution of EDTA (50 μL, 0.1 mM) and was 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 speed vac. 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 ($\sim10^5$ 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 was 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 speed vac. 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 assaying for radioactivity 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 heating at 80 °C for 5 min to induce denaturation, each solution (5 μL) was loaded onto a 15% denaturing polyacrylamide gel (42 x 34 cm, 0.4 mm thickness). The products were separated by gel electrophoresis in 1 x 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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Chapter 2

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

Introduction

The potent natural antitumor agent calicheamicin γ_1 (**1**) and the structurally related

esperamicins have been shown to initiate the cleavage of duplex DNA upon incubation

with thiols.[1] This activity was rationalized early on as the result of the generalized
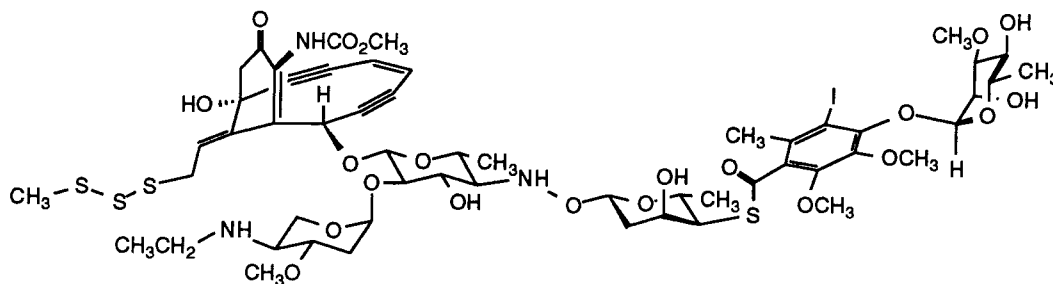
Calicheamicin γ_1 (1)

mechanism of Scheme 1 where thiol-induced cleavage of the allyl trisulfide group of **1**

produces the thiol (or thiolate) **A** which then cyclizes to form the dihydrothiophene

derivative **2** and, subsequently, the biradical **B**.[2,3] Much evidence now supports this

mechanism, to include the characterization of the products of quenching of the biradical

B with hydrogen or deuterium (e.g., **3**)[2] and the observation at low temperature of the

intermediate **2**.[4]

Scheme 1
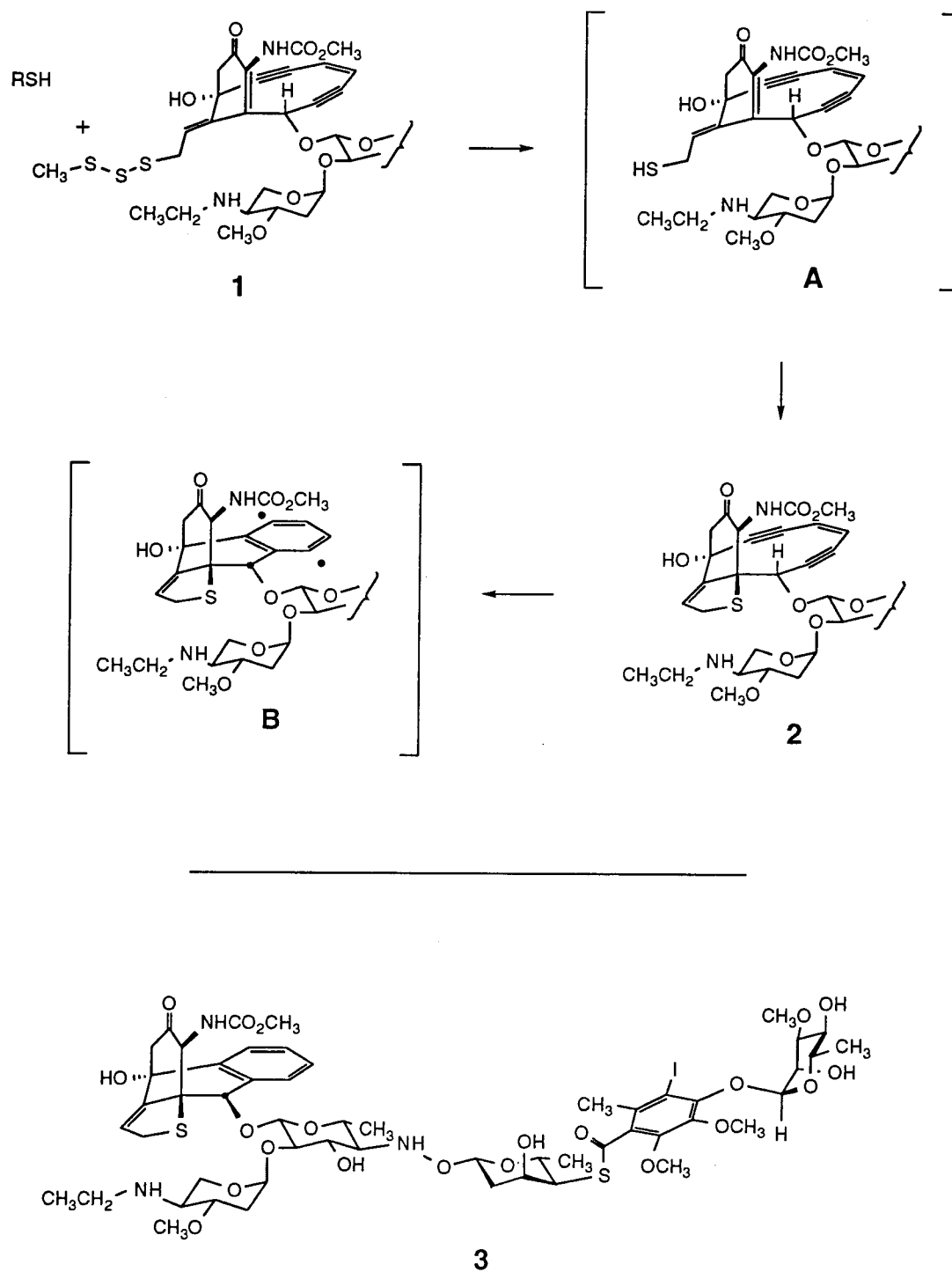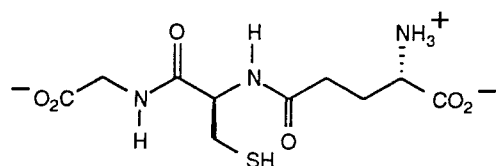
Evans and Saville have presented evidence showing that in the base-catalyzed reaction of a thiol with a symmetrical trisulfide, attack on the terminal sulfur atoms is kinetically preferred over attack on the central sulfur atom (Scheme 2).[5]

Scheme 2
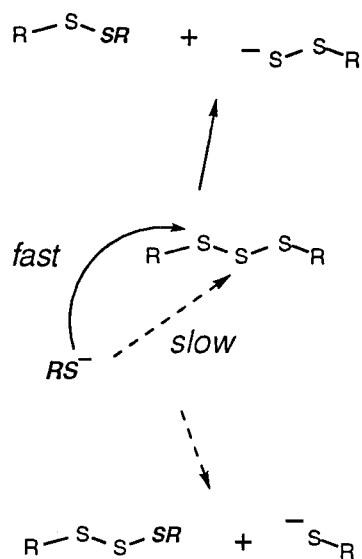
This preference was attributed to a leaving-group effect, where the presumed greater stability of the thiosulfenate anion versus the thiolate anion directs the course of reaction. Nucleophilic attack of thiols upon the unsymmetrical allyl trisulfide functional group of **1** is potentially more complex; in theory, four discrete products can be formed by single-step processes involving S-S bond cleavage and concomitant S-S bond formation, as illustrated in Scheme 3 for the specific case of the reaction of **1** with glutathione (GSH). The direct products of this reaction (A, **4-6**, Schemes 1 and 3) are

Glutathione (GSH)

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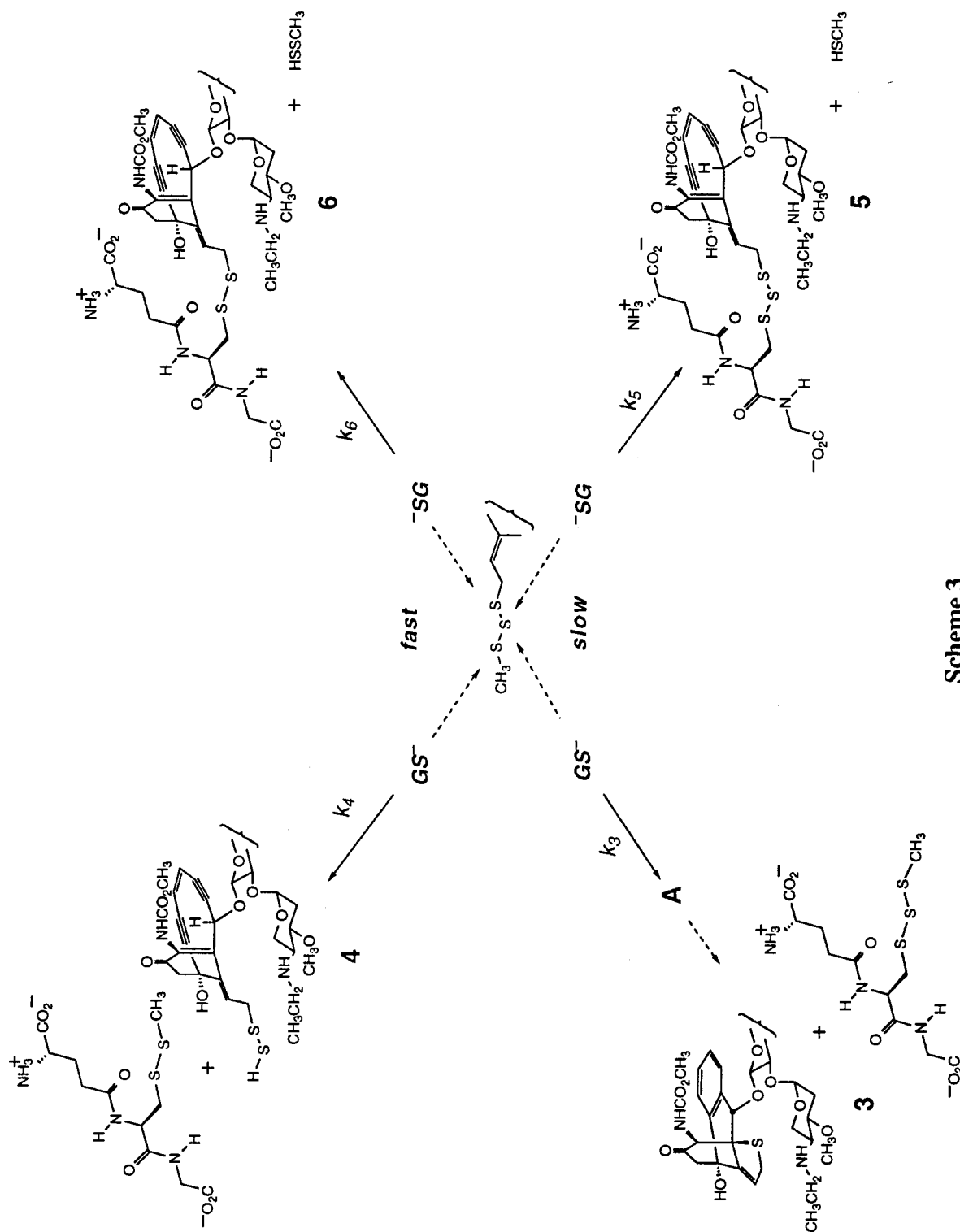
Scheme 3

not expected to be stable under the reaction conditions but are anticipated to undergo further transformations of one or more steps to produce ultimately the dihydrothiophene derivative **3** via the common final sequence **A→2→B→3** (Scheme 4). The putative intermediate **A** has never been observed; available evidence suggests that its cyclization to **2** is quite rapid, even at low temperature.[4] De Voss, Hangeland, and Townsend have measured the rate of cyclization of **2** to **B** at −10 °C from which data **2** may be estimated to have a half-life of ~20 s at 23 °C.[4] It is generally assumed that the quenching of biradicals such as **B** by hydrogen atom transfer is exceedingly rapid.[6]

Scheme 4
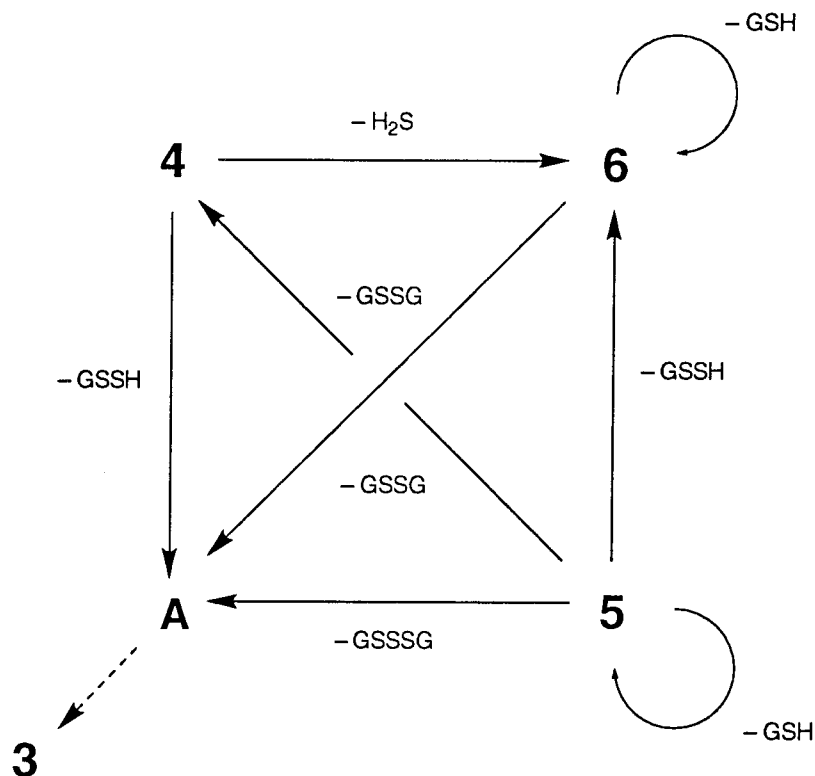
Ellestad et al. have studied the reaction of **1** with methanethiol in organic solvents and have noted that the product distribution is solvent dependent. The reaction of **1** with methanethiol in methanol is reported to form the dihydrothiophene derivative **3**, while in acetonitrile the methyl disulfide derivative **7** is produced. The methyl disulfide
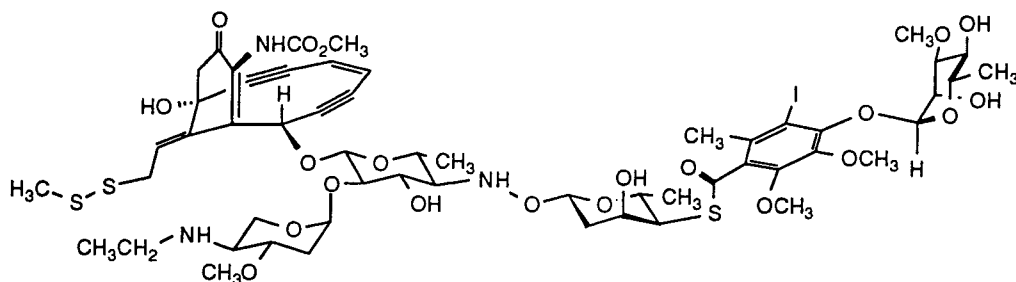
7

derivative **7** is reported to be much less reactive toward thiols than **1**, permitting its isolation from the reaction medium. In addition to the product **7**, the dimeric calicheamicin trisulfide **8** was reported to be formed in the early stages of reactions conducted in acetonitrile.[7] The formation of **8** almost certainly involves the thiosulfenic acid derivative **4** as an intermediate which, in turn, most likely arises by nucleophilic attack of methanethiolate on the methyl-terminal sulfur atom. In a study of the reaction of **1** with methyl thioglycolate in methanol containing triethylamine, Townsend and co-workers report that the initial stages of the reaction, as monitored by variable-temperature ^{1}H NMR spectroscopy, are complex, with signals for several methylthio-containing compounds visible.[4] Previous work clearly establishes a rich and potentially complex chemistry of **1** in its reactions with thiols in organic solvents. In conjunction with a parallel series of investigations of the natural antitumor agent neocarzinostatin, we have undertaken a study of the reaction of **1** with glutathione (GSH), the most abundant nonprotein thiol in eukaryotic cells and a putative cofactor in activation processes occurring in vivo.[8] In order to more closely approximate physiologically relevant conditions, our studies were conducted in water and in the presence of double-stranded calf-thymus DNA. Our goal was to learn the detailed pathway(s) by which **1** and glutathione cleave DNA and, in particular, to probe the possibility that DNA may play a role in the activation process. It is shown that the reaction of **1** with GSH in the presence of DNA is indeed complex and involves, at a
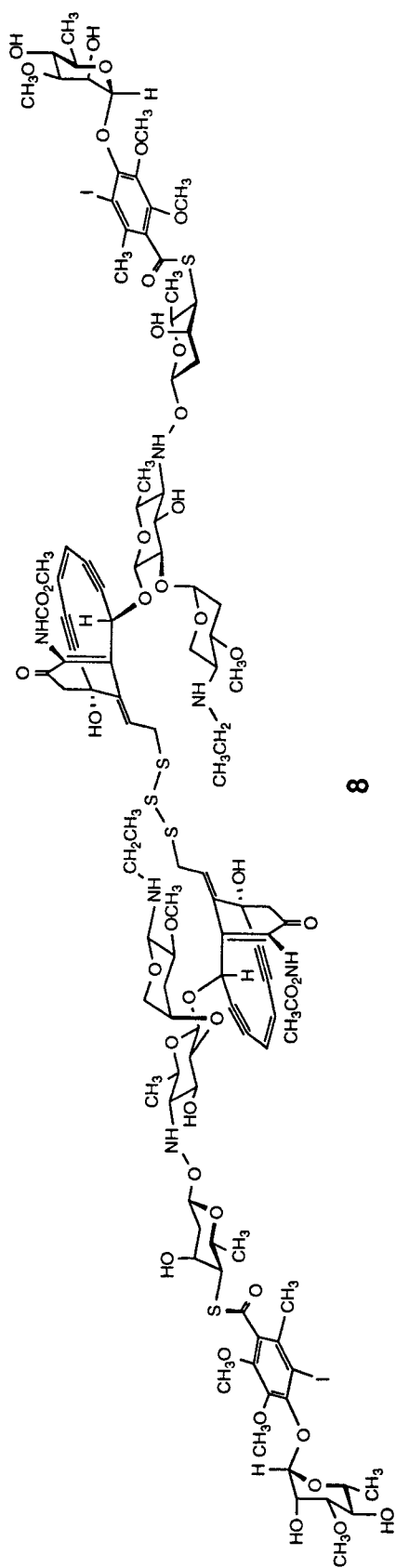
8

minimum, four distinct pathways of activation that proceed at different rates. These pathways are correlated with the observed kinetics for the cleavage of DNA by **1** and glutathione. A detailed investigation of the role of DNA in two of the primary activation processes is presented. It is shown that one of these likely proceeds as a ternary complex of drug, thiol, and DNA while the other occurs in a simple bimolecular reaction of DNA-free drug and thiol. Finally, evidence concerning the potential role of DNA as a catalyst in the thiol activation reaction and regarding the participation of the carbohydrate amino group of **1** in that process is critically evaluated.

The Reaction of Calicheamicin γ_1 with Glutathione

The reaction of calicheamicin γ_1 (**1**, 5.0 x 10^{-5} M) with glutathione (GSH, 1.0 x 10^{-3} M) was conducted in aqueous solution buffered to pH 7.5 (3.0 x 10^{-2} M tris-HCl) at 23 °C in the presence of double-stranded calf thymus DNA (5.0 x 10^{-3} M base pairs, [base pairs]/[drug] = 100) and was monitored by reverse-phase high performance liquid chromatography (rp-HPLC). These parameters define our standard reaction conditions and were employed throughout this work, unless otherwise specified. In the early stages of the reaction (t ≤ 15 min) four product peaks are apparent, as well as unreacted calicheamicin γ_1 (**1**, see Figure 1). In the following paragraphs evidence is presented to support the hypothesis that these four products correspond to the four products arising from S-S bond exchange between the calicheamicin γ_1 trisulfide group and the thiol function of glutathione, as shown in Scheme III. These products are identified as the dihydrothiophene derivative **3**, the thiosulfenic acid derivative **4**, the calicheamicin-glutathione trisulfide **5**, and the calicheamicin-glutathione disulfide **6**. While compounds **4-6** are likely direct products of the reaction of GSH with **1**, the dihydrothiophene derivative **3** presumably arises
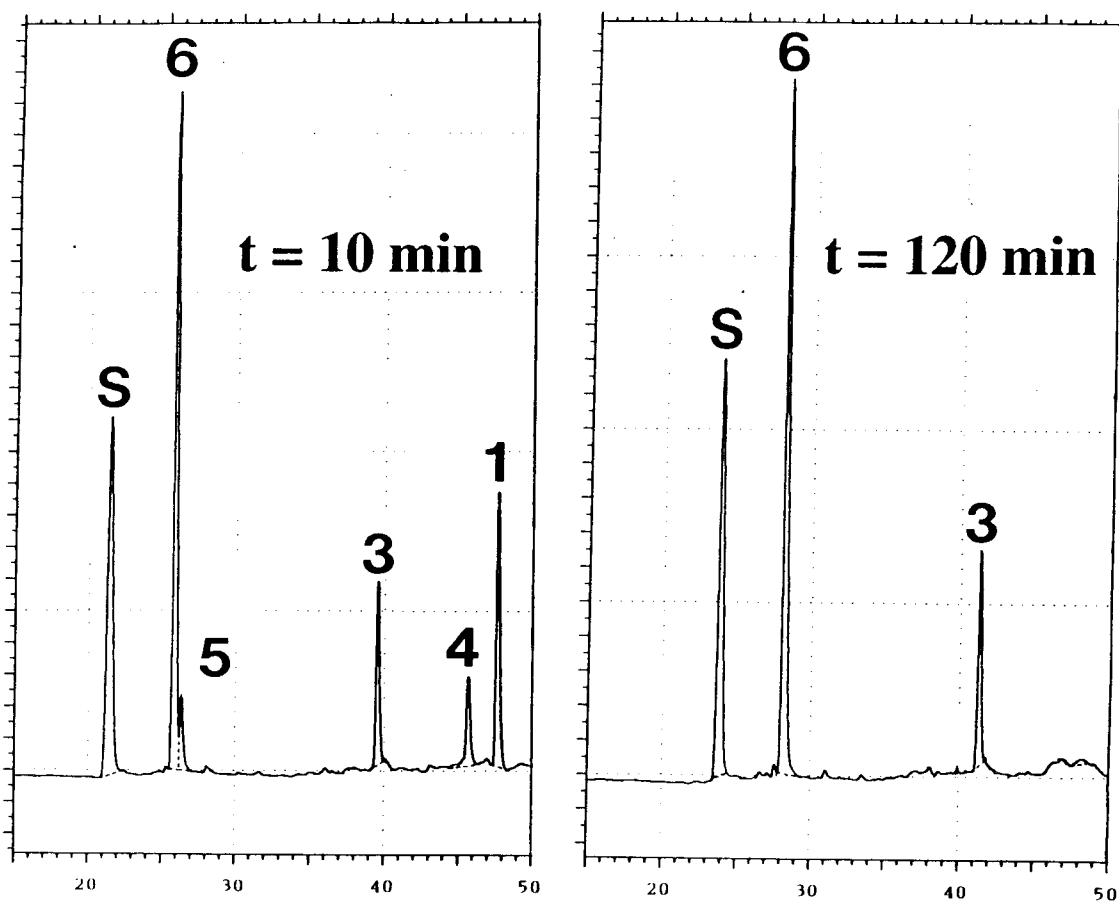
Figure 1. Reverse-phase HPLC traces of the reaction of **1** (5.0 x 10^{-5} M) with GSH (1.0 x 10^{-3} M) in the presence of DNA (5.0 x 10^{-3} M bp) at 10 and 120 minutes. The products are identified in their order of elution as: **S**, 2,5-dimethoxybenzyl alcohol (internal HPLC standard); the calicheamicin-glutathione disulfide **6**; the calicheamicin-glutathione trisulfide **5**; the dihydrothiophene derivative **3**; the thiosulfenic acid derivative **4**; calicheamicin γ_1 (**1**).

from the direct product A by the sequence A→2→B→3, as previously proposed.[2] As suggested in the introduction above, none of the products 4-6 is found to be stable to the reaction conditions; each is transformed, ultimately, to the product 3, albeit by different pathways and at different rates, as discussed below.

Product Identification

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

The similarity of the UV absorption spectra of the three remaining products with that of 1 suggests a common chromophore and thus supports the assignment of these products as structures 4-6. The most predominant (and most stable) of these was isolated by rp-HPLC and was analyzed by electrospray mass spectrometry after lyophilization. The mass of this product corresponded to that calculated for the glutathione-calicheamicin disulfide derivative 6 (calcd. for $[M + H]^+$: 1595.6; found: 1596). Consistent with the latter structural assignment, this substance was found to be
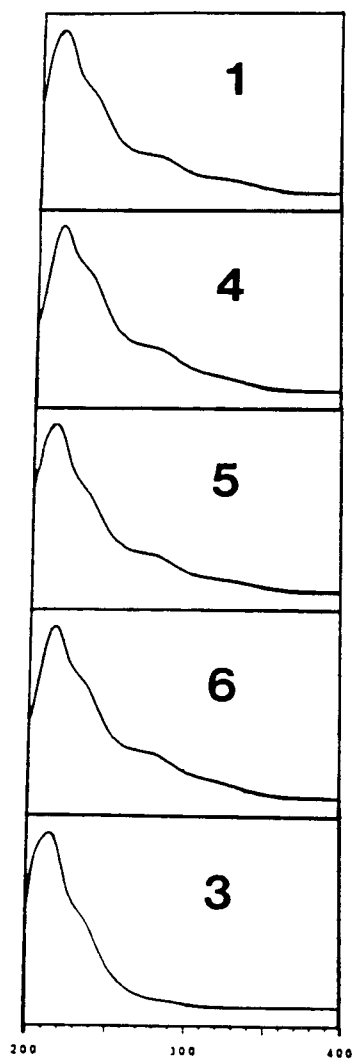
Figure 2. UV absorption spectra (200-400 nm) of **1** and products **3-6**.

substantially more polar (rp-HPLC) than 1 or 3 (see Figure 1). Also consistent with the structure 6, it was found that resubjection of this product to the standard reaction conditions led to the clean formation of the dihydrothiophene derivative 3, albeit ~2 orders of magnitude more slowly than the direct formation of 3 from 1 (vide infra). Product 6 was also found to cleave DNA in the presence of GSH, as described below in detail.

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

The remaining, unidentified component is perhaps most interesting. Careful monitoring of the early stages of the reaction shows that this component, only slightly more polar than calicheamicin γ_1 itself, is a significant product, secondary only to the glutathione disulfide 6. Like the trisulfide 5, this product is found to be highly reactive, persists only within the first several minutes of the reaction, and affords UV absorption data very similar to that of 1 and 6. HPLC fractions containing this product were frozen directly upon elution, without concentration (attempts to obtain mass spectral data on lyophilized samples were not successful); subsequent analysis of these fractions by electrospray mass spectrometry showed the product to be consistent in formulation with its assignment as the thiosulfenic acid derivative 4 (calcd. for $[M + H]^+$: 1322.2; found: 1324). The observed mass value lies within the error limits of the

instrument employed, determined using related compounds for reference (e.g., for **1**, calcd. for [M + H]$^+$: 1368.3; found: 1369 and 1370 in two separate runs). In further confirmation of the assignment, resubjection of isolated **4** to the standard reaction conditions was found to lead to the rapid formation of the disulfide **6** and the dihydrothiophene derivative **3**. This product profile, the exclusive formation of **3** and **6** by separate reaction paths (the observed rate of appearance of **3** in the latter reaction is consistent only with its direct formation from **4** and not from the secondary transformation of **6** identified above, see below), uniquely identifies the starting material as **4**, as diagrammed within Scheme 4.[9] Like the product **6, 4** is found to cleave double-stranded DNA in the presence of GSH, as described in detail below. The relative stability of product **4** as compared with **A**, the presumed precursor to **2** and **3**, is noteworthy. It is clear that internal Michael addition within **4** to form a cyclic disulfide derivative analogous to **2** is not a viable reaction pathway, perhaps due to repulsion of the adjacent sulfur lone pair orbitals in the hypothetical transition state for this cyclization.[10]

Kinetics of Primary and Secondary Activation Processes with Glutathione

Despite the complexity of the reaction of 1 and GSH in the presence of DNA, the kinetics of disappearance of 1 follows simple pseudo first-order behavior (rp-HPLC determination, $k_\psi = 7.9 \times 10^{-4} s^{-1}$, 5.0×10^{-4} M GSH; $k_\psi = 1.8 \times 10^{-3}$ s^{-1}, 1.0×10^{-3} M GSH; second-order rate constants 1.6 and 1.8 M^{-1}s^{-1}, respectively, Figure 3). This observation is consistent with the mechanism of Scheme 3 where the partitioning of 1 among the various pathways of reaction with GSH is rate-determining; k_ψ then represents the sum of k_3, k_4, k_5, and k_6, as defined within Scheme 3. Products **4** and **5** grow and decay within the first several minutes of the reaction, partitioning between
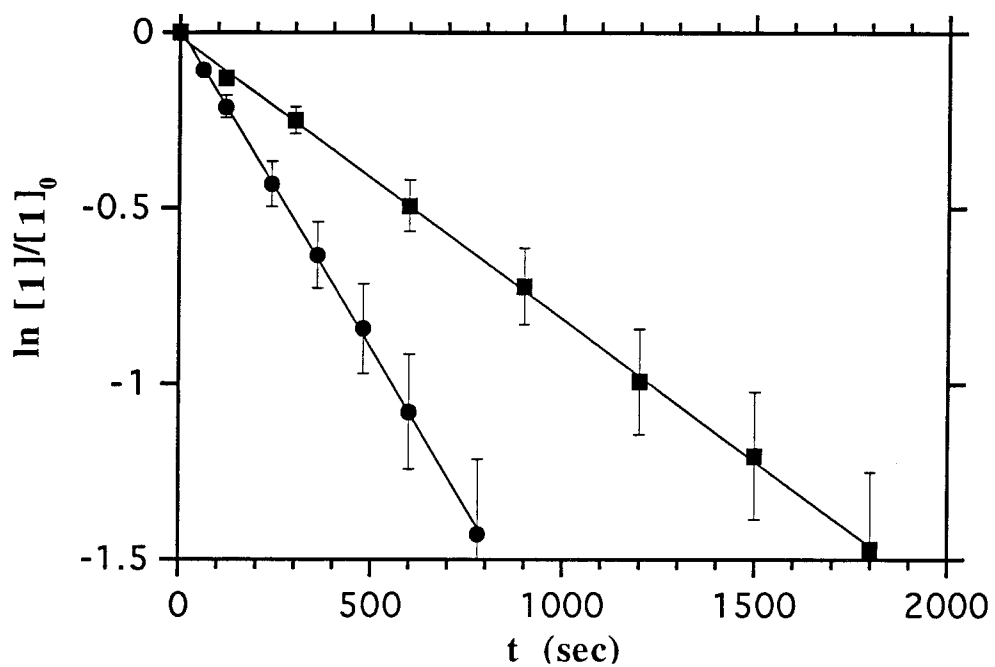
Figure 3. Logarithmic plots of the reaction of **1** (5.0 x 10^{-5} M) with GSH in the presence of DNA (5.0 x 10^{-3} M bp), as monitored by HPLC. (■): GSH = 5.0 x 10^{-4} M (k = 1.6 $M^{-1}s^{-1}$); (●): GSH = 1.0 x 10^{-3} M (k = 1.8 $M^{-1}s^{-1}$).

products **3** and **6** in one or more steps. Because of the complexity this brings to the kinetic analysis, accurate values of k_3, k_4, k_5, and k_6 have not been determined; however, on the basis of the rate of appearance of products **3**, **4**, **5**, and **6** at the onset of the reaction these rate constants may be rank-ordered as follows: $k_6 > k_4 > k_3 > k_5$ (see Figure 4). The ratio $k_6{:}k_5$ (the fastest and slowest steps, respectively) is estimated to lie between 6 and 10.

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

Following its initial stages (>1 h), the reaction may be described by a single process -- the transformation of **6** to **3**. This transformation is found to be slower than k_3-k_6 by at least two orders of magnitude under our standard reaction conditions. In order to conveniently measure the rate of formation of **3** from **6**, it was necessary to increase the concentration of glutathione by 10-fold over the corresponding reaction conducted with **1**. Under otherwise identical conditions, **6** was observed to undergo clean transformation to **3** with pseudo first-order kinetics ($k = 5.0 \times 10^{-5}$ s^{-1}, 1.0×10^{-2} M GSH; second-order rate constant 5.0×10^{-3} M^{-1}s^{-1}).[11] Comparison of the second-order
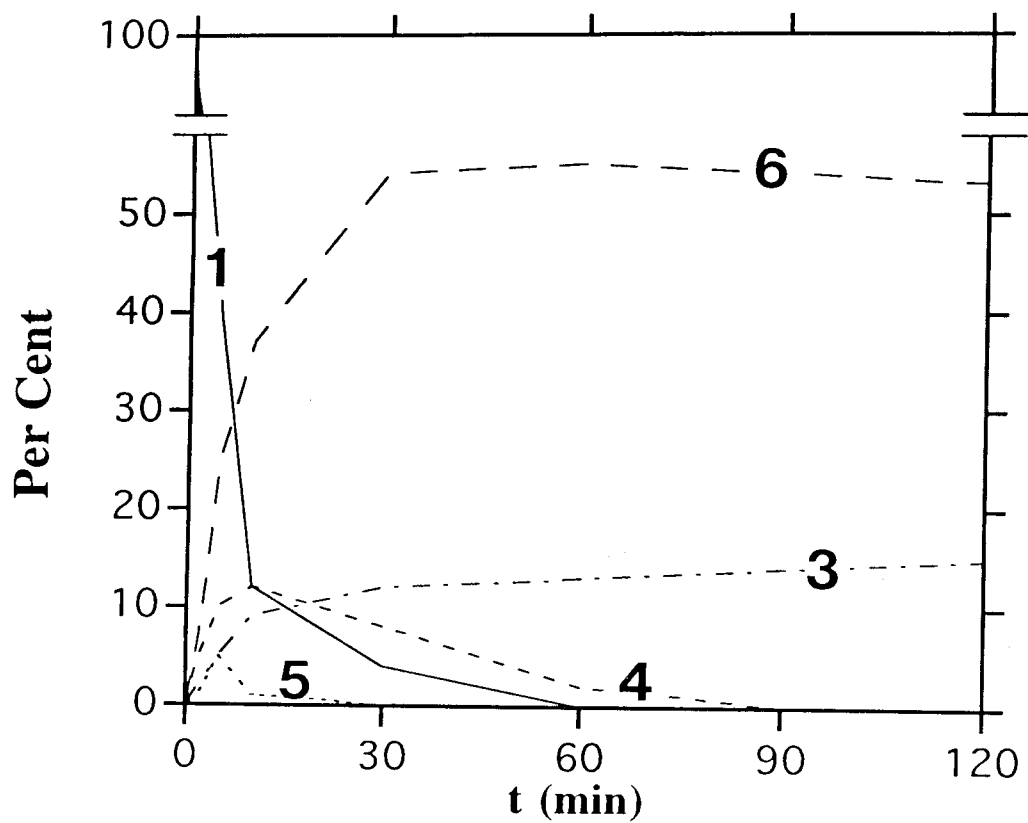
Figure 4. Time profile of the reaction of **1** (5.0×10^{-5} M) with GSH (1.0×10^{-3} M) in the presence of DNA (5.0×10^{-3} M bp) as monitored by rp-HPLC.

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

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

by studying both the kinetics and sequence specificity of DNA cleavage by **1**, **4**, and **6** in the presence of GSH, as reported below.
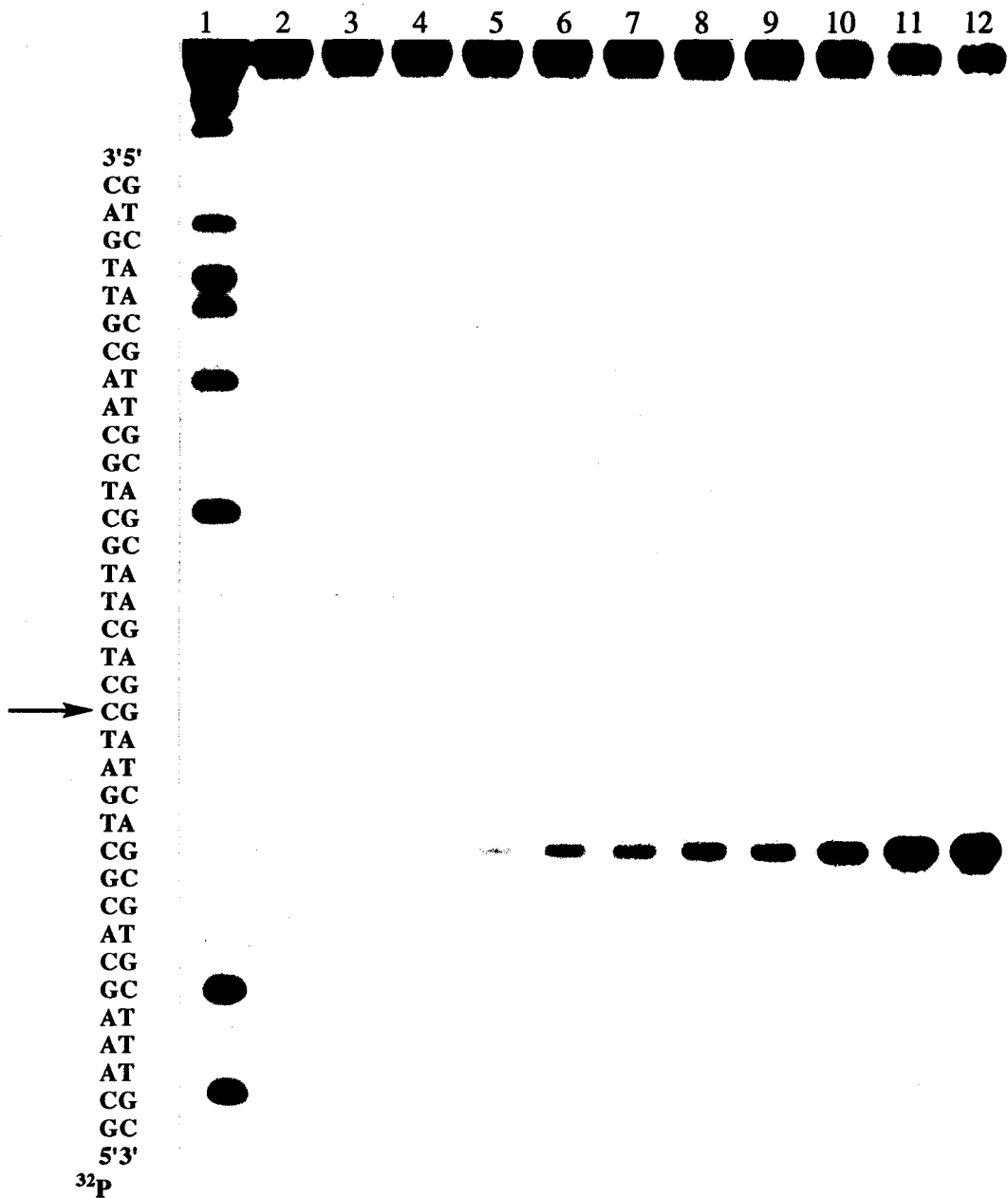
DNA Cleavage Experiments

DNA cleavage experiments were conducted under the standard reaction conditions defined above [**1**, 5.0×10^{-5} M; GSH, 1.0×10^{-3} M; calf thymus DNA, 5.0×10^{-3} M base pairs; tris-HCl buffer (pH 7.5), 3.0×10^{-2} M] with the inclusion of trace quantities of the $5'$-^{32}P-labeled synthetic 35-mer duplex DNA: $5'$-^{32}P-GCAAAGCACGCTGATCCTCTTGCTGCAACGTTGAC-$3'$. This sequence was anticipated to contain one strong cleavage site on the labeled strand ($5'$-TCCT-$3'$) on the basis of previous studies of the sequence specificity of DNA cleavage by **1**.[1a,12] Quantitative analysis of DNA cleavage by **1** and GSH was achieved by polyacrylamide gel electrophoresis (PAGE) of aliquots taken periodically throughout the course of a given reaction.[13] Figure 5 displays cleavage data from two such experiments as well as a control reaction in which GSH was omitted (lane 2). Lanes 3-9 of the gel illustrate

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

[GSH] (mM)	0	1	1	1	1	1	1	1	10	10	10
t (min)		5	15	30	60	120	300	1200	120	240	1200
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Relative Cleavage	0	10	15	23	29	33	37	40	58	90	100

the time course of a reaction containing 1.0×10^{-3} M GSH; lanes 10-12 show an identical reaction but with additional GSH incorporated after 60 min (final concentration: 1.0×10^{-2} M). As anticipated, all observed DNA cleavage was restricted to the single site 5'-T<u>C</u>CT-3'.[12] The reaction employing 1×10^{-3} M glutathione (lanes 3-9) displayed a monotonic increase in DNA cleavage over a reaction period of 5-1200 min with a cleavage efficiency of approximately 35% at 1200 min. Comparison of this reaction with lanes 10, 11, or 12 shows that even after 1200 min, maximal DNA cleavage has not been attained at the lower concentration of thiol. Complete and highly efficient (~90%) DNA cleavage is observed upon incubation of **1** with the 10-fold higher concentration of GSH (1.0×10^{-2} M, lanes 10-12). Kinetics experiments described above show that any cleavage observed after 60 min must be attributable entirely to the glutathione-calicheamicin disulfide **6**, thereby confirming the conjecture that the bulk of DNA cleavage by **1** arises from the disulfide **6**. It is also notable that the sequence specificity of cleavage does not vary with time (Figure 5, lanes 3-9, 10-12), suggesting that **1** and **6** cleave the same site within this synthetic 35-mer, a conclusion verified below.

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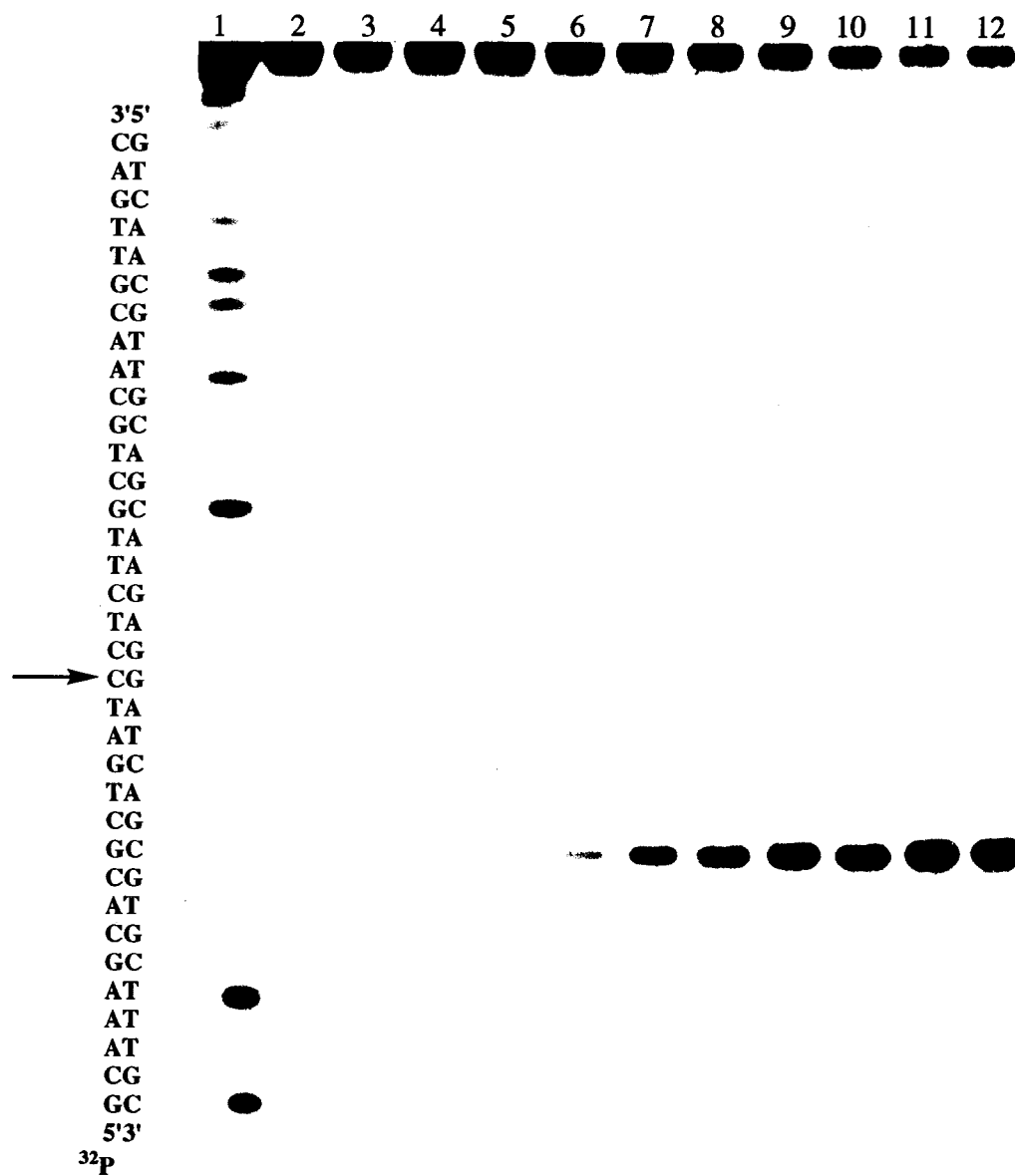
rate. The maximal cleavage efficiency attained with **6** is calculated to be approximately 90%. Comparison of the cleavage intensities of experiments employing **1** and **6** (lanes 3 and 4-12, respectively) supports the earlier conclusion that the reaction of **1** with GSH produces an initial minor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

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

Taken together, the DNA cleavage data supports the picture which arose from HPLC analysis of the reaction products derived from **1**. The reaction of **1** with GSH in the presence of DNA proceeds via 4 competing pathways, three of which are rapid and one of which is ~2 orders of magnitude slower under the standard conditions.

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

Substrate	6	1	6	6	6	6	6	6	6	6	6
[GSH] (mM)	0	1	1	10	10	10	10	10	10	10	10
t (hrs)		1	1	0.5	1	2	3	4	5	8	10

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Relative Cleavage   0   30   3   15   29   53   72   83   95   100   100

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

Substrate	1	4	4	4	4	4	4	4	4	4	4
[GSH] (mM)	1	1	1	1	1	1	1	1	10	10	10
t (min)	60	3	10	20	60	120	300	1200	120	300	1200

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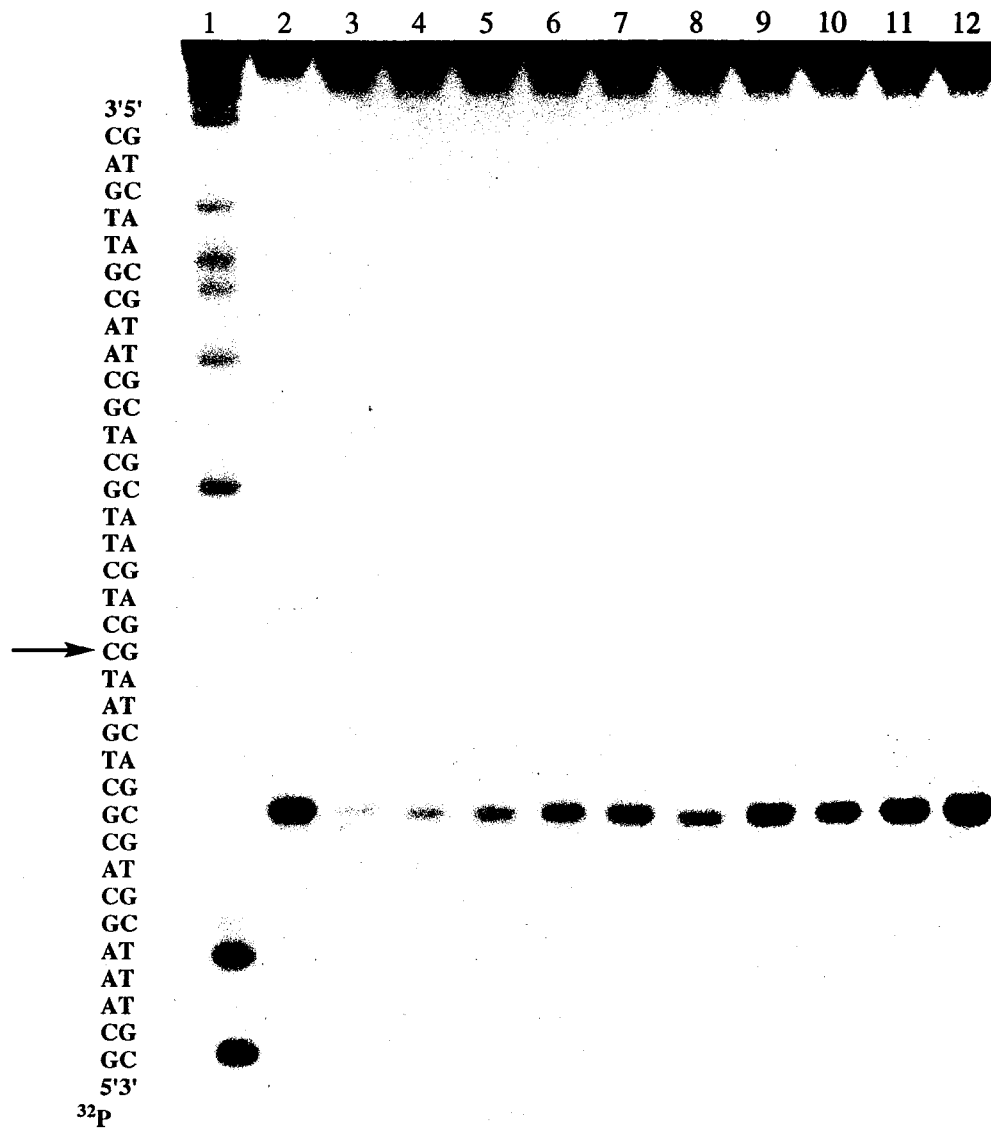
Relative Cleavage	19	32	41	45	50	54	69	59	77	100

DNA cleavage follows an essentially bimodal course initiated with a rapid burst followed by a much slower but major cleavage pathway involving the disulfide **6**. Overall, the efficiency of DNA cleavage is high; the amount of cleavage arising directly from the reaction of **1** and GSH (**1**→A→**2**→B) is conservatively estimated to be less than 25%; fully 60% of the cleavage arises indirectly via the disulfide **6**.

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

Figure 8. DNA cleavage of a $5'-^{32}P$-labeled 167-base pair restriction fragment of pBR322 (Eco RI-Rsa I digest) produced by the reaction of **1** or **6** with GSH. Lane 1: calf thymus DNA (1.0×10^{-3} M bp) alone; lane 2: cleavage products of an adenine-specific sequencing reaction;[36] lane 3: **1** (5×10^{-6} M), calf thymus DNA (1.0×10^{-3} M bp), GSH (1.0×10^{-3} M), t = 10 min; lane 4: **1** (5×10^{-6} M), calf thymus DNA (1.0×10^{-3} M bp), GSH (1.0×10^{-3} M), t = 300 min; lane 5: **1** (5×10^{-6} M), calf thymus DNA (1.0×10^{-3} M bp), GSH (1.0×10^{-3} M for the first 10 min, 1.0×10^{-2} M thereafter, t = 300 min); lane 6: **6** (5×10^{-6} M), calf thymus DNA (1.0×10^{-3} M bp), GSH (1.0×10^{-2} M), t = 300 min.

Substrate		1	1	1	6
[GSH] (mM)		1	1	10	10
t (min)		10	300	300	300

Relative Cleavage (total): 17 59 95 100

Comparison of the histograms of lanes 3 and 6 determined by phosphorimaging shows that **1** and **6** display identical sequence specificity of cleavage within this 167-base pair restriction fragment.

That **1** and **6** display identical sequence specificity of cleavage is perhaps not surprising, given that all cleavage arises ultimately from the biradical **B** and that each of these intermediates must pass through **A**, **2**, and **B** in the formation of the final product **3**. The critical issues here concern the site of activation and the dynamics of rearrangement versus equilibration among DNA binding sites for each species in the pathway. Scenarios may be envisioned where the observed cleavage specificity is kinetically determined,[14] e.g., if **2** were formed as a DNA bound intermediate and the rate of its cycloaromatization were rapid relative to its rate of equilibration among DNA binding sites. Existing data suggests that this is not likely to be the case. Townsend et al. calculate a half-life of ~20 s for the intermediate **2** (the last common intermediate arising from **1**, **4**, and **6** prior to the formation of the biradical **B**) at 23 °C.[4] Consideration of the rates of equilibration of a representative sample of non-intercalative minor groove binding drugs between DNA binding sites (Table I)[15] suggests that it is likely that **2** would have more than sufficient time to equilibrate among DNA binding sites prior to the cycloaromatization reaction that produces **B**. This hypothesis is further supported by the recent work of Walker, Murnick, and Kahne, wherein the rate of dissociation of **1** bound to an 8-mer DNA duplex was estimated to be 3.1 ± 1.1 s^{-1} at 25 °C.[16] The proposal that double helical DNA may catalyze the rearrangement of **2** to **B**[4] seems unlikely in view of the nature of the unimolecular rearrangement involved and experimental data contradicting this hypothesis has been reported.[17] The almost certain rapid quenching of the biradical **B** (relative to its equilibration among DNA binding sites) then forces the conclusion that **2**

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

Drug	Half Life (s)	T (°C)	Ref.
Distamycin A	0.173	27	15a
SN 6999	0.007	16	15b
Lexitropsin	0.016	21	15c
(4S)-(+)-Anthelvencin A	0.019	21	15d

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

Distamycin A

Lexitropsin

SN 6999

(4S)-(+)-Anthelvencin A
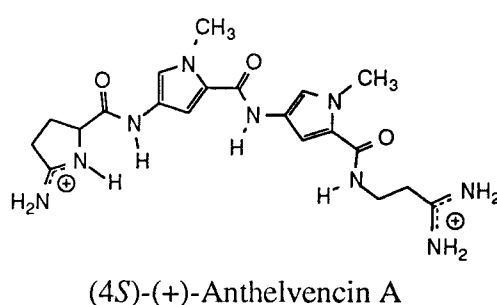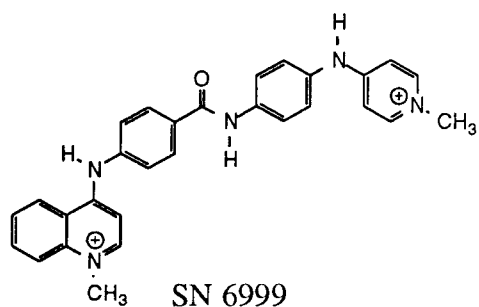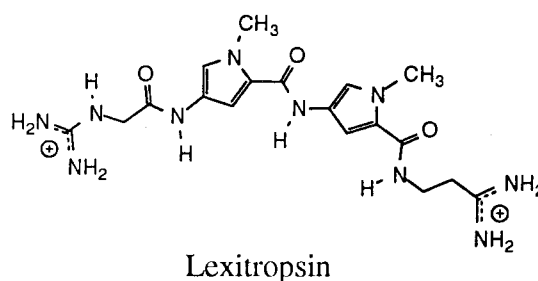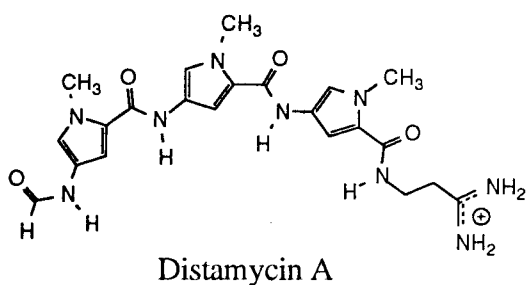
is the sequence-determining species in DNA cleavage, as previously proposed.[4] The fact that **1** and **6** display identical DNA cleavage specificity supports this hypothesis. Experiments described below will show that thiol activation of **6** occurs free in

solution, thus generating A and, presumably, 2 prior to DNA binding, whereas 1 undergoes thiol activation while bound to DNA. Thus, the product of thiol activation (2, formed by rapid cyclization of A) functions equivalently in DNA cleavage whether generated bound to DNA or free in water. Again, the implication is that 2 is the sequence-determining species in DNA cleavage.

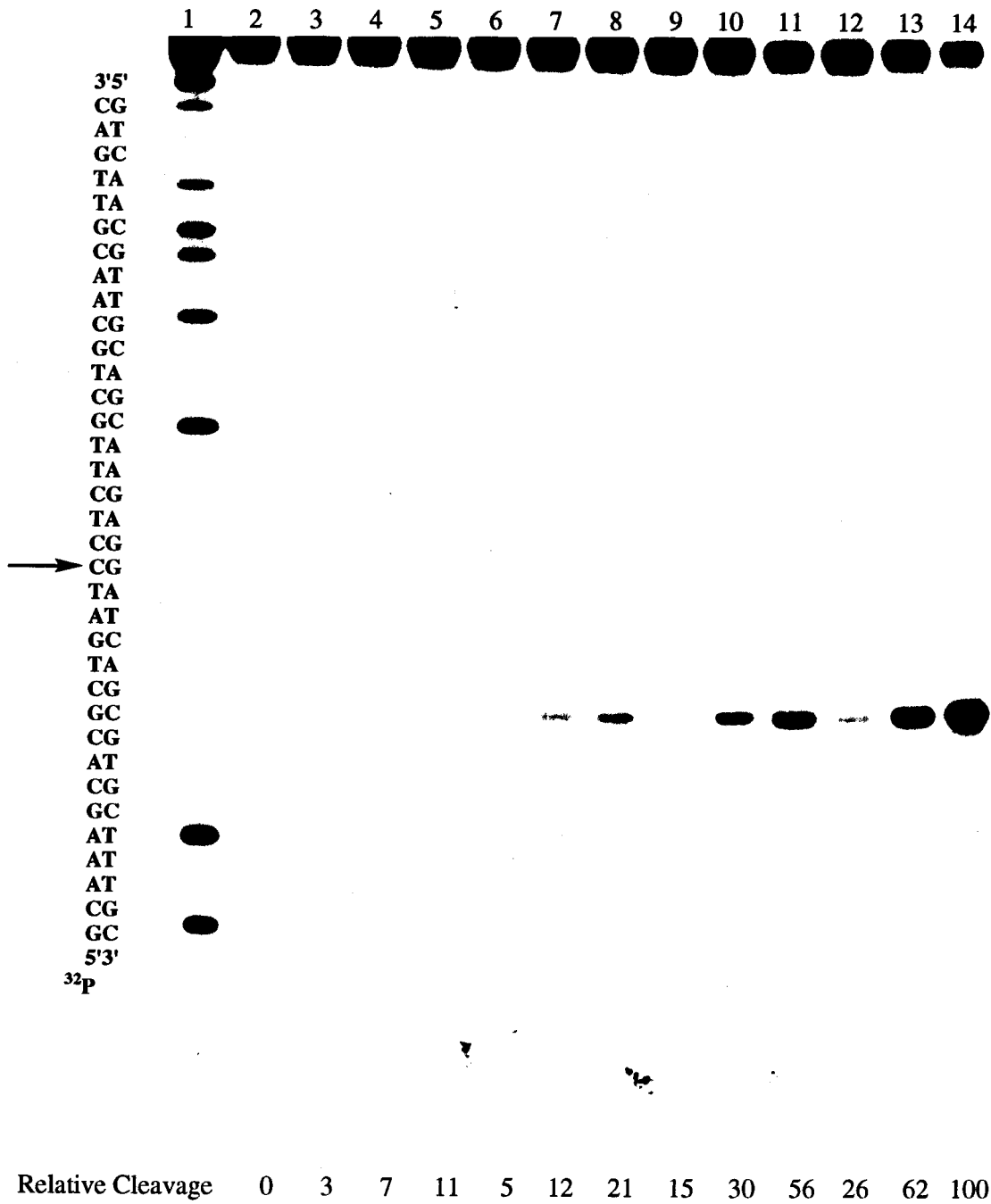## The Role of DNA in Thiol Activation of Calicheamicin γ_1

A natural question arises as to the role of DNA in the chemistry of activation of 1 by glutathione. Does the reaction occur as a ternary complex of 1, GSH, and DNA, or is 1 activated free in solution with subsequent binding of the reaction product(s) to DNA? What is the role of DNA in the primary and secondary activation steps? We have been able to address these questions, in part, by a kinetic analysis of the reaction of 1 and of 6 with GSH as a function of the concentration of DNA. The method pertains to the specific situation where drug is primarily bound to double helical DNA. By then increasing the concentration of DNA in the medium, the concentration of bound drug is changed negligibly while the concentration of free drug decreases markedly. If bound drug reacts faster than free drug, then the rate of DNA cleavage will be unaffected with increasing concentrations of DNA. The same result will obtain in the unlikely situation that bound and free drug react at identical rates. If, however, free drug reacts appreciably faster than bound drug, then the rate of DNA cleavage will decrease as the concentration of DNA is increased. The latter is precisely the situation observed with 6. Figure 9 displays the time course of DNA cleavage by 6 and GSH $(1.0 \times 10^{-2}$ M) as analyzed by PAGE at four different concentrations of DNA spanning two orders of magnitude $(4.0 \times 10^{-5}$ M to 5.0×10^{-3} M).[13] In each experiment, the concentration of 6 was varied so as to maintain a constant ratio of drug to DNA

([6]/[DNA] = 1/100). Analysis of the data of Figure 9 clearly indicates that double-stranded DNA serves to inhibit the cleavage reaction, supporting the idea that thiol activation of **6** occurs free in solution with subsequent binding of the thiol-activated product (**A** or, more likely, **2**) to DNA. These results have been confirmed by HPLC analysis of the reaction of **6** and GSH (1×10^{-2} M) with varying concentrations of calf thymus DNA (4.5×10^{-3} and 0.9×10^{-4} M bp, Figure 10). The rate of reaction of **6** is observed to increase by a factor of 4.8 upon dilution of the concentration of DNA by a factor of 5 ($k = 5.0 \times 10^{-3}$ and 2.4×10^{-2} $M^{-1}s^{-1}$, respectively), again demonstrating the inhibitory properties of DNA upon the thiol activation reaction of **6** and, consequently, upon DNA cleavage.

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

[6] (µM)		<	50	>	<	10	>	<	2	>	<	0.4	>

[DNA] (mM)		<	5.0	>	<	1.0	>	<	0.2	>	<	0.04	>

t (min)		5	15	30	5	15	30	5	15	30	5	15	30

1 2 3 4 5 6 7 8 9 10 11 12 13 14

3'5'
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^{32}P

Relative Cleavage	0	3	7	11	5	12	21	15	30	56	26	62	100
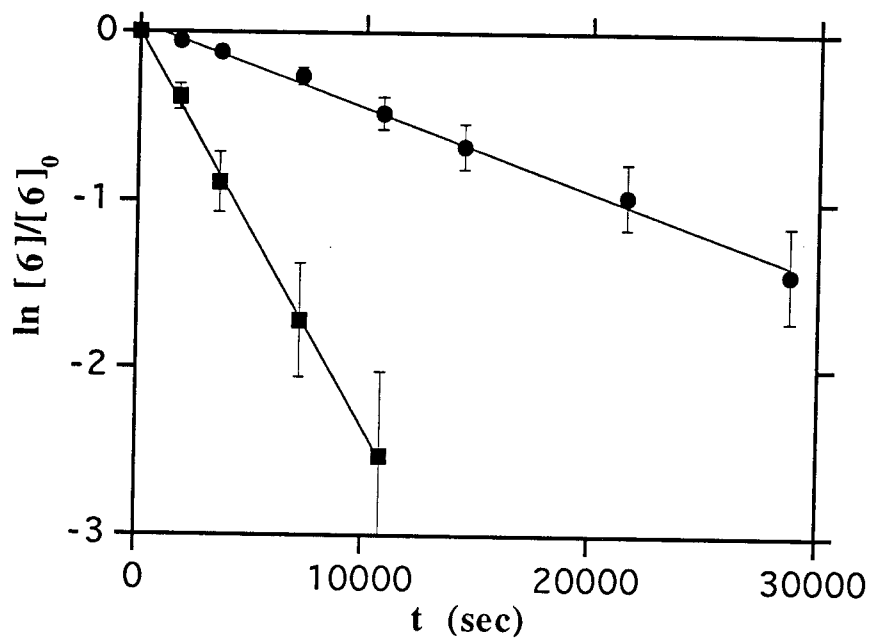
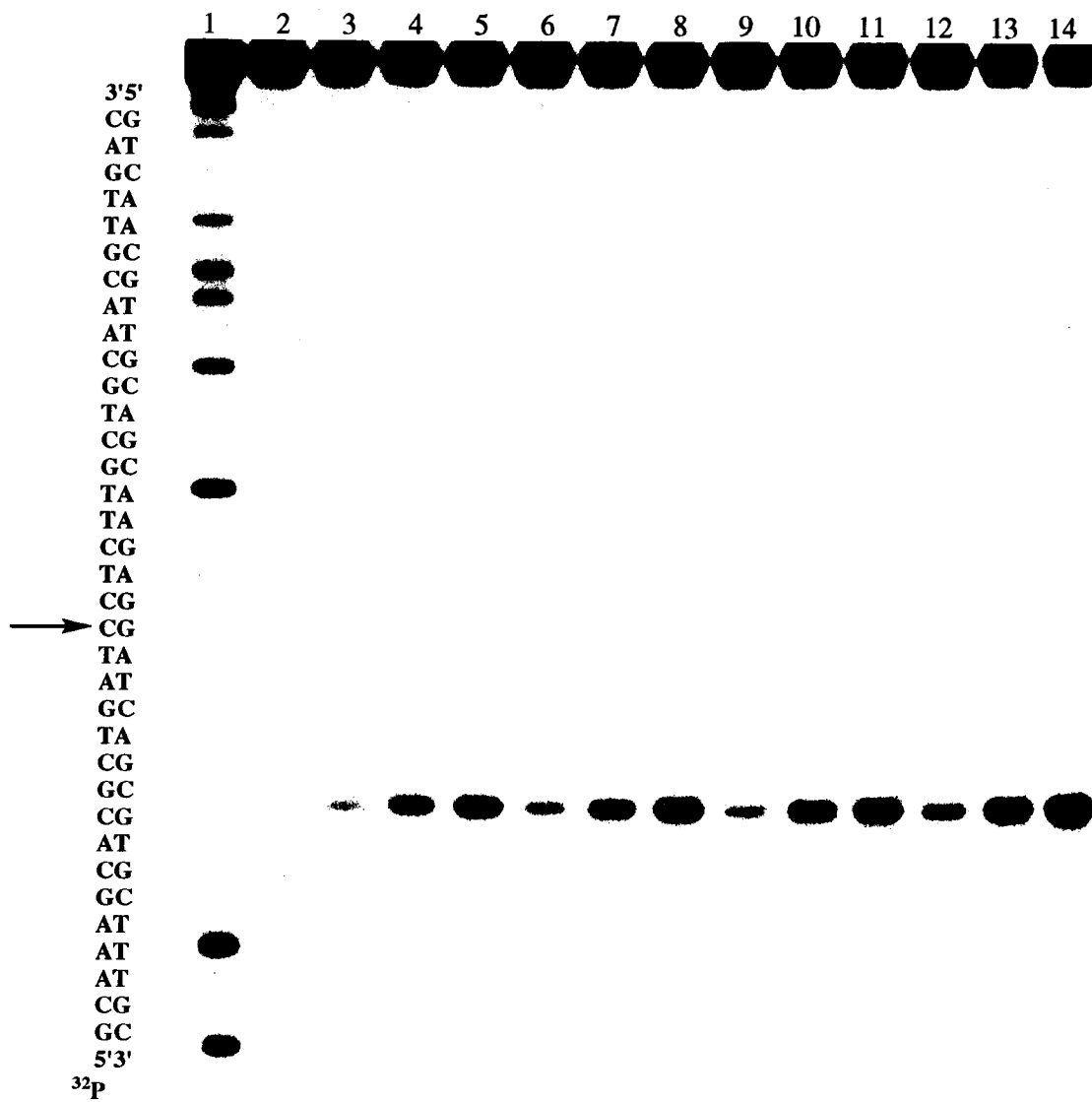**Figure 10.** Logarithmic plots of the rate of disappearance of the calicheamicin-glutathione disulfide **6** in its reaction with GSH (1 x 10^{-2} M) in the presence of DNA, as monitored by rp-HPLC. (●): **6** (4.5 x 10^{-5} M), DNA (4.5 x 10^{-3} M bp), (k = 5.0 x 10^{-3} $M^{-1}s^{-1}$); (■): **6** (9 x 10^{-6} M), DNA (9 x 10^{-4} M bp), (k = 2.4 x 10^{-2} $M^{-1}s^{-1}$).

Conceptually, the same experiment may be conducted with **1**, but analysis of the outcome of this experiment is complicated by the fact that **1** is rapidly transformed to **6**. In order to dissect the component of DNA cleavage attributable to **1** alone, DNA cleavage experiments were conducted with **1** and **6** in parallel with analysis (PAGE) restricted to the very early stages of the reaction (t ≤15 min), where cleavage by **1** dominates. An upper bound on the cleavage due to **6** as a secondary process emanating from **1** may then be roughly approximated and corrected for by quantitation of the cleavage intensity of the appropriate experiment conducted with pure **6**. As is evident from inspection of the data of Figures 9, 11 and 12, the DNA cleavage reactions mediated by **1** and **6** display very different kinetic behavior as a function of the concentration of DNA. Whereas DNA cleavage by **6** is strongly inhibited by DNA, DNA cleavage by **1** is essentially unaffected by variations in the concentration of DNA. Discounting the unlikely possibility that bound and unbound forms of **1** react with GSH at equivalent rates, the data shows that the reaction of **1** with GSH proceeds as a termolecular event, while the reaction of **6** with GSH is a simple bimolecular process.
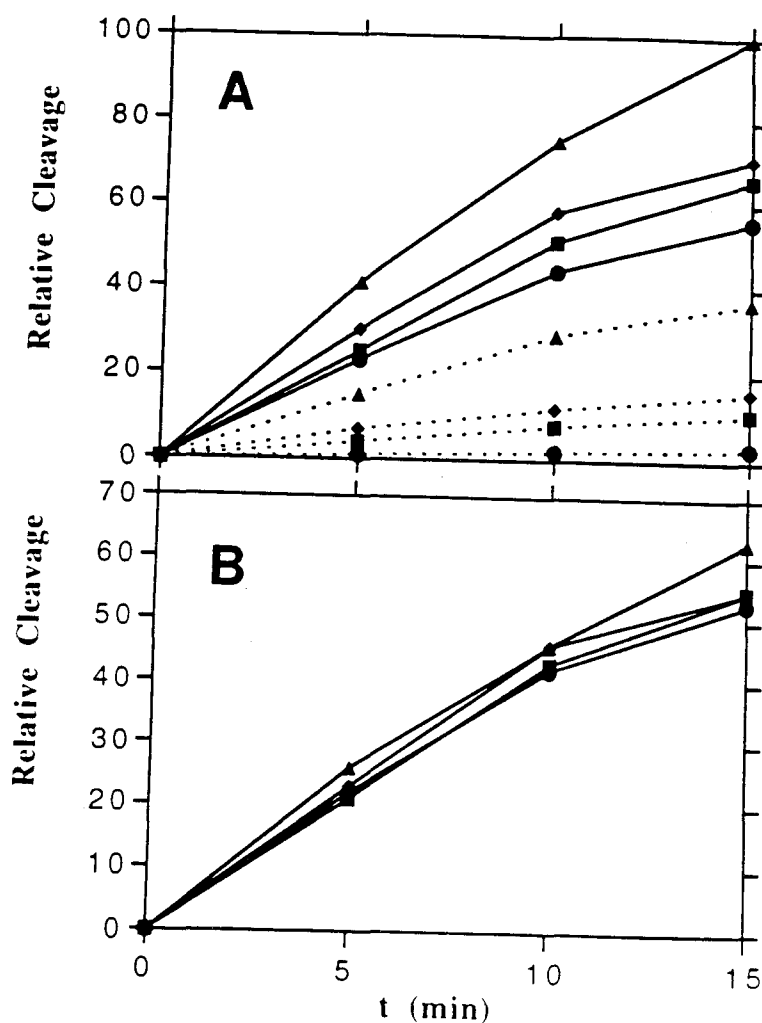

Figure 11. Kinetics of DNA cleavage produced by the reaction of calicheamicin γ_1 (**1**) with GSH at varying concentrations of DNA. Lane 1: Maxam-Gilbert G/A sequencing reaction;[35] lane 2 (control): **1** (1×10^{-4} M), DNA (5×10^{-3} M bp); no GSH, t = 60 min; lanes 3-5: **1** (1×10^{-4} M), DNA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

[1] (μM)		<	100	>	<	20	>	<	4	>	<	0.8	>
[DNA] (mM)		<	5.0	>	<	1.0	>	<	0.2	>	<	0.04	>
t (min)		5	10	15	5	10	15	5	10	15	5	10	15

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³²P

| Relative Cleavage | 0 | 23 | 44 | 56 | 25 | 51 | 66 | 30 | 58 | 71 | 41 | 75 | 100 |

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

This is an entirely reasonable outcome given the likelihood for charge repulsion in the reaction of **6** with GSH proximal to the polyanionic DNA helix. The significance of this observation is that the products of thiol activation of **1** function equivalently in the cleavage of DNA in terms of efficiency and specificity whether generated in DNA-bound form (from **1**) or free in solution (from **6**). As discussed above, this suggests that thiol-activated **1** (**A** or, more likely, **2**) is sufficiently long-lived to equilibrate among DNA binding sites or, equivalently, is the sequence-determining species in DNA cleavage by **1**. A similar conclusion has been reached regarding the cumulene product of thiol activation of neocarzinostatin chromophore.[18]

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

The possibility that DNA may catalyze the reaction of **1** with GSH is, in theory, trivially addressed. All that is necessary is to measure and compare the rates of reaction of **1** with GSH in the presence and absence of DNA. Unfortunately, our efforts to conduct this seemingly trivial experiment have been wholly unsuccessful due to the complete insolubility of **1** in aqueous media or in mixtures of water and organic solvents in the absence of DNA.[19] We have carefully studied the solubility properties of **1** in aqueous media by light-scattering analysis with a submicron particle analyzer. For example, addition of a solution of **1** in DMSO (1 x 10^{-3} M) to 19 volumes of the aqueous buffer system of our standard reaction conditions, modified by the incorporation of potassium dimethyl phosphate (1 x 10^{-2} M) in lieu of DNA to

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

Recently, the question of DNA participation in thiol activation of 1 was investigated by measuring the rates of reaction of 1 with aminoethanethiol and with GSH[20] in the presence and absence of DNA in the medium 30% methanol-aqueous tris buffer (3 x 10^{-2} M, pH 7.5). It was found that thiol activation of 1 in the presence of DNA is slightly slower than reactions conducted in its absence.[21] We have studied the solubility of 1 in the medium employed in the latter studies and find that 1 forms particulate suspensions in that medium as well. Addition of a homogeneous solution of 1 in methanol (4 x 10^{-3} M) to 79 volumes of 30% methanol-aqueous tris buffer (3 x 10^{-2} M, pH 7.5, 50 mM NaCl) produced a particulate suspension with particles of 2.5 μm maximum diameter. Centrifugation (16,000 g, 30 min) removed the larger particles (30% precipitation), affording a supernatant suspension of mean particle diameter 0.5 μm. Assuming a density of ~0.8 g/mL, these particles contain ~3 x 10^7 molecules of 1, of which <1% reside at the particle surface. The rate of the heterogeneous reaction

of GSH with particulate **1** is potentially quite different from the rate of the hypothetical, homogeneous bimolecular reaction of GSH with an isolated molecule of **1** in aqueous solution. The statement, "any possibility that the drug is not fully dissolved in the absence of DNA would only serve to increase the relative rate of solution reaction", is unfounded.[22] In studies of the rate of nucleophilic addition of GSH to DNA-bound and to free neocarzinostatin chromophore, we have found that the termolecular activation process is, in fact, slower than the bimolecular reaction by a factor of ~15 (the termolecular reaction dominates nevertheless, due to the high affinity of neocarzinostatin chromophore for DNA). Although a similar conclusion concerning the reaction of **1** with GSH is perhaps not unreasonable, it is not supported by the data available at present.

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

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

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

The Role of the Amino Group in Thiol Activation of Calicheamicin γ_1

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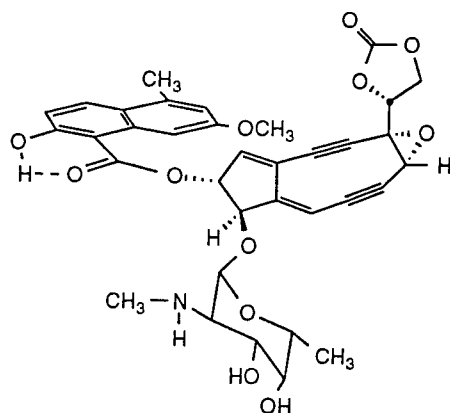
9

crystallographic study of the neocarzinostatin protein-chromophore complex, the carbohydrate amino group of 9 was found to be oriented directly above C12, the site of nucleophilic thiol addition, at a distance of ~5 Å, or approximately the van der Waals diameter of a sulfur atom.[26]

Within the context of this study of the mechanistic details of calicheamicin activation, it is worthwhile and important to question whether the carbohydrate amino group of 1 participates in the thiol activation process when 1 is bound to DNA, since this would appear to be most relevant to events occurring in vivo, on the basis of experiments described above. It is useful to define precisely the mechanisms for participation of the carbohydrate amino group in the thiol activation chemistry. Scheme 5 depicts two limiting mechanisms, 5a and 5b. Mechanism 5a involves deprotonation of the neutral thiol by the neutral amino group with concomitant attack of the developing thiolate anion upon the trisulfide (illustrated for the specific case of the generation of the intermediate **A**, the mechanism holds for the 3 alternative modes of trisulfide cleavage as well). Mechanism 5b represents the alternative extreme, involving a thiolate-ammonium ion pair, formed in a rapid pre-equilibrium step, with rate-determining attack of thiolate upon the trisulfide group. While conceptually

distinct, it is important to recognize that these two mechanisms are kinetically equivalent, and that both fall within the definition of general-base "catalysis" (the word catalysis is used somewhat loosely here due to the intramolecular nature of the reaction).[27] Thus, the protonation state of the amino group does not, per se, support or rule out mechanisms involving the participation of this group in the thiol activation step.[28]

Scheme 5
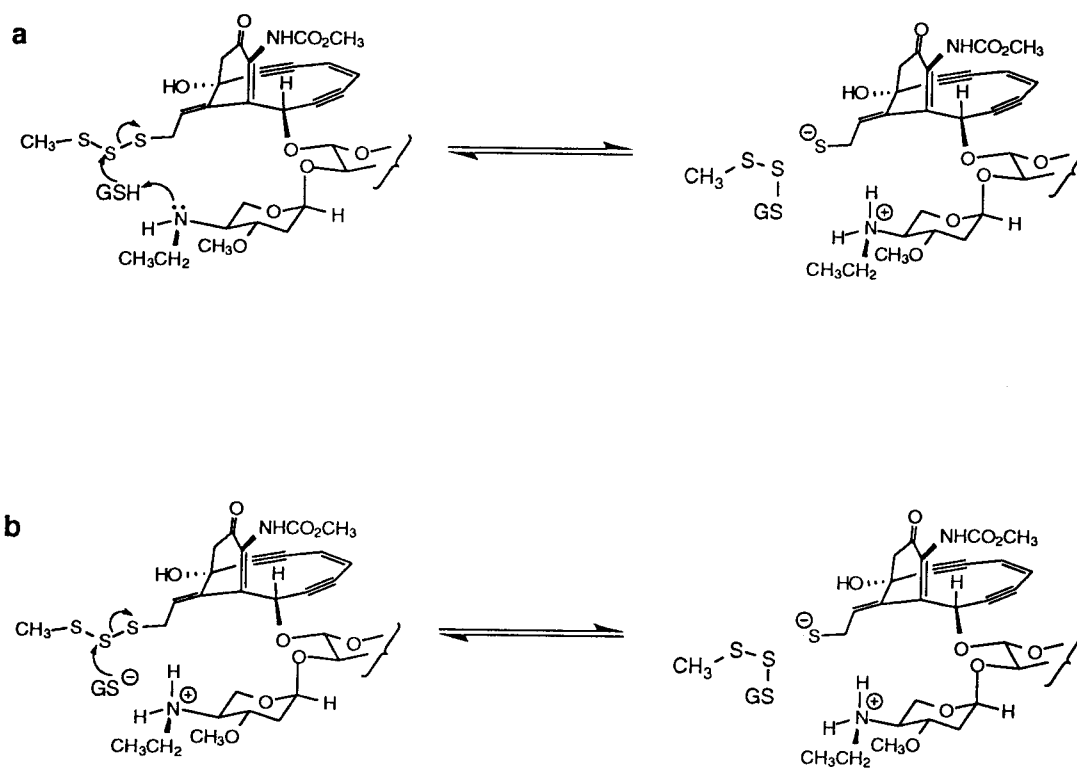
In a recent study comparing the rates of thiol activation of **1** in the presence and absence of DNA, it was concluded that DNA conferred no kinetic advantage in thiol activation, rather that the rate of activation was slightly attenuated in the presence of DNA. It was also concluded from this result that the amino sugar is not a general-base catalyst in the reaction. Our finding that **1** is insoluble in 30% methanol-aqueous tris

buffer (3×10^{-2} M, pH 7.5), the medium utilized in these studies, calls into question the validity of these conclusions. It should also be noted that, even were it true that the rate of thiol activation of **1** is slower in the presence of DNA, this would not mitigate against participation of the amino group in the reaction. In order to determine if the amino group offers a kinetic advantage in the thiol activation step, it is necessary to have a reference state -- a compound lacking the amino group. The "kinetic advantage" is then defined relative to some standard, and the validity of any conclusions arising from such a comparison is intimately connected to the "accuracy" of the model compound chosen.

In prior work, Cramer and Townsend attempt just such a comparison, using the *N*-acylated calicheamicin derivative **10** and the *des*-aminosugar derivative **11** as reference compounds.[28] Unfortunately, these experiments, which compare the rates of reaction

10

11

of **1**, **10**, and **11** with the non-biological thiol aminoethanethiol, were also conducted in the medium 30% methanol-tris buffer (30 mM, pH 7.4, 50 mM NaCl), which was

shown above to lead to precipitation of **1**. The more hydrophobic calicheamicin derivatives **10** and **11** are expected to be even less soluble in this medium. In addition, kinetics measurements were determined by a continuous UV assay without characterization of the reaction products, e.g., by rp-HPLC analysis.[20] Such an analysis obfuscates the complex trisulfide interchange chemistry which dominates calicheamicin activation, as shown above, because the glutathione disulfide **6**, the glutathione trisulfide **5**, the thiosulfenic acid **4**, and **1** are virtually indistinguishable by UV spectroscopy (Figure 2) and it is expected that the same would be true of the corresponding derivatives of aminoethanethiol.[29] Furthermore, the use of aminoethanethiol (net positive charge at physiological pH) as the activating thiol bears little resemblance to potential in vivo activation factors such as GSH (net negative charge at physiological pH) and further complicates interpretation of the data by the presence of an additional amino group.

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

Conclusions

The reaction of calicheamicin γ_1 (**1**) with glutathione (GSH), the most prevalent thiol in mammalian cells[8] and a putative cofactor in the activation of **1** in vivo, has been studied in water in the presence of double-stranded DNA and is shown to produce each of the four products of S-S bond exchange between the thiol function of GSH and the trisulfide group of **1** (**3-6**, Scheme III). The major reaction pathway produces the calicheamicin-glutathione disulfide **6** while the dihydrothiophene derivative **3**, the

thiosulfenic acid derivative **4**, and the calicheamicin-glutathione trisulfide **5** are formed in relatively minor competing processes. Products **4-6** react further, each converging upon the product **3**, albeit by different reaction paths and at different rates. Thus, the most direct pathway for biradical formation from **1** (Scheme 1) is, in fact, a minor process under conditions mimicking a physiological setting. The major reaction product (**6**) forms **3** at a rate approximately two orders of magnitude slower than the rate of formation of **3** from **1**, **4**, or **5**. The kinetics of appearance of **3** then displays a bimodal profile with an initial rapid burst as **1**, **4**, and **5** react, followed by a much slower period as **6** is transformed into **3**. This bimodal kinetic profile is reflected in the kinetics of cleavage of double-stranded DNA by **1** and GSH, as anticipated if the putative biradical precursor to **3** (**B**) were to initiate the DNA cleavage reaction.

It is found that the rate of DNA cleavage by **1** is essentially independent of the concentration of DNA whereas the rate of DNA cleavage by **6** slows markedly with increasing concentrations of DNA. Similarly, the rate of reaction of **6** with GSH in the presence of double-stranded DNA is inversely proportional to the concentration of DNA and parallels the observed rate of DNA cleavage by **6** and GSH. The most reasonable interpretation of these observations is that **1** undergoes thiol activation as a DNA-bound species while **6** is preferentially activated free in solution. Because **1** and **6** display identical sequence specificity and efficiency in DNA cleavage, one implication of these findings is that the products of thiol activation of **1** (**A**→**2**) function equivalently in the cleavage of DNA whether generated free in water or bound to DNA. This supports the idea that the sequence specificity of DNA cleavage by **1** is determined by a species formed post-activation, most probably the intermediate **2**.

The concentration of double-stranded DNA in a eukaryotic cell nucleus is estimated to range from 4 mM to ~0.5 M[30] while the concentration of nuclear GSH in cultured rat

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

Given that the reaction of **1** with GSH occurs via DNA-bound **1** in the presence of millimolar concentrations of DNA, the question then arises as to the role of DNA in this reaction. We were unable to answer the simplest question -- does DNA accelerate (catalyze) the thiol activation step? -- due to the complete insolubility of **1** in aqueous media in the absence of DNA. Previous claims in this regard are called into question for the same reason.[21] The fact that the thiol activation reaction is indiscriminate, producing all possible S-S exchange products, demonstrates that the DNA-mediated process lacks one feature that typifies many protein-catalyzed or enzymatic processes -- that of selectivity. The finding that the products of thiol activation of **1** (A→2) function equivalently in DNA cleavage whether generated free in solution or bound to DNA in some ways diminishes the importance of the DNA catalysis question because it suggests that the sequence specificity of DNA cleavage by **1** is determined after thiol activation. This hypothesis is supported by earlier estimates of the half-life of the activated intermediate **2**[24] vis-a-vis representative rates of binding and debinding of

related small molecules to and from DNA (Table 1)[15] and has been previously proposed by others.[4] The possibility that the sequence specificity of DNA cleavage by **1** is kinetically determined, reflecting DNA sequences that catalyze the activation of **1** (or the cyclization of **2**) as opposed to most-favored binding sites of the activated product **2**, appears unlikely.

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

Experimental

General. Calicheamicin γ_1 (**1**) was generously supplied by Dr. George Ellestad of the American Cyanamid Company, Lederle Laboratories. The drug was stored as a dry powder at −80 °C and was weighed with a Mettler microbalance. All manipulations of the drug were conducted with extreme caution due to its potential human toxicity. An authentic sample of the dihydrothiophene derivative **3** was kindly provided by Professor Daniel Kahne of Princeton University. All reaction solutions were prepared with ultrapure water, obtained from a Millipore Milli-Q Plus water purification system. "Double-stranded calf thymus DNA" refers to sonicated, phenol-extracted calf thymus DNA (Pharmacia) of approximately 90% double strand content, analyzed as follows. Calf thymus DNA was dissolved in sufficient aqueous sodium phosphate buffer (10 mM, pH 7.2) to prepare a solution 1 mM bp in DNA. A 50-µL aliquot of this solution was injected onto a Waters 600E HPLC system configured with a Bio-Rad Econo Pac Hydroxylapatite Cartridge (5 mL) with the following profile of elution (1 mL/min) with aqueous solutions A (10 mM sodium phosphate, pH 7.2) and B (400 mM sodium phosphate, pH 6.8), respectively; 0-2 min, 100:0 v/v A:B; 2-30 min, linear gradient from 100:0 to 20:80 v/v A:B; 31-60 min, isocratic elution with 20:80 v/v A:B. Peaks were detected by ultraviolet absorption at 220, 230, 240, and 250 nm with a Waters 994 Programmable Photodiode Detector. Aqueous solutions of DNA were adjusted to pH 7.5 by the addition of tris base (Fisher). Aqueous solutions of glutathione (GSH, Sigma, 10 or 100 mM, pH adjusted to 7.5 with tris base) were prepared just prior to use; control experiments established that these solutions were stable toward air oxidation during the time of their use.[32] A standard buffer solution (100 mM, pH 7.5) was prepared from tris base (Fisher) and 1.00 M aqueous hydrochloric acid solution. All pH measurements were determined with a Beckman ϕ40 digital pH meter equipped with a MI-410 micro-pH electrode (Microelectrodes, Inc). Unless otherwise specified,

all reactions were conducted with tris-HCl buffer solutions (30 mM, pH 7.5) and were quenched by the transfer of a specified reaction volume to an Eppendorf tube containing aqueous ammonium acetate buffer solution (10 μL, 1 M, pH 5.5) followed by rapid freezing by immersion of the tube in liquid nitrogen. Electrospray mass spectra were obtained on a Vestec 201 Electrospray Mass Spectrometer with an extended mass range of 0 - 2000 amu; meter voltage = 2000 V; electrospray current = 0.2 mamp; flow = 4 mL/min; solvent matrix 50:47:3 $CH_3OH:H_2O:CH_3CO_2H$. The spray chamber was maintained at 50 °C.

Reaction of 1 with GSH, HPLC Analysis. Reactions were performed at 23 °C in 1.5-mL Eppendorf tubes containing a total reaction volume of 1.0 mL. In a typical reaction, a 50-μL aliquot of a freshly prepared solution of **1** (1.0 mM) in DMSO containing 2,5-dimethoxybenzyl alcohol (5.0 mM) as an internal standard was combined with a freshly prepared solution of double-stranded calf thymus DNA (500 μL, 10 mM bp) in water, tris-HCl aqueous buffer solution (300 μL, 100 mM, pH 7.5), and water (50 μL). An initial ratio of **1** to 2,5-dimethoxybenzyl alcohol (internal standard) was established by HPLC analysis (50-μL injection volume) employing a Waters 600E HPLC system equipped with a Beckman Ultrasphere ODS (C_{18}, 5 μm) rp-HPLC column, 4.6 x 250 mm, flow = 0.40 mL/min with the following step gradient of acetonitrile and aqueous ammonium acetate buffer solution (10 mM, pH 6.0), respectively; 0-5 min, 27:73 v/v; 6-20 min, 40:60 v/v; 21-60 min, 60/40 v/v. Peaks were detected by ultraviolet absorption at 220, 230, 240 and 250 nm with a Waters 994 Programmable Photodiode Detector. The reaction was initiated by the addition of an aqueous solution of glutathione (100 μL, 10 mM), thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.05 mM; GSH,

1.0 mM; double-stranded calf thymus DNA, 5.0 mM bp; tris-HCl buffer, 30 mM. The reaction was monitored periodically by quenching 100-µL aliquots of the reaction solution (see **General**) with subsequent HPLC analysis, as described. Representative retention times are as follows: **1**, 48.4 min; **3**, 39.6 min; **4**, 45.7 min; **5**, 26.5 min; **6**, 25.8 min (see Figure 1). Ultraviolet absorption spectra for each component are depicted in Figure 2. The identity of component **3** was established by coinjection with an authentic sample of **3** (~0.05 mM in methanol), kindly provided by Professor Daniel Kahne of Princeton University.

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

Isolation of 4. A 100-µL aliquot of a freshly prepared solution of **1** (2.5 mM) in DMSO containing 2,5-dimethoxybenzyl alcohol (10 mM) as an internal standard was combined with a freshly prepared solution of double-stranded calf thymus DNA (500

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

Reaction of 6 with GSH, HPLC Analysis. An aqueous stock solution of the disulfide **6** (0.09 mM), 2,5-dimethoxybenzyl alcohol (internal standard, 0.45 mM), and double-stranded calf thymus DNA (9.0 mM bp, drug:DNA = 1:100) was prepared by combining a methanolic solution of **6** (50 μL, 1.0 mM, based on an assumed extinction coefficient of 75,000 at 215 nm)[33] and 2,5-dimethoxybenzyl alcohol (5.0 mM) with an aqueous solution of double-stranded calf thymus DNA (500 μL, 10 mM bp). The ratio of **6** to internal standard at time zero was determined by HPLC analysis of the stock solution. This stock solution was diluted 2-fold and 10-fold, respectively, for parallel reactions with GSH (10 mM) at a constant ratio of drug to DNA = 1:100. Thus, 350 μL of the stock solution was combined with aqueous tris-HCl buffer solution (210 μL, 100 mM, pH 7.5) and water (70 μL) and the reaction was initiated at

23 °C by the addition of an aqueous solution of GSH (70 µL, 100 mM, pH 7.5). The concentrations of solution components at the onset of this reaction were as follows: **6**, 0.045 mM; GSH, 10.0 mM; DNA, 4.5 mM; tris-HCl buffer, 30.0 mM. In a parallel incubation, 70 µL of the stock solution was combined with aqueous tris-HCl buffer solution (210 µL, 100 mM, pH 7.5) and water (350 µL) and the reaction was initiated at 23 °C by the addition of an aqueous solution of GSH (70 µL, 100 mM, pH 7.5). The concentrations of solution components at the onset of this reaction were as follows: **6**, 0.009 mM; GSH, 10.0 mM; DNA, 0.9 mM bp; tris-HCl buffer, 30.0 mM. In each of the parallel incubations at times of 0.5, 1, 2, 3, 4, 6, and 8 h, an aliquot (100 µL) was withdrawn, quenched, and analyzed by HPLC, as described above.

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

Preparation of ^{32}P-labeled 35-Base Pair Duplex DNA. The single stranded 35-base DNA oligomer 5'-GCAAAGCACGCTGATCCTCTTGCTGCAACGTTGAC-3' and its complementary sequence were synthesized on an Applied Biosystems DNA Synthesizer (1.0 μmole scale each) using standard phosphoramidite methodology.[34] Removal of protective groups was achieved by the incubation of each protected synthetic oligomer with concentrated aqueous ammonium hydroxide solution (1 mL) for 12 h at 55 °C. Each product was dissolved in formamide loading buffer solution (50 μL)[35] and the resulting solution was applied to the top of a 15% denaturing polyacrylamide gel, 1.5 mm thickness, for purification by electrophoresis. The bands containing the DNA oligomers were located by UV shadow and were excised from the gel. The oligomers were isolated by the crush and soak method[35] followed by dialysis against ultrapure water (2 d) and lyophilization. The single stranded oligomer 5'-GCAAAGCACGCTGATCCTCTTGCTGCAACGTTGAC-3' (50 pmole) was 5' end-labeled with [γ-^{32}P]-ATP (NEN, ≥5000 Ci/mmol) and polynucleotide kinase (Boehringer Mannheim) using standard procedures.[35] The labeled single stranded oligomer was purified over a 15% denaturing polyacrylamide gel, 0.4 mm thickness, and the band containing the oligomer was located by autoradiography. The band was excised from the gel, was crushed thoroughly and, after combination with aqueous Nonidet P-40 detergent solution (Sigma, 350 μL, 0.05%), was vortexed for 30 min at 23 °C. The resulting suspension was filtered through a Centrex filter (0.45 μm) and the filtrate was extracted twice with 1:1 v/v phenol:chloroform (300 μL). The labeled product was precipitated by the addition of aqueous sodium acetate buffer solution (100 μL, 0.3 M, pH 5.3) and ethanol (900 μL), followed by centrifugation at 2 °C (16,000 g, 20 min), and was then washed with aqueous ethanol (1 mL, 70%). The purified labeled fragment was dissolved in an aqueous solution of tris-acetate buffer (25 μL, 50

mM, pH 7.4) and sodium chloride (100 mM) and the complementary synthetic single stranded DNA oligomer (50 pmole) was added. The mixture was annealed by heating at 90 °C for 5 min with subsequent slow cooling to 23 °C (maintained at 23 °C for 12 h) to form the labeled duplex DNA.

Preparation of ^{32}P-labeled 167-Base Pair Restriction Fragment. Plasmid pBR322 (40 µL, 0.25 µg/µL, Boehringer Mannheim) was linearized by digestion with Eco RI (Boehringer Mannheim) according to the manufacturer's specifications. Following removal of the 5'-phosphate groups with alkaline phosphatase (Boehringer Mannheim), the DNA was 5'-end labeled with [γ-^{32}P]-ATP (NEN, ≥5000 Ci/mmol) and polynucleotide kinase (Boehringer Mannheim) using standard procedures.[35] The 5'-labeled product was digested with Rsa I (Boehringer Mannheim) according to the manufacturer's specifications, and the 167-base pair fragment was purified over a 8% non-denaturing polyacrylamide gel, 0.8 mm thickness. The band containing the 167-base pair fragment was located by autoradiography and was excised from the gel. The gel slice was crushed thoroughly and, after combination with aqueous Nonidet P-40 detergent solution (Sigma, 350 µL, 0.05%), was vortexed for 1 h at 23 °C. The resulting suspension was filtered through a Centrex filter (0.45 µm) and the filtrate was extracted twice with 1:1 v/v phenol:chloroform (300 µL). The labeled product was precipitated by the addition of aqueous sodium acetate buffer solution (100 µL, 0.3 M, pH 5.3) and ethanol (900 µL), followed by centrifugation at 2 °C (16,000 g, 20 min), then was washed with aqueous ethanol (1 mL, 70%). The purified labeled fragment was stored frozen in tris-HCl aqueous buffer solution (10 mM, pH 7.4) with EDTA (1 mM).

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

Reaction of 1 with GSH, DNA Cleavage Analysis.   Reactions were performed at 23 °C in 1.5-mL Eppendorf tubes containing a total reaction volume of ≤300 µL. A 30-µL aliquot of a freshly prepared solution of **1** (0.55 mM) in methanol was combined with a solution of double-stranded calf thymus DNA (165 µL, 10 mM bp) in water, tris-HCl aqueous buffer solution (90 µL, 100 mM, pH 7.5), water (15

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

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

reaction solution were quenched and analyzed by gel electrophoresis, as described above. The DNA cleavage reaction with **6** and GSH (1.0 mM, lane 4) was performed at 23 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 50 μL. A freshly prepared solution of **6** in methanol (5 μL, 0.5 mM) was combined with a solution of double-stranded calf thymus DNA (25 μL, 10 mM bp) in water, tris-HCl aqueous buffer solution (15 μL, 100 mM, pH 7.5), and labeled duplex (~10^5 cpm). The reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (5 μL, 10 mM, pH 7.5) to the remaining solution, thus producing the following concentrations of solution components at the onset of the reaction: **6**, 0.05 mM; GSH, 1.0 mM; double-stranded calf thymus DNA, 5.0 mM bp; tris-HCl buffer, 30 mM. At a reaction time of 60 min the reaction solution was quenched and analyzed by gel electrophoresis, as described above. The DNA cleavage reaction with **1** and GSH (1.0 mM, lane 3) was performed at 23 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 50 μL. A freshly prepared solution of **1** in methanol (5 μL, 0.5 mM) was combined with a solution of double-stranded calf thymus DNA (25 μL, 10 mM bp) in water, tris-HCl aqueous buffer solution (15 μL, 100 mM, pH 7.5), and labeled duplex (~10^5 cpm). The reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (5 μL, 10 mM, pH 7.5) to the remaining solution, thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.05 mM; GSH, 1.0 mM; double-stranded calf thymus DNA, 5.0 mM bp; tris-HCl buffer, 30 mM. At a reaction time of 60 min the reaction solution was quenched and analyzed by gel electrophoresis, as described above.

Reaction of 4 with GSH, DNA Cleavage Analysis. The DNA cleavage reaction with **4** and GSH was performed at 23 °C in a 1.5 mL Eppendorf tube containing a total reaction volume of 400 μL. The frozen solution of purified **4** (see

Isolation of 4) was thawed and its pH was adjusted to 7.5 by the addition of tris

base (~0.01 moles). An aliquot of the latter solution (200 μL, ca. 0.1 mM **4**) was

withdrawn and combined with an aqueous tris-HCl buffer solution (80 μL, 100 mM,

pH 7.5), double-stranded calf thymus DNA (1.30 mg, 0.0020 mmoles) and water (80

μL). After thorough vortexing, the reaction was initiated at 23 °C by the addition of an

aqueous solution of glutathione (40 μL, 10 mM, pH 7.5), producing the following

concentrations of solution components at the onset of the reaction: **4**, 0.05 mM; GSH,

1.0 mM; double-stranded calf thymus DNA, 5.0 mM bp; tris-HCl buffer, 40 mM. At

times of 3, 10, 20, 60, 120, 300, and 1200 min (Figure 7, lanes 3-9, respectively) 30-

μL aliquots of the reaction solution were quenched and analyzed by gel electrophoresis,

as described above. At a reaction time of 60 min, 54 μL of the reaction mixture was

transferred to a fresh 1.5-mL Eppendorf tube and was treated with an aqueous solution

of glutathione (5.4 μL, 100 mM, pH 7.5, [GSH] = 10 mM). At times of 120, 240,

and 1200 min 20-μL aliquots (lanes 10-12, respectively) of this reaction solution were

quenched and analyzed by gel electrophoresis, as described above. The DNA cleavage

reaction with **1** and GSH (1.0 mM, lane 2) was performed at 23 °C in a 1.5-mL

Eppendorf tube containing a total reaction volume of 50 μL. A freshly prepared

solution of **1** in methanol (5 μL, 0.5 mM) was combined with a solution of double-

stranded calf thymus DNA (25 μL, 10 mM bp) in water, tris-HCl aqueous buffer

solution (15 μL, 100 mM, pH 7.5), and labeled duplex (~10^5 cpm). The reaction was

initiated at 23 °C by the addition of an aqueous solution of glutathione (5 μL, 10 mM,

pH 7.5) to the remaining solution, thus producing the following concentrations of

solution components at the onset of the reaction: **1**, 0.05 mM; GSH, 1.0 mM; double-

stranded calf thymus DNA, 5.0 mM bp; tris-HCl buffer, 30 mM. At a reaction time of

60 min the reaction solution was quenched and analyzed by gel electrophoresis, as

described above.

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

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

Reaction of 1 with GSH at Varying Concentrations of DNA. An aqueous solution of 1 (0.18 mM) and double-stranded calf thymus DNA (9.0 mM, drug:DNA = 1:50) was prepared by combining a methanolic solution of 1 (20 μL, 2.0

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

Light Scattering Analysis. The solubility of **1** in aqueous solution in the absence of DNA was analyzed using a Malvern System 4700-C Submicron Particle Analyzer employing a Spectra Physics Series 2000 Argon laser (488 nm). The detector was set at an angle of 90° to the laser beam. To examine the solubility of **1** under our standard

reaction conditions (employing potassium dimethyl phosphate in lieu of DNA to maintain constant ionic strength), a solution of **1** (50 μL, 1.0 mM) in DMSO containing 2,5-dimethoxybenzyl alcohol (5.0 mM) was combined in a 1.5-mL Eppendorf tube with an aqueous solution of potassium dimethyl phosphate (500 μL, 20 mM), aqueous tris-HCl buffer solution (300 μL, 100 mM, pH 7.5), and water (150 μL) affording a total volume of 1.00 mL. The final concentrations of solution components are calculated as follows: **1**, 0.05 mM (maximum, if **1** were completely dissolved); potassium dimethyl phosphate, 10.0 mM; tris HCl buffer, 30 mM. A 50-μL aliquot of the resulting aqueous suspension was analyzed by rp-HPLC, as previously described, to establish an initial ratio of **1** to internal standard. The fine suspensions produced upon addition of solutions of **1** in DMSO to water are found to analyze as homogeneous solutions by HPLC, presumably due to the rapid dissolution of **1** in the HPLC eluent system. The aqueous suspension of **1** was transferred to a 5-mL Pyrex test tube (for placement in the sample holder) and was examined by light-scattering analysis. The Malvern System reported an average particle diameter of 5-10 μm. A 750-μL aliquot of the latter aqueous suspension was transferred into a fresh 1.5-mL Eppendorf tube and was centrifuged at 16,000 g for 30 min (23 °C). A portion of the supernatant (ca. 500 μL) was carefully withdrawn and was examined by light-scattering analysis; the Malvern system reported an average particle diameter of ~0.5 μm. Analysis of the supernatant by rp-HPLC showed that it contained approximately 20% of **1** originally added. Two controls were also examined in parallel by light-scattering analysis. Control solution 1 was prepared by combining a solution of **1** (50 μL, 1.0 mM) in DMSO containing 2,5-dimethoxybenzyl alcohol (5.0 mM) with DMSO (950 μL) to afford a solution of **1** (0.05 mM) in DMSO. Control solution 2 was prepared by combining DMSO (50 μL) with an aqueous solution of potassium dimethyl phosphate (500 μL, 20 mM), aqueous tris-HCl buffer solution (300 μL, 100 mM, pH

7.5), and water (150 µL). The control solutions were found not to scatter light. To examine the solubility of **1** at lower concentrations ($\leq$ 1 µM), three solutions of **1** (20 µM, 2 µM, and 0.2 µM) in DMSO were prepared. Aliquots (50 µL) of each of the solutions were combined in 1.5-mL Eppendorf tubes with an aqueous solution of potassium dimethyl phosphate (500 µL, 20 mM), aqueous tris-HCl buffer solution (300 µL, 100 mM, pH 7.5), and water (150 µL) to afford solutions nominally containing 10^{-6}, 10^{-7}, and 10^{-8} M **1**, respectively (maximum, if **1** were completely dissolved). These solutions were found to scatter light distinguishable from the control solutions. Solutions of lesser particle density did not scatter light distinguishable from background. To examine the solubility of **1** under the conditions of Cramer and Townsend,[28] a solution of **1** (12.5 µL, 4.0 mM) in methanol containing 2,5-dimethoxybenzyl alcohol (20 mM, internal HPLC standard) was combined in a 1.5-mL Eppendorf tube with a solution of 70:30 aqueous tris-HCl buffer (30 mM, pH 7.4, 50 mM NaCl):methanol (987 µL) to afford a solution of **1** at a nominal concentration of 0.05 mM (maximum, if **1** were completely dissolved). A 50-µL aliquot of the resulting suspension was analyzed by rp-HPLC before light-scattering analysis to establish an initial ratio of **1** to internal standard. The suspension of **1** was transferred to a 5-mL Pyrex test tube and was examined by light scattering analysis. The Malvern System reported an average particle diameter of 2.6 µm. After light-scattering analysis, 250-µL aliquots of the suspension were transferred to each of three fresh 1.5 mL Eppendorf tubes (750 µL total) and were centrifuged at 16,000 g for 30 min (23 °C). A portion of the supernatant was carefully withdrawn from each tube (200 µL). The supernatant solutions were combined (600 µL) and the resulting solution was examined by light-scattering analysis. The Malvern System reported an average particle diameter of 0.54 µm. Analysis of the supernatant solution by rp-HPLC showed that it contained

approximately 70% of **1** originally added.[37] Two controls were also examined in parallel by light-scattering analysis. Control solution1 was prepared by combining a solution of **1** (12.5 μL, 4.0 mM) in methanol containing 2,5-dimethoxybenzyl alcohol (20 mM) with methanol (987 μL) to afford a solution of **1** (0.05 mM) in methanol. Control solution 2 was prepared by combining methanol (12.5 μL) with a solution of 70:30 aqueous tris-HCl buffer (30 mM, pH 7.4, 50mM NaCl):methanol (987 μL). The control solutions were found not to scatter light.

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

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

(20) Experiments investigating the reaction of 1 with GSH in the presence of DNA, the analysis of the products of the latter reaction by rp-HPLC, kinetic monitoring of that reaction by rp-HPLC, and the identification of the calicheamicin-glutathione disulfide 6 as the primary product of that reaction, were initially conducted in our laboratories and

this information was shared with Professor Craig Townsend of The Johns Hopkins University.

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(22) See footnote 13 in reference 21.

(23) Previously, K_B for a TCCT site within a synthetic double-stranded DNA dodecamer was determined to be ~1-3 x 10^{-8} M: Drak, J.; Iwasawa, N.; Danishefsky, S.; Crothers, D. M. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 7464.

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(29) In preliminary studies, we have found that aminoethanethiol reacts more rapidly (2-3 fold) with 1 in the presence of DNA than does GSH. Due to the rapidity of transformations with aminoethanethiol versus GSH, we were unable to detect the thiosulfenic acid derivative 4 or the aminoethanethiol-calicheamicin trisulfide in the reaction of 1 with aminoethanethiol; however, we did observe the aminoethanethiol-calicheamicin disulfide as an intermediate and, in contrast to the observations of Townsend et al.,[21] we find no significant distinction in the UV absorption characteristics of this intermediate versus 1. The reaction of the aminoethanethiol-

calicheamicin disulfide with aminoethanethiol in the presence of DNA (5×10^{-3} M bp) is found to be 1-2 orders of magnitude more rapid than the reaction of **6** with GSH under identical conditions.

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

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

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

Chapter 3

The Dynamic Process of DNA Cleavage by Dynemicin A as Revealed by DNA Binding and DNA Cleavage Studies of Synthetic Analogs

Introduction

Dynemicin A (**1**), a deep violet metabolite extracted from the fermentation broth of *Micromonospora chersina*, exhibits remarkable antitumor and antibiotic properties, with LD_{50} values in the picogram/mL range against a variety of tumor cell lines.[1] Dynemicin A is unique among natural antitumor agents, possessing features of both the anthracycline and enediyne antibiotic families. The highly reactive anthraquinone fragment imbues the molecule with its deep violet color and is characteristic of the anthracyclines, while the (Z)-enediyne bridge and epoxide ring classify it among the enediyne antibiotics.[2]
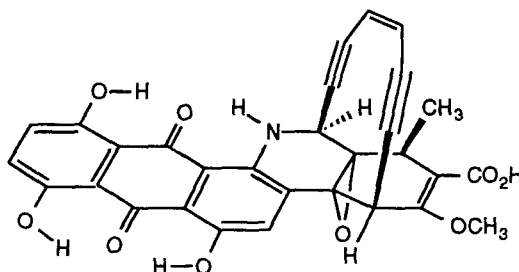
Dynemicin A (1)

In parallel with other members of the enediyne antibiotic family, dynemicin A has been proposed to function in vivo as a DNA-damaging agent. It has been demonstrated that dynemicin A cleaves double-stranded B-form DNA in vitro when in the presence of a reducing cofactor such as NADPH or methyl thioglycolate. Sugiura

Scheme 1
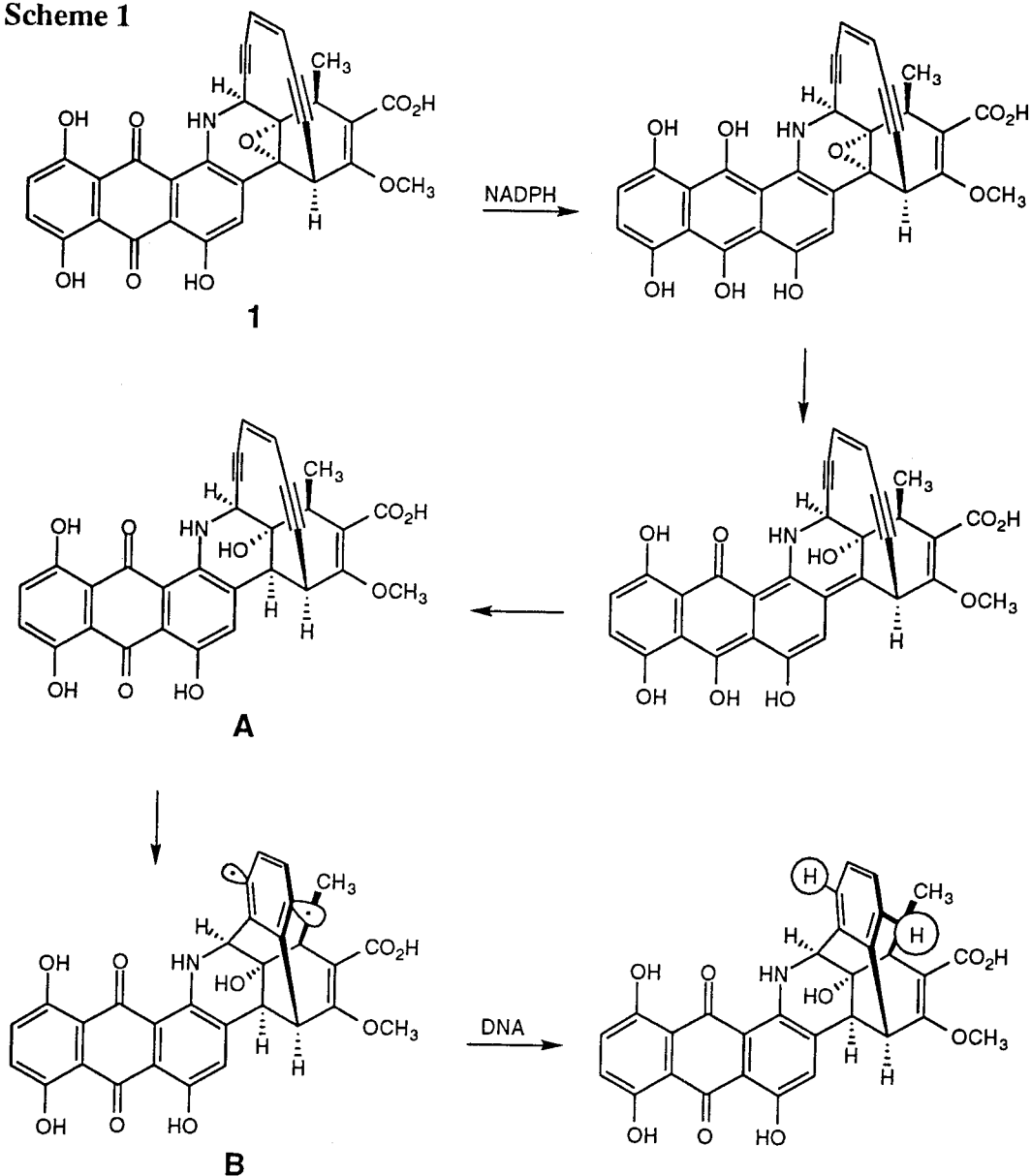
and co-workers have provided convincing evidence that **1** is transformed into the biradical intermediate **B** (Scheme 1) upon reductive activation. This proposal requires initial reduction of the anthraquinone, followed sequentially by epoxide opening, tautomerization, and Bergman cyclization of the strained (Z)-enediyne bridge of **A** (Scheme 1).[3-5]

The oxidized anthraquinone of **1** is a unique structural element among the enediyne antibiotics. In addition to serving as the site of reductive activation, the anthraquinone of **1** has been proposed to function as a DNA-binding element by intercalation into the base stack via the minor groove.[6] Two other unusual features make **1** unique among the enediyne antibiotic family. While all other enediyne antibiotics require a nucleophilic thiol for activation, **1** may be reductively activated by either a thiol or NADPH. Perhaps the most striking feature of **1** is the fact that it bears a negative charge at physiological pH, by virtue of its carboxylate group; all other members of the enediyne antibiotic family are positively charged at neutral pH,[2] as is common for molecules that bind to the DNA polyanion.[7] This becomes even more unusual when considering that the biological reducing agents glutathione (GSH) and NADPH bear negative charges of −1 and −4, respectively, at physiological pH.


Glutathione (GSH)   **NADPH**
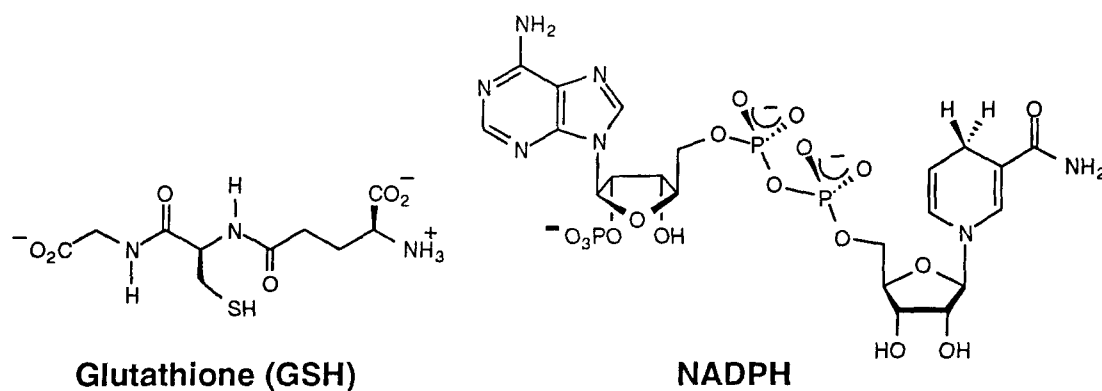
These features raise questions regarding the nature of the molecular mechanism(s) between **1**, GSH, NADPH, and DNA. What are the dynamics of the DNA-binding and reductive activation steps; can activation of the intercalated anthraquinone compete with activation of **1** free in solution? Do GSH and NADPH function equivalently in the DNA cleavage mechanism in terms of cleavage efficiency and cleavage specificity? Does the carboxylate of **1** serve a specific function? To address these questions, we have studied the DNA-binding and DNA-cleaving properties of **1** versus synthetic analogs of **1**. The dynamic process for DNA cleavage by dynemicin A and its analogs is presented. It is shown that the DNA cleaving ability of these compounds is intimately related to their DNA-binding properties, and that the carboxylate group of **1** plays a critical role in the DNA cleavage mechanism.

Binding Constants for Dynemicin A (1) and Structural Analogs 2 - 6 to Double-Stranded DNA

A convergent synthetic route to enantiomerically pure dynemicin A, recently developed in the Myers research group, has provided access to a wide range of structural analogs for study.[8] Both natural and nonnatural dynemicins may arise from the retrosynthetic fragments illustrated in Scheme 2 for a generalized dynemicin target molecule. In preparing analogs of **1** for mechanistic study, we have focused on two

Scheme 2
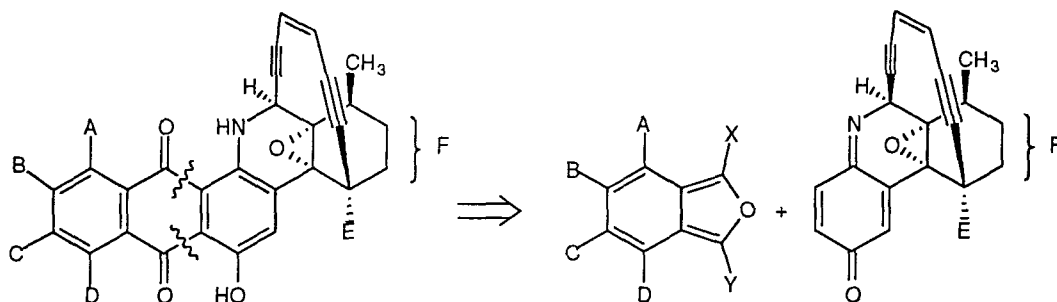
structural features of **1**: 1) the two hydroxyl groups of the E-ring and 2) the

carboxylate group. Prepared for the first time, in enantiomerically pure form, are

dideoxydynemicin A (**2**), dynemicin A methyl ester (**3**), dideoxydynemicin A methyl

ester (**4**), dynemicin A-ring analog **5**, and dideoxydynemicin A-ring analog **6** (Figure

1). Each of the molecular pairs **1** and **2**, **3** and **4**, and **5** and **6** varies only in the

presence or absence of the E-ring hydroxyl groups, whereas pairs **1** and **3**, and **2** and

4 are related as the negatively-charged carboxylate and the neutral methyl ester.

Compounds **5** and **6** may also be viewed simply as charge-neutral A-ring analogs of **1**

and **2**, respectively.

Equilibrium binding constants for the binding of compounds **1 - 6** to double-

stranded calf thymus DNA were determined by equilibrium dialysis in aqueous tris-

HCl buffer solution (30 mM, pH 7.5; NaCl, 50 mM) at 25 °C using a dialysis

membrane with molecular weight cutoff 12,000 - 14,000. The binding constants span

a range of four orders of magnitude, from 6×10^2 M^{-1} for dideoxydynemicin A (**2**,

weakest binding) to 8×10^6 M^{-1} for dynemicin A methyl ester (**3**, tightest binding,

Figure 1). Comparison of the binding constants for structure pairs **1** and **2**, **3** and **4**,

and **5** and **6** shows that the two hydroxyl groups of the anthraquinone E-ring

contribute approximately 2.7 kcal/mole in binding energy; the binding constants of the

dideoxydynemicin analogs are consistently two orders of magnitude lower than

molecules with the natural dynemicin anthraquinone substitution pattern. According to

models of the intercalative binding of dynemicin A to a 12-mer DNA duplex, these

hydroxyls penetrate into the major groove of DNA.[6] In the recent crystal structure of

nogalomycin complexed to d(TGATCA), a similarly oriented anthraquinone hydroxyl

was found to be engaged in hydrogen bonding to N4 of cytosine (separated by one

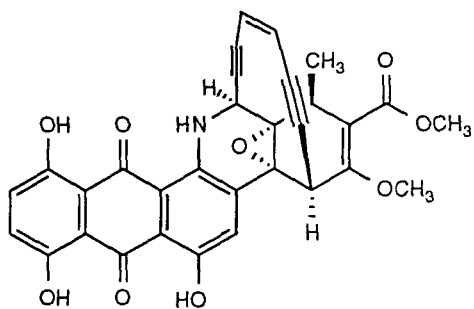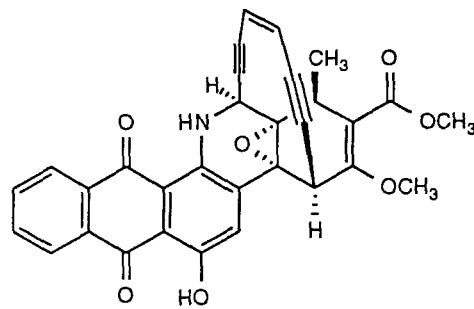base pair from the intercalated anthraquinone) mediated by a network of two water

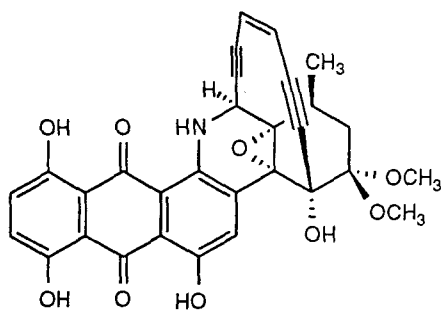**Figure 1**
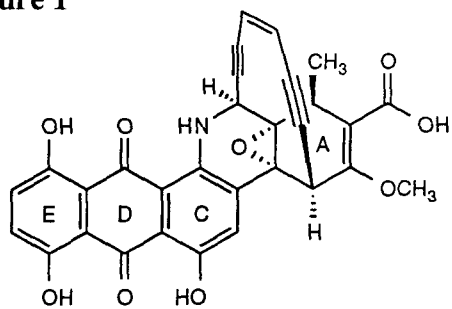
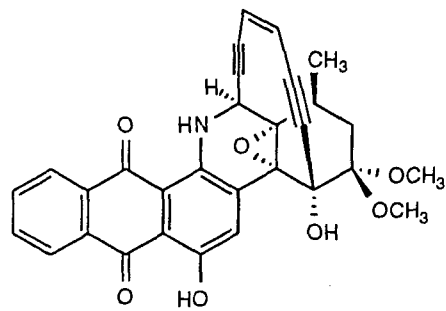**1** $K_B = (5 \pm 2) \times 10^4\,M^{-1}$
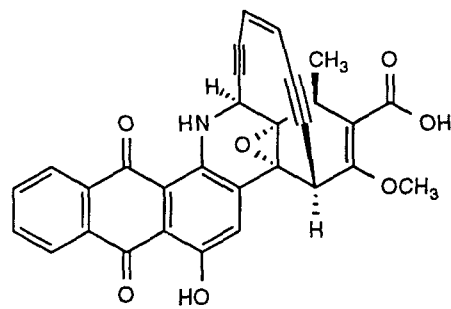
2 $K_B = (6 \pm 1) \times 10^2\,M^{-1}$

3 $K_B = (8 \pm 2) \times 10^6\,M^{-1}$

4 $K_B = (5 \pm 2) \times 10^4\,M^{-1}$

5 $K_B = (4 \pm 2) \times 10^6\,M^{-1}$

**6** $K_B = (4 \pm 1) \times 10^4\,M^{-1}$

molecules in the major groove of DNA.[9] It is not unreasonable to propose that one or both of the E-ring hydroxyl groups of dynemicin A is engaged in hydrogen bonding in the major groove of DNA.

Comparing binding constants of dynemicin A with its methyl ester (1 vs. 3) and dideoxydynemicin A with its methyl ester (2 vs. 4) reveals that neutralization of the negatively-charged carboxylate residue by methyl ester formation results in greatly increased DNA binding. This charge neutralization contributes ~3 kcal/mol of stabilization to the DNA-drug complex. Binding constants for analogs 5 and 6, charge-neutral analogs of 1 and 2, respectively, provide further support for this conclusion. Given the opportunity for stabilization of this magnitude by such a modest structural change, and one easily accommodated in nature (e.g., by esterification), it is reasonable to ask why nature has not chosen to modify dynemicin A in this way. The answer to this question is immediately apparent upon consideration of the DNA cleavage data for compounds 1 - 6.

DNA Cleavage by Dynemicin A (1) and Analogs 2 - 6

DNA cleavage reactions of a 5'-^{32}P-labeled 193 base pair restriction fragment of pBR322 (Eco RI/Ssp I digests) were carried out with compounds 1 - 6 (0.05 mM) in the presence of carrier double-stranded calf thymus DNA (1.0 mM base pairs, base pairs DNA:drug = 20:1, 37 °C, 12 h) using GSH (20 mM) or NADPH (20 mM) as activating cofactors and were analyzed by gel electrophoresis (Figure 2). Analysis of the cleavage data provides considerable insight into the mechanism of DNA cleavage by 1 and its analogs. Although the sequence selectivity of DNA cleavage by dynemicin A is poor, this selectivity is largely preserved among those analogs which cleave DNA. More importantly, comparison of GSH- and NADPH-induced cleavage

Figure 2. DNA cleavage of a 5'-labeled 193 base-pair restriction fragment of pBR322 (Eco RI/Ssp I digests) from the reaction of dynemicin A or synthetic anthraquinones with glutathione (GSH) or NADPH. Reactions were performed on a volume of 20 µL and contained calf thymus DNA (1.0 mM bp), 193 base-pair restriction fragment ($\sim 10^5$ cpm), tris-HCl buffer (30 mM, pH 7.5), sodium chloride (50 mM), dynemicin A or synthetic anthraquinone (0.05 mM), and either glutathione (GSH, 20 mM) or NADPH (20 mM), as indicated. Reactions were incubated at 37 °C for 12 h. Lane **C**: 193 bp restriction fragment alone. Lane **A**: products from an adenine-specific cleavage reaction (Iverson, B. L.; Dervan, P. B. *Nucleic Acids Res.* **1987**, *15*, 7823).
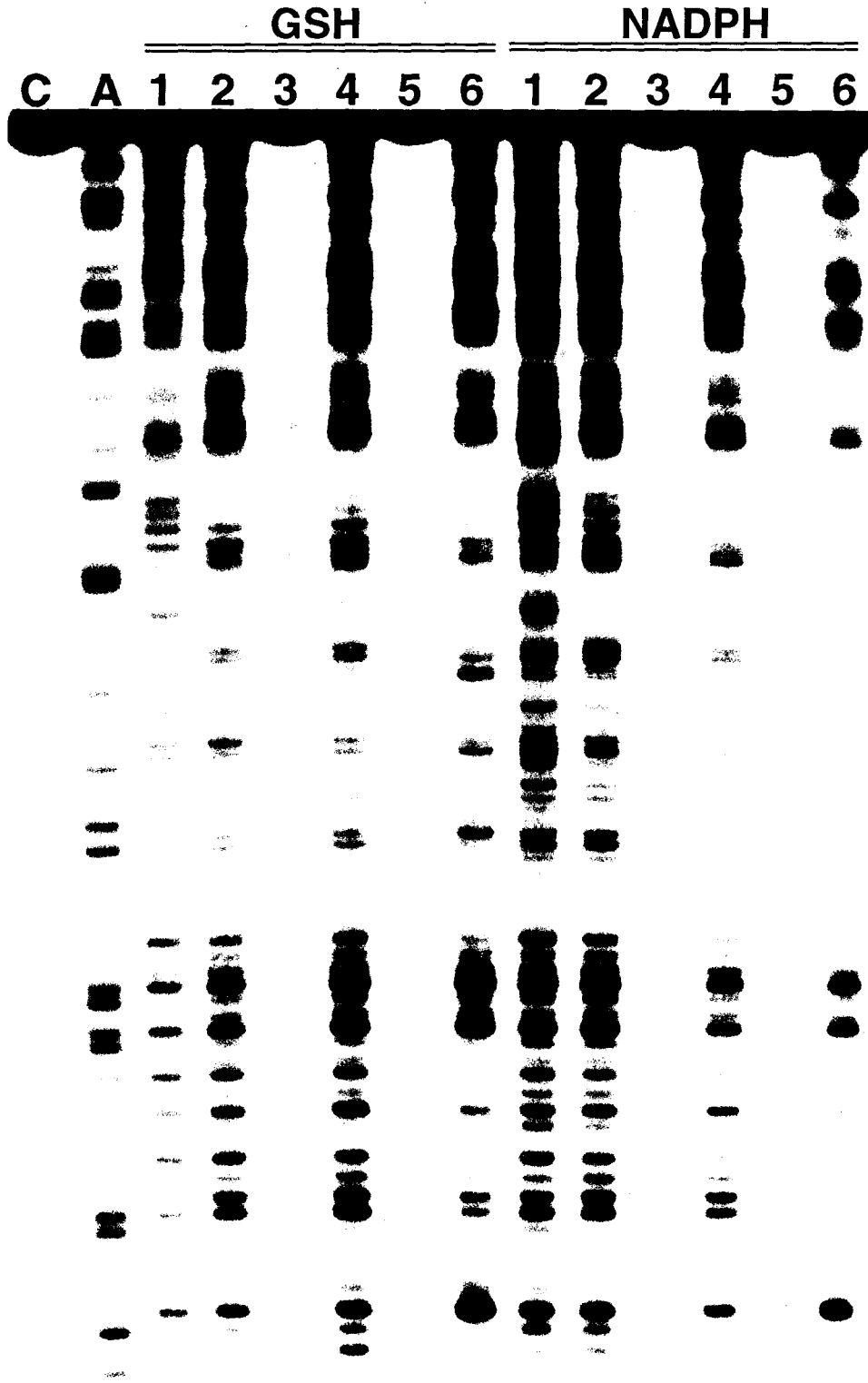
reactions for a given compound shows that both methods of activation produce identical cleavage patterns. This strongly suggests that both reductants produce a common intermediate that is sufficiently long-lived to equilibrate among DNA binding sites, a conclusion verified below.

By far, the most striking outcome of the DNA cleavage experiments is the observation that dynemicin methyl ester (3) and dynemicin analog 5 produce no detectable cleavage after a 12-h reaction period. This is a surprising result and is all the more intriguing when it is realized that 3 and 5 are the strongest DNA binders (K_B > 10^6 M^{-1}). To investigate the possibility that 3 and 5 follow an alternative pathway that does not lead to DNA cleavage, these reactions were monitored by HPLC. In both cases the dynemicin analog was found to be inert under the reaction conditions (calf thymus DNA, 1.0 mM bp; GSH or NADPH, 20 mM; 37 °C, 12 h).[10] Taken alone, these results suggest that analogs 3 and 5 do not possess the inherent reactivity towards the reductive cofactors that is characteristic of 1. However, treatment of 3 and 5 with GSH in methanol[11] in the presence of 1,4-cyclohexadiene afforded the expected Bergman-cyclized products 9 and 13 (Chart 1) in approximately 35% yield each as the major reaction product. This reactivity parallels that observed for 1 and analogs 2, 4, and 6. Treatment of 3 (0.1 mM) with GSH (5.0 mM) in methanol:water (90:10; 1,4-cyclohexadiene, 1 M; triethylamine, 0.2 M; 37 °C) leads to its complete consumption within minutes [$t_{1/2}$ (3) ~ 3 min; $t_{1/2}$ (1) ~ 15 min under identical conditions]. Products 8 - 12 were isolated by rp-HPLC and their structures confirmed by standard spectroscopic characterization methods (^1H NMR, structure 7 has been reported[5]). To account for the lack of DNA cleavage by 3 and 5 requires an understanding of the dynamics of the DNA cleavage process with dynemicin A.

Chart 1
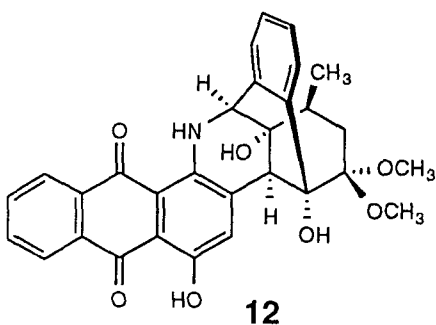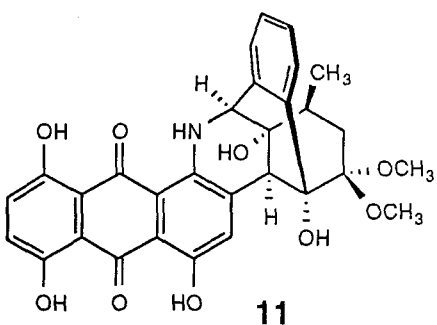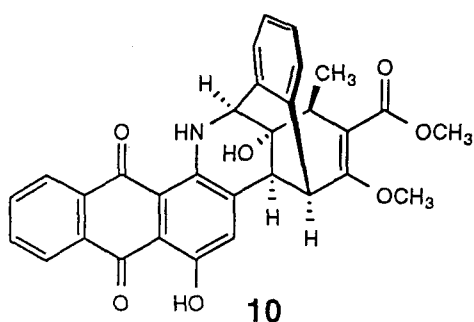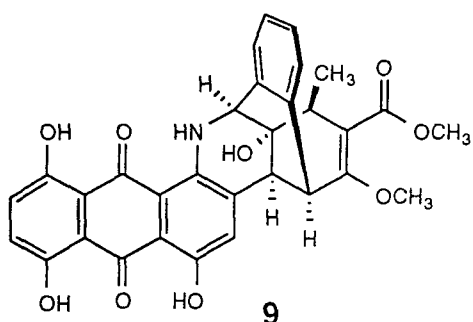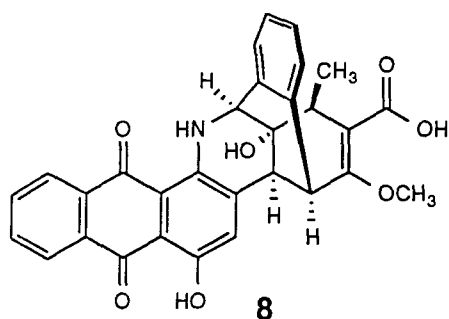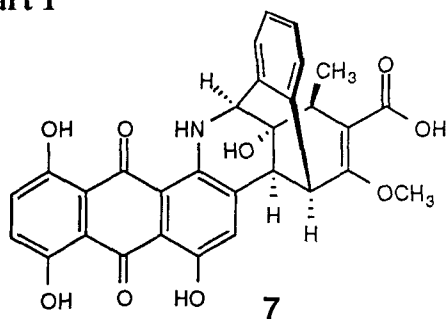
**Dynamics of the DNA Cleavage Process with Dynemicin A (1) and Analogs 2 - 6**

Although dynemicin analogs **3** and **5** may react to form the Bergman-cyclized products, they are unreactive in aqueous solution in the presence of DNA. These results suggests that the enhanced DNA binding of **3** and **5** renders these molecules inert toward reduction by GSH or NADPH: DNA inhibits DNA cleavage.

Specifically, it is proposed that dynemicin A and its analogs must dissociate from the DNA binding complex in order to undergo chemical activation; activation of compounds 1 - 6 proceeds as a bimolecular process involving drug and reductive cofactor. A similar proposal has been presented for the reaction of GSH with the calicheamicin-glutathione disulfide (the major product of the reaction of calicheamicin with GSH).[12a] To differentiate between a termolecular event of drug, DNA, and reductive cofactor, and the simple bimolecular process occurring free in solution, we have employed a kinetic analysis of the reaction of 1 with GSH or NADPH as a function of the concentration of DNA. If 1 dissociates from DNA prior to activation, then the rate of DNA cleavage should increase as the concentration of DNA is decreased. Figure 3 displays the time course of DNA cleavage by 1 with GSH and NADPH at three different concentrations of DNA (5.0, 1.0, and 0.2 mM base pairs). The data demonstrates inhibition of DNA cleavage by DNA whether GSH or NADPH is used as the activating cofactor. Thus, the intercalated anthraquinone of 1 is not reduced by GSH or NADPH at any appreciable rate. This is not a surprising outcome considering the potential for charge repulsion in the reaction of 1 with the anionic activating cofactors proximal to the DNA polyanion.

The fact that 1 is activated free in solution supports the conclusion from Figure 2 that both reductants produce a common intermediate which is sufficiently long-lived to equilibrate among DNA binding sites. It is reasonable to assume that this intermediate is that which immediately precedes Bergman cyclization (structure A). This conclusion is consistent with mechanistic studies of calicheamicin[12] and neocarzinostatin chromophore,[13] where the activated intermediates preceding biradical formation have been shown to have more than sufficient lifetimes to equilibrate among
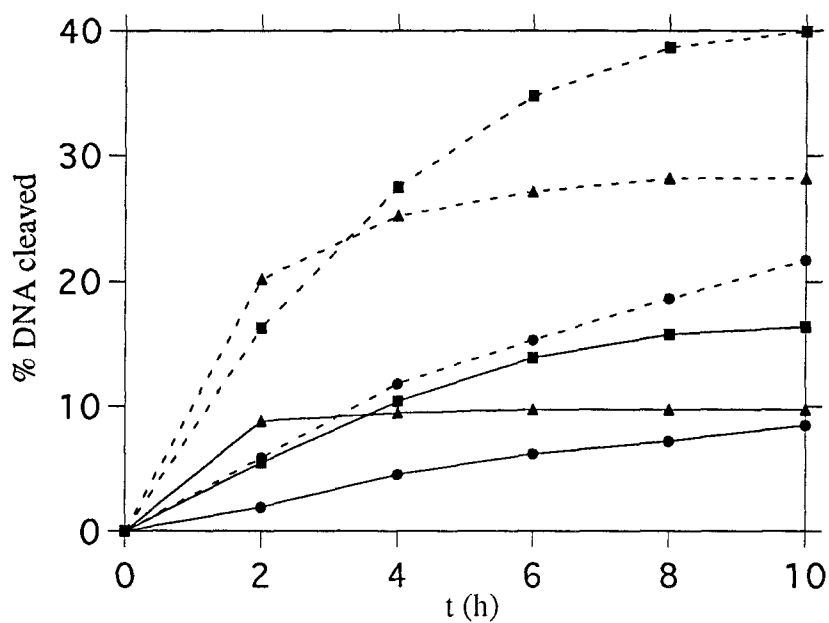
Figure 3. Kinetics of DNA cleavage from the reaction of dynemicin A (**1**) with

GSH or NADPH at varying concentrations of DNA. Reactions were performed at 37

°C in tris-HCl buffer (30 mM, pH 7.5) containing sodium chloride (50 mM) with a

constant ratio of drug to base pairs DNA (1:20): (●) **1** (0.25 mM), DNA (5.0 mM

bp); (■) **1** (0.05 mM), DNA (1.0 mM bp); (▲) **1** (0.01 mM), DNA (0.2 mM bp);

——————— reactions containing GSH (10 mM); ---------- reactions containing NADPH

(5.0 mM).

DNA binding sites. Consideration of the rates of equilibration of a representative sample of intercalative DNA binders (Table 1) suggests that intermediate **A** must have a lifetime of at least a few tenths of a second at 25 °C. Although GSH and NADPH function equivalently in the DNA cleavage process in terms of cleavage specificity, the data from Figure 3 shows that they are not equivalent in terms of cleavage efficiency.

Table 1. Rates of Equilibration of Intercalative DNA Binders[a]

Drug	Half Life (ms)	T (°C)	Ref.
Rivanol	9	25	14a
Ethidium	12	23	14a
Daunomycin	63	20	14b
4-Demethoxydaunomycin	26	20	14c

[a]Exchange rates were determined by temperature-jump relaxation techniques using absorption detection. Half lives were obtained from the first-order rate constants representing dissociation of the intercalated form of the drug.

Rivanol

Ethidium

Daunomycin

4-Demethoxydaunomycin
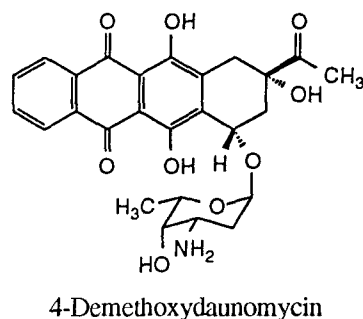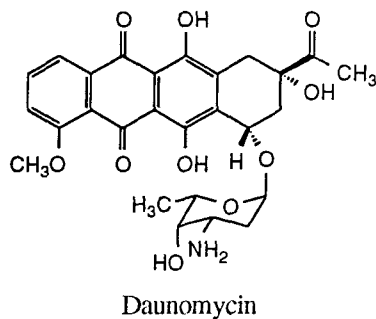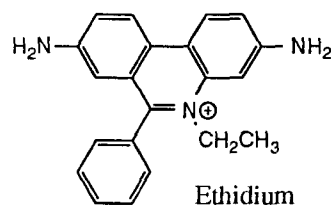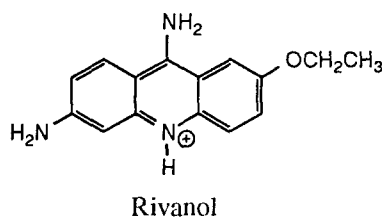
The cleavage efficiency of **1** in the presence of 1.0 mM base pairs DNA from activation with GSH is approximately 4%, compared to 9% for activation with NADPH. It can also be seen that the efficiency of DNA cleavage decreases upon lowering the concentration of DNA from 1.0 mM to 0.2 mM. This decrease in cleavage efficiency is a consequence of a smaller proportion of intermediate **A** that is in the bound form. This observation suggests that the biradical **B** does not have sufficient lifetime to equilibrate among DNA binding sites, an interpretation consistent with previous reactivity studies of biradicals of this type.[13,15] The implication is that intermediate **A** determines the sequence specificity in DNA cleavage.

Similar observations have been made for the dideoxy series of compounds. DNA cleavage by dideoxydynemicin methyl ester (**4**) is inhibited by DNA whether GSH or NADPH is used as the activating cofactor (Figure 4). Together, these findings reveal the dynamic nature of DNA cleavage by **1** and its analogs; reductive activation of the drug occurs free in solution. If the drug binds too tightly to DNA, as with compounds **3** and **5**, activation is prohibitively slow.[16]

If follows that DNA cleavage by a weak DNA binder, such as dideoxydynemicin A (**2**, $K_B \sim 6 \times 10^2$ M^{-1}), should proceed at a faster rate than **4** ($K_B \sim 5 \times 10^4$ M^{-1}). Indeed, DNA cleavage by **2** under the conditions from Figure 4 (20 mM reductive cofactors) shows that **2** is much more reactive than **4** (Figure 5). Decreasing the concentration of reductive cofactors to 2.0 mM (Figure 6) reveals that **2** is roughly two orders of magnitude more reactive than **4**. A trade-off for the high reactivity of **2** is a rapid decrease in cleavage efficiency with decreasing DNA concentration   (Figures 5,6). The cleavage efficiency for **2** with NADPH at 0.2 mM base pairs DNA is less than 1%, compared to ~7% for **1**.
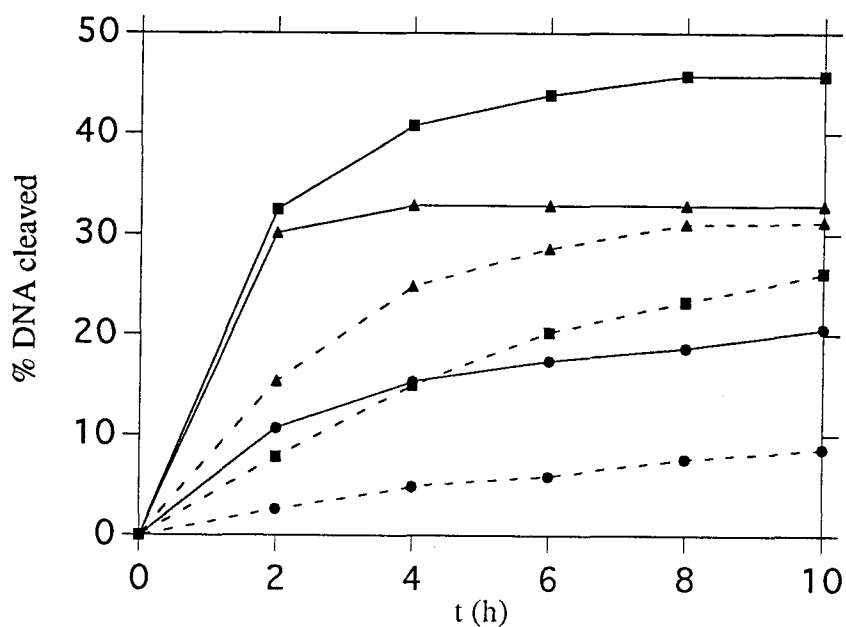
Figure 4. Kinetics of DNA cleavage from the reaction of dideoxydynemicin A methyl ester (**4**) with GSH or NADPH at varying concentrations of DNA. Reactions were performed at 37 °C in tris-HCl buffer (30 mM, pH 7.5) containing sodium chloride (50 mM) with a constant ratio of drug to base pairs DNA (1:20): (●) **1** (0.25 mM), DNA (5.0 mM bp); (■) **1** (0.05 mM), DNA (1.0 mM bp); (▲) **1** (0.01 mM), DNA (0.2 mM bp); ————— reactions containing GSH (20 mM); ---------- reactions containing NADPH (20 mM).
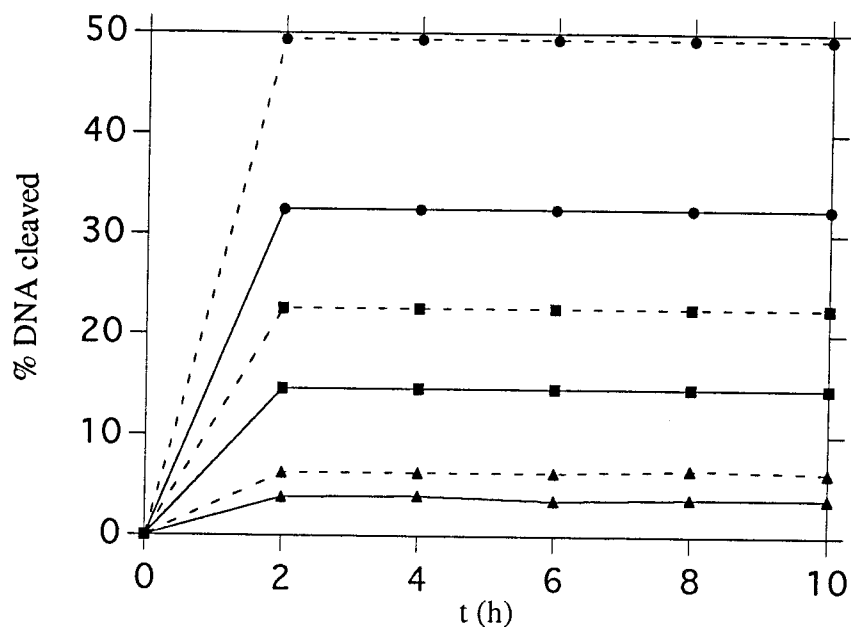
Figure 5. Kinetics of DNA cleavage from the reaction of dideoxydynemicin A (**2**) with GSH or NADPH at varying concentrations of DNA. Reactions were performed at 37 °C in tris-HCl buffer (30 mM, pH 7.5) containing sodium chloride (50 mM) with a constant ratio of drug to base pairs DNA (1:20): (●) **1** (0.25 mM), DNA (5.0 mM bp); (■) **1** (0.05 mM), DNA (1.0 mM bp); (▲) **1** (0.01 mM), DNA (0.2 mM bp); ——————— reactions containing GSH (20 mM); ---------- reactions containing NADPH (20 mM).
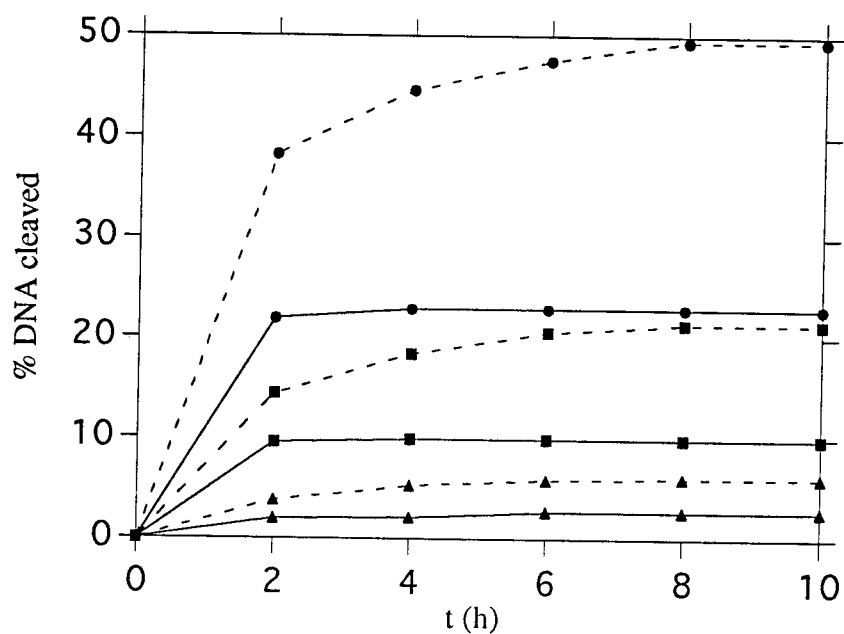
Figure 6. Kinetics of DNA cleavage from the reaction of dideoxydynemicin A (**2**) with GSH or NADPH at varying concentrations of DNA. Reactions were performed at 37 °C in tris-HCl buffer (30 mM, pH 7.5) containing sodium chloride (50 mM) with a constant ratio of drug to base pairs DNA (1:20): (●) **1** (0.25 mM), DNA (5.0 mM bp); (■) **1** (0.05 mM), DNA (1.0 mM bp); (▲) **1** (0.01 mM), DNA (0.2 mM bp); —————— reactions containing GSH (2.0 mM); ---------- reactions containing NADPH (2.0 mM).

The inverse relationship of binding affinity and reactivity is also supported by inspection of the bimolecular rate constants for the reaction of compounds 1 and 2 with GSH and NADPH, obtained by monitoring the pseudo-first-order disappearance of 1 and 2 by rp-HPLC (Table 1). Dynemicin A (1) is some 50-fold less reactive than the weaker-binding 2.

Table 2. Second Order Rate Constants for the Reactions of Dynemicin A (1) and Dideoxydynemicin A (2) with GSH and NADPH in the Presence of Double-Stranded DNA[a]

Drug	Cofactor	$k \, (M^{-1}s^{-1})$
1	GSH	$(9 \pm 2) \times 10^{-3}$
2	GSH	$(5 \pm 1) \times 10^{-1}$
1	NADPH	$(1 \pm 0.2) \times 10^{-2}$
2	NADPH	$(4 \pm 1) \times 10^{-1}$

[a]Rate constants obtained by monitoring the pseudo-first-order disappearance of drug by rp-HPLC. Reactions were performed in tris-HCl buffer (30 mM, pH 7.5; NaCl, 50 m M) with calf thymus DNA (1.0 mM base pairs) at 37 °C.

Dynemicin A: A Balance Between Binding Affinity and Reactivity

The series of dynemicins 1 - 6 span several orders of magnitude in binding affinity and reactivity. The binding affinity and reactivity of 1 lie in the middle of the range for the values observed, and points to a critical role for the carboxylate: the carboxylate of 1 is necessary to destabilize the DNA-drug binding complex to an extent sufficient for appreciable reactivity. This conclusion is supported by the fact that dynemicin A, in contrast to all other enediyne antibiotics, does not bear a positive charge at

physiological pH. In designing dynemicin A, Nature appears to have achieved a balance between high reactivity with weak binding (dideoxydynemicin A, **2**) and low reactivity with tight binding (dynemicin A methyl ester, **3**).

Experimental

General. Dynemicin A (**1**) and analogs **2-6**, synthesized as described previously, were obtained as purple films. All manipulations of the drugs were conducted with caution due to their potential human toxicity. Compounds **2, 4**, and **6** were typically manipulated as solutions in methanol; compounds **1, 3**, and **5** were manipulated as solutions in dimethylsulfoxide owing to their poor solubility in methanol. The concentrations of drug solutions were determined by UV using the following extinction coefficients: for compounds **2, 4**, and **6**, $\varepsilon(540 \text{ nm}) = 6000 \text{ M}^{-1}\text{cm}^{-1}$; for compounds **1, 3**, and **5**, $\varepsilon(560 \text{ nm}) = 10000 \text{ M}^{-1}\text{cm}^{-1}$. UV spectra were obtained on a Beckman D-640 spectrophotometer using a quartz UV cell, 10 mm path length. All reaction solutions were prepared with ultrapure water, obtained from a Millipore Milli-Q Plus water purification system. "Double-stranded calf thymus DNA" refers to sonicated, phenol-extracted calf thymus DNA (Pharmacia). Aqueous solutions of glutathione (GSH, Sigma, pH adjusted to 7.5 with tris base) and NADPH (Sigma) were prepared just prior to use. A standard buffer solution (100 mM, pH 7.5) was prepared from tris base (Fisher) and 1.00 M aqueous hydrochloric acid solution. All pH measurements were determined with a Beckman ϕ40 digital pH meter equipped with a Beckman Futura Plus gel-filled electrode. Unless otherwise specified, all reactions were conducted in tris-HCl aqueous buffer solution (30 mM, pH 7.5) containing sodium chloride (50 mM).

Equilibrium Dialysis, General. All dialysis experiments utilized Spectra Por #2 dialysis membrane (molecular weight cutoff = 12,000-14000, flat tube diameter = 10 mm). The dialysis membrane was soaked in water for at least 1 h prior to use. One end of the dialysis membrane (20 cm) was sealed by tying a loop knot. The drug/DNA

solution was carefully added (~3 cm dialysis membrane/mL drug/DNA solution), then the dialysis membrane was sealed with a loop knot. All dialysis experiments were performed at 25 °C in a standard dialysis buffer (tris-HCl aqueous buffer solution, 30 mM, pH 7.5; sodium chloride, 50 mM). Following equilibration, the concentrations of all solution components were determined by UV, using the following extinction coefficients: for compounds **2**, **4**, and **6**, $\varepsilon(540 \text{ nm}) = 6000 \text{ M}^{-1}\text{cm}^{-1}$; for compounds **1**, **3**, and **5**, $\varepsilon(560 \text{ nm}) = 10000 \text{ M}^{-1}\text{cm}^{-1}$; for calf thymus DNA, $\varepsilon(260 \text{ nm}) = 12000$ $\text{M}^{-1}\text{cm}^{-1}$. The binding constants were obtained from the equation $K_B =$ [DNA•drug]/[DNA][drug$_{free}$]. The concentration of drug that diffuses through the dialysis membrane represents [drug$_{free}$]. The concentration of drug that is retained within the dialysis membrane represents the sum [DNA•drug] + [drug$_{free}$].

Equilibrium Dialysis of Dynemicin A (1). A solution containing dynemicin A (0.2 mM) and calf thymus DNA (1.0 mM bp) was prepared on a volume of 800 μL by mixing an 80-μL aliquot of a solution of calf thymus DNA (10 mM bp) with standard dialysis buffer (560 μL), followed by the addition of a 160-μL aliquot of a solution of dynemicin A (1.0 mM) in DMSO. A 400-μL aliquot of this solution was diluted to a volume of 2.0 mL by the addition of dialysis buffer (1.6 mL), affording a more dilute solution containing dynemicin A (0.04 mM) and calf thymus DNA (0.2 mM bp). This solution was transferred into a dialysis membrane, sealed, then placed into a 15-mL polypropylene centrifuge tube containing dialysis buffer (5 mL). The tube was shaken (~100 rpm) in a horizontal position for 8 h at 25 °C, and the two compartments were analyzed by UV. A 1.0-mL aliquot of the solution within the dialysis membrane was diluted to a volume of 2.0 mL by the addition of dialysis buffer (1.0 mL) prior to UV analysis; the concentration of free drug was measured directly. In a separate

experiment, a 200-µL aliquot of the dynemicin A (0.2 mM)/DNA (1.0 mM bp) solution was diluted to a volume of 2.0 mL by the addition of dialysis buffer (1.8 mL), affording a more dilute solution containing dynemicin A (0.02 mM) and calf thymus DNA (0.1 mM bp). This solution was transferred into a dialysis membrane, sealed, then placed into a 15-mL polypropylene centrifuge tube containing dialysis buffer (5 mL). This tube was dialyzed and analyzed in parallel with the above experiment.

Equilibrium Dialysis of Dideoxydynemicin (2). A solution containing dideoxydynemicin (0.2 mM) and calf thymus DNA (1.0 mM bp) was prepared on a volume of 3.00 mL by mixing a 300-µL aliquot of a solution of calf thymus DNA (10 mM bp) with standard dialysis buffer (2.58 mL), followed by the addition of a 120-µL aliquot of a solution of dideoxydynemicin (5 mM) in DMSO. A 2.0-mL aliquot of the resulting drug/DNA solution was transferred into a dialysis membrane, sealed, then placed into a 15-mL polypropylene centrifuge tube containing dialysis buffer (5 mL). The tube was shaken (~100 rpm) in a horizontal position for 8 h at 25 °C, and the two compartments were analyzed by UV. A 200-µL aliquot of the solution within the dialysis membrane was diluted to a volume of 2.0 mL by the addition of dialysis buffer (1.8 mL) prior to UV analysis; the concentration of free drug was measured directly. In a separate experiment, a 1.0-mL aliquot of the drug/DNA solution was diluted to a volume of 2.0 mL by the addition of dialysis buffer (1.0 mL), affording a more dilute solution containing dideoxydynemicin (0.1 mM) and calf thymus DNA (0.5 mM bp). This solution was transferred into a dialysis membrane, sealed, then placed into a 15-mL polypropylene centrifuge tube containing dialysis buffer (5 mL). This tube was dialyzed and analyzed in parallel with the above experiment.

Equilibrium Dialysis of Dynemicin Methyl Ester (3). A solution containing dynemicin methyl ester (0.2 mM) and calf thymus DNA (1.0 mM bp) was prepared on a volume of 500 μL by mixing an 50-μL aliquot of a solution of calf thymus DNA (10 mM bp) with standard dialysis buffer (350 μL), followed by the addition of a 100-μL aliquot of a solution of **3** (1.0 mM) in DMSO. A 300-μL aliquot of this solution was diluted to a volume of 3.0 mL by the addition of dialysis buffer (2.7 mL), affording a more dilute solution containing **3** (0.02 mM) and calf thymus DNA (0.1 mM bp). This solution was transferred into a dialysis membrane, sealed, then placed into a 500-mL pyrex bottle containing dialysis buffer (400 mL). The solution was stirred magnetically for 12 h at 25 °C, and the two compartments were analyzed by UV. The concentrations of components within the dialysis membrane were measured directly. To determine the concentration of free drug, a 300-mL aliquot of the external dialysis buffer was removed and extracted four times with ethyl acetate (50 mL). The extracts were combined and concentrated to dryness by rotary evaporation. The residue was dissolved in methanol (1.5 mL) and analyzed by UV. In a separate experiment, a 150-μL aliquot of the **3** (0.2 mM)/DNA (1.0 mM bp) solution was diluted to a volume of 3.0 mL by the addition of dialysis buffer (2.85 mL), affording a more dilute solution containing **3** (0.01 mM) and calf thymus DNA (0.05 mM bp). This solution was transferred into a dialysis membrane, sealed, then placed into a 500-mL pyrex bottle containing dialysis buffer (400 mL). The solution was stirred magnetically for 12 h at 25 °C. This solution was dialyzed and analyzed in parallel with the above experiment.

Equilibrium Dialysis of Dideoxydynemicin Methyl Ester (4). A solution containing **4** (0.2 mM) and calf thymus DNA (1.0 mM bp) was prepared on a volume of 800 μL by mixing an 80-μL aliquot of a solution of calf thymus DNA (10 mM bp)

with standard dialysis buffer (560 μL), followed by the addition of a 160-μL aliquot of a solution of **4** (1.0 mM) in DMSO. A 400-μL aliquot of this solution was diluted to a volume of 2.0 mL by the addition of dialysis buffer (1.6 mL), affording a more dilute solution containing **4** (0.04 mM) and calf thymus DNA (0.2 mM bp). This solution was transferred into a dialysis membrane, sealed, then placed into a 15-mL polypropylene centrifuge tube containing dialysis buffer (5 mL). The tube was shaken (~100 rpm) in a horizontal position for 8 h at 25 °C, and the two compartments were analyzed by UV. A 1.0-mL aliquot of the solution within the dialysis membrane was diluted to a volume of 2.0 mL by the addition of dialysis buffer (1.0 mL) prior to UV analysis; the concentration of free drug was measured directly. In a separate experiment, a 200-μL aliquot of the **4** (0.2 mM)/DNA (1.0 mM bp) solution was diluted to a volume of 2.0 mL by the addition of dialysis buffer (1.8 mL), affording a more dilute solution containing **4** (0.02 mM) and calf thymus DNA (0.1 mM bp). This solution was transferred into a dialysis membrane, sealed, then placed into a 15-mL polypropylene centrifuge tube containing dialysis buffer (5 mL). This tube was dialyzed and analyzed in parallel with the above experiment.

Equilibrium Dialysis of Dynemicin Analog 5. A solution containing **5** (0.2 mM) and calf thymus DNA (1.0 mM bp) was prepared on a volume of 500 μL by mixing an 50-μL aliquot of a solution of calf thymus DNA (10 mM bp) with standard dialysis buffer (350 μL), followed by the addition of a 100-μL aliquot of a solution of **5** (1.0 mM) in DMSO. A 300-μL aliquot of this solution was diluted to a volume of 3.0 mL by the addition of dialysis buffer (2.7 mL), affording a more dilute solution containing **5** (0.02 mM) and calf thymus DNA (0.1 mM bp). This solution was transferred into a dialysis membrane, sealed, then placed into a 500-mL pyrex bottle

containing dialysis buffer (400 mL). The solution was stirred magnetically for 12 h at 25 °C, and the two compartments were analyzed by UV. The concentrations of components within the dialysis membrane were measured directly. To determine the concentration of free drug, a 300-mL aliquot of the external dialysis buffer was removed and extracted four times with ethyl acetate (50 mL). The extracts were combined and concentrated to dryness by rotary evaporation. The residue was dissolved in methanol (1.5 mL) and analyzed by UV. In a separate experiment, a 150-μL aliquot of the 5 (0.2 mM)/DNA (1.0 mM bp) solution was diluted to a volume of 3.0 mL by the addition of dialysis buffer (2.85 mL), affording a more dilute solution containing 5 (0.01 mM) and calf thymus DNA (0.05 mM bp). This solution was transferred into a dialysis membrane, sealed, then placed into a 500-mL pyrex bottle containing dialysis buffer (400 mL). The solution was stirred magnetically for 12 h at 25 °C. This solution was dialyzed and analyzed in parallel with the above experiment.

Equilibrium Dialysis of Dynemicin Analog 6. A solution containing 6 (0.2 mM) and calf thymus DNA (1.0 mM bp) was prepared on a volume of 800 μL by mixing an 80-μL aliquot of a solution of calf thymus DNA (10 mM bp) with standard dialysis buffer (560 μL), followed by the addition of a 160-μL aliquot of a solution of 6 (1.0 mM) in DMSO. A 400-μL aliquot of this solution was diluted to a volume of 2.0 mL by the addition of dialysis buffer (1.6 mL), affording a more dilute solution containing 6 (0.04 mM) and calf thymus DNA (0.2 mM bp). This solution was transferred into a dialysis membrane, sealed, then placed into a 15-mL polypropylene centrifuge tube containing dialysis buffer (5 mL). The tube was shaken (~100 rpm) in a horizontal position for 8 h at 25 °C, and the two compartments were analyzed by UV. A 1.0-mL aliquot of the solution within the dialysis membrane was diluted to a volume

of 2.0 mL by the addition of dialysis buffer (1.0 mL) prior to UV analysis; the concentration of free drug was measured directly. In a separate experiment, a 200-μL aliquot of the **6** (0.2 mM)/DNA (1.0 mM bp) solution was diluted to a volume of 2.0 mL by the addition of dialysis buffer (1.8 mL), affording a more dilute solution containing **6** (0.02 mM) and calf thymus DNA (0.1 mM bp). This solution was transferred into a dialysis membrane, sealed, then placed into a 15-mL polypropylene centrifuge tube containing dialysis buffer (5 mL). This tube was dialyzed and analyzed in parallel with the above experiment.

Preparation of 5'-^{32}P-labeled 193-Base Pair Restriction Fragment.
Plasmid pBR322 (40 μL, 0.25 μg/μL, Boehringer Mannheim) was precipitated by the addition of aqueous ammonium acetate buffer solution (20 μL, 8 M, pH 7) and ethanol (180 μL), followed by centrifugation at 2 °C (16,000 g, 30 min), then was washed with aqueous ethanol (100 μL, 70%). The DNA pellet was dried on a Savant rotary speed vac, then was dissolved in water (90 μL). Digestion buffer H (10 μL, Boehringer Mannheim) was added, and the plasmid was digested with Eco RI (5 μL, 50 units, Boehringer Mannheim) at 37 °C for 6 h. The digestion was quenched by extracting the solution twice with phenol:chloroform (100 μL, 1:1 v/v), and the DNA was precipitated by the addition of aqueous ammonium acetate buffer solution (50 μL, 8 M, pH 7) and ethanol (400 μL), followed by centrifugation at 2 °C (16,000 g, 30 min). The DNA pellet was washed with aqueous ethanol (100 μL, 70%), then was dried. The DNA was dissolved in water (90 μL) and dephosphorylation buffer (10 μL, Boehringer Mannheim). The 5'-phosphate groups were removed by incubation with alkaline phosphatase (2 μL, 2 units, Boehringer Mannheim) at 37 °C for 1 h. Aqueous EDTA solution (10 μL, 200 mM) was added, and the solution was incubated at 60 °C

for 30 min to deactivate the alkaline phosphatase. Water (90 µL) was added, and the

alkaline phosphatase was further deactivated by three extractions with

phenol:chloroform:amyl alcohol (200 µL, 50:48:2). The DNA was precipitated by the

addition of aqueous ammonium acetate buffer solution (50 µL, 8 M, pH 7) and ethanol

(700 µL), followed by centrifugation at 2 °C (16,000 g, 30 min). The DNA pellet was

washed with aqueous ethanol (100 µL, 70%), then was dried. The DNA was

dissolved in water (25 µL), phosphorylation buffer (5 µL, Boehringer Mannheim), [γ-

^{32}P]-dATP 20 µL, 200 µCi, NEN, ≥6000 Ci/mmol), and 5'-labeled with

polynucleotide kinase (3 µL, 30 units, Boehringer Mannheim) at 37 °C for 3 h. The

reaction solution was eluted through a NICK column (Pharmacia) to remove

unincorporated ^{32}P-dATP, and the eluent containing the labeled fragment was extracted

twice with phenol:chloroform (300 µL, 1:1), then concentrated to a volume of 100 µL.

The labeled fragment was precipitated by the addition of aqueous ammonium acetate

buffer solution (50 µL, 8 M, pH 7) and ethanol (400 µL), followed by centrifugation at

2 °C (16,000 g, 30 min), then was washed with aqueous ethanol (100 µL, 70%) and

dried. The DNA was dissolved in water (90 µL). Digestion buffer H (10 µL,

Boehringer Mannheim) was added, and the DNA was digested with Ssp I (5 µL, 50

units, Boehringer Mannheim) at 37 °C for 6 h. The DNA was precipitated by the

addition of aqueous ammonium acetate buffer solution (50 µL, 8 M, pH 7) and ethanol

(400 µL), followed by centrifugation at 2 °C (16,000 g, 30 min). The DNA pellet was

washed with aqueous ethanol (100 µL, 70%), then was dried. The DNA was

dissolved in water (20 µL) and ficoll loading buffer (5 µL), then was purified over an

8% non-denaturing polyacrylamide gel, 0.8 mm thickness.[17] The band containing the

193-base pair fragment was located by autoradiography and was excised from the gel.

The gel slice was crushed thoroughly and, after combination with aqueous Nonidet P-

40 detergent solution (700 μL, 0.05%, Sigma), was vortexed for 6 h at 23 °C. The resulting suspension was filtered through a Centrex filter (0.45 μm) and the filtrate was extracted twice with phenol:chloroform (400 μL, 1:1 v/v), then was concentrated to a volume of 100 μL. The labeled product was precipitated by the addition of aqueous ammonium acetate buffer solution (50 μL, 8 M, pH 7) and ethanol (400 μL), followed by centrifugation at 2 °C (16,000 g, 30 min), then was washed with aqueous ethanol (100 μL, 70%). The purified labeled fragment was stored frozen in tris-HCl aqueous buffer solution (10 mM, pH 7.4) with EDTA (1 mM). This procedure typically yielded 5 - 7 million cpm labeled fragment.

Preparation of 3'-^{32}P-labeled 193-Base Pair Restriction Fragment.
Plasmid pBR322 (40 μL, 0.25 μg/μL, Boehringer Mannheim) was precipitated by the addition of aqueous ammonium acetate buffer solution (20 μL, 8 M, pH 7) and ethanol (180 μL), followed by centrifugation at 2 °C (16,000 g, 30 min), then was washed with aqueous ethanol (100 μL, 70%). The DNA pellet was dried on a Savant rotary speed vac, then was dissolved in water (90 μL). Digestion buffer H (10 μL, Boehringer Mannheim) was added, and the plasmid was digested with Eco RI (4 μL, 40 units, Boehringer Mannheim) and Ssp I (4 μL, 40 units, Boehringer Mannheim) at 37 °C for 6 h. The digestion was quenched by extracting the solution twice with phenol:chloroform (100 μL, 1:1 v/v), and the DNA was precipitated by the addition of aqueous ammonium acetate buffer solution (50 μL, 8 M, pH 7) and ethanol (400 μL), followed by centrifugation at 2 °C (16,000 g, 30 min). The DNA pellet was washed with aqueous ethanol (100 μL, 70%), then was dried. The DNA was dissolved in water (18 μL), then was mixed with aqueous dithiothreitol solution (4 μL, 100 mM), Sequenase 2.0 buffer (8 μL, USB), and [α-^{32}P]-dATP (10 μL, 200 μCi, NEN,

≥6000 Ci/mmol). The restriction fragment was 3'-labeled with Sequenase Version 2.0 (2 μL, 25 units, USB) at 23 °C for 3 h, then was treated with aqueous dATP solution (5 μL, 10 mM) and Sequenase Version 2.0 (2 μL, 25 units, USB) and incubated further at 23 °C for 2 h. The reaction solution was eluted through a NICK column (Pharmacia) to remove unincorporated ^{32}P-dATP, and the eluent containing the labeled fragment was concentrated to a volume of 100 μL. The labeled fragment was precipitated by the addition of aqueous ammonium acetate buffer solution (50 μL, 8 M, pH 7) and ethanol (400 μL), followed by centrifugation at 2 °C (16,000 g, 30 min), then was washed with aqueous ethanol (100 μL, 70%) and dried. The 193-base pair fragment was dissolved in water (20 μL) and ficoll loading buffer (5 μL), then was purified over an 8% non-denaturing polyacrylamide gel, 0.8 mm thickness, and isolated as described above.

Analysis of DNA Cleavage Products, General. The products from a given DNA cleavage reaction were precipitated by the addition of aqueous ammonium acetate buffer solution (50 μL, 8 M, pH 7) and ethanol (300 μL), followed by centrifugation at 2 °C (16,000 g, 20 min). The resulting product pellet was washed with aqueous ethanol (100 μL, 70%), was dried, then was dissolved in formamide loading buffer (10 μL), and the resulting solution was transferred to a 0.65-mL Eppendorf tube. After assaying for radioactivity with a Beckman LS 6000SC scintillation counter, the solution was diluted with additional formamide loading buffer so as to produce a radiation density of 3000 cpm/μL. After heating at 80 °C for 3 min to induce denaturation, the solution (5 μL) was analyzed by gel electrophoresis. Cleavage products were loaded onto an 8% denaturing polyacrylamide gel (42 x 34 cm x 0.4 mm thickness) and were separated by electrophoresis in 1 x TBE buffer at 2000 V for 15 min and then at 1500 V

until the bromophenol blue dye had migrated off the gel.[17] The gel was exposed to a storage phosphor plate and the DNA cleavage products were quantified with a molecular Dynamics 400-S PhosphorImager.

Analysis of DNA Cleavage by Dynemicin A (1) and Synthetic Analogs 2-6.

Reactions were performed at 37 °C in 1.5-mL Eppendorf tubes containing a total reaction volume of 20 μL. A 2-μL aliquot of a solution of dynemicin A (0.5 mM) in DMSO was combined with a solution of double-stranded calf thymus DNA (2 μL, 10 mM bp) in water, tris-HCl aqueous buffer solution (6 μL, 100 mM, pH 7.5), aqueous sodium chloride solution (2 μL, 500 mM), water (6 μL), and 5'-labeled restriction fragment (~100,000 cpm). The reaction was initiated at 23 °C by the addition of an aqueous solution of glutathione (2 μL, 200 mM, pH 7.5) to the remaining solution, thus producing the following concentrations of solution components at the onset of the reaction: Dynemicin A, 0.05 mM; GSH, 20 mM; double-stranded calf thymus DNA, 1.0 mM bp; tris-HCl buffer, 30 mM; sodium chloride, 50 mM. The reaction solution was incubated at 37 °C for 12 h. Water (80 μL) was added, and the cleavage products were then precipitated and analyzed as described above. DNA cleavage reactions employing the synthetic dynemicin analogs 2-6 were conducted in an identical manner, employing a solution of the respective dynemicin analogue (0.5 mM) in DMSO in lieu of dynemicin A. DNA cleavage reactions employing NADPH were conducted in an identical manner, employing an aqueous solution of NADPH (200 mM) in lieu of GSH.

Preparation of Product 8.

A solution of dynemicin analog 2 (5 mg, 0.01 mmol, 1 equiv) in methanol (7.5 mL) containing 2,5-dimethoxybenzyl alcohol (1 mM, internal

HPLC standard) was treated sequentially with 1,4-cyclohexadiene (1.0 mL, 10 mmol, 1000 equiv), triethylamine (0.28 mL, 204 mg, 2.0 mmol, 200 equiv), and aqueous glutathione solution (1.0 mL, 20 mM, 0.02 mmol, 2.0 equiv), thus producing the following concentrations of solution components at the onset of the reaction: **2**, 1 mM; 1,4-cyclohexadiene, 1 M; triethylamine, 200 mM; glutathione, 2.0 mM. The solution was incubated at 37 °C for 12 h, during which time its color changed from violet to dark purple. The product solution was concentrated to a volume of ~1 mL, then was diluted with water (3 mL). Purification of the product was achieved by reverse-phase HPLC, using 4 separate 1-mL injection volumes, on a Beckman Ultrasphere ODS (C_{18}, 5 μm) rp-HPLC column, 10 x 250 mm, as part of a Beckman HPLC system, flow = 2.00 mL/min, with a linear gradient of acetonitrile:aqueous ammonium acetate buffer solution (10 mM, pH 6.0): 5:95 v/v acetonitrile:aqueous ammonium acetate buffer to 70:30 v/v acetonitrile:aqueous ammonium acetate buffer over a period of 50 min. Peaks were detected by ultraviolet absorption at 240 nm with a Beckman 168 Programmable Photodiode Detector. The purple fractions containing product **8** (retention time t_R ~35 min) were collected and pooled. Acetonitrile was removed by rotary evaporation and the resulting concentrated aqueous solution was lyophilized. Product **8** was obtained as a dark purple film (1.5 mg, 30%, as determined by integration against the internal standard). ^{1}H NMR (500 MHz, 19:1 C_6D_6:DMSO-d_6), δ: 14.02 (s, 1 H, aryl OH), 10.90 (d, 1 H, J = 4.9 Hz, NH), 8.31 (dd, 1 H, J = 0.8, 7.9 Hz, o-C(O)-aryl **H**), 8.20 (dd, 1 H, J = 0.8, 7.7 Hz, o-C(O)-aryl **H**), 8.14 (s, 1 H, m-aryl **H**), 7.18 (td, 1 H, J = 1.1, 7.3 Hz, 1.1, 7.3 Hz, m-C(O)-aryl **H**), 7.11 (td, 1 H, J = 1.1, 7.3 Hz, m-C(O)-aryl **H**), 6.99 (t, 1 H, J = 7.6 Hz, aromatized core), 6.96 (t, 1 H, J = 7.1 Hz, aromatized core), 6.93 (td, 1 H, J = 0.8, 7.3 Hz, aromatized core), 6.83 (td, 1 H, J = 1.3, 6.2 Hz, aromatized core), 4.89 (br s, 1 H, C≡CC**H**),

4.65 (d, 1 H, J = 5.1 Hz, NCH), 3.91 (s, 1 H, C(OH)CH), 3.83 (q, 1 H, J = 7.1 Hz, CHCH$_3$), 3.60 (s, 1 H, OCH$_3$), 1.42 (d, 1 H, J = 7.3 Hz, CHCH$_3$).

Preparation of Product 9. A solution of dynemicin analog **3** (2.5 mg, 0.0045 mmol, 1 equiv) in DMSO (1 mL) was treated sequentially with methanol (7.2 mL) containing 2,5-dimethoxybenzyl alcohol (1 mM, internal HPLC standard), 1,4-cyclohexadiene (1.0 mL, 10 mmol, 2200 equiv), triethylamine (0.28 mL, 204 mg, 2.0 mmol, 440 equiv), and aqueous glutathione solution (0.50 mL, 30 mM, 0.015 mmol, 3.3 equiv), thus producing the following concentrations of solution components at the onset of the reaction: **3**, 0.45 mM; 1,4-cyclohexadiene, 1 M; triethylamine, 200 mM; glutathione, 1.5 mM. The solution was incubated at 37 °C for 12 h. The product solution was concentrated to a volume of ~5 mL, then was diluted with water (5 mL). Purification of the product was achieved by reverse-phase HPLC, using 10 separate 1-mL injection volumes, on a Beckman Ultrasphere ODS (C$_{18}$, 5 µm) rp-HPLC column, 10 x 250 mm, as part of a Beckman HPLC system, flow = 2.00 mL/min, with a linear gradient of acetonitrile:aqueous ammonium acetate buffer solution (10 mM, pH 6.0): 30:70 v/v acetonitrile:aqueous ammonium acetate buffer to 70:30 v/v acetonitrile:aqueous ammonium acetate buffer over a period of 50 min. Peaks were detected by ultraviolet absorption at 240 nm with a Beckman 168 Programmable Photodiode Detector. The blue fractions containing product **9** (retention time t_R ~56 min) were collected and pooled. Acetonitrile was removed by rotary evaporation and the resulting concentrated aqueous solution was lyophilized. Product **9** was obtained as a dark blue film (1 mg, 40%, as determined by integration against the internal standard). ^{1}H NMR (500 MHz, C$_6$D$_6$), δ: 13.35 (s, 1 H, aryl OH), 12.75 (s, 1 H, aryl OH), 12.43 (s, 1 H, aryl OH), 9.62 (d, 1 H, J = 4.6 Hz, NH), 7.72 (s, 1 H, *m*-

aryl H), 6.91 (d, 1 H, J = 7.6 Hz, m-aryl H), 6.86 (td, 1 H, J = 0.9, 8.5 Hz,

aromatized core), 6.83 (m, 2 H, aromatized core), 6.74 (m, 2 H, m-aryl H, aromatized

core), 4.12 (d, 1 H, J = 4.6 Hz, NCH), 3.74 (q, 1 H, J = 7.2 Hz, CHCH$_3$), 3.65 (s,

1 H, C(OH)CH), 3.49 (s, 3 H, OCH$_3$), 3.36 (s, 3 H, OCH$_3$), 3.20 (br s, 1 H,

C≡CCH), 2.86 (br s, 1 H, OH), 1.14 (d, 3 H, J = 7.2 Hz, CHCH$_3$).

Preparation of Product 10. A solution of dynemicin analog **4** (5 mg, 0.01 mmol,

1 equiv) in methanol (7.5 mL) containing 2,5-dimethoxybenzyl alcohol (1 mM, internal

HPLC standard) was treated sequentially with 1,4-cyclohexadiene (1.0 mL, 10 mmol,

100 equiv), triethylamine (0.28 mL, 204 mg, 2.0 mmol, 200 equiv), and aqueous

glutathione solution (1.0 mL, 20 mM, 0.02 mmol, 2.0 equiv), thus producing the

following concentrations of solution components at the onset of the reaction: **4**, 1 mM;

1,4-cyclohexadiene, 1 M; triethylamine, 200 mM; glutathione, 2.0 mM. The solution

was incubated at 37 °C for 12 h, during which time its color changed from violet to

dark purple. The product solution was concentrated to a volume of ~1 mL, then was

diluted with water (3 mL). Purification of the product was achieved by reverse-phase

HPLC, using 4 separate 1-mL injection volumes, on a Beckman Ultrasphere ODS

(C$_{18}$, 5 μm) rp-HPLC column, 10 x 250 mm, as part of a Beckman HPLC system,

flow = 2.00 mL/min, with a linear gradient of acetonitrile:aqueous ammonium acetate

buffer solution (10 mM, pH 6.0): 20:80 v/v acetonitrile:aqueous ammonium acetate

buffer to 80:20 v/v acetonitrile:aqueous ammonium acetate buffer over a period of 50

min. Peaks were detected by ultraviolet absorption at 240 nm with a Beckman 168

Programmable Photodiode Detector. The purple fractions containing product **10**

(retention time t_R ~45 min) were collected and pooled. Acetonitrile was removed by

rotary evaporation and the resulting concentrated aqueous solution was lyophilized.

Product **10** was obtained as a dark purple film (1.5 mg, 30%, as determined by integration against the internal standard). ^{1}H NMR (500 MHz, C$_6$D$_6$), δ: 13.68 (s, 1 H, aryl OH), 10.06 (d, 1 H, J = 4.2 Hz, NH), 8.22 (dd, 1 H, J = 1.1, 7.9 Hz, o-C(O)-aryl H), 8.12 (dd, 1 H, J = 1.1, 8.0 Hz, o-C(O)-aryl H), 7.83 (s, 1 H, m-aryl H), 7.09 (td, 1 H, J = 1.4, 7.4 Hz, m-C(O)-aryl H), 7.04 (td, 1 H, J = 1.2, 7.8 Hz, m-C(O)-aryl H), 6.93 (d, 1 H, J = 7.4 Hz, aromatized core), 6.83 (m, 2 H, aromatized core), 6.74 (td, 1 H, J = 1.4, 7.5 Hz, aromatized core), 4.05 (d, 1 H, J = 4.5 Hz, NCH), 3.71 (q, 1 H, J = 7.2 Hz, CHCH$_3$), 3.69 (s, 1 H, C(OH)CH), 3.49 (s, 3 H, OCH$_3$), 3.36 (s, 3 H, OCH$_3$), 2.86 (br s, 1 H, C≡CCH), 2.75 (br s, 1 H, OH), 1.11 (d, 3 H, J = 7.3 Hz, CHCH$_3$).

Preparation of Product 11. A solution of dynemicin analog **5** (2.5 mg, 0.0045 mmol, 1 equiv) in DMSO (0.5 mL) was treated sequentially with methanol (7.2 mL) containing 2,5-dimethoxybenzyl alcohol (1 mM, internal HPLC standard), 1,4-cyclohexadiene (1.0 mL, 10 mmol, 2200 equiv), triethylamine (0.28 mL, 204 mg, 2.0 mmol, 440 equiv), and aqueous glutathione solution (1 mL, 200 mM, 0.2 mmol, 40 equiv), thus producing the following concentrations of solution components at the onset of the reaction: **5**, 0.45 mM; 1,4-cyclohexadiene, 1 M; triethylamine, 200 mM; glutathione, 20 mM. The solution was incubated at 37 °C for 12 h. The product solution was concentrated to a volume of ~5 mL, then was diluted with water (5 mL). Purification of the product was achieved by reverse-phase HPLC, using 10 separate 1-mL injection volumes, on a Beckman Ultrasphere ODS (C$_{18}$, 5 μm) rp-HPLC column, 10 x 250 mm, as part of a Beckman HPLC system, flow = 2.00 mL/min, with a linear gradient of acetonitrile:aqueous ammonium acetate buffer solution (10 mM, pH 6.0): 30:70 v/v acetonitrile:aqueous ammonium acetate buffer to 70:30 v/v

acetonitrile:aqueous ammonium acetate buffer over a period of 50 min. Peaks were detected by ultraviolet absorption at 240 nm with a Beckman 168 Programmable Photodiode Detector. The blue fractions containing product 11 (retention time t_R ~50 min) were collected and pooled. Acetonitrile was removed by rotary evaporation and the resulting concentrated aqueous solution was lyophilized. Product 11 was obtained as a dark blue film (0.8 mg, 30%, as determined by integration against the internal standard). ^{1}H NMR (500 MHz, C_6D_6), δ: 13.60 (s, 1 H, aryl OH), 12.92 (s, 1 H, aryl OH), 12.53 (s, 1 H, aryl OH), 9.98 (d, 1 H, J = 4.6 Hz, NH), 7.38 (dd, 1 H, J = 1.2, 7.8 Hz, aromatized core), 7.21 (s, 1 H, m-aryl H), 6.97 (td, 1 H, J = 1.5, 7.4 Hz, aromatized core), 6.92 (td, 1 H, J = 1.4, 7.4 Hz, aromatized core), 6.91 (d, 1 H, J = 9.2 Hz, m-C(O)-aryl H), 6.83 (d, 1 H, J = 9.2 Hz, m-C(O)-aryl H), 6.81 (dd, 1 H, J = 1.2, 7.5, aromatized core), 3.87 (dd, 1 H, J = 1.8, 4.5, NCH), 3.43 (d, 1 H, J = 1.8 Hz, C(OH)-CH-C(OH)), 3.08 (s, 3 H, OCH_3), 2.97 (s, 3 H, OCH_3), 2.68 (s, 1 H, OH), 2.57 (s, 1 H, OH), 2.37 (m, 1 H, $CHCH_3$), 1.56 (dd, 1 H, J = 5.9, 14.3 Hz, gem-CH_2), 0.94 (d, 1 H, J = 6.8 Hz, $CHCH_3$), 0.52 (t, 1 H, J = 14.0 Hz, gem-CH_2).

Preparation of Product 12. A solution of dynemicin analog 6 (6 mg, 0.012 mmol, 1 equiv) in methanol (9 mL) containing 2,5-dimethoxybenzyl alcohol (1 mM, internal HPLC standard) was treated sequentially with 1,4-cyclohexadiene (1.0 mL, 10 mmol, 830 equiv), triethylamine (0.30 mL, 220 mg, 2.2 mmol, 180 equiv), and aqueous glutathione solution (1.0 mL, 200 mM, 0.20 mmol, 20 equiv), thus producing the following concentrations of solution components at the onset of the reaction: 6, 1 mM; 1,4-cyclohexadiene, 1 M; triethylamine, 200 mM; glutathione, 20 mM. The solution was incubated at 37 °C for 12 h, during which time its color changed from

violet to dark purple. The product solution was concentrated to a volume of ~0.5 mL, then was diluted with water (1 mL). Purification of the product was achieved by reverse-phase HPLC, using 3 separate 500-μL injection volumes, on a Beckman Ultrasphere ODS (C_{18}, 5 μm) rp-HPLC column, 10 x 250 mm, as part of a Beckman HPLC system, flow = 2.00 mL/min, with a linear gradient of acetonitrile:aqueous ammonium acetate buffer solution (10 mM, pH 6.0): 10:90 v/v acetonitrile:aqueous ammonium acetate buffer to 90:10 v/v acetonitrile:aqueous ammonium acetate buffer over a period of 50 min. Peaks were detected by ultraviolet absorption at 240 nm with a Beckman 168 Programmable Photodiode Detector. The purple fractions containing product **12** (retention time t_R ~45 min) were collected and pooled. Acetonitrile was removed by rotary evaporation and the resulting concentrated aqueous solution was lyophilized. Product **12** was obtained as a dark purple film (3 mg, 50%, as determined by integration against the internal standard). ^{1}H NMR (500 MHz, C_6D_6), δ: 13.80 (s, 1 H, aryl OH), 10.48 (d, 1 H, $J = 4.3$ Hz, NH), 8.27 (dd, 1 H, $J = 1.0$, 8.0 Hz, o-C(O)-aryl H), 8.15 (dd, 1 H, $J = 1.2$, 7.8 Hz, o-C(O)-aryl H), 7.41 (dd, 1 H, $J = 1.1$, 8.0 Hz, aromatized core), 7.25 (s, 1 H, m-aryl H), 7.12 (td, 1 H, $J = 1.3$, 7.8 Hz, m-C(O)-aryl H), 7.04 (td, 1 H, $J = 1.3$, 8.5 Hz, m-C(O)-aryl H), 6.96 (td, 1 H, $J = 1.2$, 7.4 Hz, aromatized core), 6.92 (td, 1 H, $J = 1.7$, 7.3 Hz, aromatized core), 6.85 (dd, 1 H, $J = 1.3$, 7.0 Hz, aromatized core), 3.91 (dd, 1 H, $J = 1.9$, 4.4 Hz, NCH), 3.50 (d, 1 H, $J = 1.7$ Hz, C(OH)-CH-C(OH)), 3.11 (s, 3 H, OCH_3), 3.00 (s, 3 H, OCH_3), 2.93 (s, 1 H, OH), 2.66 (s, 1 H, OH), 2.43 (m, 1 H, $CHCH_3$), 1.58 (dd, 1 H, $J = 5.9$, 14.5 Hz, gem-CH_2), 0.96 (d, 3 H, $J = 6.8$ Hz, $CHCH_3$), 0.55 (t, 1 H, $J = 13.9$ Hz, gem-CH_2); FTIR (neat), cm^{-1}: 3446 (m, br), 2954 (m), 2923 (s), 2851 (w), 1615 (m), 1585 (s), 1492 (m), 1354 (s), 1251 (s), 1051 (s); HRMS (FAB): calcd for $C_{30}H_{28}NO_7$ [MH]$^+$ 514.1866, found 514.1869.

Reaction of Dynemicin Methyl Ester (3) with GSH in the Presence of DNA. The reaction with GSH was performed at 37 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 100 µL. A 10-µL aliquot of a solution of **3** (1.0 mM) in DMSO containing 2,5-dimethoxybenzyl alcohol (0.5 mM, internal HPLC standard) was combined with a solution of double-stranded calf thymus DNA (10 µL, 10 mM bp) in water, tris-HCl aqueous buffer solution (30 µL, 100 mM, pH 7.5), aqueous sodium chloride solution (10 µL, 500 mM), and water (30 µL). The reaction was initiated by the addition of an aqueous solution of GSH (10 µL, 200 mM, pH 7.5,), thus producing the following concentrations of solution components at the onset of the reaction: **3**, 0.1 mM; GSH, 20 mM; double-stranded calf thymus DNA, 1.0 mM bp; tris-HCl buffer, 30 mM; sodium chloride, 50 mM. The reaction solution was incubated at 37 °C for 10 h, followed by HPLC analysis (40-µL injection volume) employing a Beckman HPLC system equipped with a Beckman Ultrasphere ODS (C_{18}, 5 µm) rp-HPLC column, 4.6 x 250 mm, flow = 0.40 mL/min with a linear gradient of acetonitrile:aqueous ammonium acetate buffer solution (10 mM, pH 6.0): 10:90 v/v acetonitrile:aqueous ammonium acetate buffer to 100% acetonitrile over a period of 50 min. Peaks were detected by ultraviolet absorption at 240 nm with a Beckman 168 Programmable Photodiode Detector (**3**, retention time $t_R \sim 54$ min; internal standard, retention time $t_R \sim 27$ min). An initial ratio of **3** to 2,5-dimethoxybenzyl alcohol was established by HPLC analysis of the solution of **3** in DMSO containing the internal standard. Compound **3** was observed to be inert towards GSH under the reaction conditions.

Reaction of Dynemicin Analog 5 with GSH in the Presence of DNA. The reaction with GSH was performed at 37 °C in a 1.5-mL Eppendorf tube containing

a total reaction volume of 100 μL. A 10-μL aliquot of a solution of **5** (1.0 mM) in DMSO containing 2,5-dimethoxybenzyl alcohol (0.5 mM, internal HPLC standard) was combined with a solution of double-stranded calf thymus DNA (10 μL, 10 mM bp) in water, tris-HCl aqueous buffer solution (30 μL, 100 mM, pH 7.5), aqueous sodium chloride solution (10 μL, 500 mM), and water (30 μL). The reaction was initiated by the addition of an aqueous solution of GSH (10 μL, 200 mM, pH 7.5), thus producing the following concentrations of solution components at the onset of the reaction: **5**, 0.1 mM; GSH, 20 mM; double-stranded calf thymus DNA, 1.0 mM bp; tris-HCl buffer, 30 mM; sodium chloride, 50 mM. The reaction solution was incubated at 37 °C for 10 h, followed by HPLC analysis (40-μL injection volume) employing a Beckman HPLC system equipped with a Beckman Ultrasphere ODS (C_{18}, 5 μm) rp-HPLC column, 4.6 x 250 mm, flow = 0.40 mL/min with a linear gradient of acetonitrile:aqueous ammonium acetate buffer solution (10 mM, pH 6.0): 10:90 v/v acetonitrile:aqueous ammonium acetate buffer to 100% acetonitrile over a period of 50 min. Peaks were detected by ultraviolet absorption at 240 nm with a Beckman 168 Programmable Photodiode Detector (**5**, retention time t_R ~ 54 min; internal standard, retention time t_R ~ 27 min). An initial ratio of **5** to 2,5-dimethoxybenzyl alcohol was established by HPLC analysis of the solution of **5** in DMSO containing the internal standard. Compound **5** was observed to be inert towards GSH under the reaction conditions.

Reaction of Dynemicin A (1) with GSH in Methanol. The reaction with GSH was performed at 37 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 150 μL. A 20-μL aliquot of a solution of **1** (1.0 mM) in DMSO was combined with methanol (135 μL), 1,4-cyclohexadiene (10 μL), triethylamine (5 μL),

and a solution of 2,5-dimethoxybenzyl alcohol (10 μL, 5 mM, internal HPLC standard) in methanol. A 45-μL aliquot of the resulting solution was removed and an initial ratio of **1** to the internal standard was established by HPLC analysis as described above. The reaction solution was pre-incubated for 30 min at 37 °C, then the reaction was initiated at 37 °C by the addition of an aqueous solution of GSH (15 μL, 50 mM), thus producing the following concentrations of solution components at the onset of the reaction: **1**, 0.1 mM; GSH, 5 mM; 1,4-cyclohexadiene, 500 mM; triethylamine, 250 mM. The reaction was monitored at times of 10 and 60 min by transfer of a 50-μL aliquot to an Eppendorf tube followed by rapid freezing by immersion of the tube in liquid nitrogen. Aliquots were analyzed by HPLC as described above. (**1**, retention time t_R ~ 36 min; **7**, retention time t_R ~ 30 min; internal standard, retention time t_R ~ 27 min).

Reaction of Dynemicin Methyl Ester (3) with GSH in Methanol. The reaction with GSH was performed at 37 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 150 μL. A 20-μL aliquot of a solution of **3** (1.0 mM) in DMSO was combined with methanol (135 μL), 1,4-cyclohexadiene (10 μL), triethylamine (5 μL), and a solution of 2,5-dimethoxybenzyl alcohol (10 μL, 5 mM, internal HPLC standard) in methanol. A 45-μL aliquot of the resulting solution was removed and an initial ratio of **3** to the internal standard was established by HPLC analysis as described above. The reaction solution was pre-incubated for 30 min at 37 °C, then the reaction was initiated at 37 °C by the addition of an aqueous solution of GSH (15 μL, 50 mM), thus producing the following concentrations of solution components at the onset of the reaction: **3**, 0.1 mM; GSH, 5 mM; 1,4-cyclohexadiene, 500 mM; triethylamine, 250 mM.. The reaction was monitored at times of 10 and 60

min by transfer of a 50-μL aliquot to an Eppendorf tube followed by rapid freezing by immersion of the tube in liquid nitrogen. Aliquots were analyzed by HPLC as described above. (3, retention time t_R ~ 54 min; 9, retention time t_R ~ 46 min; internal standard, retention time t_R ~ 27 min).

Reaction of Dynemicin Analog (5) with GSH in Methanol. The reaction with GSH was performed at 37 °C in a 1.5-mL Eppendorf tube containing a total reaction volume of 100 μL. A 10-μL aliquot of a solution of **5** (1.0 mM) in DMSO containing 2,5-dimethoxybenzyl alcohol (0.5 mM, internal HPLC standard) was combined with methanol (70 μL), 1,4-cyclohexadiene (5 μL), and triethylamine (5 μL). The reaction was initiated by the addition of an aqueous solution of GSH (10 μL, 200 mM), thus producing the following concentrations of solution components at the onset of the reaction: **5**, 0.1 mM; GSH, 20 mM; 1,4-cyclohexadiene, 500 mM; triethylamine, 500 mM.. The reaction was incubated at 37 °C for 10 h and analyzed by HPLC as described above. (**5**, retention time t_R ~ 54 min; **11**, retention time t_R ~ 46 min; internal standard, retention time t_R ~ 27 min).

DNA Cleavage by Dynemicin A (1) with GSH and NADPH at Varying Concentrations of DNA. A solution containing dynemicin A (0.5 mM), double-stranded calf thymus DNA (10 mM bp), and ~2 x 10^7 cpm 3'-^{32}P-labeled restriction fragment in 4:1 v/v water:DMSO (130 μL) was prepared as follows. A 130-μL aliquot of an aqueous solution of double-stranded calf thymus DNA (10 mM bp) was mixed with ~2 x 10^7 cpm labeled fragment, and the resulting solution was concentrated to a volume of 105 μL. A 25-μL aliquot of a solution of dynemicin A (2.6 mM) in DMSO was then added to afford a final volume of 130 μL. A 25-μL aliquot of this solution

was diluted to a volume of 125 µL by the addition of water (80 µL) and DMSO (20 µL), affording a second, more dilute solution containing dynemicin A (0.1 mM) and calf thymus DNA (2 mM bp). A 25-µL aliquot of this second solution was diluted to a volume of 125 µL by the addition of water (80 µL) and DMSO (20 µL), affording a third solution containing dynemicin A (0.02 mM) and calf thymus DNA (0.4 mM bp). Three parallel reactions of dynemicin A with GSH (10 mM) at a constant ratio of drug:calf thymus DNA:^{32}P-labeled DNA were performed at 37 °C in 1.5-mL Eppendorf tubes on a total reaction volume of 100 µL (9:1 water:DMSO) by combining 50 µL of each respective solution of drug/DNA with aqueous tris-HCl buffer solution (30 µL, 100 mM, pH 7.5) and aqueous sodium chloride solution (10 µL, 500 mM). The resulting solutions were pre-incubated at 37 °C for 30 min, and the reactions were initiated at 37 °C by the addition of an aqueous solution of glutathione (10 µL, 100 mM, pH 7.5, 37 °C). The concentrations of dynemicin A and calf thymus DNA, respectively, at the onset of these reactions were, in order of decreasing concentrations, as follows: (1) dynemicin A, 0.25 mM; DNA, 5.0 mM bp; (2) dynemicin A, 0.05 mM; DNA, 1.0 mM bp; (3) dynemicin A, 0.01 mM; DNA, 0.2 mM; each reaction solution also contained tris-HCl buffer (30 mM, pH 7.5), sodium chloride (50 mM), and glutathione (10 mM). At reaction times of 2, 4, 6, 8, and 10 h, an 18-µL aliquot from each reaction solution was transferred to a 1.5-mL Eppendorf tube containing aqueous ammonium acetate buffer solution (50 µL, 2 M, pH 5.5), and immediately frozen in liquid nitrogen. Reactions employing NADPH were conducted in an identical manner, employing an aqueous solution of NADPH (50 mM) in lieu of GSH. Following completion of the reactions, the cleavage products were precipitated and analyzed as described above. A control solution was prepared on a volume of 20 µL by combining a 10-µL aliquot of the solution of dynemicin A (0.02 mM) and DNA (0.4 mM) with

tris-HCl aqueous buffer solution (6 μL, 100 mM, pH 7.5), aqueous sodium chloride solution (2 μL, 500 mM), and water (2 μL), thus producing the following concentrations of reaction components: dynemicin A, 0.01 mM; calf thymus DNA, 0.2 mM bp; tris-HCl buffer, 30 mM; sodium chloride, 50 mM. The control solution was incubated in parallel with the cleavage reactions at 37 °C for 10 h. The amount of background in the control lane (typically 3-5% of the total radioactivity in the lane) was subtracted from each cleavage lane before calculation of the amount of DNA cleaved. The amount of DNA cleaved is defined as the amount of radioactivity corresponding to cleavage products divided by the total radioactivity in the lane.

DNA Cleavage by Dideoxydynemicin (2) with GSH and NADPH at Varying Concentrations of DNA. A solution containing dideoxydynemicin (0.5 mM), double-stranded calf thymus DNA (10 mM bp), and ~2 x 10^7 cpm 3'-^{32}P-labeled restriction fragment in 4:1 v/v water:DMSO (130 μL) was prepared as follows. A 130-μL aliquot of an aqueous solution of double-stranded calf thymus DNA (10 mM bp) was mixed with ~2 x 10^7 cpm labeled fragment, and the resulting solution was concentrated to a volume of 105 μL. A 25-μL aliquot of a solution of dideoxydynemicin (2.6 mM) in DMSO was then added to afford a final volume of 130 μL. A 25-μL aliquot of this solution was diluted to a volume of 125 μL by the addition of water (80 μL) and DMSO (20 μL), affording a second, more dilute solution containing dideoxydynemicin (0.1 mM) and calf thymus DNA (2 mM bp). A 25-μL aliquot of this second solution was diluted to a volume of 125 μL by the addition of water (80 μL) and DMSO (20 μL), affording a third solution containing dideoxydynemicin (0.02 mM) and calf thymus DNA (0.4 mM bp). Three parallel reactions of dideoxydynemicin with GSH (2 mM) at a constant ratio of drug:calf

thymus DNA:^{32}P-labeled DNA were performed at 37 °C in 1.5-mL Eppendorf tubes on a total reaction volume of 100 μL (9:1 water:DMSO) by combining 50 μL of each respective solution of drug/DNA with aqueous tris-HCl buffer solution (30 μL, 100 mM, pH 7.5) and aqueous sodium chloride solution (10 μL, 500 mM). The resulting solutions were pre-incubated at 37 °C for 30 min, and the reactions were initiated at 37 °C by the addition of an aqueous solution of glutathione (10 μL, 20 mM, pH 7.5, 37 °C). The concentrations of dideoxydynemicin and calf thymus DNA, respectively, at the onset of these reactions were, in order of decreasing concentrations, as follows: (1) dideoxydynemicin, 0.25 mM; DNA, 5.0 mM bp; (2) dideoxydynemicin, 0.05 mM; DNA, 1.0 mM bp; (3) dideoxydynemicin, 0.01 mM; DNA, 0.2 mM; each reaction solution also contained tris-HCl buffer (30 mM, pH 7.5), sodium chloride (50 mM), and glutathione (2 mM). At reaction times of 2, 4, 6, 8, and 10 h, an 18-μL aliquot from each reaction solution was transferred to a 1.5-mL Eppendorf tube containing aqueous ammonium acetate buffer solution (50 μL, 2 M, pH 5.5), and immediately frozen in liquid nitrogen. Reactions employing NADPH were conducted in an identical manner, employing an aqueous solution of NADPH (20 mM) in lieu of GSH. Following completion of the reactions, the cleavage products were precipitated and analyzed as described above. A control solution was prepared on a volume of 20 μL by combining a 10-μL aliquot of the solution of dideoxydynemicin (0.02 mM) and DNA (0.4 mM) with tris-HCl aqueous buffer solution (6 μL, 100 mM, pH 7.5), aqueous sodium chloride solution (2 μL, 500 mM), and water (2 μL), thus producing the following concentrations of reaction components: dideoxydynemicin, 0.01 mM; calf thymus DNA, 0.2 mM bp; tris-HCl buffer, 30 mM; sodium chloride, 50 mM. The control solution was incubated in parallel with the cleavage reactions at 37 °C for 10 h. The amount of background in the control lane (typically 3-5% of the total radioactivity

in the lane) was subtracted from each cleavage lane before calculation of the amount of DNA cleaved.

DNA Cleavage by Dynemicin Methyl Ester (3) with GSH and NADPH at Varying Concentrations of DNA. A solution containing dynemicin analog **3** (5 µM), double-stranded calf thymus DNA (50 µM bp), and ~2 x 10^7 cpm 3'-^{32}P-labeled restriction fragment in water (130 µL) was prepared as follows. A 10-µL aliquot of an aqueous solution of double-stranded calf thymus DNA (10 mM bp) was mixed with a 4-µL aliquot of a solution of **3** (2.6 mM) in DMSO, and the resulting solution was diluted to a total volume of 100 µL by the addition of water (86 µL), affording a solution containing calf thymus DNA (1 mM) and **3** (0.1 mM). A 6.5-µL aliquot of this solution was mixed with ~2 x 10^7 cpm 3'-^{32}P-labeled restriction fragment and water (123.5 µL) to afford the desired solution containing calf thymus DNA (50 µM bp) and **3** (5 µM) in water (130 µL). A 25-µL aliquot of this solution was diluted to a volume of 125 µL by the addition of water (100 µL), affording a second, more dilute solution containing **3** (1 µM) and calf thymus DNA (10 µM bp). A 25-µL aliquot of this second solution was diluted to a volume of 125 µL by the addition of water (100 µL), affording a third solution containing **3** (0.2 µM) and calf thymus DNA (2 µM bp). Three parallel reactions of **3** with GSH (50 mM) at a constant ratio of drug:calf thymus DNA:^{32}P-labeled DNA were performed at 37 °C in 1.5-mL Eppendorf tubes on a total reaction volume of 100 µL by combining 50 µL of each respective solution of drug/DNA with aqueous tris-HCl buffer solution (30 µL, 100 mM, pH 7.5) and aqueous sodium chloride solution (10 µL, 500 mM). The resulting solutions were pre-incubated at 37 °C for 30 min, and the reactions were initiated at 37 °C by the addition of an aqueous solution of glutathione (10 µL, 500 mM, pH 7.5, 37 °C). The

concentrations of **3** and calf thymus DNA, respectively, at the onset of these reactions were, in order of decreasing concentrations, as follows: (1) **3**, 2.5 μM; DNA, 25 μM bp; (2) **3**, 0.5 μM; DNA, 5 μM bp; (3) **3**, 0.1 μM; DNA, 1 μM bp; each reaction solution also contained tris-HCl buffer (30 mM, pH 7.5), sodium chloride (50 mM), and glutathione (50 mM). At reaction times of 2, 4, 6, 8, and 10 h, an 18-μL aliquot from each reaction solution was transferred to a 1.5-mL Eppendorf tube containing a solution of calf thymus DNA (50 μL, 0.1 mM bp, to assist precipitation) in aqueous ammonium acetate buffer (2 M, pH 5.5), and immediately frozen in liquid nitrogen. Reactions employing NADPH were conducted in an identical manner, employing an aqueous solution of NADPH (500 mM) in lieu of GSH. Following completion of the reactions, the cleavage products were precipitated and analyzed as described above. A control solution was prepared on a volume of 20 μL by combining a 10-μL aliquot of the solution of **3** (0.2 μM) and DNA (2 μM bp) with tris-HCl aqueous buffer solution (6 μL, 100 mM, pH 7.5), aqueous sodium chloride solution (2 μL, 500 mM), and water (2 μL), thus producing the following concentrations of reaction components: **3**, 0.1 μM; calf thymus DNA, 1 μM bp; tris-HCl buffer, 30 mM; sodium chloride, 50 mM. The control solution was incubated in parallel with the cleavage reactions at 37 °C for 10 h. The amount of background in the control lane (typically 3-5% of the total radioactivity in the lane) was subtracted from each cleavage lane before calculation of the amount of DNA cleaved.

DNA Cleavage by Dideoxdynemicin Methyl Ester (4) with GSH and NADPH at Varying Concentrations of DNA. A solution containing dynemicin analog **4** (0.5 mM), double-stranded calf thymus DNA (10 mM bp), and ~2 x 10^7 cpm 3'-^{32}P-labeled restriction fragment in 4:1 v/v water:DMSO (130 μL) was prepared as

follows. A 130-μL aliquot of an aqueous solution of double-stranded calf thymus DNA (10 mM bp) was mixed with ~2 x 10^7 cpm labeled fragment, and the resulting solution was concentrated to a volume of 105 μL. A 25-μL aliquot of a solution of **4** (2.6 mM) in DMSO was then added to afford a final volume of 130 μL. A 25-μL aliquot of this solution was diluted to a volume of 125 μL by the addition of water (80 μL) and DMSO (20 μL), affording a second, more dilute solution containing **4** (0.1 mM) and calf thymus DNA (2 mM bp). A 25-μL aliquot of this second solution was diluted to a volume of 125 μL by the addition of water (80 μL) and DMSO (20 μL), affording a third solution containing **4** (0.02 mM) and calf thymus DNA (0.4 mM bp). Three parallel reactions of **4** with GSH (20 mM) at a constant ratio of drug:calf thymus DNA:^{32}P-labeled DNA were performed at 37 °C in 1.5-mL Eppendorf tubes on a total reaction volume of 100 μL (9:1 water:DMSO) by combining 50 μL of each respective solution of drug/DNA with aqueous tris-HCl buffer solution (30 μL, 100 mM, pH 7.5) and aqueous sodium chloride solution (10 μL, 500 mM). The resulting solutions were pre-incubated at 37 °C for 30 min, and the reactions were initiated at 37 °C by the addition of an aqueous solution of glutathione (10 μL, 200 mM, pH 7.5, 37 °C). The concentrations of **4** and calf thymus DNA, respectively, at the onset of these reactions were, in order of decreasing concentrations, as follows: (1) **4**, 0.25 mM; DNA, 5.0 mM bp; (2) **4**, 0.05 mM; DNA, 1.0 mM bp; (3) **4**, 0.01 mM; DNA, 0.2 mM; each reaction solution also contained tris-HCl buffer (30 mM, pH 7.5), sodium chloride (50 mM), and glutathione (10 mM). At reaction times of 2, 4, 6, 8, and 10 h, an 18-μL aliquot from each reaction solution was transferred to a 1.5-mL Eppendorf tube containing aqueous ammonium acetate buffer solution (50 μL, 2 M, pH 5.5), and immediately frozen in liquid nitrogen. Reactions employing NADPH were conducted in an identical manner, employing an aqueous solution of NADPH (200 mM) in lieu of

GSH. Following completion of the reactions, the cleavage products were precipitated and analyzed as described above. A control solution was prepared on a volume of 20 μL by combining a 10-μL aliquot of the solution of **4** (0.02 mM) and DNA (0.4 mM) with tris-HCl aqueous buffer solution (6 μL, 100 mM, pH 7.5), aqueous sodium chloride solution (2 μL, 500 mM), and water (2 μL), thus producing the following concentrations of reaction components: **4**, 0.01 mM; calf thymus DNA, 0.2 mM bp; tris-HCl buffer, 30 mM; sodium chloride, 50 mM. The control solution was incubated in parallel with the cleavage reactions at 37 °C for 10 h. The amount of background in the control lane (typically 3-5% of the total radioactivity in the lane) was subtracted from each cleavage lane before calculation of the amount of DNA cleaved.

Reaction of Dynemicin A (1) with GSH and NADPH in the Presence of DNA, HPLC Analysis. Reactions were performed at 37 °C in 1.5-mL Eppendorf tubes containing a total reaction volume of 250 μL. In a typical reaction, a 30-μL aliquot of a solution of **1** (1.0 mM) in DMSO was combined with a solution of double-stranded calf thymus DNA (30 μL, 10 mM bp) in water, tris-HCl aqueous buffer solution (90 μL, 100 mM, pH 7.5), aqueous sodium chloride solution (30 μL, 500 mM), aqueous 1,2-bis(hydroxymethyl)-1,4-cyclohexadiene (30 μL, 500 mM, a hydrogen atom source), aqueous 1-bromo-2-naphthoic acid (30 μL, 0.2 mM, internal HPLC standard), and water (30 μL), affording a volume of 270 μL. A 45-μL aliquot of the resulting solution was removed and an initial ratio of **1** to the internal standard was established by HPLC analysis (40-μL injection volume) employing a Beckman HPLC system equipped with a Beckman Ultrasphere ODS (C_{18}, 5 μm) rp-HPLC column, 4.6 x 250 mm, flow = 0.40 mL/min with a linear gradient of acetonitrile:aqueous ammonium acetate buffer solution (10 mM, pH 6.0): 10:90 v/v

acetonitrile:aqueous ammonium acetate buffer to 100% acetonitrile over a period of 50 min. Peaks were detected by ultraviolet absorption at 240 nm with a Beckman 168 Programmable Photodiode Detector (1, retention time $t_R \sim$ 36 min; 7, retention time $t_R \sim$ 30 min; internal standard, retention time $t_R \sim$ 22 min). The reaction solution was pre-incubated for 30 min at 37 °C, and the reaction was initiated at 37 °C by the addition of an aqueous solution of GSH (25 μL, 100 mM, pH 7.5, 37 °C), thus producing the following concentrations of solution components at the onset of the reaction: 1, 0.1 mM; GSH, 10 mM; calf thymus DNA, 1.0 mM bp; tris-HCl buffer, 30 mM; sodium chloride, 50 mM; 1,2-bis(hydroxymethyl)-1,4-cyclohexadiene, 50 mM. At times of 20, 40, 60, and 80 min, an aliquot (50 μL) was quenched by transfer to an Eppendorf tube containing aqueous ammonium acetate buffer solution (10 μL, 2 M, pH 5.5) followed by rapid freezing by immersion of the tube in liquid nitrogen. Aliquots were analyzed by HPLC as described above. The reaction with NADPH was conducted in an identical manner, employing an aqueous solution of NADPH (25 μL, 100 mM, 37 °C) in lieu of GSH.

Reaction of Dideoxydynemicin A (2) with GSH and NADPH in the Presence of DNA, HPLC Analysis. Reactions were performed at 37 °C in 1.5-mL Eppendorf tubes containing a total reaction volume of 250 μL. In a typical reaction, a 30-μL aliquot of a solution of 2 (1.0 mM) in DMSO was combined with a solution of double-stranded calf thymus DNA (30 μL, 10 mM bp) in water, tris-HCl aqueous buffer solution (90 μL, 100 mM, pH 7.5), aqueous sodium chloride solution (30 μL, 500 mM), aqueous 1,2-bis(hydroxymethyl)-1,4-cyclohexadiene (30 μL, 500 mM, a hydrogen atom source), aqueous 1-bromo-2-naphthoic acid (30 μL, 0.2 mM, internal HPLC standard), and water (30 μL), affording a volume of 270 μL. A 45-μL

aliquot of the resulting solution was removed and an initial ratio of **2** to the internal standard was established by HPLC analysis (40-μL injection volume) employing a Beckman HPLC system equipped with a Beckman Ultrasphere ODS (C_{18}, 5 μm) rp-HPLC column, 4.6 x 250 mm, flow = 0.40 mL/min with a linear gradient of acetonitrile:aqueous ammonium acetate buffer solution (10 mM, pH 6.0): 10:90 v/v acetonitrile:aqueous ammonium acetate buffer to 100% acetonitrile over a period of 50 min. Peaks were detected by ultraviolet absorption at 240 nm with a Beckman 168 Programmable Photodiode Detector (**2**, retention time t_R ~ 34 min; **8**, retention time t_R ~ 29 min; internal standard, retention time t_R ~ 22 min). The reaction solution was pre-incubated for 30 min at 37 °C, and the reaction was initiated at 37 °C by the addition of an aqueous solution of GSH (25 μL, 20 mM, pH 7.5, 37 °C), thus producing the following concentrations of solution components at the onset of the reaction: **2**, 0.1 mM; GSH, 2.0 mM; calf thymus DNA, 1.0 mM bp; tris-HCl buffer, 30 mM; sodium chloride, 50 mM; 1,2-bis(hydroxymethyl)-1,4-cyclohexadiene, 50 mM. At times of 2, 4, 6, and 8 min, an aliquot (50 μL) was quenched by transfer to an Eppendorf tube containing aqueous ammonium acetate buffer solution (10 μL, 2 M, pH 5.5) followed by rapid freezing by immersion of the tube in liquid nitrogen. Aliquots were analyzed by HPLC as described above. The reaction with NADPH was conducted in an identical manner, employing an aqueous solution of NADPH (25 μL, 20 mM, 37 °C) in lieu of GSH.

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(16) Under forcing conditions, we were able to observe DNA cleavage by dynemicin methyl ester (**3**). These conditions (see experimental) involved the use of dilute solutions of DNA ($1 - 25 \times 10^{-6}$ M), an increased raio of drug to base pairs DNA (1:10), and an increased concentration of reductant (5.0×10^{-2} M).

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Chapter 4

The Enediyne Antibiotics:
A Structurally Diverse Class
with Common Mechanistic Behavior

Introduction

The enediyne antibiotics are characterized by the presence of a strained enediyne ring system and the requirement for a thiol activating cofactor to initiate the transformation of the enediyne unit to a carbon-centered biradical species (Scheme 1). A clear understanding of the chemistry of the enediyne antibiotics is necessary if we are to rationalize their remarkable antitumor activity. Does the chemistry of these drugs vary as much as their structure, or are there consistent observations to be made for the class as a whole? The chemical reactivity and mechanistic behavior of three enediyne antibiotics have been examined in this thesis, each employing a different experimental approach. Experiments with neocarzinostatin chromophore have relied on the trapping and subsequent utilization of a postulated intermediate. The complexity of calicheamicin γ_1 has been revealed by a thorough product and kinetic characterization. Quantitation of kinetic and thermodynamic parameters of synthetic dynemicin analogs has led to conclusions for dynemicin A. Despite the different experimental approaches and the structural diversity of these compounds, several consistencies in their mechanistic behavior have been observed.

Scheme 1

Neocarzinostatin
Chromophore (1) → 1b → 1c

Calicheamicin γ_1 (2) → 2b → 2c

Dynemicin A (3) → 3b → 3c
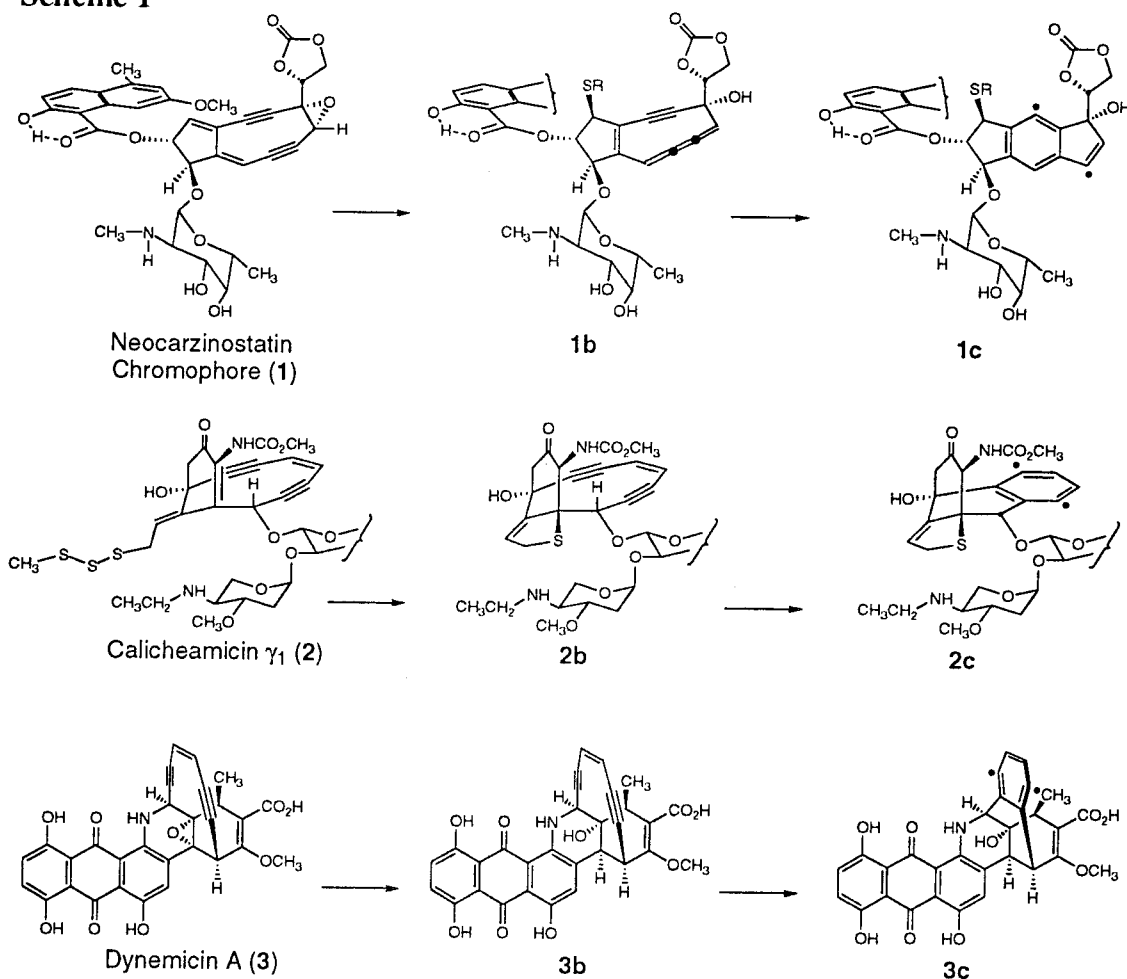
Relevance of Experiments in Organic Solvents

All of the enediyne antibiotics require activation by a thiol cofactor to initiate their chemical cascade (Scheme 1). Prior to our studies, much of the chemistry of these compounds had been studied in organic solvents. Products from drug activation had been isolated and mechanisms involving biradical intermediates proposed on the basis of the product structure. Organic solvents have proven to be useful for elucidating the sequence leading to biradical formation due to the increased solubility and stability of the drugs in such media. Neocarzinostatin chromophore decomposes within minutes in

aqueous solution, and calicheamicin γ_1 has essentially no water solubility in the absence of DNA. Furthermore, solvents such as methanol or tetrahydrofuran serve as hydrogen atom donors to efficiently trap radical intermediates that may form during the course of a reaction; water is an inferior trapping agent for these radical species. Experiments in organic solvents have also allowed for the observation of intermediates preceding biradical formation at low temperature, such as the neocarzinostatin cumulene (**1b**).[1] A major goal of our studies was to evaluate the relevance of observations in organic solvents to the chemistry occurring in aqueous solution in the presence of DNA.

For the three drugs indicated in Scheme 1, the product observed from activation in organic solvents is the same as the product observed in aqueous solution in the presence of DNA. Thus, using experiments conducted in organic solvents to formulate a mechanism for activation and biradical formation serves as a useful first step in understanding the enediyne chemistry with DNA. However, several of our results suggest that little additional mechanistic information can be extracted from such experiments. In all three studies, it has been shown that the kinetics of drug activation and biradical formation (biradical formation parallels DNA cleavage) in organic solvents are vastly different from the corresponding kinetics occurring in aqueous solution with DNA. This difference is manifested primarily as a change in the rate-determining step for the net transformation of drug to product. In THF/AcOH at –70 °C, methyl thioglycolate readily adds to neocarzinostatin chromophore (**1**) to form the cumulene (**1b**). The cumulene is stable indefinitely at this temperature, indicating that the rate-determining step in product formation is the unimolecular rearrangement to the biradical (**1c**). In contrast to these observations, it is the thiol activation that is the rate-determining step in aqueous solution with DNA.

Similar observations have been made for calicheamicin γ_1; thiol activation is rate-determining in aqueous solution, but not in methanol.[2] The product distribution from S–S bond interchange differs greatly as well. The reaction sequence of Scheme 1, which is the most direct pathway to DNA cleavage and is typically presented in the literature, is actually a minor pathway in the presence of DNA. We have shown that the major product from reaction of calicheamicin γ_1 with GSH is the calicheamicin-glutathione disulfide, a compound 2 – 3 orders of magnitude less reactive than calicheamicin γ_1.

Formulation of a mechanism from activation and cycloaromatization of the drug in an organic solvent is simplified due to the absence of DNA. Not surprisingly, the absence of DNA further limits correlation of the kinetics observed in organic media to the kinetics observed in aqueous solution with DNA. This was vividly demonstrated in the studies with dynemicin A and its methyl ester. In methanol, both compounds react within minutes with GSH to form the expected Bergman-cyclized products (see 3c). In fact, dynemicin A methyl ester is even more reactive than dynemicin A under these conditions. In the presence of DNA, however, dynemicin methyl ester was shown to be virtually inert relative to dynemicin A.

With each enediyne antibiotic studied, it has been demonstrated that the biradical species generated in organic media is the same as that generated in aqueous solution in the presence of DNA. It is the kinetics of formation of this biradical species, which presumably mediates DNA cleavage, that lacks correlation between the two experimental conditions. These differences can, in part, be attributed to the choice of thiol cofactor used in the respective experiments, as discussed below.

Relevance of Experiments with Non-Biological Thiols

Thiols that have been employed in the enediyne literature (Table 1) vary in their acidity (pKa of the sulfhydryl group), solubility properties, and net charge at physiological pH. As with organic solvents, non-biological thiols have their distinct advantages under certain conditions. Because biological thiols cysteine and glutathione (GSH) are insoluble in organic solvents, the non-biological thiols of Table 1 are useful for conducting initial experiments in organic solvents to isolate the Bergman-cyclized product or for observing an intermediate at low temperature. Our experimental data support the conclusion that the net transformation of drug to biradical to product is the same regardless of the thiol chosen.

Table 1. Thiols used in enediyne chemistry.

Thiol	pKa	Net charge[a]	Ref.
2-aminoethanethiol	8.3	+1	3a
dithiothreitol	8.3	0	3a
methyl thioglycolate	7.8	0	3b
cysteine	8.6	0	3c
glutathione	8.6	−1	3c

[a]Net charge of the thiol in aqueous solution at physiological pH.

Upon review of the literature, one finds that nearly all experiments, whether conducted in organic or aqueous media, are conducted with non-biological thiols instead of GSH. Despite the fact the thiols of Table 1 promote the same net transformation of drug to product, we have observed significant differences in the rate at which this transformation occurs. There are two variables among the thiols that may

account for the observed differences: the pKa of the thiol function and the net charge of the thiol at physiological pH. Since the range of thiol acidity is only 0.8 pKa units, the variation in the ratio of thiol to thiolate is less than a factor of ten, insufficient to account for our contrasting results observed with GSH. The more significant variable, and the one to which we have attributed the differing kinetic behavior, is the net charge. Only GSH bears a net negative charge at physiological pH, an important consideration when it is realized that the drugs have significant binding affinity for DNA, a polyanion.

The effect of net charge of the thiol on the reactivity in the presence of DNA is illustrated with calicheamicin γ_1. Published experiments serving as the foundation for our work employed aminoethanethiol, which bears a net positive charge at pH 7.5.[4] Aminoethanethiol is anticipated to have affinity for the DNA polyanion, increasing the rate of formation of the calicheamicin-aminoethanethiol disulfide relative to formation of the calicheamicin-glutathione disulfide, as was observed. A larger difference in rates was observed for subsequent activation of the respective calicheamicin-thiol disulfides. Activation of the calicheamicin-aminoethanethiol disulfide is likely facilitated proximal to the DNA helix relative to the process occurring free in solution. Conversely, it was demonstrated that the calicheamicin-glutathione disulfide must dissociate from DNA in order to under go activation by another molecule of GSH. This behavior is attributed to the negative charge repulsion proximal to the DNA helix. The use of aminoethanethiol resulted in different molecular dynamics and a rate increase of 2 – 3 orders of magnitude relative to GSH.

Dynamics of Drug Activation and Biradical Formation

The molecular dynamics of the DNA cleavage process addresses several fundamental issues: (1) Does drug activation occur as a termolecular process involving drug, DNA, and thiol, or is activation a bimolecular process occurring free in solution?

(2) Is the sequence specificity of DNA cleavage determined by the parent drug or by a later intermediate, and is this process thermodynamic or kinetic in nature? (3) Does DNA catalyze the activation process? Understanding the molecular dynamics of the enediyne antibiotics requires consideration of three species: the parent drug, the activated intermediate preceding biradical formation, and the biradical itself. The rates of equilibration of these species among DNA binding sites, the rate of unimolecular rearrangement of the activated intermediate to the biradical, and the rate of activation free in solution relative to activation bound to DNA must also be considered.

We have observed wide variation in the dynamics of activation, from a termolecular process involving DNA-bound neocarzinostatin chromophore to the bimolecular process with dissociated dynemicin A. The most direct means for assessing the nature of this process is to measure the rate of activation in the presence and absence of DNA, as was done for neocarzinostatin chromophore. Although activation of free chromophore is faster than activation of bound chromophore by approximately one order of magnitude, this modest rate difference is offset by the larger proportion of bound drug relative to free drug, a factor of about 10^5. Only in the case of neocarzinostatin chromophore was it possible to measure directly the rate of activation for the free drug. Calicheamicin γ_1 and dynemicin A do not have sufficient water solubility in the absence of DNA for kinetic analysis.

To circumvent the lack of solubility of calicheamicin γ_1 and dynemicin A in the absence of DNA, we have relied on an experimental technique developed in the course of our studies that has been invaluable in elucidating the dynamics of these drugs. The technique entails monitoring the rate of reaction as a function of the concentration of DNA. As the concentration of DNA is increased, the proportion of bound drug remains relatively constant compared to the significant decrease in the proportion of free drug. If activation occurs through the bound form, then the rate of reaction will remain

constant as a function of the concentration of DNA. If, however, free drug reacts significantly faster than bound drug, then the rate of reaction will display an inverse dependence on the concentration of DNA. This technique does not require reactions performed in the absence of DNA or a knowledge of absolute rate constants.

The utility of this technique was first demonstrated by comparing the behavior of calicheamicin γ_1 to the calicheamicin-glutathione disulfide. Calicheamicin γ_1 is activated by GSH in the bound form, the calicheamicin-glutathione disulfide free in solution. Although the behavior of the calicheamicin-glutathione disulfide was surprising and unexpected, later work with dynemicin A supported the idea that activation free in solution is clearly a viable pathway among the enediyne class. Without performing any experiments in the absence of DNA, this technique has led to the conclusion that activation of dynemicin A free in solution must be at least two orders of magnitude faster than activation of the intercalated drug. The attenuated reactivity of the bound forms of these drugs is likely a result of negative charge repulsion between the DNA polyanion and GSH.

The observation that activation may proceed free in solution implies that the sequence specificity of the parent drug is lost in the DNA cleavage process; the site of DNA cleavage is determined by a later intermediate. A recurring proposal in this thesis is that the intermediate preceding biradical formation has sufficient lifetime to equilibrate among DNA binding sites and is the species that determines the sequence specificity of DNA cleavage. The ability of the neocarzinostatin cumulene to equilibrate among DNA binding sites prior to biradical formation was demonstrated unequivocally by generating the cumulene independently in a NMR tube and then adding the cumulene to DNA. The DNA cleavage from externally generated cumulene was identical to that from incubation of chromophore and thiol in terms of specificity and efficiency. This rather

elaborate experiment effectively affords the same experimental evidence as demonstrating that the drug is activated free in solution, as was done for the calicheamicin-glutathione disulfide and dynemicin A. Each of the three activated intermediates encountered in our studies (**1b, 2b,** and **3b,** Scheme 1) has been shown to have sufficient lifetime to equilibrate among DNA binding sites. The results are consistent with known kinetic rates of equilibration among DNA binding sites of these drugs and their unimolecular rearrangement to the biradical. Binding and de-binding generally occurs on the time scale of milliseconds, whereas biradical formation occurs on the time scale of seconds.

To establish that the intermediate preceding biradical formation determines the sequence specificity of DNA cleavage, it is necessary to demonstrate that the biradical does not have sufficient lifetime (milliseconds) to equilibrate among DNA binding sites. In support of this, it was shown that molar concentrations of a water-soluble cyclohexadiene trap (bond dissociation energy 73 kcal/mol)[5] is as effective as millimolar DNA (deoxyribose bond dissociation energies estimated at 85–95 kcal/mol) at quenching the biradical from neocarzinostatin chromophore. The interpretation of this result is that the biradical must be generated as a DNA-bound species to induce DNA cleavage. This interpretation is further supported by reactivity studies of aryl radicals, where bimolecular rate constants with hydrogen atom donors range from 10^5 to 10^7 $M^{-1}s^{-1}$,[6] corresponding to a lifetime in the microsecond range at most, much less than the milliseconds required for equilibration. A slow rate of biradical formation relative to the rate of equilibration is essential to enediynes such as dynemicin A that are activated free in solution. If biradical formation was so rapid as to occur prior to DNA binding, then dynemicin A might find other self-destructive pathways free in solution; in other words, dynemicin A would conceivably not cleave DNA at all.

The ability of the activated intermediates to equilibrate among DNA binding sites diminishes the importance of DNA catalysis in enediyne activation. The sequence specificity of DNA cleavage is determined through thermodynamic selection rather than kinetic. Even if activation proceeding through the bound form were kinetically favored (catalyzed) on the basis of DNA sequence, this kinetic effect would be lost once the activated intermediate dissociated from DNA. For the drugs that are activated free in solution, the possibility of DNA catalysis is not an issue; DNA serves to inhibit these reactions.

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