Kinetic *vs* Thermodynamic Determinants in the Sequence Selectivity of DNA Cleavage by Calicheamicin

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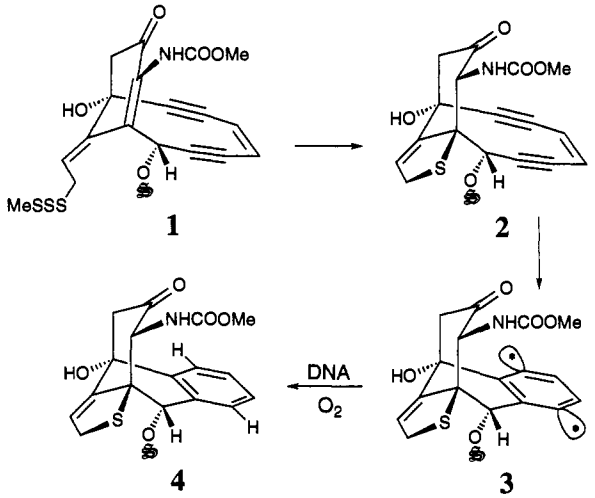
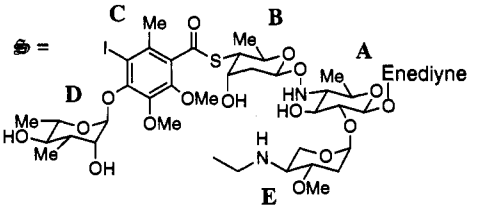
The appreciable half-life of dihydrothiophene **2** (4.5 ± 1.5 s in MeOH at 37 °C) formed upon activation of calicheamicin γ_1^1 (CLMγ_1^1, **1**) suggested that this intermediate may be responsible for the notable sequence selectivity of DNA cleavage by this drug.[1] In addition, the electrocyclic nature of the Bergman rearrangement[2] of **2** to the 1,4-diyl **3**, the key, highly reactive agent that initiates DNA cleavage, gave rise to the idea that encounter of dihydrothiophene **2** with variations in DNA structure (*e.g.*, minor groove narrowing, bending, or kinking) could lead to favored cutting.[1] That is, steric compression at certain sites, for example, could be envisioned to lower the activation energy of this rearrangement and, hence, accelerate cutting at these sequences. Support for this kinetic view of recognition and cleavage could be argued from the behavior of structurally simpler cometabolites of **1** and esperamicin A_1, which showed lower affinity for DNA but unchanged sequence selectivity of scission.[3,4]





However, DNA cutting experiments with the racemic aglycone of CLM[5] and CLM T (analogous to **1** but containing only sugars A and E)[6] and footprinting experiments with the calicheamicin side chain lacking the enediyne[7,8] clearly point to the importance of the aryl-linked carbohydrate to site selectivity of cleavage. The contribution of thermodynamic binding effects implied in these experiments to cleavage selectivity was more tangibly revealed in hydroxyl radical footprinting studies of CLMϵ (**4**), where sequences protected mirrored those cleaved, despite differences in concentration between the two experimental protocols.[9] In this paper we compare directly the rates of DNA cleavage by the dihydrothiophene at a variety of sequences and establish that interaction of **2** with the DNA helix provides little or no kinetic component to the site selection of cleavage.

The role of the dihydrothiophene **2** as the kinetically significant species responsible for the site-selective recognition and cleavage of DNA was demonstrated by comparing the sequence selectivity and relative cleavage intensities of fragments resulting from reaction of a *Bam*HI-*Nar*I restriction fragment of the plasmid pUC18 with **1** by reduction to that treated with the dihydrothiophene **2** itself. The latter was prepared by treatment of **1** in methanol (37 μM, −78 °C) with an equal volume of 3.7 mM tributylphosphine in methanol.[1] After 10 min, the solution of **2** was transferred to a −10 °C bath, and to it was added a 5-fold excess volume of ^{32}P-labeled restriction fragment and calf thymus DNA (830 μM in base pairs) at a concentration to ensure single-hit statistics.[10] In each case, the reaction was allowed to proceed for 2 h. The reactions were quenched by the addition of an excess of ethanol, and the precipitated DNA was examined by gel electrophoresis and autoradiography. While a variety of sites gave rise to strand cutting, it is important to note that the cleavage patterns under the two conditions appeared identical. This apparent identity was confirmed quantitatively by densitometry[11] of the resolved portions of the autoradiogram as shown in Figure 1.

To examine the effect of sequence on the rate of DNA cleavage by the dihydrothiophene **2**, hence the rate of electrocyclization of **2** to **3**,[1] an oligodeoxynucleotide was designed containing two preferred cleavage sites to allow comparisons to be accurately made by internal competition. The well-studied TCCT/AGGA motif[12] was chosen as one site to serve as an internal control, while the other, NNNN, was varied to give a run of four pyrimidines.[6,9,13] The principal sites of cleavage in the synthetic 20-mer **5** are shown in bold:

5′ ^{32}P-CCCGG T**C**CT ATCG N**N**NN AAG 3′
3′ CGGCC AGGA TAGC MMMM TTC 5′

5

The dihydrothiophene **2** (20 μM) was prepared as above and diluted 5-fold into a solution of the 5′ ^{32}P-end labeled oligonucleotide **5** (NNNN = TCCT, TTTT, CTCT, TCTC, CCCC)

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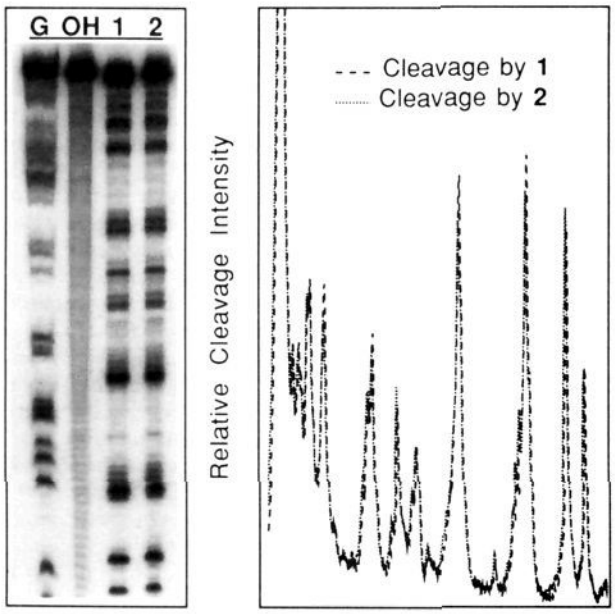

Figure 1. Autoradiogram of a DNA sequencing gel (8%) showing products obtained upon treatment of a 267 base pair 5′ ^{32}P-end labeled *Bam*HI-*Nar*I restriction fragment of plasmid pUC18 with Maxam–Gilbert G-specific reaction[20] (**G**), hydroxyl radical cleavage[21] (**OH**), 3.7 μM calicheamicin γ_1^I, with 3.7 mM tributylphosphine (**1**), and 3.7 μM dihydrothiophene (**2**) in 30% MeOH/70% 30 mM Tris, pH 7.50, 50 mM NaCl. An overlay of one-dimensional scans of lanes **1** and **2** from the gel is shown alongside.

and calf thymus DNA at –10 °C. Concentration of the latter was adjusted to achieve single-hit kinetics. Reaction mixtures (calf thymus DNA 2 mM in base pairs, final volume 50 μL, 30% MeOH/70% 30 mM Tris, pH 7.50, 50 mM NaCl) were quenched with ethanol at various time points, and the extent of cleavage was quantitated by gel electrophoresis and PhosphorImager analysis.[14] Determination of the percent cleavage per site as a function of time fit smoothly to the first-order appearance of fragments.[15] The results of these experiments are summarized in Table 1.

The identity of sites and relative intensities of DNA cleavage by CLM (**1**) activated *in situ* and by dihydrothiophene **2** prepared independently (Figure 1) establishes that the latter is the species responsible for the appearance of site-selective cleavage by the drug. This view is in keeping with the rate of dissociation of **1** from a drug–oligonucleotide complex measured by NMR methods[16] (*ca.* 3/s at 25 °C), which is well within the lifetime of the dihydrothiophene[1] (presuming the DNA exchange behavior of **2** is similar to **1**). However, the marked similarity of the measured rate constants (Table 1) irrespective of sequence context is striking despite substantial differences in the extents of cleavage

Table 1. First-Order Rate Constants for Cleavage Within Oligonucleotide **5** on Reaction with Dihydrothiophene **2**[a]

sequence (TCCT)	$k \times 10^4$ s^{-1}	extent of cleavage, %	sequence (NNNN)	$k \times 10^4$ s^{-1}	extent of cleavage, %
TCCT	7.3 ± 0.5	13	TCCT	7.5 ± 0.7	6
TCCT	8.1 ± 0.8	14	TTTT	8.3 ± 0.8	2
TCCT	8.6 ± 1.4	10	CTCT	6.6 ± 1.6	1
TCCT	8.6 ± 1.2	11	TCTC	ND	<0.3
TCCT	6.1 ± 0.6	15	CCCC	ND	<0.1

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

as NNNN is varied.[17] For example, even in the first experiment, the same TCCT cassette embedded in dissimilar flanking sequences experiences a 2-fold difference in cutting efficiency. The ordering of four pyrimidine bases NNNN = CTCT *vs* TCTC results in significantly altered cleavage facility. Finally, cutting in the homopyrimidine NNNN = C_4 is sharply disfavored relative to NNNN = T_4. Therefore, interaction of the dihydrothiophene with DNA imparts minimal, if any, kinetic enhancement to the rearrangement of **2** to **3** and, consequently, to strand scission despite variations in cleavage efficiency that can be very great.

As the rates of cleavage at several DNA cutting sites remain essentially unchanged, the appearance of differing intensities of cleavage must be largely, if not entirely, governed by the relative (and different) binding affinities of the activated drug at these sequences. The experiments here show that species to be the dihydrothiophene **2**, whose high chemical reactivity, yet sufficiently long lifetime, suggests that equilibrium binding has been achieved before hydrogen abstraction and DNA cleavage significantly occurs. This kinetic view that DNA interaction does not significantly affect the rate of electrocyclization of **2** to **3** is in accord with the structural view of the interaction of **1** with DNA deduced by NMR.[16] It is the duplex that accommodates the drug rather than the reverse, affirming the view that DNA is neither a static nor an invariant structure. Departures from classical B form DNA are well recognized from X-ray structures, and these conformational changes can be quite dramatic on complexation with proteins.[18] The absence of measurable effect on the rate of electrocyclization and cleavage by dihydrothiophene **2** suggests that deformation of the host rather than of the guest occurs and is driven largely, if not entirely, by the thermodynamics of binding. This dynamic behavior of DNA is further manifest in CD[10b,19] and footprinting/cleavage[9,13] experiments which show that binding occurs most favorably at those sites capable of deformation to accommodate good fits to the drug.

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(14) ImageQuant software resident on a Model 400E, Molecular Dynamics, Sunnyvale, CA.

(15) IGOR, Wavemetrics, Portland, OR. First-order rate constants are reported as the weighted averages ± standard error for three runs of NNNN = TCCT, two runs of all other duplexes. Minimal cleavage (<1%) was observed elsewhere in the test oligonucleotides.

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