Role of the Aryl Iodide in the Sequence-Selective Cleavage of DNA by Calicheamicin. Importance of Thermodynamic Binding *vs* Kinetic Activation in the Cleavage Process

Moneesh Chatterjee, Stanley C. Mah, Thomas D. Tullius,* and Craig A. Townsend*

Contribution from the Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218

Received January 13, 1995[⊗]

Abstract: Calicheamicin γ_1^I (CLMγ_1^I) is one of the diynene antitumor antibiotics known to show comparatively high sequence selectivity in its cleavages of DNA. The origins of this discrimination and the proposed role of the side-chain aryl iodide interaction with the helix are addressed. While drug activation with thiols is complex, the rate-limiting rearrangement of the dihydrothiophene intermediate in this process is sufficiently slow for it to serve as the species responsible for the sequence selection common to all reaction pathways leading to DNA cleavage. Internal competition experiments between a TCCT·AGGA sequence, known to be favorable for selective cutting, and a series of other cleavage sites of equal size have been conducted with the dihydrothiophene. Although the extents of reaction differ at the variable four-base pair cassette, the rates of reaction are identical within experimental error. These results indicate that equilibrium binding of the dihydrothiophene is achieved before substantial strand scission has taken place. Therefore, kinetic effects owing, for example, to steric compression to enhance the rate of Bergman cyclization at certain sites do not occur. Sequence selection is governed by thermodynamic effects favoring binding of the dihydrothiophene at these sequences. Free energy differences relative to the TCCT site can, therefore, be calculated. Knowing this, inosine (I) substitutions for guanosine have been made within the complementary AGGA to give AIGA and AGIA to examine the effect of replacing the 2-amino group of guanosine with the hydrogen of inosine. Analogous competition experiments permit the thermodynamic advantage conferred by aryl iodide interaction with the 5′-guanosine to be estimated as approximately 1 kcal/mol. The magnitude of this stabilization was compared to the total binding of CLMγ_1^I and CLMϵ at a TCCT·AGGA sequence by isothermal titration calorimetry and quantitative hydroxyl radical footprint titration, respectively, to give an overall view of the energetics of calicheamicin binding to DNA and the role played by the side-chain aryl iodide.

Among the diynene antitumor antibiotics calicheamicin γ_1^I (CLMγ_1^I, **1**) is remarkably selective in the DNA sequences it cleaves. The first studies of CLMγ_1^I reaction with DNA noted a limited number of cleavage sites contained within restriction fragments of the plasmids pUC18 and pBR322 and a *streptomyces* promoter region.[1] The sequence TCCT·AGGA was identified in this early work as particularly favorable for scission. Such a sequence is intriguing since the G·C base pair is not typically recognized by small molecules that bind to the DNA minor groove.[1,2] Influenced by this observation, Hawley *et al.*[3] made the interesting suggestion based on molecular modeling that the side-chain aryl iodide of CLM confers selective binding at TCCT·AGGA through noncovalent interaction with the C-2 amino substituent of guanine(s) in the minor groove. This proposed interaction is depicted in Figure 1.

It should be borne in mind, however, that the original report of CLMγ_1^I cleavages of bacterial DNA noted that sequences other than TCCT·AGGA were identified by the natural product as well,[1] an important finding that was amplified by subsequent work. Most telling among these studies was the observation of comparable extents of cleavage at runs of thymines four or greater in length.[4−7] In particular, these sequences lack the exocyclic 2-amino group of a G·C base pair and argue for the general case that aryl iodide interactions with this function cannot be the primary reason for site selectivity of CLM-induced cleavage. Nonetheless, footprinting studies of synthetic samples of the aryl-linked carbohydrate side chain containing an iodine substituent or replacing it by, for example, hydrogen suggest that the presence of the large halogen atom contributes to the DNA binding selectivity seen in these experiments and, therefore, of CLM.[8−10] Similarly, NMR studies of CLMγ_1^I with an ACCT·TGGA sequence incorporated into an octomer duplex show that the aryl iodide could contact the 3′-guanine but not the 5′-guanine.[11−13]

To test the importance of CLM interaction with the 2-amino substituent of guanine to the sequence selectivity of DNA

[⊗] Abstract published in *Advance ACS Abstracts*, July 15, 1995.

(1) Zein, N.; Sinha, A. M.; McGahren, W. J.; Ellestad, G. A. *Science* **1988**, *240*, 1198−1201.

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

(3) Hawley, R. C.; Kiessling, L. L.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1105−1109.

(4) Walker, S.; Landovitz, R.; Ding, W. D.; Ellestad, G. A.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4608−4612.

(5) Mah, S. C.; Townsend, C. A.; Tullius, T. D. *Biochemistry* **1994**, *33*, 614−621.

(6) Mah, S. C.; Price, M. A.; Townsend, C. A.; Tullius, T. D. *Tetrahedron* **1994**, *50*, 1361−1378.

(7) Discussions of the mutually interesting behavior of calicheamicin cleavage of DNA containing A-tracts are acknowledged with Professor D. E. Kahne dating from a research seminar at Princeton University in April 1991.

(8) Nicolaou, K. C.; Tsay, S. C.; Suzuki, T.; Joyce, G. F. *J. Am. Chem. Soc.* **1992**, *114*, 7555−7557.

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

(10) Aiyar, J.; Danishefsky, S. J.; Crothers, D. M. *J. Am. Chem. Soc.* **1992**, *114*, 7552−7554.

(11) Walker, S.; Murnick, J.; Kahne, D. *J. Am. Chem. Soc.* **1993**, *115*, 7954−7961.

0002-7863/95/1517-8074$09.00/0

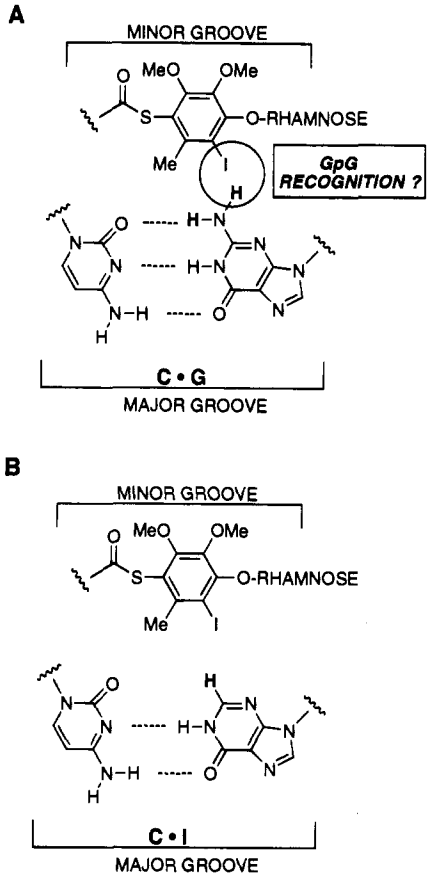
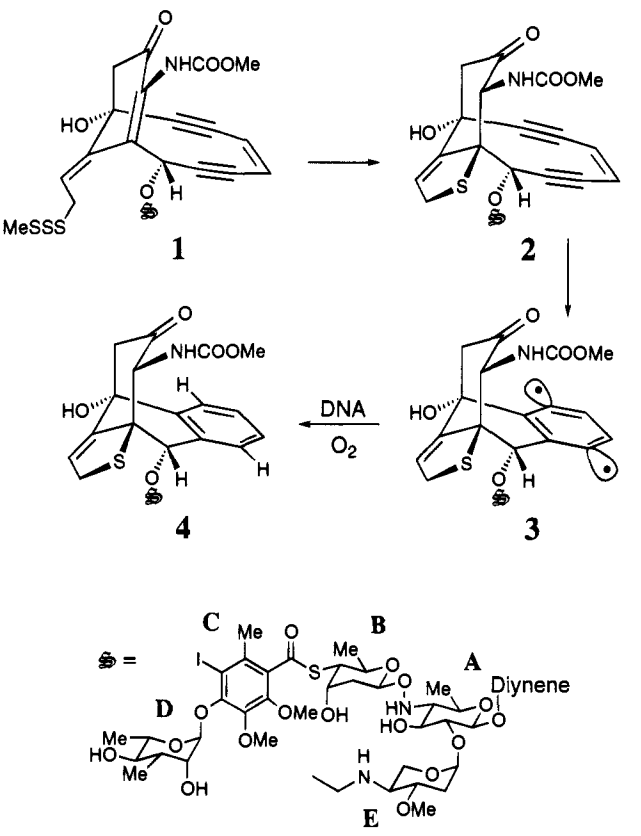


Figure 1. Proposed interaction of the aryl iodide of calicheamicin with (A) guanine and (B) inosine in the binding site.

cleavage, synthetic oligonucleotides were prepared containing two TCCT·AGGA sites. By keeping one of these recognition sites unchanged, inosine (I) was substituted in turn for each of the two guanines in the other AGGA sequence, *i.e.* AIGA and AGIA. The structure of inosine is identical to that of guanine but the 2-amino group of the latter is replaced by hydrogen and thus removes a potential interaction with the aryl iodide in the minor groove. In Figure 1, the C·I base pair (B) is compared to the C·G base pair (A) in this proposed interaction with CLM. Extents of cleavage by CLM were then determined in each of these inosine-substituted oligonucleotides to allow highly accurate comparisons to be made by internal competition. Finally, these were normalized to a control containing two unaltered TCCT·AGGA sites. Surprisingly, inosine substitution resulted in both decreased and increased cleavage by CLM depending upon the locus of base substitution.

To understand these results the larger question of DNA cleavage selectivity by CLM needed to be addressed. The conventional view would hold that CLMγ_1^I site selectivity results from thermodynamic affinity of the natural product for certain DNA sequences. That is, discrimination in binding is manifested in selectivity of cleavage. On the other hand, the pivotal chemical step initiating actual cleavage of the helix is a Bergman[14-16] rearrangement of the dihydrothiophene **2** to the 1,4-diyl **3** poised in the minor groove of DNA for what is now known to be impressively specific hydrogen abstraction from the sugar–phosphate backbone.[17,18] Is the rate of this critical

Scheme 1



electrocyclization enhanced by DNA binding, particularly at certain sites? For example, the onset of steric compression as the activated form of CLM tracks along the helix could be visualized to lower the activation energy of this fundamentally thermal rearrangement process. Site selectivity of CLM cleavage could be the consequence of kinetic acceleration of the electrocyclization step and, thus, function as a potential probe of DNA structure sensitive to groove narrowing, bending, or kinking. Such a kinetic component to the observation of site selectivity is precedented in the covalent reaction of other minor groove binders to N-3 of adenine.[19,20]

In this paper, we demonstrate the significance of the dihydrothiophene **2** to the overall activation of CLM for DNA cleavage as suggested by earlier variable-temperature NMR experiments.[2] Direct kinetic measurements of the rate of electrocyclization (**2** to **3**) of this species upon DNA interaction were found to be constant within experimental error.[21] The origin of sequence-selective cleavage by **2**, therefore, must arise by equilibrium binding among preferred sequences prior to substantial rearrangement to 1,4-diyl **3** and DNA cleavage. This being so, it is possible to calculate the relative free energy advantage/disadvantage of a cleaved sequence with respect to others. From the internal competition experiments carried out in the present work, a quantitative evaluation of the CLM aryl iodide–guanine interaction in the minor groove has been possible. To place the magnitude of this contribution to binding

(12) Walker, S. L.; Andreotti, A. H.; Kahne, D. E. *Tetrahedron* **1994**, *50*, 1351–1360.
(13) Paloma, L. G.; Smith, J. A.; Chazin, W. J.; Nicolaou, K. C. *J. Am. Chem. Soc.* **1994**, *116*, 3697–3708.
(14) Bergman, R. G. *Acc. Chem. Res.* **1973**, *6*, 697–699.
(15) Mayer, J.; Sondheimer, F. *J. Am. Chem. Soc.* **1966**, *88*, 602–603.
(16) Darby, N.; Kim, C. U.; Salaun, J. A.; Shelton, K. W.; Takada, S.; Masamune, S. *J. Chem. Soc., Chem. Commun.* **1971**, 1516–1517.
(17) 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.
(18) Hangeland, J. J.; De, V. J. J.; Heath, J. A.; Townsend, C. A.; Ding, W. D.; Ashcroft, J. S.; Ellestad, G. A. *J. Am. Chem. Soc.* **1992**, *114*, 9200–9202.
(19) Baker, B. F.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 2700–2712.
(20) Warpehoski, M. A.; Harper, D. E. *J. Am. Chem. Soc.* **1995**, *117*, 2951–2952.
(21) Chatterjee, M.; Cramer, K. D.; Townsend, C. A. *J. Am. Chem. Soc.* **1994**, *116*, 8819–8820.

in context, comparisons have been made to the overall thermodynamics of CLM binding to DNA determined by two independent methods, isothermal titration calorimetry and quantitative hydroxyl radical footprint titration. From these data, a comprehensive picture can be composed of the interplay of *thermodynamics and kinetics to the DNA cleavage selectivity* by CLM and the role played by the aryl iodide.

Results and Discussion

Role of the Dihydrothiophene. The discovery that CLMγ_1^I (**1**) could be treated at low temperature with a phosphine to rapidly afford the comparatively stable dihydrothiophene **2** led to the following test of the proposal that this reactive intermediate was sufficiently long-lived to be the species responsible for site-selective cleavage of DNA.[2] A methanol solution of **1** at −78 °C was reacted with excess tributylphosphine, and the resulting solution of dihydrothiophene **2** was transferred to a −10 °C bath. A 5-fold volume excess of a 267 base pair 5′-^{32}P end-labeled restriction fragment of the plasmid pUC18 and calf thymus DNA was added to **2**. The concentration of calf thymus DNA was adjusted to ensure single-hit kinetics.[22] To control for the effect of added phosphine in the DNA cleavage reaction, **1** was allowed to preassociate with the above mixture of labeled restriction fragment and calf thymus DNA before reaction was initiated at −10 °C by the addition of an *equal* concentration of tributylphosphine. The sites and relative intensities of cleavage were examined by electrophoresis and autoradiography of the DNA products. Densitometry scans revealed that the cleavage patterns under these two reaction conditions were superimposable.[21]

The issue of the key role played by the dihydrothiophene **2** in the site selectivity of DNA cleavage was pressed further. The above experiments were repeated in parallel with reactions where CLMγ_1^I activation was initiated by the addition of an equivalent concentration of glutathione rather than phosphine. Identical concentrations of CLMγ_1^I (**1**, 4 μM final concentration), radiolabeled restriction fragment, and carrier DNA were reacted as before at −10 °C with 500 equiv of glutathione or tributylphosphine. The reactions were run for 3 h to allow essentially complete conversion to CLMϵ (**4**). The DNA reaction products were precipitated by the addition of ethanol, and the distribution of cleavage products was examined by gel electrophoresis (Figure 2).

Activation of CLM with thiol is more complex than reaction with phosphine, and several routes to the dihydrothiophene are available.[2,23,24] These differences notwithstanding, the relative intensity and location of cleavage sites are virtually identical for CLMγ_1^I activated by phosphorus or sulfur reductants (methyl thioglycolate, 2-mercaptoethanol, aminoethanethiol, and dithiothreitol were also used as the activating thiol and gave the same result—data not shown). As each of these reactions passes through the dihydrothiophene **2** prior to 1,4-diyl **3** formation and, indeed, gives reactions indistinguishable from those caused by this relatively long-lived intermediate itself, it is the selectivity of this species in its interaction with DNA that determines the site selectivity of DNA cleavage.[25]

Effect of DNA Sequence on Electrocyclization Rate. To examine the effect of sequence on the rate of DNA cleavage by the dihydrothiophene **2** and thus address the central question of kinetic acceleration by various duplex environments, the oligonucleotide **5** was designed. This 20-mer contained a

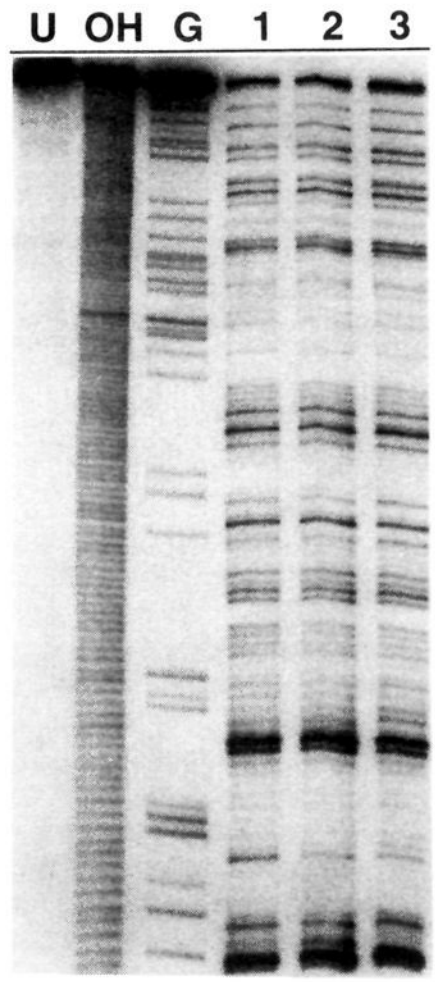


Figure 2. Autoradiogram of a DNA sequencing gel (6%) showing products obtained upon treatment of a 267 base pair *Bam*HI-*Nar*I restriction fragment of plasmid pUC19 (**U**) with hydroxyl radical cleavage (**OH**), Maxam−Gilbert guanine-specific reaction (**G**), calicheamicin γ_1^I and tributylphosphine (**1**), dihydrothiophene (**2**), and calicheamicin γ_1^I and glutathione (**3**).

```
5' CCCGGTCCTATCGNNNNAAG 3'
3' GGGCCAGGATAGCMMMMTTC 5'
```

5

TCCT·AGGA site, a favorable and well-studied cleavage sequence,[27,17,18] and a NNNN·MMMM cassette that could be systematically varied, *e.g.* with runs of pyrimidine bases to construct a second cleavage site.[4−6] The dihydrothiophene **2** was prepared as before, and the absolute rates of cleavage of the test 20-mers were monitored by PhosphorImager as a function of time at constant temperature.

The rate of hydrogen abstraction from the DNA backbone by the diradical **3** is rapid relative to the rate of electrocyclization of **2** to **3**. Therefore, the appearance of cleavage products arising from 5′-abstraction[18,28,29] typically obtained in runs of pyrimidines[5,6] provides a good measure of the rate of the slower reaction, that is, of the thermal rearrangement of the dihydrothiophene to the 1,4-diyl. Rate differences in cleavage required by the kinetic model owing to interaction of **2** with different DNA sequences should be manifest in the measured rates of cleavage at the changing NNNN site. The experiment, therefore, allows absolute rates to be monitored at a variety of

(22) Brenowitz, M.; Senear, D. F.; Shea, M. A.; Ackers, G. K. *Methods Enzymol.* **1986**, *130*, 132−181.

(23) 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.

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

(25) As an aside, it should be noted that molecular modeling studies to date have ignored the role of the dihydrothiophene **2** in DNA cleavage and have concentrated on the interaction of the γ_1^I form (see refs 3, 26, and 27).

(26) Langley, D. R.; Doyle, T. W.; Beveridge, D. L. *Tetrahedron* **1994**, *50*, 1379−1396.

(27) Zein, N.; Poncin, M.; Nilakantan, R.; Ellestad, G. A. *Science* **1989**, *244*, 697−699.

(28) While the rate of 4′-hydrogen abstraction from the complementary strand is surely virtually identical, the efficiency with which DNA cleavage products appear depends upon the conditions of DNA treatment prior to electrophoresis. On the other hand, 5′-abstraction leads directly to strand cutting irrespective of, for example, piperidine treatment.

(29) Dedon, P. C.; Salzberg, A. A.; Xu, J. *Biochemistry* **1993**, *32*, 3617−3622.

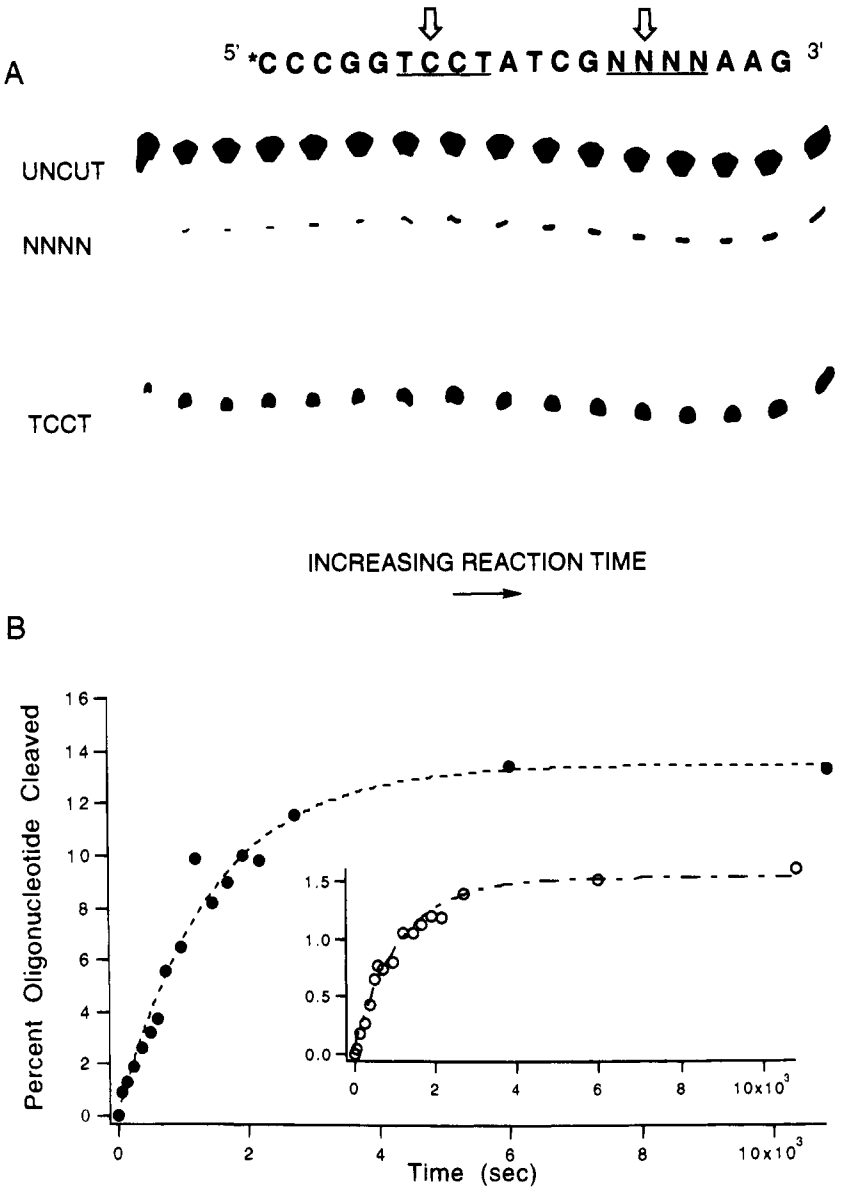


Figure 3. (A) PhosphorImager scan of a sequencing gel (25%) showing products obtained upon treatment of oligodeoxynucleotide duplex **5** (NNNN = TTTT) with dihydrothiophene **2** over increasing time (1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 45, 90, and 180 min). (B) Data obtained from the phosphorimager scan quantitated using ImageQuant software and plotted as a function of the percent of oligonucleotide cleaved *vs* time. The data were fit to a single exponential (Igor, Wavemetrics, OR) to obtain the rate of reaction at each site of cleavage: TCCT (●) and NNNN (○).

cleavage sites and, more accurately, to be determined by internal competition relative to a constant TCCT site. A representative run is shown below in Figure 3A for NNNN = TTTT. Analysis of the data by PhosphorImager and plotting the fraction of cutting at each site as a function of total radiolabeled DNA/lane *vs* time gave the well-behaved first-order appearance of cleavage products as shown in Figure 3B.

In each instance, the labeled oligonucleotide **5** was diluted with calf thymus DNA whose concentration was adjusted to achieve single-hit kinetics and >95% saturation.[22,30] The experiments were carried out in duplicate or triplicate for NNNN = TCCT, TTTT, CTCT, TCTC, and CCCC. The reactions were run for 3 h at −10 °C to fully expend the cleavage agent and allow accurate fits of the cleavage data to be computed. From these calculations the final extent of cleavage was determined from an estimated infinite time point. The results of these experiments are summarized in Table 1.

(30) It is assumed that the association constant for the TCCT sequence is *ca.* 10^6 (see text) and an average binding constant for calf thymus DNA is 10^5.

Table 1. First-Order Rate Constants for Cleavage within Oligonucleotide **5** on Reaction with Dihydrothiophene **2**

	TCCT Reference Site		Variable Site (NNNN)			
expt	10^4k (s^{-1})	extent of cleavage	NNNN	10^4k (s^{-1})	extent of cleavage	$\Delta\Delta G$ (kcal/mol)
1	7.3 ± 0.5	13%	TCCT	7.5 ± 0.7	6%	+0.5
2	8.1 ± 0.8	14%	TTTT	8.3 ± 0.8	1.2%	+1.3
3	8.6 ± 1.4	10%	CTCT	6.6 ± 1.6	1.3%	+1.2
4	8.6 ± 1.2	11%	TCTC	N.D.[a]	<0.3%	>+1.9
5	6.1 ± 0.6	15%	CCCC	N.D.	<0.1%	>+2.7

[a] For low extents of cleavage, rate data could not be determined (N.D.).

The data in Table 1 are striking on two accounts. First, despite experimental error, the absolute rates of DNA cleavage are notably similar irrespective of sequence. Within a run, agreement between the TCCT reference sequence and the NNNN variable test sequence is experimentally indistinguishable. Second, although the rates of cleavage are essentially

Table 2. First-Order Rate Constants for Cleavage within Oligonucleotide **6** on Reaction with Dihydrothiophene **2**

expt	sequence (site 1)	10^4k (s^{-1})	extent of cleavage	sequence (site 2)	10^4k (s^{-1})	extent of cleavage	$\Delta\Delta G$ (kcal/mol)
6	TCCT AGGA	5.7 ± 1.5	11%	TCCT AGGA	4.3 ± 0.9	13%	
7	TCCT AGGA	6.2 ± 0.3	10%	TCCT AGIA	7.0 ± 0.8	1.4%	+1.1
8	TCCT AGGA	7.4 ± 0.6	10%	TCCT AIGA	5.7 ± 0.4	22%	−0.4
9	TCCT AGIA	7.4 ± 1.3	1.4%	TCCT AGGA	7.0 ± 1.2	8%	+0.9
10	TCCT AIGA	6.2 ± 0.7	17%	TCCT AGGA	6.4 ± 0.7	11%	−0.4

identical, the extents of cutting are found to vary significantly, being particularly unfavorable in experiments 4 and 5 for TCTC and CCCC sequences. These results show clearly no kinetic differentiation among these polypyrimidine sites. Differences in the extents of cleavage must, therefore, correspond to differences in thermodynamic favorability of binding of the dihydrothiophene **2**. While **2** is both highly reactive and yet sufficiently long-lived, these observations strongly suggest that the binding equilibrium of **2** has been attained prior to significant Bergman rearrangement and DNA scission. That is, diffusion of **2** along the helix must be fast[31] relative to the rate of further chemical reaction to **3**, a diyl that abstracts DNA hydrogens exceedingly rapidly to initiate strand scission.[2] In conclusion, the thermodynamic determinants of dihydrothiophene binding to DNA control its local concentration and, hence, the appearance of site-selective cleavage by calicheamicin. In a less quantitative way, the importance of this effect has been recognized previously by Kahne.[32]

The role of equilibrium binding in these experiments makes possible the simple calculation of the free energy advantage or disadvantage ($\Delta G = -RT \ln K_{eq}$) relative to the internal TCCT sequence of these five possible arrangements of pyrimidine bases at NNNN. For example, in experiment 1, a 2-fold difference in the extent of cleavage exists between two TCCT sites. This discrimination in binding corresponds to about 0.5 kcal/mol that must owe to the effect of the different flanking sequences. In experiments 2−5, a steady decrease in association of **2** is seen between TTTT and CCCC, a quite unfavorable sequence for binding.

Role of the Aryl Iodide. At an early stage in understanding the interaction of calicheamicin with DNA it was proposed that the side-chain aryl iodide played a key role in the sequence selectivity of CLM cleavage of DNA.[3] Interaction of the aryl iodide with the C-2 amino groups of the d(GpG) step of a TCCT·AGGA sequence was accorded sufficient strength to be an important determinant of molecular recognition. Subsequently it was shown[4,5] that runs of thymines four bases and greater[6] confer sites of quite favorable cutting. Clearly, aryl iodide recognition of G·C pairs alone cannot be essential for good binding. As a variety of DNA sequences are bound and cut, other features of DNA structure must be primarily responsible for CLM binding and cleavage.[4−6,33,34] Nonetheless, calicheamicin is interesting among minor groove binding small molecules for selecting both A·T-rich and G·C-containing sites.

The dominant role of thermodynamic binding of dihydrothiophene **2** established above makes possible the evaluation of the free energy advantage gained or lost in the proposed aryl iodide interaction. Substitution of inosine successively for one guanosine and then the other replaces the 2-amino group with hydrogen in the AGGA recognition site of an otherwise unchanged duplex.[35−37] Using again the experimental approach of competition between two sites, the test oligonucleotide **5** was redesigned to **6** in order to make the sequences flanking the

site 1 site 2

5' CGCGCGCGGTCCTATGCGCGGTCCTATGC 3'
3' GCGCGCGCCAZYATACGCGCCAXWATACG 5'

6

reference TCCT site and the NNNN cassette more alike and enlarge their separation. The oligonucleotides incorporated two tetranucleotide sites, TCCT·AWXA and TCCT·AYZA, which were tested for calicheamicin cleavage. The two test sequences are based on the TCCT·AGGA site, but with nucleotides W, X, Y, or Z either guanine or inosine. This gives rise to a series of five oligonucleotides: a control molecule with both sites AGGA and four others with inosine incorporated at one of the guanines. This experimental design allows measurement of cleavage against an internal AGGA standard in each case.

As shown in Table 2, the extents of cleavage in the TCCT·AGGA control (experiment 6) were more nearly equal than seen earlier for **5** (Table 1, experiment 1). Inosine substitution was carried out in turn at each G residue and checked by reaction at both site 1 and site 2 relative to an internal TCCT·AGGA sequence. Normalizing the similar extents of cleavage (11% at site 1 and 13% at site 2, experiment 6) in the TCCT·AGGA control and computing a modified extent of cleavage readily led to a $\Delta\Delta G$ for binding of the dihydrothiophene at those sites as a function of inosine substitution. Excellent agreement can be seen in the paired experiments, *i.e.* experiments 7/9 and 8/10. In the latter pair, inosine substitution is favorable by 0.4 kcal/mol; that is, interaction of CLM with the 2-amino group of the 3′-guanosine is disadvantageous to binding, presumably owing to steric interference. In contrast, the adjacent guanosine 2-amino group in the 5′-direction in the 3′-AGGA-5′ cassette is significantly favorable to binding. Substitution with inosine results in a 6−7-fold decrease in dihydrothiophene affinity, or a relative stabilization for AGGA of approximately 1 kcal/mol.

A related inosine substitution experiment has been carried out but involving a more complex analysis. The latter difference in binding energy was deduced to be 2.3 kcal/mol between the iodinated and non-iodinated forms of the ether-linked carbohydrate side chain in the course of competitive binding/cleavage

(31) von Hippel, P. H.; Berg, O. C. *J. Biol. Chem.* **1989**, *264*, 675−678.
(32) Walker, S.; Landovitz, R.; Ding, W.-D.; Ellestad, G. A.; Kahne, D. *Proc. Nat. Acad. Sci. U.S.A.* **1992**, *89*, 4608−4612.
(33) Uesugi, M.; Sugiura, Y. *Biochemistry* **1993**, *32*, 4622−4627.
(34) Krishnamurthy, G.; Ding, W.; O'Brien, L.; Ellestad, G. A. *Tetrahedron* **1994**, *50*, 1341−1349.
(35) While to a first-order approximation the helix structure is unchanged by this substitution, inosine does alter duplex behavior in small but detectable ways (see refs 36 and 37).
(36) Xuan, J.-C.; Weber, I. T. *Nucleic Acids Res.* **1992**, *20*, 5457−5464.
(37) Reich, N. O.; Sweetnam, K. R. *Nucleic Acids Res.* **1994**, *22*, 2089−2093.

Table 3. Comparision of Thermodynamic Constants for CLMγ_1^I (**1**) and Netropsin with Synthetic Oligodeoxynucleotides

compd	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	log K (M^{-1})
CLM/12-mer	−8.17	−6.47	+1.7	6
netropsin/25-mer[a]	−10.50	−7.90	+2.6	8.1

[a] Values from ref 41.

Table 4. Comparision of Binding Constants for CLMϵ (**4**) with Oligodeoxynucleotides **6**

sequence (site 1)	$10^4 K_a$ (M^{-1})	$\Delta\Delta G$ (kcal/mol)
TCCT AGGA	6.3 ± 1.0	
TCCT AIGA	29.1 ± 2.3	−0.9
TCCT AGIA	1.9 ± 0.4	+0.7

experiments with CLMγ_1^I.[9] It is correctly noted that this value far exceeds that that can be attributed to a single hydrogen bond interaction. The present experiments make direct comparison to an unperturbed TCCT·AGGA site and place the magnitude of the aryl iodide/guanosine 2-amine interaction lower at approximately 1 kcal/mol. We believe that the internal competition method more accurately estimates the free energy contribution to complex formation and reflects hydrogen bonding as well as van der Waals interaction of the large, polarizable halogen in the minor groove. Energetically favorable interaction of the aryl iodide with the 5′-guanosine is consistent with the picture of CLM·DNA binding emerging from NMR studies.[11-13]

Calicheamicin Binding to DNA. Having estimated the free energy contribution of aryl iodide interaction in the minor groove within TCCT·AGGA, comparison was sought to the total binding affinity of the dihydrothiophene **2** to this sequence. However, the short half-life of **2** makes determination of its binding constant by direct methods problematic. Nonetheless presuming its complex with DNA (**2**·DNA) is sufficiently similar to that of CLMγ_1^I (**1**·DNA), nondestructive methods have been employed to measure a binding constant for the latter. CD titration of **1** with dodecamer **7** afforded the value $K_a = 9 \times 10^5$ M^{-1}.[34] This duplex provides a single cleavage site whose behavior has been thoroughly characterized in densitometry and atom transfer experiments.[18]

```
5' CCCGGTCCTAAG 3'
3' GGGCCAGGATTC 5'
```
7

We have determined the binding constant of CLMγ_1^I (**1**) with dodecamer **7** as well, but using isothermal titration calorimetry (ITC). The heat evolved during the stepwise addition of **7** to a solution of **1** was measured. The data were then fit to eq 5 (see the Experimental Section) using a nonlinear minimization protocol[38-40] (after correcting for the slope and intercept) to provide a binding curve. The advantage of this highly sensitive method, apart from giving the binding constant, is that the enthalpy of binding (ΔH) is also obtained. From these values, ΔG and ΔS of binding can be readily calculated. These thermodynamic constants (at 25 °C) are shown in Table 3 and compared with those recently described for netropsin and a synthetic duplex (at 5 °C).[41] For the present discussion it is important to note that two quite different physical methods when applied to the binding of CLMγ_1^I to dodecamer **7** give effectively the same result, $K_a = 10^6$.

A second comparison for dihydrothiophene **2** binding to DNA can be made by the use of CLMϵ (**4**), although electrocyclization of **2** and hydrogen transfer make this benzene derivative a less compelling model of the diynene in the minor groove (however, **4** is more similar to the very short-lived 1,4-diyl **3**). CD titration experiments using **4** and dodecamer **7** analogous to those carried out above for **1** gave $K_a = (5 \pm 2) \times 10^4$ M^{-1}.[34]

The hydroxyl radical footprinting method[42] has been used previously to compare the sites and intensities of CLMγ_1^I (**1**) cleavage in a DNA restriction fragment with those sequences protected by CLMϵ (**4**).[5] The footprinting method can be quantitatively extended to observe hydroxyl radical cleavage under identical conditions for a series of reactions in the presence of varying concentrations of CLMϵ.[43] The relative extent of protection can be measured within the binding site as a function of CLMϵ concentration. From the resulting saturation curve, a binding constant can be estimated. Oligonucleotide **6** was used containing two TCCT sites (sites 1 and 2) and two fully complementary AGGA sequences. A representative phosphorimage of a hydroxyl radical footprint titration of this duplex after polyacrylamide gel electrophoresis is shown in Figure 4. A relative decrease in intensity for all bands within the TCCT motif is discernable, particularly at the bottom of the gel (site 1) for both 3′-phosphate and 3′-phosphoglycolate ends corresponding to each fragment.[44]

The 5′-cytosine revealed the greatest change in intensity as the concentration of CLMϵ (**4**) increased. Therefore, this base in site 1 was selected to provide the protection data. These were quantitated using a PhosphorImager and fitted to eq 6 using a nonlinear, least-squares analysis (Figure 5). An association constant $K_a = (6.7 \pm 0.9) \times 10^4$ M^{-1} was obtained. Solving this equation in its rearranged linear form and fitting the data where CLMϵ is 10−90% bound (Figure 5, inset) gives a value of $(5.9 \pm 0.4) \times 10^4$ M^{-1}. Owing to the inherent experimental limitations, the errors associated with this method are larger than those obtained in the cleavage assays above. Nonetheless, this figure is in quite good agreement with that reported by CD titration.[34]

The titration experiments were repeated for oligonucleotide **6** where inosine (I) substitutions in the AZYA cassette were tested for their effect on CLMϵ binding. The results of these experiments for AZYA = 3′-AIGA-5′ and 3′-AGIA-5′ are shown in Table 4 and qualitatively parallel those in Table 2 based on the extent of reaction by the dihydrothiophene **2**.

In the event, nondestructive assays of CLMγ_1^I and CLMϵ affinity have been obtained using methodologically distinct techniques that show impressive agreement. CD titration[34] and isothermal titration calorimetry of CLMγ_1^I and dodecamer **7** under slightly different solvent conditions give values of $K_a = 9 \times 10^5$ and 1×10^6 M^{-1}, respectively. CLMϵ (**4**) gives $K_a = (5 \pm 2) \times 10^4$ M^{-1} by CD titration[34] with **7**, while hydroxyl radical titration footprinting gives $K_a = (6 \pm 1) \times 10^4$ M^{-1} for the TCCT-containing oligonucleotide **6**. Thus, the γ_1^I form of calicheamicin binds at this sequence approximately 15-fold better than the reduced form of CLM.

(38) Johnson, M. L. *Biophys. J.* **1983**, *44*, 101−106.
(39) Johnson, M. L. *Anal. Biochem.* **1985**, *148*, 471−478.
(40) Friere, E.; Mayorga, O. L.; Straume, M. *Anal. Chem.* **1990**, *62*, 950A-959A.
(41) Rentzeperis, D.; Marky, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 1645−1650.
(42) Dixon, W. J.; Hayes, J. J.; Levin, J. J.; Weidner, M. F.; Dombroski, B. A.; Tullius, T. D. *Methods Enzymol.* **1991**, *208*, 380−413.
(43) Dabrowiak, J. C.; Goodisman, J.; Kissinger, K. *Biochemistry* **1990**, *29*, 6139−6145.
(44) 3′-Phosphoglycolate cleavage products are typically observed in hydroxyl radical reactions with DNA and correspond to the faster migrating band in each fragment pair. Pogozelski, W. K.; McNeese, T. J.; Tullius, T. D. *J. Am. Chem. Soc.* **1995**, *117*, 6428−6433.
(45) Drak, J.; Iwasawa, N.; Danishefsky, S.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7464−7468.

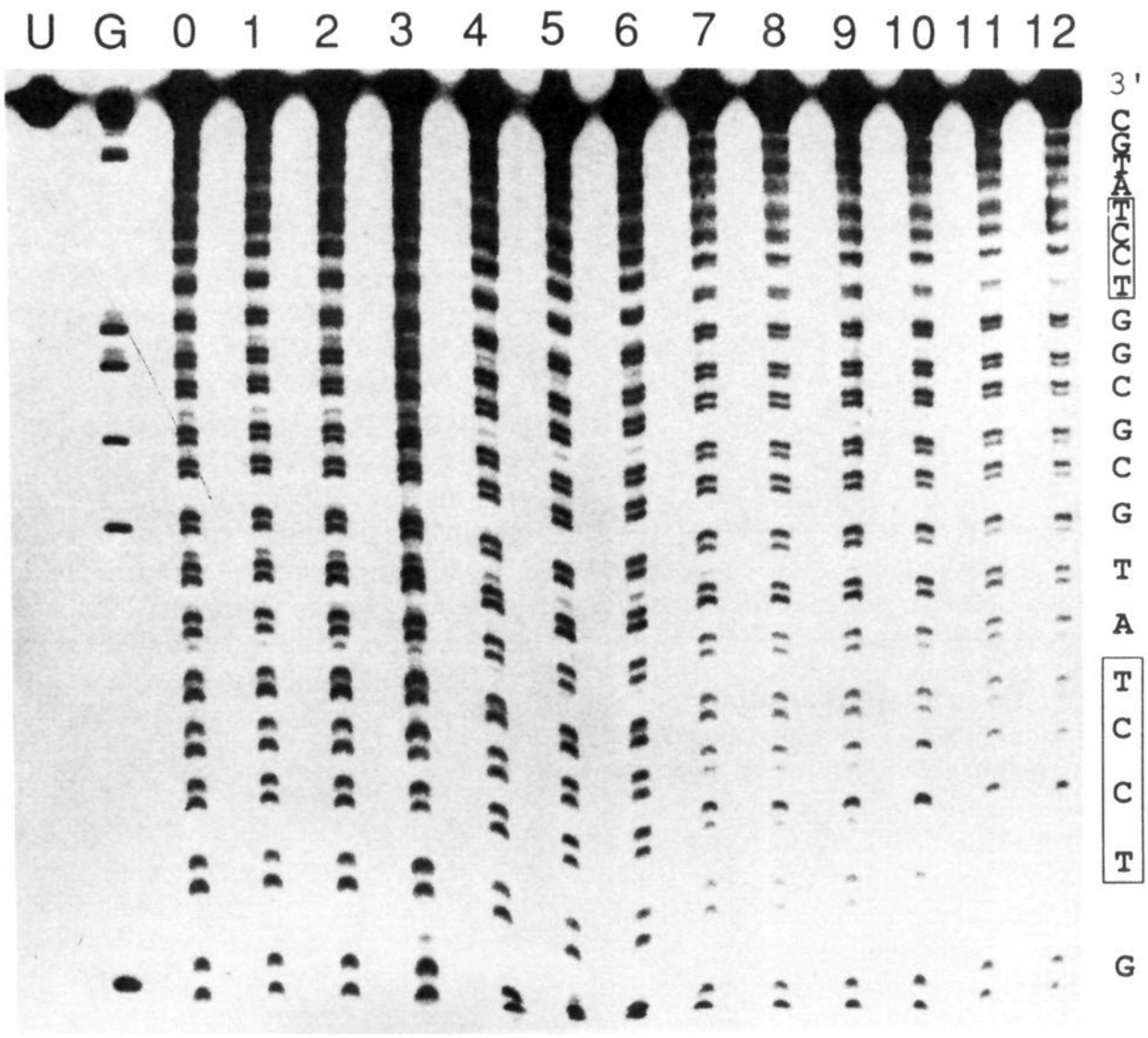


Figure 4. Representative phosphorimage of a DNA sequencing gel (25%) showing products of hydroxyl radical cleavage of oligonucleotide **6** (control AGGA sequence) that was bound to CLMϵ. Concentrations of CLMϵ used in the experiment are as follows: **U**, uncut DNA; **G**, Maxam–Gilbert guanine-specific reaction; 0, no CLMϵ; 1, 0.66 μM; 2, 1.32 μM; 3, 3.30 μM; 4, 4.95 μM; 5, 6.60 μM; , 13.2 μM; 7, 33 μM; 8, 49.5 μM; 9, 66 μM; 10, 220 μM; 11, 440 μM; 12, 660 μM CLMϵ.

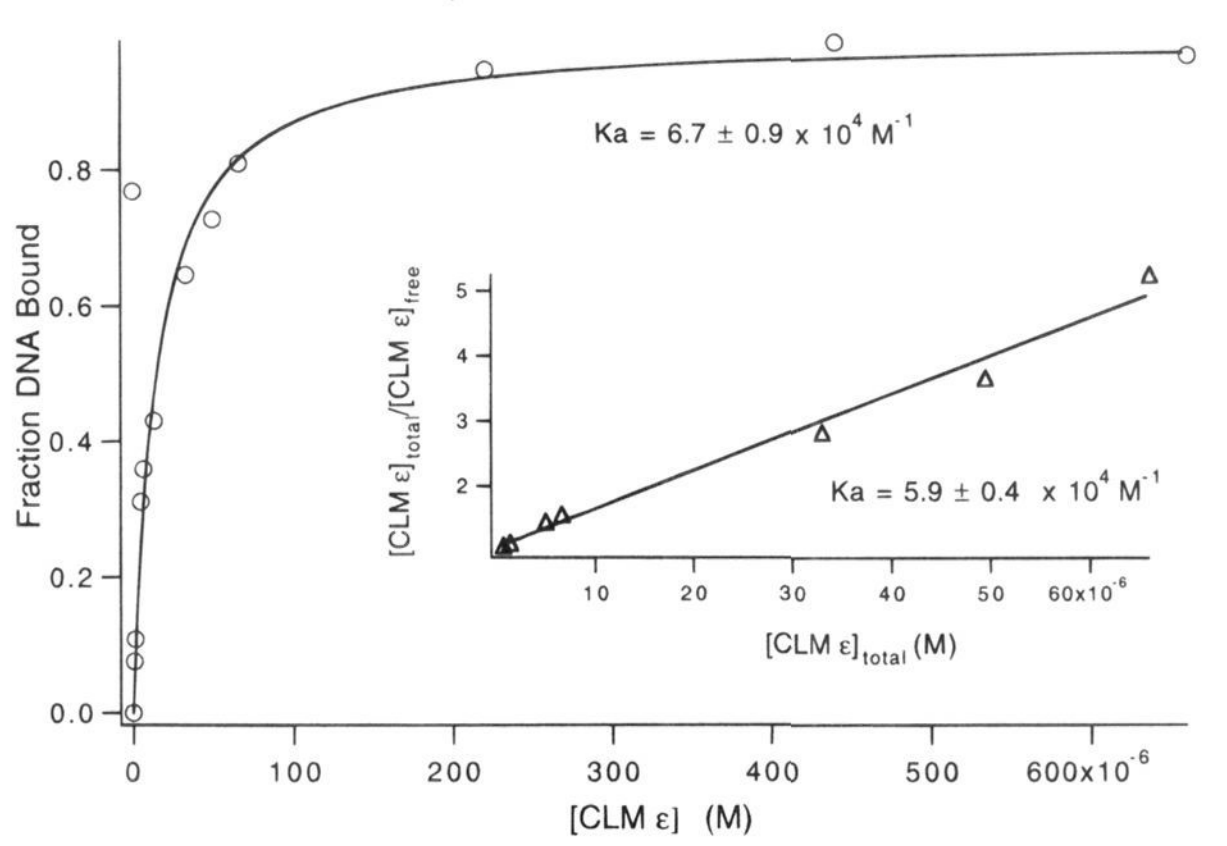


Figure 5. Binding curve generated for CLMϵ bound to duplex **6** (control AGGA sequence). The data were fit to eq 6 using Igor.

Several association constants for calicheamicin ranging from 10^6 to 10^8 M^{-1} have been determined in kinetic assays based on DNA cleavage.[45,46,9] It is recognized now that thiol activation of CLMγ_1^I is very complicated, involving several modes of thiolate attack on the allylic methyl trisulfide.[2,24] The major course of reaction (by several paths) results in mixed disulfide formation, a relatively stable intermediate. At least one minor reaction pathway, however, gives virtually instantaneously the allylic thiolate, which is known to give exceedingly rapidly the dihydrothiophene **2**. For the most part, this route likely accounts for the "burst kinetics" in the DNA cleavage experiments reported by Joyce *et al.*[9] The difficulty in extracting binding information from these experiments is that a considerable number of species is present in the early phases of reaction, many short-lived,[24] and the concentrations of each, how they partition between DNA and solution, and their rates of reaction on and off the helix are not known. The information gained in a cleavage assay with CLMγ_1^I is a composite of all these events. Nonetheless, the values trend higher than 10^6 M^{-1}, suggesting, but not proving, that the dihydrothiophene **2** may

(46) Ding, W. D.; Ellestad, G. A. *J. Am. Chem. Soc.* **1991**, *113*, 6617–6620.

exhibit stronger binding to DNA than the unactivated γ_1^I form of calicheamicin itself. It can be imagined that shortened distance between the acetylenic termini of the diynene and an overall reduced molecular volume on dihydrothiophene formation could favor DNA interaction. In the event, the free energy contribution of the aryl iodide to a correctly placed guanosine in a TCCT·AGGA motif is something less than one-eighth of the total for calicheamicin (for $K_a = 10^6\ M^{-1}$, $\Delta G = 8.16$ kcal/mol)

Experimental Section

All buffer and solutions used in the following sections were prepared as described by Sambrook, Fritsch, and Maniatis.[47] The cleavage buffer was 30 mM Tris·HCl/50 mM NaCl (pH 7.5). Tris(hydroxymethyl)-aminomethane (Tris), glutathione (reduced form), and calf thymus DNA were obtained from the Sigma Chemical Co. (St. Louis, MO). A stock solution of glutathione (100 mM) was made in cleavage buffer (30 mM Tris/50 mM NaCl (pH 7.5)) and the pH of the solution adjusted to 7.5 with Tris. CLMγ_1^I was a gift from the Lederle Research Laboratories and was used as a solution in the indicated concentrations in methanol. Tributylphosphine was obtained from the Aldrich Chemical Co. (Milwaukee, WI) and used without further purification as a 40 or 100 mM solution in methanol. Calf thymus DNA was dissolved in cleavage buffer by sonnication, filtered through a 0.45 μM filter, and diluted to attain a concentration of 1.3 mg/mL (27 OD/mL) as determined by the A_{260} (1 OD/mL = 50 μg).

UV−vis spectrophotometry employed a Beckman DU 70 spectrophotometer (Fullerton, CA). Radioactive measurements were made with a Beckman LS 5801 liquid scintillation counter using Opti-Fluor O scintillation cocktail. PhosphoImager analysis was performed using a Molecular Dynamics 300E PhosphorImager (Sunnyvale, CA) equipped with ImageQuant software. Sonnicaton was carried out with a Heat Systems-Ultrasonics, Inc. W225-R Sonicator (Plainview, NY).

Synthesis and Purification of Oligonucleotides. Oligonucleotides **5** and **7** were obtained in the "trityl-off" form from the Protein/Peptide/DNA facility at The Johns Hopkins University, School of Medicine, Department of Biological Chemistry, and were ^{32}P-labeled and purified using standard procedures.[47] Oligonucleotide **6** was synthesized on a Milligen Biosearch 8600 DNA Synthesizer using phosphoramidite chemistry. Reagents were purchased from Milligen with the exception of inosine, which was obtained from BioGenex (San Ramon, CA). [γ-^{32}P]ATP was bought from the Amersham Corp. (Arlington Heights, IL) as the triethylammonium salt at an activity of 3000 Ci/mmol. Enzymes were purchased from New England Biolabs (Beverly, MA) or Stratagene (La Jolla, CA).

The gels were run so that the percentage of polyacrylamide was 20 or 25 with 7 M urea as stated in the figures. Gels were 0.25 mm thick and were typically run for 3 h at 60 W. In the case of the BamHI-NarI restriction fragment from pUC19, a 6% polyacrylamide gel (7 M urea) was run for 90 min at 60 W.

Oligonucleotides were 5′-end-labeled with ^{32}P using the following method: 1 μL of oligonucleotide (15 pmol), 1 μL of 10X PNK buffer, 2 μL of doubly distilled water (dd H_2O), 5 μL of [γ-^{32}P]ATP (50 mCi), and 1 μL of T-4 polynucleotide kinase (New England Biolabs) were mixed in an Eppendorf tube. The reaction mixture was incubated at 37 °C for 0.5−1 h and applied to a gel after diluting into an equal volume of formamide loading buffer[47] and denaturing at 90 °C for 3 min. The position of the ^{32}P-labeled deoxyoligonucleotide was then determined by exposing a sheet of Kodak XAR5 X-ray film to the gel for 1 min. The labeled oligonucleotide was eluted overnight from a gel slice by the "crush and soak" method.[47] The solid was removed from the elution buffer by centrifugation, and the purified oligonucleotide was precipitated from the supernatant by the addition of sodium acetate (0.5 M final concentration) and ethanol (80% final concentration).

Formation of Duplex DNA. The labeled DNA strand was annealed to the desired complementary strand at a ratio of labeled DNA to unlabeled DNA of 1 to 10 in 10 mM NaCl. To anneal the complementary strands, the samples were heated to 70 °C for 10 min and slowly cooled to room temperature over 2−3 h. Each sample was diluted to achieve a radioactive count of *ca.* 10 000 cpm/μL. The samples were then stored at 4 °C for further use.

Dihydrothiophene Kinetics. Calicheamicin γ_1^I was prepared as a 40 μM solution in methanol. The DNA stock solution contained a trace amount of ^{32}P-labeled oligonucleotide **5**, **6**, or **7** in a solution of 0.5 mM calf thymus DNA in 10% methanol.

Eppendorf microcentrifuge tubes were prepared with each containing 5 μL of calicheamicin solution. The tubes were placed into a −78 °C bath, and the reaction was initiated at the appropriate time intervals with 5 μL of tributylphosphine solution (40 mM, precooled to −78 °C). Each tube was allowed to stand for 10 min, and the solution of dihydrothiophene **2** was transfered into the DNA stock (40 μL, 10 000 cpm) solution at −10.0 ± 0.1 °C (maintained by a Lauda RM6 constant temperature circulating bath). The reactions were stopped consecutively at 1−180 min intervals by the addition of 400 μL of ethanol at −10 °C, followed by rapid mixing and placement into a −78 °C bath. Each tube was allowed to stand for at least 1 h at −78 °C. The DNA was pelleted by centrifugation at −5 °C for 20 min. The ethanol was decanted and the pellet lyophilized to dryness. Each pellet was dissolved in 3.5 μL of formamide loading buffer, heated in a 90 °C block for 1 min, rapidly cooled in an ice bath, and loaded onto a denaturing polyacrylamide gel.

After running and drying the gel, it was placed on to a phosphorimager plate overnight. The cleavage data were analyzed using the Ellipse-Volume determination method on the Molecular Dynamics PhosphorImager using the ImageQuant software. In each lane of the gel, the volume of the uncleaved DNA band and each cleavage band was determined. In addition, the background value was determined for an equal volume near each band. The percentage of cleavage for each band was established by dividing the volume of the band of interest by the total volume of all the bands in that lane (after correction for background of each band). The resulting data (time versus the percentage of cleavage) were fit to a single exponential using Igor (Wavemetrics, Portland, OR). From this plot, the rate constant for dihydrothiophene cyclization and the extent of cleavage at each site (at infinite time) were determined.

Calculations of $\Delta\Delta G$:

$$\Delta G_1 = -RT \ln K_{\text{eq (site 1)}} \quad \text{and} \quad \Delta G_2 = -RT \ln K_{\text{eq (site 2)}}$$

$$\Delta\Delta G = \Delta G_2 - \Delta G_1 = -RT \ln\{K_{\text{eq (site 2)}}/K_{\text{eq (site 1)}}\} \quad \text{where}$$

$$\{K_{\text{eq (site 2)}}/K_{\text{eq (site 1)}}\} = [\text{site 2·CLM}][\text{site 1}]/[\text{site 1·CLM}][\text{site 2}]$$

The concentrations of CLM bound at site 1 and site 2 were determined by the extents of cleavage at infinite time (expressed as a percentage) at sites 1 and 2, respectively. The concentrations of free site 1 and site 2 were then calculated as (100 − %cleaved) at each site. It should be noted that the concentration of free CLM in solution is identical for both sites.

Reaction of NarI-BamHI Restriction Fragment with Calicheamicin. Preparation of Radiolabeled Restriction Fragment. A 267 base pair NarI-BamHI fragment from pUC19 was ^{32}P 5′-labeled and isolated using standard procedures.[47] Plasmid pUC19 (10 μL, 10 μg) was cleaved using BamHI (10 U, Stratagene), and the DNA precipitated by the addition of sodium acetate (5.6 μL, 3 M) and 400 μL of cold ethanol. The 5′-phosphate was removed by treatment with calf intestine alkaline phosphatase (CIAP, 1.2 μL, 12 U, New England Biolabs) and labeled at the 5′-end using [γ-^{32}P]ATP and polynucleotide kinase. The DNA was once again pelleted as described above and treated with NarI (2 U) prior to loading on a 6% nondenaturing polyacrylamide (29:1) gel for purification. The gel (20 × 32 cm) was run for 2 h at 200 V and exposed to Kodak XAR5 X-ray film for 30 s. Two major bands were apparent: the slower running was the 2.4 kb fragment and the faster was the desired 267 base pair fragment. The latter was purified by the crush and soak procedure to yield 200 μL of purified restriction fragment having a radioactivity of 4000 cpm/μL.

Cleavage of the Bam I-Nar I Restriction Fragment. Four solutions were prepared for the cleavage reaction: 40 μM CLM in methanol, 100 mM tributylphosphine in methanol, 100 mM glutathione in cleavage buffer, and a DNA stock solution consisting of 40 μL of

(47) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, A Laboratory Manual*; Cold Spring Harbor Press: Plainview, NY, 1989.

a 0.5 mg/mL calf thymus DNA, 40 μL of the pUC19 fragment (160 000 cpm), 20 μL of cleavage buffer, and 20 μL of methanol.

The CLM solution (5 μL) was placed in each of three 1.5 mL Eppendorf tubes. One tube was placed in a −78 °C bath, and 10 μL of the tributylphosphine solution was added. After 10 min at −78 °C, the tube was transferred to the −10 °C bath and 30 μL of the DNA solution and 10 μL of cleavage buffer (pre-equilibrated at −10 °C) were immediately added. The second tube was placed in the −10 °C bath, and 30 μL of the DNA solution, 5 μL of methanol, and 10 μL of cleavage buffer were added. The mixture was allowed to stand for 10 min at −10 °C, and 5 μL of the tributylphosphine solution (also at −10 °C) was added. To the third tube were added 30 μL of the DNA solution and 10 μL of MeOH. The mixture was placed at −10 °C, and 10 μL of glutathione was added. In each case, the reaction was allowed to proceed for 3 h. To each tube was added 750 μL of ethanol at −10 °C. After being vortexed vigorously, the tubes were placed in a −20 °C freezer overnight and the precipitated DNA was isolated by centrifugation, the ethanol was decanted, and the pellet was dried. At the same time, the 267 base pair fragment from pUC19 was subjected to the Maxam−Gilbert G-specific reaction and a hydroxy radical reaction, both of which were performed under the conditions previously described.[47,42] All reactions were run on a 32 × 20 cm vertical 6% denaturing gel at 60 W for 1.5 h. The gel was then dried (60 min, 80 °C) and quantitated by phosphorimager analysis using ImageQuant software.

Isothermal Titration Calorimetry. Calorimetric experiments were performed using an Omega Titration Microcalorimeter (Microcal Inc., Northampton, MA). A Keithley 181 nanovoltmeter was used as a preamplifier to improve the signal to noise ratio of the instrument. The reaction cell (volume 1.365 mL) was filled with a 20 μM solution of CLM in buffer (70% Tris/30% methanol), and a series of 10 μL injections of DNA duplex **7** (160 mM in duplex)[48] solution were made (total of 20 injections). Analysis of the data was performed using software developed in the Biocalorimetric Center at The Johns Hopkins University, Department of Biology, as described previously.[38-40]

The heat absorbed or released for each injection is given by

$$q = V\Delta H\Delta[\mathrm{DNA_b}] \quad (1)$$

where q is the heat associated with the change in bound DNA concentration, $\Delta[\mathrm{DNA_b}]$ is the change in bound DNA concentration, ΔH is the enthalpy of binding, and V is the reaction volume. The total cumulative heat for this phenomenon, Q, is then given by

$$Q = V\Delta H\Sigma\Delta[\mathrm{DNA_b}] = V\Delta H[\mathrm{DNA_b}] \quad (2)$$

where $[\mathrm{DNA_b}]$ is the concentration of bound DNA. The concentration of bound DNA can also be given by

$$[\mathrm{DNA_b}] = [\mathrm{CLM}]\{nK[\mathrm{DNA}]/1 + K[\mathrm{DNA}]\} \quad (3)$$

where [DNA] is the concentration of DNA, [CLM] is the concentration of free CLM, K is the binding constant, and n is the number of binding sites. By substituting eq 3 in eq 2, we get

$$Q = V[\mathrm{CLM}]\{n\Delta HK[\mathrm{DNA}]/1 + K[\mathrm{DNA}]\} \quad (4)$$

Since the independent variable is total DNA concentration, $[\mathrm{DNA_t}]$, and $[\mathrm{DNA_t}] = [\mathrm{DNA}] + [\mathrm{DNA_b}]$, by substituting in eq 4, we get

$$Q^2(K/V\Delta H) + Q(-1-[\mathrm{CLM}]nK - K[\mathrm{DNA_t}]) + ([\mathrm{CLM}]V\Delta HnK[\mathrm{DNA_t}]) = 0 \quad (5)$$

The data were then fit to eq 5 (keeping the number of binding sites as $n = 1$) in order to to generate the values for K and ΔH. It was necessary in our experiments to provide for an intercept and slope correction since the first injection always showed an abnormally high heat associated with it. In order to do so, the data from a reference experiment (DNA duplex into buffer not containing CLM) were first fitted to a polynomial. This provided the slope and intercept correction for the reference.

All samples were prepared in an *identical* fashion. Cleavage buffer (70%) was mixed with spectroscopic grade MeOH (30%), adjusted to pH 7.50, and filtered through a 0.45 mM sterile filter. DNA duplex **7** was prepared in cleavage buffer by mixing the two strands in equimolar quantities,[48] heating to 90 °C, and slowly cooling to room temperature (4−6 h). The duplex was then precipitated in 80% ethanol and dried. The duplex was resuspended in the cleavage buffer/MeOH mixture and once again reannealed by heating to 70 °C and slowly cooling to room temperature. The concentration of the duplex was determined by measuring its absorbance at 260 nm and diluting to the required concentration with the buffer/MeOH mixture.

Hydroxyl Radical Footprint Titration. The conversion of CLMγ_1^I to CLMϵ and hydroxyl radical footprinting of the CLMϵ/DNA complex were performed as previously described.[5] The concentration of CLMϵ was determined by UV spectroscopy (ϵ at 280 nm = 4850 M^{-1} cm^{-1}).

Analysis and Quantitation of the DNA Sequencing Gels. Products of hydroxyl radical footprinting experiments were separated by 25% polyacrylamide gel electrophoresis. The dried sequencing gels were then exposed to phosphorimaging plates to quantitate the amount of radioactivity corresponding to each band on the gel using ImageQuant software. Hydroxyl radical cleavage of 5′-end-labeled DNA results in two visible products for attack at every nucleotide position when resolved under these conditions. The faster-migrating band of each pair contains a 3′-phosphoglycolate end resulting from C-4′ hydrogen abstraction by the hydroxyl radical.[44] Therefore, the 3′-phosphoglycolate band corresponding to the 5′-cytosine within the TCCT cleavage site was integrated for the calculation of the binding isotherms.

A "region" was drawn around the phosphoglycolate band described in the preceding section. Identical regions were used for each lane of the sequencing gel. The volume integrals were determined. The background was calculated by integrating the volume of the selected "region" in an area outside the lanes of the gel. To normalize for loading error within each lane and for differences in levels of hydroxyl radical cleavage, the volume integral of the bands corresponding to the unbound sequence CGC between the two TCCT sites was determined. The percent difference between the normalized integral of the phosphoglycolate band for each lane relative to that in the absence of CLM (lane 0, Figure 4) was calculated.

To obtain the binding constant, K_a, the data were fit to eq 6 using Igor, where x is the concentration of CLMϵ and y is the fraction of DNA bound.

$$y(x) = [(xK_a)/(xK_a + 1)] \quad (6)$$

Acknowledgment. We are grateful to Lederle Laboratories for providing a sample of calicheamicin γ_1^I and to the NIH for financial support (CA54421). We are pleased to acknowledge use of the PhosphorImager maintained by the Institute for Biophysical Research on Macromolecular Assemblies at Johns Hopkins, which is supported by an NSF Biological Research Centers award (DIR 8721059) and a grant from the W. M. Keck Foundation. We thank Marina Kasimova and the Biocalorimetry Center in the Department of Biology, a Biomedical Resource Technology Research Center sponsored by the NIH (P41 RR044328) at Johns Hopkins, and Jodie Franklin and Peter Kebbekus for synthesis of the oligonucleotides used. Dr. P. J. Smith is thanked for his critical evaluation of the data and his comments during preparation of the manuscript.

JA950130L

(48) Fasman, G. D. *CRC Handbook of Biochemistry and Molecular Biology*; CRC Press: Boca Raton, FL, 1975; p 589.