The Role of the Aminosugar and Helix Binding in the Thiol-Induced Activation of Calicheamicin for DNA Cleavage

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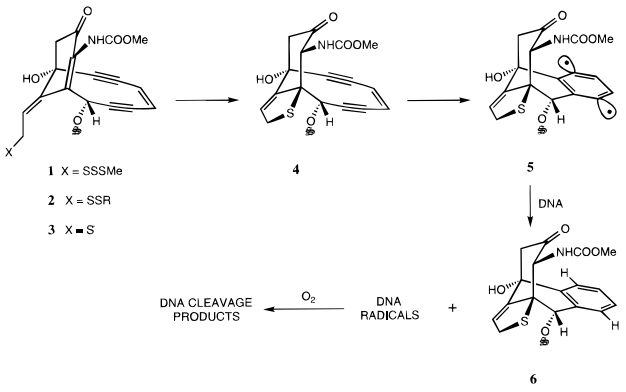
Abstract: The diynene antitumor antibiotic calicheamicin γ_1^I (CLMγ_1^I) cleaves DNA in the presence of thiols and molecular oxygen. The proposal that interaction of CLMγ_1^I with DNA enhances the rate of this cleavage process has been addressed. The kinetics of CLM activation for DNA cutting by aminoethanethiol and glutathione (GSH) have been investigated for the drug free in solution and primarily bound to DNA. The second-order rate constants for the disappearance of the trisulfide CLMγ_1^I and for the slower reaction of the principal disulfide intermediate in the activation process have been determined and reveal that both of these reactions are *slower* in the presence of DNA. In earlier solution studies the second-order reaction rate of CLMγ_1^I was compared to the rates measured for *N*-acetylCLM and CLMα_3, derivatives lacking an internal free amine. Little difference was observed among these rates, a finding inconsistent with the contention that the ethylamino sugar serves as a general base in the activation process. The absence of intramolecular amine participation in thiol activation concluded from these rate comparisons has been reinforced by reactions of these CLM derivatives in the presence of DNA. Again no comparative rate advantage was seen for CLMγ_1^I. The validity of the continuous UV assay used in these experiments to monitor the reaction of the intermediate GSH-CLM disulfide was confirmed by direct kinetic measurements of the mixed disulfide itself and by independent PAGE cleavage assays. Recent claims that the calicheamicins are not soluble under the conditions used in these experiments are refuted by four independent experimental means including light scattering, UV spectral comparisons, centrifugation experiments and adherence to Beer's Law. The present studies permit a much simpler picture to be drawn of the reductive activation process and the roles played by the aminosugar and DNA interaction than previously proposed.

The extreme potency of calicheamicin (CLM) and other diynene antitumor antibiotics in causing DNA damage[1] has animated the belief that interaction with the helix enhances the rate of drug activation and, consequently, that destruction of the duplex by CLM is favored compared to fruitless reaction in solution. This conviction implies that, beyond the intrinsic affinity of the drug for DNA, there exist effects that amplify the overall rate of CLM activation, hence strand scission, when the drug is bound to DNA as opposed to free in solution. There are two stages in the activation cascade where such a kinetic acceleration could be visualized to occur.

The first of these is the overall conversion of the methyltrisulfide of CLMγ_1^I (**1**) to the allylic thiolate **3**. In the presence of thiols this process is known to be quite complex from variable temperature NMR experiments[2] and recent time-course studies at low drug concentrations.[3] Nonetheless, it is these early steps that constitute the overall rate-determining activation of the drug as will become clear in this discussion. Intramolecular β-addition of the thiolate **3** to the enone system on the other hand, despite down-regulation of its electrophilicity by the carbamate,[4] is still rapid at −78 °C[2] and not rate limiting in the overall drug activation process. The swiftness of this intramolecular addition owes presumably to the entropic advantage conferred by the allylic double bond configuration and the kinetic favorability of five-membered ring formation.

Scheme 1



Electrocyclization[5] of **4** to the 1,4-diyl **5**, the signal transformation of this class of natural products, is the second step at which kinetic effects of DNA binding could play a role in the appearance of site-selective DNA cleavage by the drug. Variable temperature NMR experiments allowed the half-life of the dihydrothiophene **4** to be estimated (4.5 ± 1.5 s at 37 °C) in methanol.[2] This relatively slow rate of decomposition must be contrasted to the abstraction of alkane or ether C−H bonds by phenyl radicals, a process known to be exceedingly rapid.[6] Hence, homolytic removal of carbon-bound hydrogen atoms from DNA by CLM[7,8] to initiate strand scission is doubtless a comparably fast process. Therefore, on the basis of the

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(1) *Enediyne Antibiotics as Antibiotic Agents*; Borders, D. B., Doyle, T. W., Eds.; Marcel Dekker, Inc.: New York, 1995.

(2) De Voss, J. J.; Hangeland, J. J.; Townsend, C. A. *J. Am. Chem. Soc.* **1990**, *112,* 4554−4556.

(3) Myers, A. G.; Cohen, S. B.; Kwon, B. M. *J. Am. Chem. Soc.* **1994**, *116,* 1255−1271.

(4) Haseltine, J. N.; Cabal, M. P.; Mantlo, N. B.; Iwasawa, N.; Yamashita, D. S.; Coleman, R. S.; Danishefsky, S. J.; Schulte, G. K. *J. Am. Chem. Soc.* **1991**, *113,* 3850−3866.

(5) Bergman, R. G. *Acc. Chem. Res.* **1973**, *6,* 697−699. Mayer, J.; Sondheimer, F. *J. Am. Chem. Soc.* **1966**, *88,* 602−603. Darby, N.; Kim, C. U.; Salaun, J. A.; Shelton, K. W.; Takada, S.; Masamune, S. *J. Chem. Soc., Chem. Commun.* **1971**, 1516−1517.

(6) Scaiano, J. C.; Stewart, L. C. *J. Am. Chem. Soc.* **1983**, *105,* 3609−3614. Janzen, E. G.; Nutter, Jr., D. E.; Evans, C. A. *J. Phys. Chem.* **1975**, *79,* 1983−1984.

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relatively long half-life of **4**, it was first suggested that this species could be responsible for identifying favored binding/cleavage sites.[2] Comparisons of the CLM cleavage pattern of a restriction fragment of plasmid DNA by drug activated *in situ* by a variety of reductants and by independently prepared dihydrothiophene **4** have shown superimposable patterns of strand scission.[9,10] This observation is in complete accord with the view that **4** is the common intermediate in all of these reactions, and its interaction with DNA is the pivotal event in the overall activation/cleavage process where *site selection* in DNA scission is determined.

The intriguing corollary proposal[2] that certain sequences in DNA can enhance the *rate* of Bergman rearrangement and, therefore, kinetically favor cleavage at these sites in preference to others, however, has been shown not to hold. In careful competition experiments the measured rates at a series of cleavage sites ranging from favorable to unfavorable were found to be *identical* within experimental error.[9,10] Therefore, the evident sequence selectivity of DNA cutting by CLM devolves to the distribution of the dihydrothiophene **4** along the helix governed largely, if not entirely, by thermodynamic binding effects. The comparatively long lifetime of this reactive intermediate allows equilibrium binding to be achieved before substantial Bergman rearrangement and diradical formation occurs to cause strand scission. With respect to the overall activation of the drug for DNA cleavage, it may be concluded that interaction of dihydrothiophene **4** with the helix provides no kinetic component to this rate-limiting step in DNA cleavage.[9,10]

While the rate of electrocyclization of **3** to **4** is unaffected by differences in DNA sequence, the early rate-determining reaction of CLMγ_1^I (**1**) with thiols, presumably glutathione *in vivo*, remains an unresolved issue that is addressed in this paper.[3,11] Mixed disulfides as **2** can be synthesized in good yield[12] and their further reaction with thiol constitutes the critical rate-determining step in the major drug activation process. The fundamental question of a kinetic effect upon DNA interaction for both the formation of this key intermediate and its critical reaction to give allylic thiolate **3** is examined. Similarly, the role of the aminosugar of CLM in this activation cascade is considered. The combined observations made in these experiments allow a comprehensive evaluation of these factors to be made on the complex rate-determining sulfur chemistry that initiates DNA cleavage by calicheamicin.

Role of the Aminosugar: Preliminary Studies of CLM in Solution

The first suggestion that the aminosugar of CLMγ_1^I (**1**) could play a special role in drug activation arose in the observation that **1** in acetonitrile reacted with added thiols, while other members of the calicheamicin series lacking the aminosugar did not.[12,13] However, in the presence of added triethylamine all were reactive, and the rate for **1** was greatly increased. An interesting interpretation could be placed on these findings that the ethylamino sugar of **1** served as a general base to assist thiol deprotonation and enhance the rate of thiolate attack on the methyltrisulfide and, hence, increase the rate of drug activation. Amine functions are observed among many, but not all, of the diynene antitumor antibiotics and could be visualized to provide a common means of rate acceleration.

As a preliminary test of this notion, the second-order rate of CLMγ_1^I (**1**) reaction with thiol(ate) was compared to those determined for *N*-acetylCLM (**7**) and CLMα_3 (**8**). Aminoethanethiol (AET) was chosen for these reactions owing to its similar pK_a (8.3)[14] to that of the predominant intracellular thiol glutathione (pK_a = 8.5)[15] and its easily manipulable crystalline form. While CLM is readily solubilized in the presence of DNA, addition of organic modifiers was found necessary to achieve solutions of the drug in aqueous buffer. At the drug concentrations to be used, 30% methanol/70% Tris buffer (30 mM Tris·HCl, pH 7.4, 50 mM NaCl; defined as "buffer/methanol") was sufficient. Finally, the complexity of thiolate addition to the allylic trisulfide was known from variable-temperature NMR studies,[2] so an assay was sought that would be sensitive to the disappearance of the diynene chromophore and the appearance of the disubstituted phenyl ring of CLMϵ (**6**). While not the wavelength of maximum absorbance change during this structural transformation, 315 nm was chosen to minimize the contribution of DNA absorption anticipated in later experiments and for the value of a sensitive, continuous assay.

Rates of reaction of **1**, **7**, and **8** were determined under pseudo first-order conditions ([AET] $\geq$ 100 $\times$ [CLM]) where the formation of the mixed disulfide **2** was very rapid, and its decay to CLMϵ (**6**) was monitored. Replotting the individual observed rates of reaction as a function of thiol concentration gave the second-order rate constants. Two important observations were made. First, the rate of reaction of **2** to **6** was sensitive to thiol concentration in keeping with the overall rate-determining nature of allylic thiolate **3** formation.[16] Second, there was little difference among the second-order rate constants determined for **1**, **7**, and **8**, an observation *inconsistent* with the ethylamino sugar serving as a general base. The prior observations in acetonitrile most probably owe to the inability of thiol deprotonation to take place in the absence of a base, but does so, albeit slowly, in the presence of substrate amine. In buffered aqueous conditions where the ionic state of added thiol is governed by its pK_a relative to the solution pH, this effect disappeared.[17]

The Role of the Aminosugar: Detailed Experiments in the Presence and Absence of DNA

While these studies were being extended to analogous kinetic measurements in the presence of DNA, a paper appeared in which thiol activation of the neocarzinostatin (NCS) chromophore was examined in acetic acid/THF (1:9) and compared to the corresponding compound whose carbohydrate amino group had been modified to the less basic nitrosamine. Reaction

(7) De Voss, J. J.; Townsend, C. A.; Ding, W.-D.; Morton, G. O.; Ellestad, G. A.; Zein, N.; Tabor, A. B.; Schreiber, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 9669−9670.
(8) Hangeland, J. J.; De Voss, J. J.; Heath, J. A.; Townsend, C. A.; Ding, W. D.; Ashcroft, J.; Ellestad, G. A. *J. Am. Chem. Soc.* **1992**, *114*, 9200−9202.
(9) Chatterjee, M.; Cramer, K. D.; Townsend, C. A. *J. Am. Chem. Soc.* **1994**, *116*, 8819−8820.
(10) Chatterjee, M.; Mah, S. C.; Tullius, T. D.; Townsend, C. A. *J. Am. Chem. Soc.* **1995**, *117*, 8074−8082.
(11) Chatterjee, M.; Cramer, K. D.; Townsend, C. A. *J. Am. Chem. Soc.* **1993**, *115*, 3374−3375.
(12) Ellestad, G. A.; Hamann, P. R.; Zein, N.; Morton, G. O.; Siegel, M. M.; Pastel, M.; Borders, D. B.; McGahren, W. J. *Tetrahedron Lett.* **1989**, *30*, 3033−3036.
(13) Zein, N.; Poncin, M.; Nilakantan, R.; Ellestad, G. A. *Science* **1989**, *244*, 697−699.
(14) Cramer, K. D.; Townsend, C. A. *Tetrahedron Lett.* **1991**, *32*, 4635−4638.
(15) Danehy, J. P.; Parameswaran, K. N. *J. Chem. Eng. Data* **1968**, *13*, 368−389.
(16) This finding is consistent with the measured rate of Bergman rearrangement of **4** to **5** deduced from low-temperature NMR experiments.[2]
(17) It is well-known that disulfide exchange occurs by nucleophilic attack of thiolate anion on the sulfur−sulfur bond in a conventional S_N2 fashion: Whitesides, G. M.; Houk, J.; Patterson, M. S. K. *J. Org. Chem.* **1983**, *48*, 112−115. Wilson, J. M.; Bayer, R. J.; Hupe, D. J. *J. Am. Chem. Soc.* **1977**, *99*, 7922−7926.

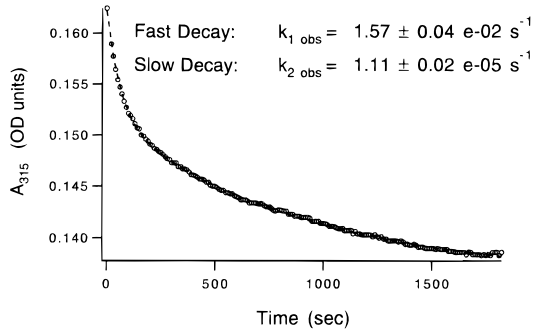

Figure 1. Plot of absorbance (315 nm) as a function of time for the reaction of 10 μM CLM with 2.5 mM AET in the presence of calf thymus DNA. Open circles are the experimental data, and the dashed line is the computer-generated fit using eq 1 corresponding to the rate constants shown.

of the latter with methyl thioglycolate was seen to be significantly slower.[18] This finding was taken to suggest an important catalytic role for the *N*-methylfucosamine residue in NCS activation (and by analogy the aminosugar in CLM) and, moreover, to support the view that in aqueous solution thiolate addition would be accelerated when the drug is bound to, or in the proximity of, DNA.

Unclear about the relevance of these experiments in acetic acid/THF to our previous findings in aqueous buffer/methanol and uncertain of the conclusions drawn from this work, we decided to examine more closely the roles, if any, played by the aminosugar of CLM and binding to DNA in the thiol-induced activation of the drug for DNA cleavage. The concentrations of both CLM and the reacting thiol were decreased relative to the preliminary experiments above to slow the overall activation process. In these early experiments mixed disulfide formation was effectively complete at the start of the kinetic measurements, and only the decomposition of **2** was observed. Aminoethanethiol (AET) was used as before, and glutathione (GSH) was selected to mimic the putative activation *in vivo* at concentrations more closely reflecting physiological conditions. The concentration of calf thymus DNA was adjusted to ensure that CLMγ_1^I (**1**) was >95% bound. Excess thiol was used to achieve pseudo first-order conversions whose progress was monitored by the continuous UV assay developed previously.[14] For reactions in 70% buffer/30% methanol in the presence or absence of calf thymus DNA two sequential pseudo first-order processes were observed. A representative experiment is shown in Figure 1. Computer fitting of these data (see dotted line in Figure 1) and those obtained at a series of thiol concentrations resolved the sulfur activation chemistry into the relatively fast decomposition of trisulfide **1** to mainly the mixed disulfide **2**[12] followed by its slower release of the allylic thiolate **3** (Figure 2). As a control, the relatively fast formation of the disulfide **2** from CLMγ_1^I (**1**) was followed by HPLC and confirmed this kinetic identification. Under the conditions of the reaction all steps subsequent to the formation of **3** are fast, and, therefore, the observation of CLMϵ (**6**) provides a kinetic measure of thiolate **3** formation. While initially unexpected, the decrease in absorption at 315 nm could be used not only to monitor the transformation of the diynene to the benzenoid chromophore present in CLMϵ (**6**) but also to detect the complex processes that carry trisulfide **1** principally to disulfide **2**.[2,3] It is known that as the number of sulfur atoms is reduced in a polysulfide, the λ_{max} and molar absorbtivity decrease as well.[19] For the present case that proportion of the absorption at 315 nm contributed by the trisulfide chromophore **1** shifts toward lower wavelength in the disulfide **2** to provide an accurate measure of this reaction process.[11,12] The UV spectra of **1** and **2** are compared in Figure 3 where the difference in molar absorbtivity at 315 nm is apparent.

(18) Myers, A. G.; Harrington, P. M.; Kwon, B. M. *J. Am. Chem. Soc.* **1992**, *114*, 1086−1087.

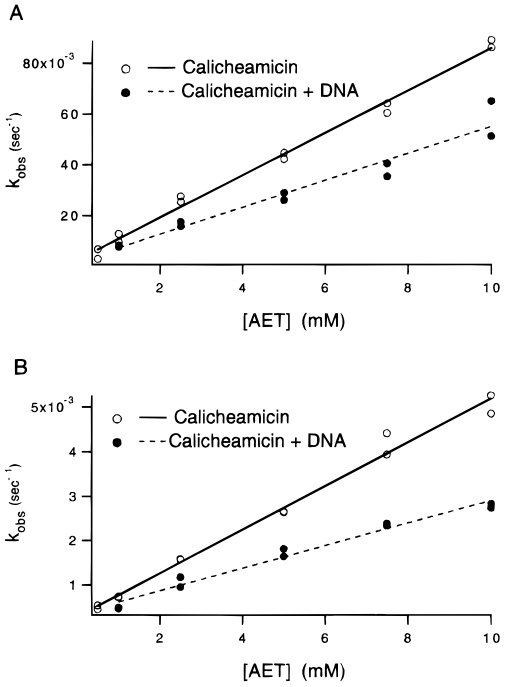

Figure 2. Pseudo first-order rate constants for reaction of **1** (10 μM) with AET as a function of thiol concentration in the presence (·) and absence (o) of calf thymus DNA. The rates for decomposition of the trisulfide (**A**) and for decomposition of the mixed disulfide (**B**) are shown. The slopes of these lines equal the second-order rate constants for the given reactions.

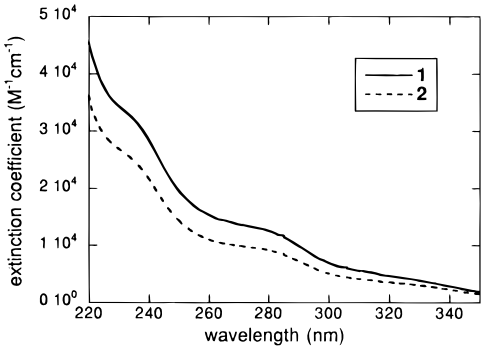

Figure 3. Plots of extinction coefficient as a function of wavelength for **1** (solid line) and **2** (dashed line) at 25.0 °C in 70/30 Tris buffer/methanol. A 24.1 μM solution of **1** and a 24.9 μM solution of **2** were used to obtain the data shown.

The kinetic experiments were repeated at somewhat higher concentrations of CLM with the less acidic thiol GSH to attain experimentally convenient rates. Replotting the observed pseudo first-order rate constants as a function of thiol concentration gave second-order rate constants for the reactions of CLMγ_1^I (**1**) with AET and GSH, each in the presence and absence of DNA. These data are presented in Table 1.[9] Rate comparisons for both the reaction of trisulfide **1** and the rate-determining decomposition of **2** show that thiol activation of CLMγ_1^I (**1**) is actually *slowed slightly* when bound to DNA rather than accelerated as had been suggested from experiments under nonaqueous conditions (see also Figure 2).[12,13,18]

The question of aminosugar participation was readdressed in analogous fashion. *N*-AcetylCLM (**7**) and CLMα_3 (**8**, see

Table 1. Bimolecular Rate Constants Determined for Reaction of **1** with AET and GSH Measuring Its Reaction to Mixed Disulfide **2** and Conversion of the Latter to **6**

	k (s^{-1} mM^{-1}) × 10^3		k (s^{-1} mM^{-1}) × 10^4	
	−DNA **1** → **2**	+DNA **1** → **2**	−DNA **2** → **6**	+DNA **2** → **6**
AET	8.31 ± 0.23[a]	5.29 ± 0.44	4.91 ± 0.16	2.55 ± 0.14
GSH	6.90 ± 0.71	2.07 ± 0.27	1.04 ± 0.06	0.29 ± 0.03

[a] Errors are standard deviations from the slope upon a linear fit of the data.

Chart 1

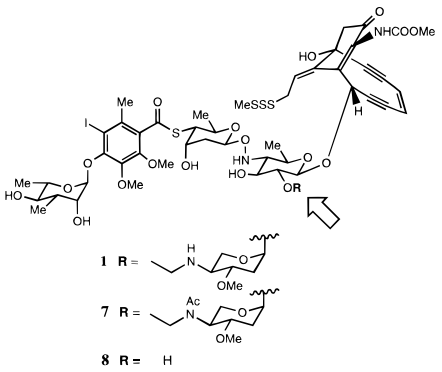

Chart 1) both lack the free ethylamine base of CLMγ_1^I (**1**). While their binding constants to DNA are not known and, hence, the >95% bound conditions may not have been met, these structural analogues were reacted in the presence of higher concentrations of calf thymus DNA (5 mM *vs* 2.5 mM DNA). Once again biphasic kinetics were observed following closely the pattern established for CLMγ_1^I (**1**). Bimolecular rate constants for each corresponding to the decomposition of **1** and subsequent reaction of the corresponding disulfide **2** are shown in Table 2 (columns 1 and 2). These experiments, in the presence of DNA, show that when **1**, **7**, and **8** were reacted with GSH, those compounds without an internal base generally reacted *slightly faster* than the γ_1^I form **1**. These experiments are in accord with earlier solution reactions in the *absence* of DNA which showed that reaction of **1** is not sigificantly faster than **7** and **8**.[14] If the proposed role of the aminosugars as intramolecular bases in the activation of the diynene antitumor antibiotics were correct, then **1** should demonstrate higher rates of reaction with thiols than analogues lacking the aminosugar. This rate enhancement was not observed.

Preparation and Reaction of Mixed Disulfides

To underscore the validity of the UV assay developed to obtain the results summarized in Table 1, two tests of the method were devised. In the first of these the mixed disulfides of **1**, **7**, and **8** were prepared by treatment with excess GSH. Each of the mixed GSH-disulfides proved to be readily separable by HPLC and was isolated on a semipreparative scale. These were in turn reacted with GSH in the presence of calf thymus DNA under the conditions above to give simple, clean pseudo first-order plots in keeping with the expected transformation of **2** to product. The second-order rate constants for each of the three disulfides are shown in Table 2, column 3. The agreement between these values and the corresponding slower reactions deduced from the sequential kinetics (columns 2 and 3) is excellent reaffirming the discrimination of the UV assay and the accuracy of the numerical analysis (Table 2).

The reductive decomposition of CLMγ_1^I (**1**), while characterized overall by pseudo first-order kinetics in the presence of excess thiol, is chemically quite complex.[2,3] For example, among the minor reactions that occur during the first encounters of the allylic trisulfide **1** with solution thiolate is the direct generation of allylic thiolate **3** and, as a consequence, virtually instantaneous closure to the dihydrothiophene **4**. The rapid reaction of this latter species with DNA most likely accounts for the "burst kinetics" in the DNA cleavage experiments reported by Joyce *et al.*[20] and similarly observed by Myers and co-workers.[3] In the event, several species exist in the early phases of reaction with GSH, many short-lived[2,3] and the concentrations of each, how they partition between DNA and solution, and their rates on and off the helix are not known (and experimentally difficult to determine). The information gained in a DNA cleavage assay with CLMγ_1^I (**1**) is a composite of all of these events. Nonetheless, the major process by far (occurring by several routes) is disulfide **2** formation. Its further reaction to give **3** is the rate-determining event in the entire activation pathway. In contrast to the chemistry of its formation, the further reaction of **2** is chemically far simpler involving S_N2 displacement at the sulfur−sulfur bond by thiolate to liberate the allylic thiolate **3**.[17]

Table 2. Bimolecular Rate Constants Obtained upon Treatment of CLM and Its Analogs with GSH for the Fast Decomposition of the Allylic Trisulfides (Column 1) Followed by the Slower Decomposition of the Mixed Disulfides (Column 2) or for Reaction of the Mixed Disulfides Alone (Column 3)

compd	k_{fast} (mM^{-1} s^{-1}) × 10^4	k_{slow} (mM^{-1} s^{-1}) × 10^5 [a]	k_{slow} (mM^{-1} s^{-1}) × 10^5 [b]
1	20.7 ± 2.7	2.9 ± 0.3	3.3 ± 0.1
7	4.6 ± 0.3	8.7 ± 0.4	8.7 ± 0.1
8	28.9 ± 0.2	12.1 ± 0.3	12.5 ± 0.4

[a] From trisulfide. [b] From mixed disulfide.

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5' CCCGGTCCTAAG 3'
3' GGGCCAGGATTC 5'
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9

This being so, it was thought that determining the actual rate of DNA cleavage by the disulfide **2** and simultaneously monitoring its conversion to **6** by the UV assay under identical conditions would provide a meaningful comparison between the rate data derived by each method. The cleavage behavior of dodecamer **9** has been thoroughly investigated in atom transfer experiments carried out in this laboratory and is known to cleave with 98 ± 1% probability at the TCCT site.[7,8] The strand containing this polypyrimidine motif was 5′-end labeled with ^{32}P and combined with radioinactive calf thymus DNA as carrier to ensure single-hit kinetics.[20] Reaction with the GSH-CLM disulfide **2** and excess GSH was carried out, and aliquots of the reaction mixture were withdrawn over time and quenched. The cleavage products were separated from uncut DNA by gel electrophoresis (Figure 4A) and analyzed using a PhosphorImager. The fraction of total radioactivity appearing in the labeled cleavage product was plotted as a function of time and gave a well-behaved first-order fit (Figure 4B). A pseudo first-order rate constant of $k_{CLV} = 2.2 \pm 0.2 \times 10^{-4}$ s^{-1} was obtained. In a second test of the continuous UV assay, the parallel reaction lacking only the trace of radiolabeled dodecamer **9** gave the rate $k_{UV} = 2.5 \pm 0.1 \times 10^{-4}$ s^{-1} in excellent agreement with the independent cleavage assay (Figure 4C).

(19) Decker, Q. W.; Post, H. W. *J. Org. Chem.* **1957**, *22*, 145−146. Nakabayashi, T.; Tsurugi, J.; Yabuta, T. *J. Org. Chem.* **1964**, *29*, 1236−1238. Field, L. In *Organic Sulfur Chemistry*; Oae, S., Ed.; Plenum Press: New York, 1977; pp 337−343.

(20) Li, T.; Zeng, Z.; Estevez, V. A.; Baldenius, K. U.; Nicolaou, K. C.; Joyce, G. F. *J. Am. Chem. Soc.* **1994**, *116*, 3709−3715.

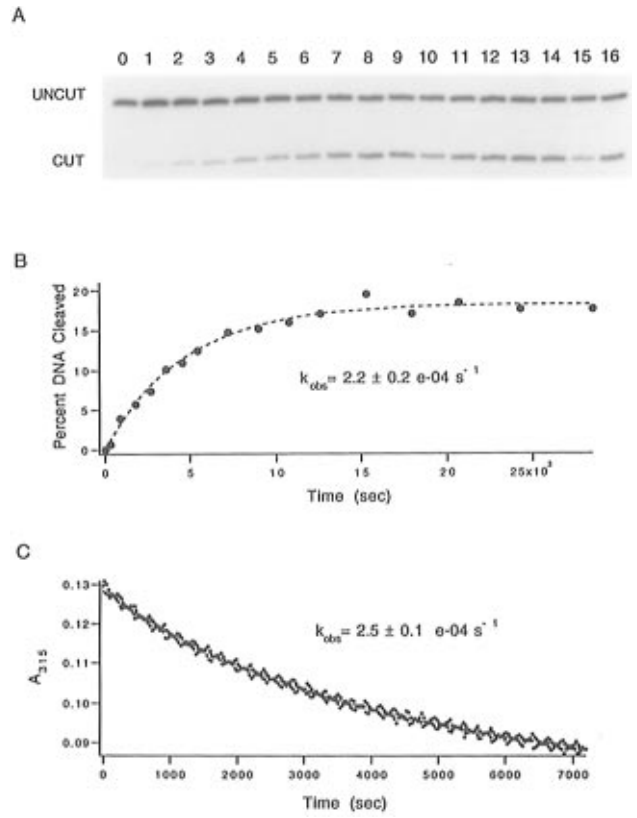

Figure 4. (**A**) PhosphorImager scan of a sequencing gel (25%) showing the reaction products obtained upon treatment of dodecamer **9** with mixed disulfide **2** (40 μM) and excess GSH (10 mM) over increasing time (0, 5, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 255, 300, 345, 405, and 475 min). (**B**) The fraction of DNA cleaved in each lane was plotted as a function of time (·) and the data were fit to a single exponential (eq 2) to obtain the rate of reaction. (**C**) Plot of absorbance (315 nm) as a function of time of **2** (40 μM) and GSH (10 mM) in the presence of 5 mM calf thymus DNA. The data were fit to a single exponential (eq 2) to obtain the rate of reaction.

The Role of DNA in the Thiol Activation of CLM

From the forgoing experiments it may be concluded that under conditions where CLMγ_1^I (**1**) is >95% bound to DNA, the rate of thiol activation in buffer/methanol is *slightly slower* than that for the drug free in solution. This effect is seen for both the initial decomposition of the trisulfide **1** and for the subsequent (slower) reaction of disulfide **2** to allylic thiolate **3**. Its magnitude is small, a factor of 1.5−4-fold, and presumably owes minimally to increased solvent viscosity in the presence of DNA, but more significantly to steric retardation and charge repulsion of thiolate approach to the polyanionic DNA helix harboring the bound drug.

The question of DNA participation in thiol activation of CLM has been approached by Myers *et al*. in a less direct manner.[3] In their experiments DNA cleavage was monitored by gel electrophoresis at varying DNA concentrations but at constant CLM:DNA ratio. They reasoned that with increasing DNA, the concentration of drug bound to DNA would change negligibly while that of drug free in solution would decrease markedly. If bound CLM reacted faster or at the same rate as drug free in solution, then the rate of DNA cleavage would be unaffected by increasing DNA concentration. On the other hand, if free drug reacted appreciably faster than bound, the rate of DNA cleavage would decrease as the DNA concentration was raised. Behavior of the latter sort is claimed by Myers and co-workers for the CLM·GSH disulfide **2**. Apart from the fact that this contention is the opposite of that asserted earlier for the interaction of CLM with DNA,[18] the experiment conducted to test this proposal was fundamentally flawed. While the amount of cleavage was monitored by gel electrophoresis and autoradiography for three fixed times as a function of increasing calf thymus DNA concentration at constant drug·DNA ratio, the amount of ^{32}P-labeled oligonucleotide added to each reaction mixture was constant. As the cleavage assay measures only the fate of the radioactive molecules, it comes without surprise that as dilution with radioinactive calf thymus DNA was increased, the apparent cleavage decreased.

Table 3. Calculated Fraction Bound and Concentration of Unbound **1** at Several DNA and Drug Concentrations[a]

ratio DNA:**1**	$[DNA]_t$, mM[b,c]	$[\mathbf{1}]_t$, μM[c]	fraction **1** bound[d]	$[\mathbf{1}]_f$, μM[e]
100	5.0	50	99.89	0.055
100	1.0	10	99.48	0.052
100	0.1	1	95.01	0.050
50	5.0	100	99.89	0.11
50	1.0	20	99.45	0.11
50	0.1	2	94.77	0.11

[a] Quantities were calculated using the Hostest program[22] and a binding constant[10] of 10^6 M^{-1} assuming a five base pair binding site and no cooperativity. [b] Concentration of DNA is in base pairs. [c] The subscript "t" denotes total concentration (bound + unbound). [d] Mole fraction of **1** bound to DNA. [e] Concentration of **1** which is not bound to DNA ("free").

Assuming, as do Myers *et al.*, the reasonable binding constant $K_a = 10^6$ M^{-1},[10] the amount of drug free in solution may be calculated as shown in Table 3.[22] Knowing the concentration of DNA is significantly greater than that of CLM and assuming five base pair binding sites and the absence of cooperativity,[23] the concentration of drug free in solution and the fraction bound can be readily calculated as shown in Table 3. Contrary to the seemingly intuitive arguments of Myers, for each of the drug·DNA ratios used (1:50 and 1:100), the variations in DNA concentration have virtually no effect on the *absolute concentration* of drug free in solution. The *percent* free and bound to DNA does change but only marginally. As the GSH concentration in each experiment was constant (10 mM), the second-order reaction of the free drug perforce should also be substantially constant despite variations in DNA concentration. Thus unpersuaded by Myers' experiments, the reactions of the CLM·GSH disulfide **2** were further examined at several concentrations of CLM and DNA, including those used previously.[3] Rather than measuring extents of reaction at a small number of time points, actual rate constants were determined as above. In our hands the reactions of disulfide **2** took a different course.

The GSH-CLM disulfide **2** was prepared as before and purified by semipreparative HPLC. 5′-^{32}P End-labeled oligonucleotide **9** was combined with calf thymus DNA to give concentrations of 5, 1, and 0.1 mM (in base pairs). In keeping with the experiments reported earlier,[3] the disulfide was added to maintain drug/DNA ratios of 1:50 and 1:100. Reactions were initiated by the addition of GSH (10 mM), and DNA cleavage was monitored as a function of time by gel electrophoresis and phosphorimaging (a representative example is shown in Figure 5). The fraction of total radioactivity in each lane present as the cleavage product was plotted as a function of time. These data smoothly fit the pseudo first-order appearance of product and gave an associated rate constant. These data are shown in Table 4 for three DNA concentrations and two drug/DNA ratios.

(21) Brenowitz, M.; Senear, D. F.; Shea, M. A.; Ackers, G. K. *Methods Enzymol.* **1986**, *130*, 132−181.
(22) Cowart, M. D.; Suchloleiki, I.; Bukownik, R. R.; Wilcox, C. S. *J. Am. Chem. Soc.* **1988**, *110*, 6204−6210.

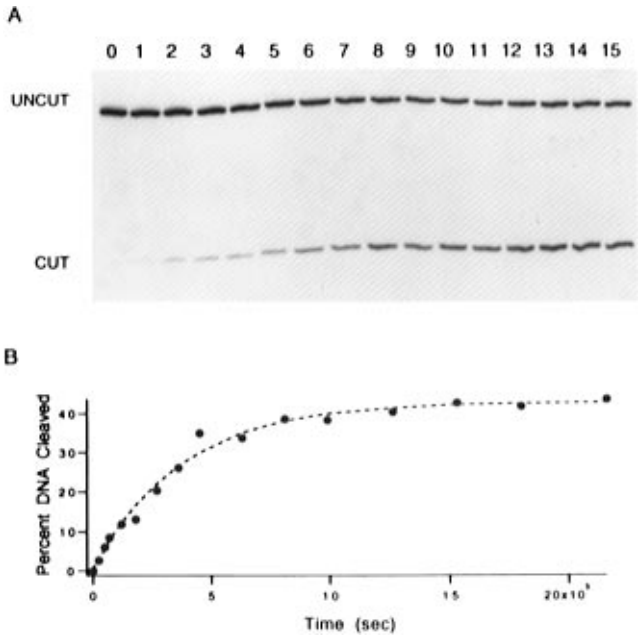

Figure 5. (**A**) PhosphorImager scan of a sequencing gel (25%) showing the reaction of ^{32}P-labeled **9** and 5 mM calf thymus DNA with mixed disulfide **2** (100 μM) and excess GSH (10 mM) over increasing time (0, 4, 8, 12, 20, 30, 45, 60, 75, 105, 135, 165, 210, 255, 300, and 360 min). (**B**) The fraction of DNA cleaved in each lane was plotted as a function of time (·) and the data were fit to a single exponential (eq 2) to obtain the rate of reaction.

Table 4. Pseudo First-Order Rate Constants for the Reaction of **2** with GSH[a]

ratio DNA:**2**	[DNA], mM	[**2**], μM	$k \times 10^4$ (s^{-1}) [b]
100	5.0	50	2.2 ± 0.2
100	1.0	10	2.7 ± 0.4
100	0.1	1	2.1 ± 0.3
50	5.0	100	2.2 ± 0.1
50	1.0	20	2.2 ± 0.1
50	0.1	2	2.4 ± 0.1

[a] Reactions were carried out with 10 mM GSH at room temperature (*ca.* 25 °C). [b] Values for experiments in which the DNA:**2** ratio is 100 are from single runs ± the standard deviation; values for experiments in which the DNA:**2** ratio is 50 are reported as the weighted mean ± the standard error of at least three runs.

At the same GSH concentration, each of these measured rates is effectively the same. While the results contradict the findings of Myers *et al.* with respect to the disulfide **2**, they agree with the kinetic measurements shown in Table 1. In this assay changes in the concentration of DNA do not affect the rate of CLM activation by thiol. However, given the small variation in CLM concentration free in solution in these experiments (see Table 3), such an outcome would be expected from first principles—particularly for a reaction having very similar rates bound to DNA and free in solution (*cf.* Table 1).

Using the same method of fixed-time assays, Myers *et al.* also attempted to examine the significantly more difficult behavior of CLMγ_1^I (**1**) itself upon activation with GSH in the presence of varying concentrations of DNA. Despite the complexity of the chemical events that take place in the initial phase of activation with thiolate, they,[3] as have we (see Table 1),[11] observed pseudo first-order disappearance of the trisulfide **1**. To correct for that component of cleavage contributed by **2** and not from more direct reaction of **1**, Myers *et al.* subtracted the amount of cleavage obtained in a control reaction of an equal concentration of disulfide **2** for each time point.[3] Having made this correction, no effect of changes in DNA concentration is seen in the reaction of CLM with DNA. While this conclusion is fully in accord with our findings, it too is drawn from a flawed experiment. The experiment as designed[3] conceived the reaction of **1** and **2** as *parallel* processes where the reaction of **2** can be subtracted from **1** to isolate the contribution to cleavage by **1** alone. This is clearly incorrect as the concentration of **2** at the start of reaction is zero, not equal to the concentration of **1**, as has been assumed. The formation of **2** is a *sequential* reaction from **1** and grows in a probably very closely first-order manner in the presence of excess thiol but decomposes, as we have shown in the preceeding, in a slower first-order process. Determining its concentration at any time in the reaction requires a considerably more complex treatment than described. However, the experiment as reported does yield the correct conclusion, although for reasons that we do not understand.

Solubility of CLM in Aqueous Buffer/Methanol

As we have demonstrated, experiments to vary DNA concentration that result in only very small changes in the fraction of CLM bound to the helix prove relatively incapable of answering the question of the role, if any, DNA may play in enhancing the rate of CLM activation by thiol. A much sharper probe of this effect is comparison of the actual rates of reaction in the presence and absence of DNA. As summarized earlier in Table 1 and discussed in previous communications,[11,14] this comparison has been made and shows that DNA binding slightly inhibits reaction.

An unfortunate confusion has appeared in the literature attempting to cast doubt on these kinetic experiments claiming that while CLMγ_1^I (**1**) is readily solubilized by DNA, it is insoluble in mixtures of water and organic solvents in general and in 70% Tris buffer/30% methanol in particular.[3] This solvent mixture has been used in most of the experiments in the present paper as well as in prior studies published from this laboratory.[11,14] Were this statement true, then comparisons to the solution reactions of CLM would be meaningless. Similarly, the issue of the role of the aminosugar of CLM in these reactions has depended upon comparative measurements using *N*-acetyl-CLM (**7**) and CLMα_3 (**8**).[14] Myers has discounted our preliminary findings using these CLM derivatives on the basis of their greater hydrophobicity and his expectation, therefore, of even lower solubility than **1** in buffered aqueous methanol.[3] This assumption about the solubility behavior of **7** and **8** is incorrect, as we show in the following section, as is the specious argument that CLMγ_1^I (**1**) is not soluble at the concentrations in question.

Light Scattering Experiments. The assertion that CLM is "completely" insoluble in mixtures of water and organic solvents rests on the observation of light scattering from mixtures of **1** in 5% DMSO in aqueous potassium dimethyl phosphate (10 mM) using a submicron particle analyzer. By estimating the mean particle size, an upper bound of the solubilty of CLMγ_1^I (**1**) in this medium was calculated to be $\leq$10 nM, a rather low level.[3] Light scattering was reported from a mixture of **1** in 70% Tris buffer/30% methanol as well, the solvent system used in this laboratory, although no quantitative estimate of CLM solubility in this medium was provided.

We have performed light scattering measurements on the same Malvern instrument used by Myers *et al.* but employing a lower power laser (5 mW). Solutions (60 μM) of **1**, **7**, and **8**, the highest concentrations of CLM used in any of the experiments reported in Table 1 and previously, were prepared in the customary 70% Tris/30% methanol, pH 7.4, solvent system. Two control solutions were prepared. The first was simply the buffer/methanol mixture alone, and the second was 60 μM solutions of **1**, **7**, and **8** in pure methanol. Relative to both sets

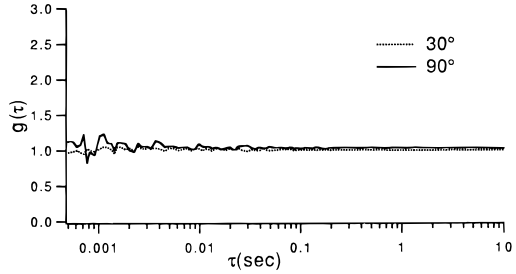

Figure 6. The autocorrelation function $g(\tau)$ plotted as a function of τ obtained with a 60 μM sample of **1** in 70/30 Tris buffer/methanol, pH 7.4, at 30° (dashed line) and at 90° (solid line) using a 50 mW HeNe laser at 633 nm.

of controls, modestly increased levels of light scattering were indeed observed from each of the CLM samples in buffer/methanol. However, dust particles are difficult to fully remove, especially from aqueous solutions, and are a notorious bane of light scattering measurements. Suspecting that this was the case in the present experiments, all solutions were filtered through 0.2 micron inorganic membrane filters (Anotop, Whatman) and re-examined. The elipticities of the samples (as a sensitive measure of concentration) were checked by circular dichroism (CD) spectroscopy at the maxima near 270 and 315 nm before and after filtration. The filtrates were all >98% of the original concentrations. Light scattering determinations, however, for the test solutions in buffer/methanol and each of the similarly filtered controls were quite low and essentially equivalent. So little suspended material was present at this point that the low "percent merit" (signal/noise) associated with these measurements indicated that random variation in the readings was a consequence of very low signal.

While these experiments were highly encouraging that **1**, **7**, and **8** were completely soluble at the level of 60 μM in 70% Tris/30% methanol, the low sensitivity of this instrument for the solutions after filtration led us to a more demanding test of the light scattering behavior of calicheamicin. Static and dynamic light scattering measurements were performed on 60 μM solutions of **1** in 70% Tris/30% methanol using an ALV-5000 instrument with either a 50 mW HeNe ($\lambda = 633$ nm) laser or a 100 mW Ar^+ laser ($\lambda = 488$ nm). In the dynamic experiment, the autocorrelation function of the scattered light [$g(\tau)$] was accumulated in the homodyne mode at several angles. Typical results are shown in Figure 6, where $g(\tau)$ obtained at 30° and 90° is plotted as a function of τ. These data were obtained using the HeNe laser. No inflection was observed for the data in the range of $\tau = 5 \times 10^{-4}$ to $\tau = 10$ s, indicating that there are no particles that scatter light with diameters in the range of 60 Å to 6 μm.[24] If 0.5 μm diameter particles of CLM were present, as reported by Myers *et al.*,[3] then inflection points at $\tau = 0.043$ s ($\theta = 30°$) and at $\tau = 0.0058$ s ($\theta = 90°$) should be present (see eq 6, Experimental Section), with the value of $g(\tau)$ changing from 1.0 to 2.0 with decreasing τ. A static light scattering experiment was also conducted with the above CLM sample. In this case, the absolute intensity of scattered light was measured at angles from 20° to 160° to determine if any scattering was due to a large number of relatively small particles (real aggregates) or from a few large particles (dust). Both the CLM sample and a blank solution of buffer/methanol showed only background levels of scattering and trace amounts of dust.

The dynamic and static light scattering experiments clearly establish that 60 μM solutions of **1**, **7**, and **8** can be readily attained in 70% Tris/30% methanol. Light scattering properties of these solutions result not from insolubility of the diynenes but from dust and trace amounts of insoluble impurities derived from their isolation. Notwithstanding, three other methodologically distinct tests have been performed to unequivocally establish the solubility of the three calicheamicins under the conditions used in the kinetics experiments described in Tables 1 and 2 and in earlier publications from this laboratory.[11,14]

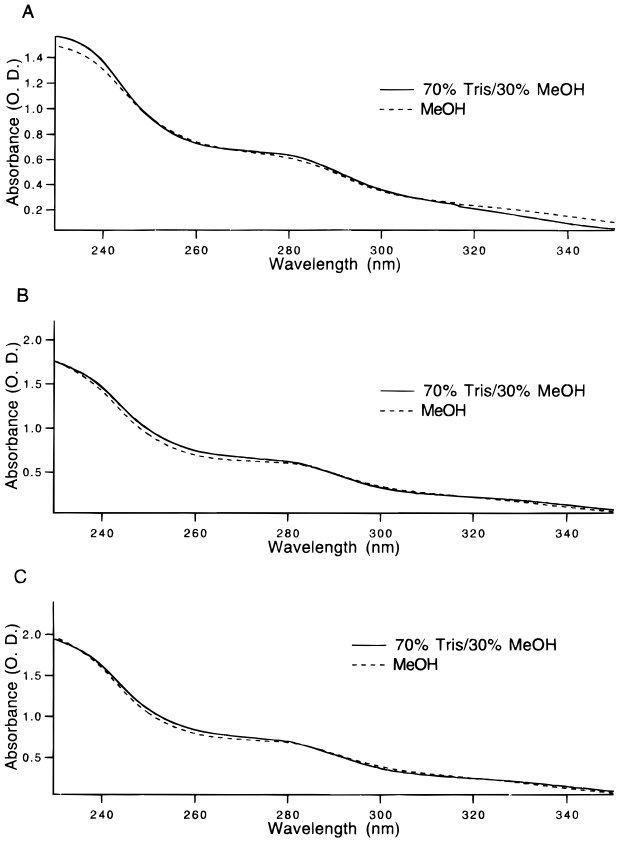

Figure 7. UV/visible absorption spectra for (**A**) 80 μM **1**, (**B**) 60 μM **7**, and (**C**) 60 μM **8** in methanol (dashed line) and in 70/30 Tris buffer/methanol (solid line) at 25.0 °C.

Spectroscopic Comparisons. There can be no disagreement that the calicheamicins **1**, **7**, and **8** are soluble in methanol, DMSO and other organic solvents at the concentrations used to prepare the low drug concentrations used in the kinetics experiments. If the solubility of CLMγ_1^I (**1**) were on the order of 10^{-8} M as claimed by Myers *et al.* in aqueous DMSO,[3] then a simple comparison of the UV−vis spectrum of CLM in organic solvent to that of the same drug concentration in a aqueous buffer/organic modifier should differ greatly. This comparison was made by dilution of stock solutions of **1**, **7**, and **8** in both methanol and 70% Tris/30% methanol. The UV spectra from 220−350 nm were recorded and overlaid in pairwise fashion as reproduced in Figure 7. For each diynene the spectra were very similar suggesting that all three were soluble in 70% buffer/30% methanol, at least up to 60 μM.

Circular dichroism (CD) spectroscopy has been shown to be highly sensitive to aggregation of chiral molecules in aqueous solvents, so a similar comparison was made using this technique.[25] The spectra of **1**, **7**, and **8** were recorded in methanol and in 70% Tris buffer/30% methanol at concentrations of 10.7, 13.8, and 13.8 μM, respectively at 25 °C (data not shown). The spectra of all three compounds were found to be very similar, having three prominant bands between 210 and 380 nm; these

(23) Mah, S. C.; Townsend, C. A.; Tullius, T. D. *Biochemistry* **1994**, *33*, 614−620. The possibility of cooperativity in the interaction of CLM with DNA has been suggested recently, although the Hill coefficients of these experiments were not reported: Krishnamurthy, G.; Brenowitz, M. D.; Ellestad, G. A. *Biochemistry* **1995**, *34*, 1001−1010.

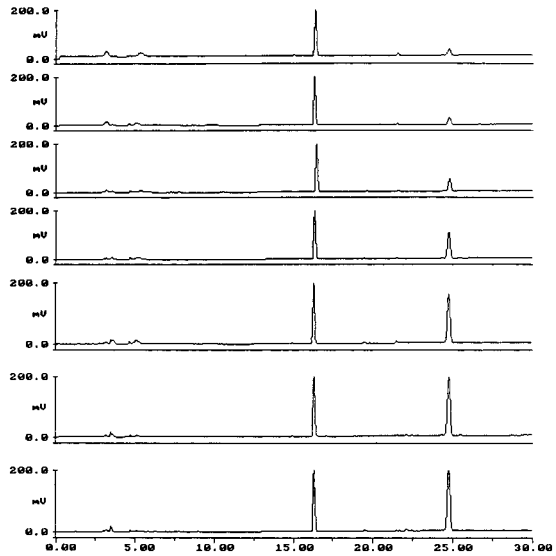

Figure 8. HPLC traces (detection at 280 nm) for centrifuged samples of 60 μM **7** in 5, 10, 15, 20, 25, 30, and 35% methanol in Tris buffer (top to bottom, respectively). The peak at 24 min corresponds to **7** and the peak at 16 min corresponds to nocardicin A, the internal standard.

are a positive band at 211−214 nm, a second positive band at 270−280 nm, and a negative band between 310 and 320 nm. As was the case with the corresponding UV/visible absorption spectra, there was very little difference between the CD spectra in methanol and in 70% Tris buffer/30% methanol. As an additional test, the CD spectrum of a 14.9 μM solution of CLMγ_1^I (**1**) was recorded at different temperatures, based on the observation that the CD spectrum of daunomycin is strongly temperature dependent due to aggregation.[26] It was found that spectra of **1** at 10, 25, and 40 °C in 70% Tris buffer/30% methanol were superimposable from 210 to 380 nm.

Centrifugation Experiments. When an insufficient amount of organic modifier is present, the calicheamicins form fine suspensions that are visible to the eye and can be readily precipitated by centrifugation. A more accurate measure of the solubility for each of the calicheamicins was attained with the following assay. Fixed concentrations of **1** (80 μM), **7**, and **8** (each 60 μM) and an internal standard were mixed thoroughly with Tris buffer containing increasing proportions of methanol (5−35%). The internal standard used was nocardicin A, a β-lactam antibiotic having two hydroxyphenyl rings.[27] Half of the sample was analyzed directly by HPLC in order to determine the ratio of peak areas at 280 nm of the drug and internal standard. The other half of the sample was centrifuged (16 000 $\times$ *g*, 30 min) to pellet undissolved CLM, and the supernatant was analyzed by HPLC. Figure 8 depicts a series of HPLC chromatograms of centrifuged samples of *N*-acetylCLM (**7**) in increasing proportions of methanol.

The fine suspensions of undissolved CLM are easily drawn up into a syringe and injected into the HPLC flow system. The mobile phase quickly dissolves these small particles, and the HPLC chromatograms show essentially unchanged ratio to the internal standard irrespective of the proportion of organic modifier in the original solvent mixture. In contrast, centrifugation deposits the particulate drug, and the HPLC chromatograms show increasing solubility of the respective calicheamicins as the methanol content of the solvent increases to a plateau at

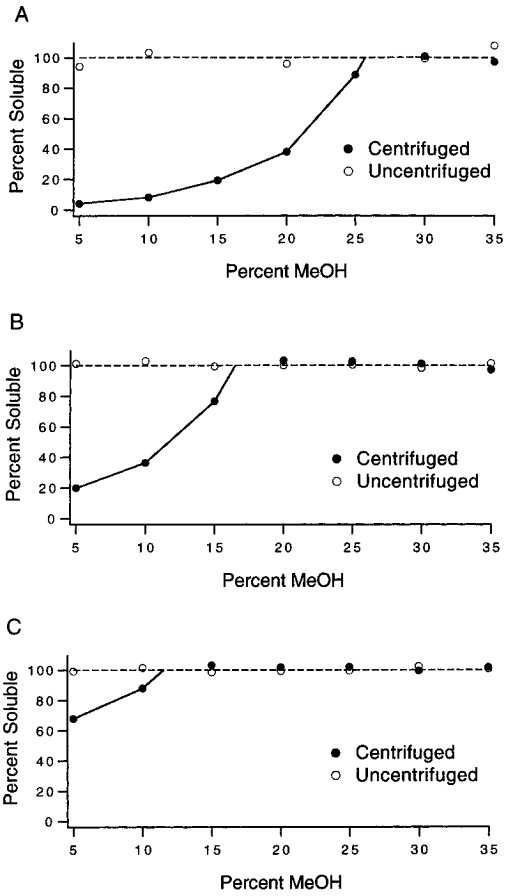

Figure 9. Percent of drug present in solution after centrifugation (•) relative to an uncentrifuged aliquot of the same solution (o) as a function of percent methanol in Tris buffer. Drug concentrations prior to centrifugation are (**A**) 80 μM **1**, (**B**) 60 μM **7**, and (**C**) 60 μM **8**.

complete solubility (Figure 9). Clearly **1**, **7**, and **8** are soluble in 70% Tris buffer/30% methanol at the concentrations used in the kinetics experiments.

Observation of Beer's Law. The linear correlation between absorbance and concentration of a species in solution is well-known to chemists. Deviation from Beer's Law comes about when solute molecules aggregate and when they precipitate from solution. The concentration dependent absorption of CLMγ_1^I (**1**) was, therefore, considered to provide a useful measure of the solubility limit in 70% Tris buffer/30% methanol. Individual drug solutions at a series of concentrations were prepared, and the UV spectra were recorded after equilibrating the samples to 25 °C. The results show that the absorbances measured at 280 nm increased linearly up to a concentration of 72 μM (Figure 10A). Above this concentration, the sample absorbances show a positive deviation from this linear relationship (Figure 10B). This is likely the result of light scattering by aggregates which form at concentrations above the solubility limit of **1**, and, in fact, a fine suspension was visible in the cuvette at the highest concentrations examined (151 and 201 μM). This result indicates that CLMγ_1^I (**1**) is soluble in 70% Tris buffer/30% methanol up to at least 72 μM at 25 °C.

As noted earlier, CD spectroscopy has been shown to be highly sensitive to aggregation of chiral molecules in aqueous solvents.[25] In a final test of the solubilities of CLM **1**, **7**, and **8** in 70% Tris buffer/30% methanol, the change in elipticity at the positive and negative CD maxima for each was determined

(24) Pecora, R. *Dynamic Light Scattering; Applications of Photon Correlation Spectroscopy*; Plenum Press: New York, 1985. Berne, B. J.; Pecora, R. *Dynamic Light Scattering with Applications to Chemistry, Biology and Physics*; John Wiley & Sons: New York, 1976.

(25) Balakrishnan, A. R.; Easwaran, K. R. K. *Biochim. Biophys. Acta* **1993**, *1148*, 269−277.

(26) Barthelemy-Clavey, V.; Maurizot, J.-C.; Dimicoli, J.-L. Sicard, P. *FEBS Lett.* **1974**, *46*, 5−10.

(27) Hashimoto, M.; Komori, T.; Kamiya, T. *J. Am. Chem. Soc.* **1976**, *98*, 3023−3025.

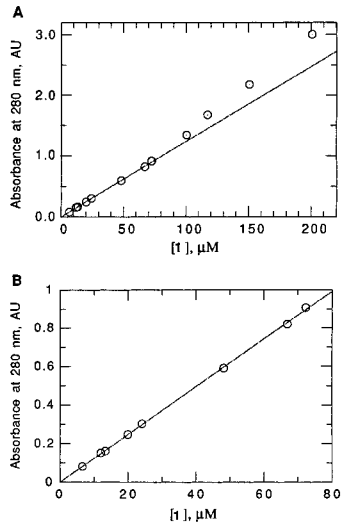

Figure 10. Absorbance at 280 nm as a function of the concentration of **1** in 70/30 Tris buffer/methanol at 25.0 °C. All data are included in panel **A** (6.7 μM ≤ [**1**] ≤ 201 μM). Panel **B** is an expansion of part of the graph in panel **A** (6.7 μM ≤ [**1**] ≤ 72 μM). The straight line drawn through the points in both graphs is the least squares fit for the data shown in panel **B** (slope = ϵ at 280 nm = 12 400 M^{-1} cm^{-1}, correlation coefficient = 1.000, measured absorbances did not exceed 1.0 AU).

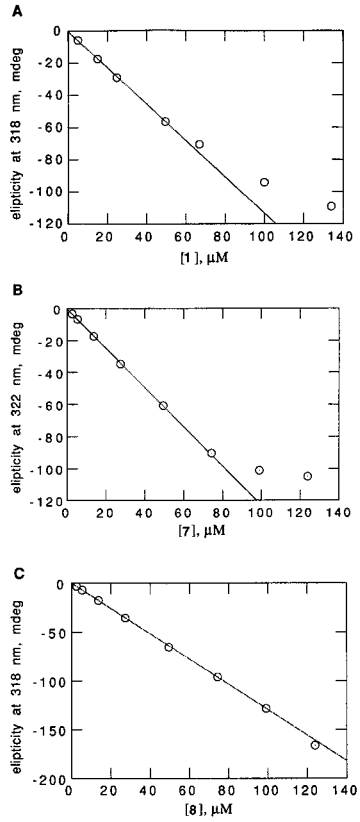

Figure 11. Ellipticity as a function of concentration for (**A**) **1**, (**B**) **7**, and (**C**) **8** in 70/30 Tris buffer/methanol at 25.0 °C. The straight lines shown are the least squares fits for data up to 49.6 μM **1**, 74.4 μM **7**, and 99.2 μM **8** (panels **A**, **B**, and **C**, respectively).

as a function of increasing drug concentrations at 25 °C. These data are presented in Figure 11. In all instances the Beer's Law plots are linear at least to 60 μM drug, the highest concentration used in the kinetic experiments in Table 1. Contrary to earlier predictions,[3] CLMγ_1^I (**1**) is actually the least soluble of the three derivatives examined. CLMα_3 showed no deviation from linearity up to 124 mM, the highest drug concentration measured.[28]

Conclusions

The roles of the aminosugar and duplex binding in the thiol activation of CLM for DNA cleavage have been examined by direct kinetic measurements. Comparisons of the second-order reaction rates of CLMγ_1^I (**1**) with *N*-acetylCLM (**7**) and CLMα_3 (**8**), close structural analogues lacking the free amine of the former, reveal no significant differences in buffered aqueous/organic solvent that could be attributed to aminosugar participation as a general base in either the presence or absence of DNA. Attempts to distinguish[3] between aminosugar deprotonation of thiol or ion pairing of thiolate anion and the carbohydrate ammonium ion have been shown to be unimportant in aqueous solution. For both the initial decomposition of the allylic methyltrisulfide in the presence of thiol and the subsequent, major rate-determining liberation of the allylic thiolate **3** from the mixed disulfide **2** of CLM, neither reaction is enhanced in rate by binding to DNA. On the contrary, the rates of both reactions are slowed by a factor of 1.5−4 compared to those of CLM free in solution. Similarly, the parallel behavior of aminoethanethiol (AET) and glutathione (GSH) in these experiments demonstrates that the kinetics of reductive activation of CLM are not uniquely invalid for "nonbiological" thiols as has been implied elsewhere.[3] Further, the statement that calicheamicins **1**, **7**, and **8** are insoluble under the conditions used in these and previous experiments[11,14] is incorrect. The complex activation dynamics that have been attributed to CLM in the presence of GSH and DNA[3] were based on experiments shown here to be flawed in design. The counterintuitive picture drawn from these data that **1** reacts with thiol as a ternary complex with DNA but the disulfide **2** must dissociate from DNA before it can undergo further reaction with thiol to yield dihydrothiophene **4** is erroneous.[29] That DNA binding modestly affects the reaction rates of both **1** and **2** with GSH or AET (or likely any thiol) is in accord with chemical expectation and is demonstrably the case as established by the data in Table 1.

The thiol activation of the neocarzinostatin chromophore (NCS) and its cleavages of DNA are mechanistically related, but not identical, to the corresponding reactions of calicheamicin.[30] The same questions of aminosugar participation and the effects of interaction with DNA have been posed for NCS as they have for CLM. Kinetic differences observed in organic solvents[18] and circumstantial evidence based on the proximity of the carbohydrate methylamine to the site of thiolate attack as interpreted from the X-ray structure of the NCS holoprotein[31] have been taken to support the contention that the aminosugars of both NCS and CLM participate in the thiol activation step.[3] Despite the persistent advocacy of this view, studies in aqueous solvent of NCS with thiols, while they leave the issue of amine participation unanswered experimentally, establish quite the opposite that DNA binding does not stimulate but slows the rate of thiol activation and cleavage of the duplex.[32] This finding is in complete accord with the results detailed here and earlier for CLM.[11,14] Similarly, the critical cumulene intermediate of the thiol activation process is shown to be the species capable of equilibration among binding sites on DNA and

(28) In this connection we thank Dr. G. P. Royer for the winning wager: Which has the greater solubility in water—phenylalanine or tyrosine?

(29) Although lacking in experimental detail, this criticism may likewise apply to recently reported experiments with dynemicin A: Myers, A. G.; Cohen, S. B.; Tom, N. J.; Madar, D. J.; Fraley, M. E. *J. Am. Chem. Soc.* **1995**, *117*, 7574−7575.

(30) For a review see: Goldberg, I. H. *Accts. Chem. Res.* **1991**, *24*, 191−198.

(31) Kim, K.-H., Kwon, B.-M.; Myers, A. G.; Rees, D. C. *Science* **1993**, *262*, 1042−1045.

(32) Myers, A. G.; Cohen, S. B.; Kwon, B. M. *J. Am. Chem. Soc.* **1994**, *116*, 1670−1682.

responsible for site-selection in drug-induced cleavage.[32] This role is identical to that determined for the dihydrothiophene of CLM[9,10] as suggested by earlier temperature-dependent NMR experiments.[2]

Experimental Section

General Methods. Calicheamicin γ_1^I (**1**), *N*-acetylcalicheamicin (**7**), and calicheamicin α_3 (**8**) were generous gifts from Drs. P. R. Hamann and G. A. Ellestad of the Lederle Laboratories, American Cyanamid Co. (American Home Products). These compounds were weighed out and dissolved in MeOH to provide 1−4 mM stock solutions, which were further diluted with MeOH as needed. All solutions were prepared with distilled deionized water or HPLC grade MeOH (Fisher; Pittsburgh, PA). Tris(hydroxymethyl)aminoethane (Tris), aminoethanethiol hydrochloride, glutathione (reduced form), and calf thymus DNA were obtained from the Sigma Chemical Company (St. Louis, MO). "Tris buffer" refers to 30 mM Tris·HCl/50 mM NaCl, pH 7.4, unless otherwise noted. Aminoethanethiol was prepared as a 1 M stock solution in MeOH and diluted as needed. Stock solutions of glutathione were prepared in Tris buffer immediately prior to use; the pH of these solutions was adjusted with NaOH. Calf thymus DNA was dissolved in either Tris buffer or 70/30 Tris buffer/MeOH by sonication, filtered through a Millipore 0.45 μM filter, and diluted to the desired concentration as determined by the A_{260} (1 OD/mL = 50 μg). Oligonucleotide **9** was synthesized, purified, and ^{32}P-endlabeled by the methods previously described.[10] Nocardicin A was a gift from Fujisawa Pharmaceutical Company Ltd. (Osaka, Japan) and was used as a 1.5 mg/mL solution in water.

A Beckman DU 70 spectrophotometer (Fullerton, CA) equipped with a Lauda circulating constant-temperature bath was used for all UV/visible absorption spectrophotometry. For kinetic experiments, the temperature inside the cell was determined by a Cole-Palmer (Chicago, IL) thermister-thermometer. Phosphoimager analysis was performed using a Molecular Dynamics 300E PhosphorImager (Sunnyvale, CA) equipped with ImageQuant software. All HPLC experiments employed a Hitachi L-6200 Intelligent Pump/L-6000 Diode Array detector (San Jose, CA) using a 250 mm × 4.6 mm Spherex C-18 reverse phase column (Phenomenex; Torrance, CA). Data were processed using the Diode Array Detector Manager computer program (Hitachi). Initial light scattering measurements were made using a Malvern 4700C submicron particle analyzer equipped with a helium-neon laser (λ = 633 nm, 5 mW), and data were processed using Automeasure software (Malvern; Southborough, MA). Subsequent static and dynamic light scattering measurements were performed using an ALV-5000 instrument (ALV Laser; Langen, Germany) equipped with either a 50 mW HeNe (λ = 633 nm) laser or a 100 mW Ar^+ laser (λ = 488 nm). CD spectra were recorded using a Jasco J-710 spectropolarimeter (Easton, MD) equipped with a a Haake F3 circulating bath for temperature control (25.0 ± 0.1 °C). Centrifugation experiments employed a Brinkmann Eppendorf Centrifuge, Model 5414 (Westbury, NY). Sonication was carried out with a Heat Systems-Ultrasonics, Inc. W225-R Sonicator (Plainview, NY).

Reaction of 1 with Aminoethanethiol or Glutathione in the Presence and Absence of DNA. These experiments were conducted at pH 7.5. **Glutathione.** Reaction solutions contained 2.5−7.5 μL of **1** (4 mM, 20−60 μM final concentration), 470 μL of 70/30 Tris buffer/MeOH, with or without 2.5 mM calf thymus DNA (2.35 mM DNA final concentration), and 3.75−20 μL of glutathione (1 M, 7.5−40 mM). Additional Tris buffer was added such that all final reaction volumes were 500 μL. Solutions less glutathione were equilibrated to 25.0 ± 0.1 °C in a 1.0 cm quartz cuvette. Reactions were initiated by the addition of glutathione, which had also been equilibrated to 25.0 °C.

Aminoethanethiol. Reaction solutions were prepared by combining up to 15 μL of a stock solution of **1** in MeOH (10 μM final concentration) and a volume of MeOH such that the total was 15 μL, 470 μL of 70/30 Tris buffer/MeOH, with or without 2.5 mM calf thymus DNA (2.35 mM DNA final concentration), and 0.5−5 μL of aminoethanethiol (1 M, 1−10 mM). The remainder of the solution was made up by adding Tris buffer such that all final reaction volumes were 500 μL. Solutions less aminoethanethiol were equilibrated to 25.0 ± 0.1 °C in a 1.0 cm quartz cuvette. Reactions were initiated by the addition of aminoethanethiol, which had also been equilibrated to 25.0 °C.

For all reactions, the absorbance at 315 nm was recorded as a function of time, and absorbance data from a blank reaction where MeOH was substituted for the drug solution was subtracted. The first-order rate constants were obtained from fitting these data to equation 1 using the computer program IGOR (Wavemetrics, Portland, Oregon). A plot of pseudo first-order rate constants as a function of thiol concentration provided the second order rate constants shown in Table 1.

$$y = k_0 + k_1 \cdot \exp(-k_2 \cdot x) + k_3 \cdot \exp(-k_4 \cdot x) \quad (1)$$

Reactions of 7 and 8 with Glutathione in the Presence of DNA. For reactions with compound **7**, 470−485 μL calf thymus DNA in 70/30 Tris buffer/MeOH (5.0 mM, 4.7−4.85 mM final concentration) and 10 μL of **7** (2 mM, 40 μM final concentration) were combined in a quartz cuvette and equilibrated to 25.0 °C. The reaction was initiated by the addition of 5−20 μL of glutathione (0.5 M, 5−20 mM final concentration) which had also been equilibrated to 25.0 °C. For reactions with compound **8**, 465−477.5 μL of calf thymus DNA in 70/30 Tris buffer/MeOH (5.0 mM, 4.65−4.77 mM final concentration) and 20 μL of **8** (1 mM, 40 μM final concentration) were combined in a quartz cuvette and equilibrated to 25.0 °C. The reaction was initiated by the addition of 2.5−20 μL of glutathione (1.0 M, 5−40 mM final concentration) which had also been equilibrated to 25.0 °C. In both cases, the absorbance at 315 nm was recorded as a function of time, and absorbance data from a blank reaction where MeOH was substituted for the drug solution was subtracted. Second order rate constants were obtained from these data as described in the previous section.

Preparation of the Glutathione Mixed Disulfides of 1, 7, and 8. Glutathione (2 mg, 6.5 μmol) was dissolved in MeOH (365 μL) containing TEA (2 μL). An equal volume of a 2 mM solution of **1**, **7**, or **8** in MeOH was added to this. The reaction was allowed to proceed for 10 min at room temperature, and the entire reaction mixture was injected onto a C-18 column for purification. The following gradient gave a clean separation: 20% to 75% acetonitrile in 50 mM triethylammonium acetate over 20 min (1 mL/min flow rate). Under these conditions, the mixed disulfides eluted at *ca*. 13 min, while the reduced forms of the drug and starting materials eluted at *ca*. 17 and *ca*. 22 min, respectively. Conversion to the corresponding mixed disulfide was about 65% and to spent drug about 10%. The solvent was evaporated using a Speed-Vac concentrator (Savant; Farmingdale, NY). The mixed disulfides were then resuspended in 100 μL of distilled water and again evaporated to dryness; this was repeated a total of three times to remove all traces of triethylammonium acetate. The resulting solids were dissolved in MeOH to a concentration of *ca*. 2 μM using a molar absorbtivity at 280 nm = 9200 M^{-1} cm^{-1}. The mixed disulfides were characterized by FAB-MS: CLMγ_1^I (M + H, calcd = 1369, found = 1369); CLMγ_1^I-GSH (M + H, calcd = 1595, found = 1596); *N*-AcCLM-GSH (M + H, calcd = 1636, found = 1637); CLMα_3-GSH (M + H, calcd = 1437, found = 1439).

The sample of **2** used for UV spectroscopic comparison to **1** (Figure 3) was prepared as described above and initially isolated using the same HPLC protocol. However, in order to insure that the sample was free of any residual triethylammonium acetate, the sample was dried down, dissolved in *ca*. 50% aqueous methanol and reinjected onto the column using the following gradient: 20% acetonitrile in water for 5 min followed by a linear gradient to 75% acetonitrile in water over 20 min (retention time of **2** in this case was approximately 5 min longer than with the above gradient). The solvent was removed using a Speed-Vac concentrator, and the resulting solid was dried under high vacuum. A specimen was then weighed using a Cahn Electrobalance, transferred to a microcentrifuge tube, and dissolved in methanol. An aliquot of this methanol solution was used to prepare the sample in 70/30 Tris buffer/methanol.

Reaction of the Mixed Disulfides of 1, 7, and 8 with Glutathione, UV Assay. Reaction solutions contained 240 μL calf thymus DNA in Tris buffer (11 mM, 5.3 mM final concentration), 10 μL of mixed disulfide (*ca*. 2 mM, 40 μM final concentration), 55−218 μL glutathione stock solution (92 mM, 10−40 mM final concentration), and the appropriate amount of Tris buffer to bring the total volume to 500 μL. Final solvent composition was 2% MeOH in Tris buffer. Reaction solutions less either glutathione or mixed disulfide were combined in

a quartz cuvette and equilibrated to 25.0 ± 0.1 °C in the cell holder; a solution of the missing component, which had also been equilibrated to 25.0 °C was added to initiate the reaction. The absorbance of the sample *vs* time at 315 nm was acquired. For each measurement the absorbance of a blank solution in which MeOH was substituted for the mixed disulfide in MeOH was subtracted, and the resulting data were fit to a single exponential (eq 2) using the computer program IGOR (Wavemetrics; Portland, Oregon). A plot of first order rate constant as a function of thiol concentration provided second order rate constants.

$$y = k_0 + k_1 \cdot \exp(-k_2 \cdot x) \tag{2}$$

Cleavage of Oligonucleotide 9 by 2. For experiments with a 1:50 ratio of **2** to DNA, a DNA/drug stock was prepared by combining 151 μL of calf thymus DNA in Tris buffer (8.6 mM), 13 μL of **2** in MeOH (2 mM), and 56 μL of Tris buffer. Reaction solutions containing 5 mM DNA/100 μM **2**, 1 mM DNA/20 μM **2**, and 100 μM DNA/2 μM **2**, respectively, were prepared in Eppendorf microcentrifuge tubes from this stock as follows: 170 μL DNA/drug stock, 10 μL **9**, and 20 μL glutathione (100 mM); 35 μL DNA/drug stock, 10 μL **2**, 135 μL Tris buffer, and 20 μL glutathione (100 mM); 3.4 μL DNA/drug stock, 10 μL **2**, 166.6 μL Tris buffer, and 20 μL glutathione (100 mM). Total ^{32}P radioisotope present as tracer quantities of **9** in each reaction (200 μL) was *ca*. 160 000 dpm. Reaction solutions less glutathione were equilibrated to 25.0 °C in a constant-temperature bath; reactions were initiated by addition of the glutathione solution, which had been equilibrated to the same temperature. Aliquots (10 μL) were removed throughout the course of the reactions and quenched by addition to tubes containing 20 μL of 0.5 M triethylammonium acetate, pH 5.5, and 400 μL of ethanol. The DNA products were isolated and quantitated as previously described.[10] From these data, rate constants were obtained using nonlinear curve fitting as above.

Light Scattering Experiments. For initial experiments (Malvern instrument), a 75 μL aliquot **1** in MeOH (4.0 mM) was diluted to 1500 μL with MeOH and this was then added to 3500 μL of Tris buffer with constant mixing using a vortex mixer (final concentration 60 μM CLM, 30% MeOH). Tris buffer was freshly prepared and filtered through a 0.45 μm cellulose acetate filter (Corning; Corning, NY) prior to use. The resulting solution was transfered to a quartz cuvette and both CD and light scattering measurements were made. The solution was then taken up in a syringe and filtered through a 0.2 μm Anotop filter (Whatman; Clifton, NJ) directly into a second quartz cuvette and the CD spectrum and light scattering were again measured. An analogous experiment was carried out using **1** (60 μM) in 100% MeOH.

For the second set of experiments (ALV instrument), 60 μM solutions of **1** were prepared as described above followed by filtration through a 0.2 μm Anotop filter into cylindrical glass cuvettes, which were flame-sealed. The autocorrelation function of the scattered light [$g(\tau)$] was accumulated in the homodyne mode at several angles. The average particle radius was calculated as follows: The translational diffusion coefficient

$$D = kT/6\pi\eta R_H \tag{3}$$

where k = Boltzman constant, T = temperature, η = viscosity, and R_H = average particle radius. The scattering vector

$$q = 4\pi n_D \sin(\theta/2)/\lambda \tag{4}$$

where n_D = refractive index, θ = angle at which measurement is made, and λ = wavelength of laser. The diffusion coefficient, D, is also equal to

$$1/\tau q^2 \tag{5}$$

Substituting the values for eqs 3 and 4 in eq 5 and rearranging we get

$$R_H = kT\tau 16\pi^2 (n_D)^2 \{\sin(\theta/2)\}^2/\lambda^2 6\pi\eta \tag{6}$$

Solubility of 1, 7, and 8 as Determined by Centrifugation. Tris buffer was mixed with MeOH to give the desired final concentrations (5, 10, 15, 20, 25, 30 and 35% v/v). **1** (10 μL, 80 μM final concentration, pH 7.4), **7**, or **8** (7.5 μL, 60 μM final concentration, pH 7.5), and nocardicin A (10 μL) were added to the appropriate buffer to provide a series of 500 μL samples. The solutions were vortexed and divided in two. One half was centrifuged (16 000 × *g* for 30 min at room temperature), while the other half was used as a control. The supernatant from the centrifuged fraction (100 μL) and the control sample (100 μL) were analyzed by HPLC using the following gradient for **7** and **8**: isochratic 5% acetonitrile in 0.1% aqueous trifluoroacetic acid for 5 min, followed by a linear gradient to 100% acetonitrile over 20 min. The conditions for **1** (retention time 18 min) were as previously described.[11] The integrated peak areas of **7** and **8** (retention times 24 and 23 min, respectively), normalized to nocardicin A (retention time 16 min), from the centrifuged *vs* the noncentrifuged samples were used to determine solubility.

Concentration Dependent Absorbance of 1. Samples (1000 μL) of the various concentrations of **1** in 70/30 Tris buffer/MeOH were prepared as follows: 50−300 μL of either a 669 or a 241 μM stock solution of **1** in MeOH was transferred to a series of 1.5 mL Eppendorf microcentrifuge tubes. The required amount of MeOH was added to each to bring each solution to 300 μL. To each of these was added 700 μL of Tris buffer. All transfers were made using Hamilton syringes. The samples were vigorously vortexed and in turn transferred to either a 1.0 or a 0.2 cm pathlength quartz cuvette. Samples were equilibrated in the sample compartment at 25.0 °C for 10 min prior to recording spectra. In all cases the measured absorbance at 280 nm was less than 1.0 AU; absorbances for samples obtained using the 0.2 cm cell were multiplied by 5 to obtain the data in Figure 10.

Concentration Dependent Elipticities of 1, 7, and 8. Samples were prepared essentially as described in the preceding section. In this case, however, 400 μL of sample was prepared for measurements using a 0.2 cm pathlength cell whereas 1800 μL was prepared for measurements using a 1.0 cm pathlength cell. The concentrations of the MeOH stock solutions used were as follows: **1**, 2.68 mM and 0.178 mM; **7**, 992 and 99.2 μM; **8**, 992 and 99.2 μM. The observed elipticities for samples obtained using the 0.2 cm cell were multiplied by 5 to obtain the data in Figure 11.

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