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DNA Alkylation Properties of the Duocarmycins: (+)-Duocarmycin A, *Epi*-(+)-Duocarmycin A, *Ent*-(-)-Duocarmycin A and *Epi,Ent*-(-)-Duocarmycin A

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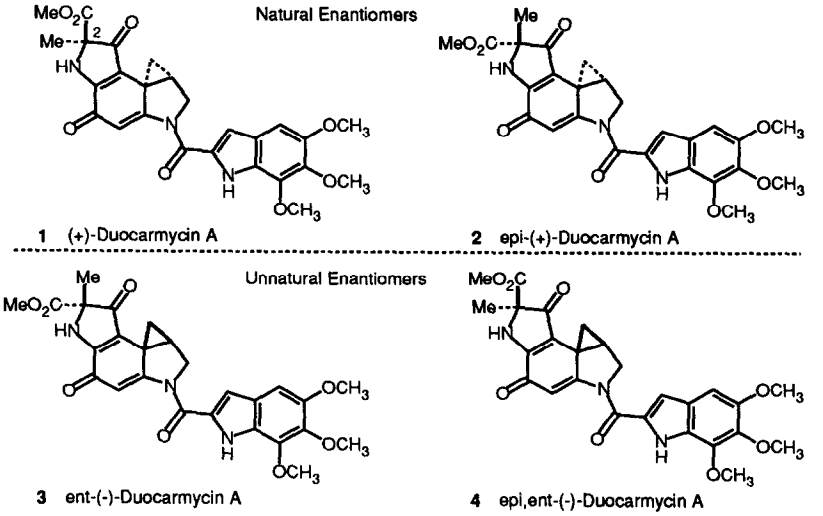
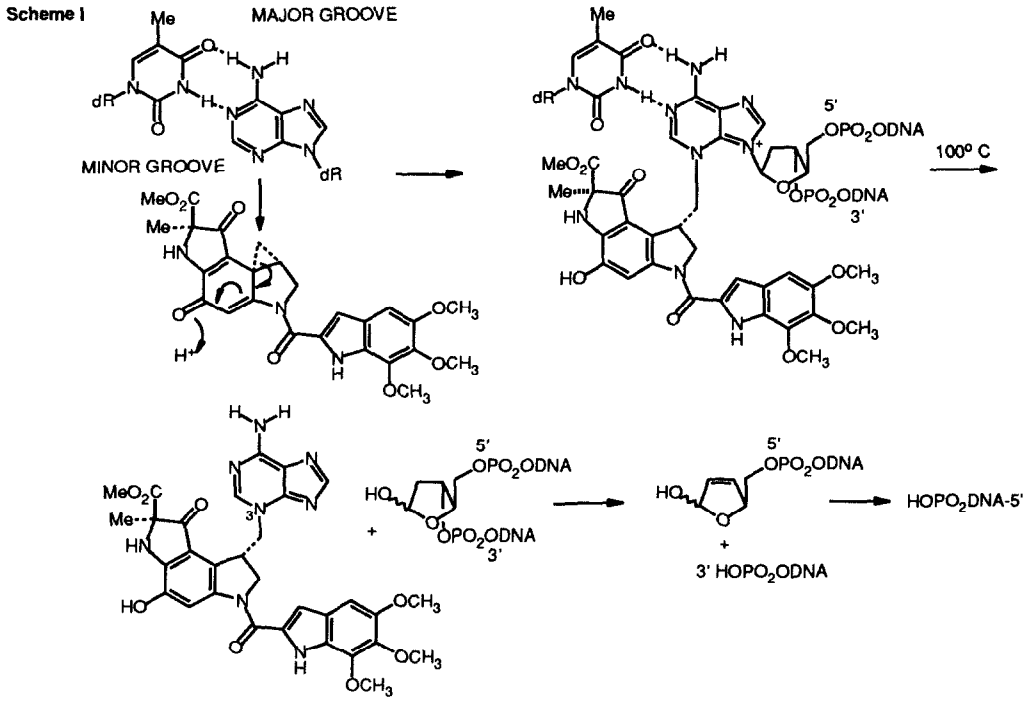
Abstract. A study of the comparative in vitro cytotoxic activity and DNA alkylation properties of both enantiomers of the two diastereomers of (+)-duocarmycin A are detailed. The DNA alkylation efficiency and in vitro cytotoxic potency of the natural enantiomers ((+)-duocarmycin A > *epi*-(+)-duocarmycin A, 3-8x) exceed those of the unnatural enantiomers (*ent*-(-)-duocarmycin A, *epi,ent*-(-)-duocarmycin A) by at least 100x.

In recent efforts, the independent isolation and identification of (+)-duocarmycin A (**1**)[1,2] and pyrindamycins A and B[3] have provided the initial members of a new class of exceptionally potent antitumor antibiotics. In initial studies, the relative and absolute stereochemistry of pyrindamycin A (duocarmycin C_2) was unambiguously established in a single-crystal X-ray structure determination[3] and through chemical correlation has provided the relative and absolute stereochemical assignments for the full class of agents. At the time of the disclosure of the agents, we initiated efforts to demonstrate the event[4] and selectivity[5] of duplex DNA alkylation by the duocarmycins which provided support for the proposal that their biological properties are derived from their irreversible DNA alkylation. In these studies, the thermally-induced strand cleavage of double-stranded DNA after exposure to the agents was employed to demonstrate the event and sites of minor groove adenine N-3 alkylation and to define the DNA alkylation selectivity.[5,6] The subsequent isolation and unambiguous structural characterization of the duocarmycin - adenine adduct derived from the thermal DNA depurination reaction along with the quantification of its formation (86-92% based on **1**) established the predominant DNA alkylation reaction as that which proceeds in the minor groove with adenine N3 addition to the unsubstituted cyclopropane carbon of duocarmycin A,[7-9] Scheme I. Herein, we detail the results of a comparative study of natural (+)-duocarmycin A (**1**) with its enantiomer *ent*-(-)-duocarmycin A (**3**)[10] as well as *epi*-(+)-duocarmycin A (**2**)[10] and *epi,ent*-(-)-duocarmycin A (**4**)[10] conducted in efforts to establish the relative importance of the structural and stereochemical features of **1**.



1 (+)-Duocarmycin A
2 epi-(+)-Duocarmycin A
3 ent-(-)-Duocarmycin A
4 epi,ent-(-)-Duocarmycin A



DNA Alkylation Properties. The DNA alkylation properties of **1-4** were examined within the w794 and w836 clones of M13mp10 containing complementary SV40 nucleosomal DNA[6,11] since comparative data was available for the natural duocarmycins from our earlier studies.[4-6,8-9] Following our earlier protocol, the two singly 5′ ^{32}P-end-labeled segments of duplex DNA derived from clones w794 and w836 were treated with the four agents under a range of concentration and incubation conditions (4°C and 25°C, 24 - 48 h). Following incubation, the unbound agent was removed by ethanol precipitation of the DNA, the isolated DNA-agent complexes were redissolved in buffer, and warmed to 100°C (30 min) to induce strand cleavage at the sites of alkylation. Electrophoresis of the resulting DNA under denaturing conditions adjacent to Sanger dideoxynucleotide sequencing reactions followed by autoradiography permitted the identification of the sites of DNA alkylation.

Natural Enantiomers: (+)-Duocarmycin A and *epi*-(+)-Duocarmycin A. The natural agent (+)-**1** and its C2 epimer possessing the natural absolute configuration of the cyclopropane, *epi*-(+)-duocarmycin A (**2**), were found to effectively alkylate DNA with the same profile of selectivity within w794 and w836 duplex DNA, Figures 1 and 2. A summary of the observed alkylation selectivity and the resultant definition of the high affinity consensus sequence for alkylation is provided in Figure 3. Notably, this summary of duocarmycin alkylation selectivity is derived from a statistical treatment[5,8] of the data which includes a consideration of the sites *not* alkylated by the agents and readily detects the distinctions between high and low affinity alkylation sites.[8] Although the profile of DNA alkylation selectivity is not distinguishable for **1** and **2**, the efficiency of DNA alkylation by the two agents differ. At identical concentrations, natural or synthetic (+)-duocarmycin A exhibits an intensity of DNA alkylation that is 3-6x greater than that of *epi*-(+)-duocarmycin A. As detailed latter herein, this corresponds nicely to the observation that (+)-duocarmycin A is 3-8x more potent than *epi*-(+)-duocarmycin A in cytotoxic assays. The relative efficiency of DNA alkylation could be readily quantitated by scanning densitometry of the autoradiograms. The relative efficiency of DNA alkylation by the two agents did not differ significantly under alternative incubation conditions (4°C and 25°C, 2 - 48 h) nor did they appear to exhibit a distinguishable time dependency. Under the conditions detailed in the legends of Figures 1 and 2 the alkylation reactions appear to have proceeded to completion and no apparent change was observed with longer reaction periods.

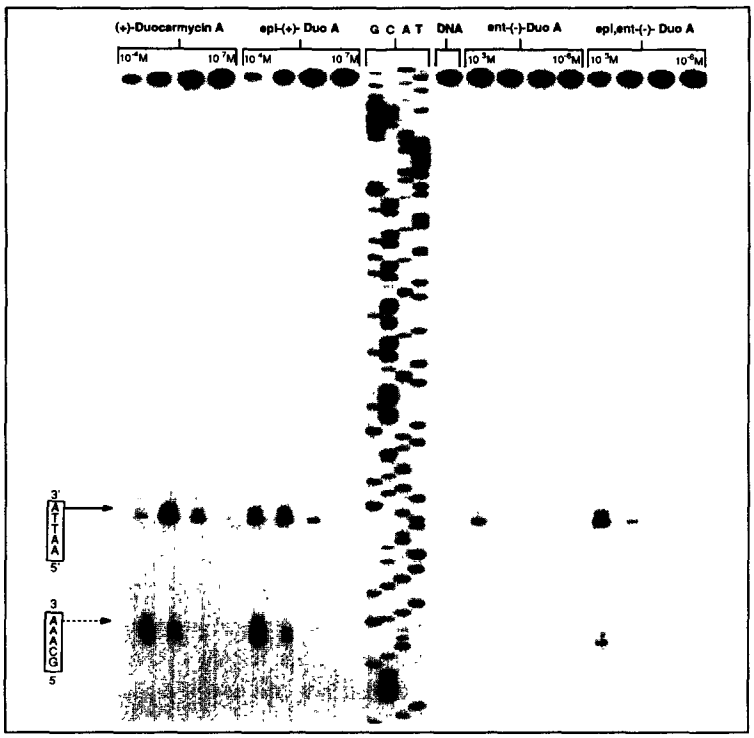


Figure 1. Thermally induced strand cleavage of double-stranded DNA (SV40 DNA fragment, 144 bp; nucleotide no. 5238-138, clone w794) after 24 h incubation of agent-DNA at 25°C followed by removal of unbound agent and 30 min incubation at 100°C; 8% denaturing polyacrylamide gel and autoradiography. Lanes 1-4, (+)-duocarmycin A (1 x 10^{-4}M to 1 x 10^{-7}M); lanes 5-8, *epi*-(+)-duocarmycin A (1 x 10^{-4}M to 1 x 10^{-7}M); lanes 9-12, Sanger G, C, A and T reactions; lane 13, control DNA; lanes 14-17, *ent*-(-)-duocarmycin A (1 x 10^{-3}M to 1 x 10^{-6}M); lanes 18-21, *epi,ent*-(-)-duocarmycin A (1 x 10^{-3}M to 1 x 10^{-6}M).

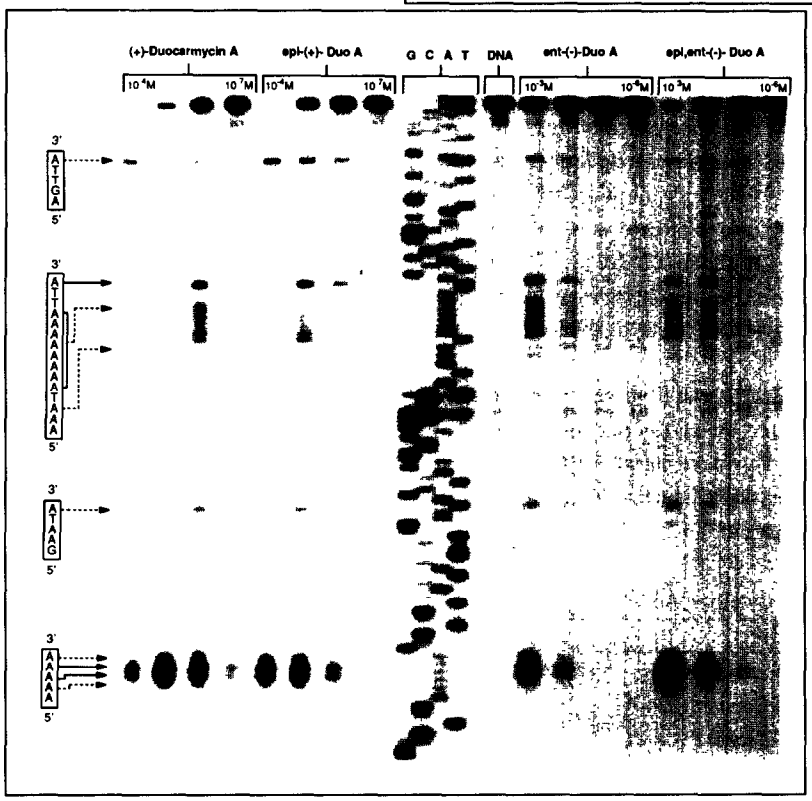
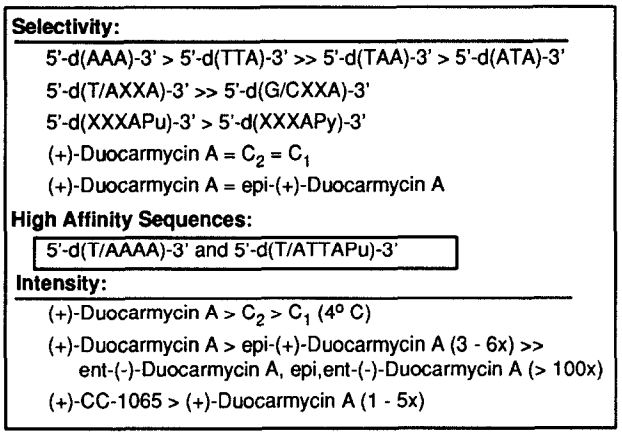


Figure 2. Thermally induced strand cleavage of double-stranded DNA (SV40 DNA fragment, 145 bp; nucleotide no. 5189-91, clone w836) after 24 h incubation of agent-DNA at 4°C followed by removal of unbound agent and 30 min incubation at 100 °C; 8% denaturing polyacrylamide gel and autoradiography. Lanes 1-4, (+)-duocarmycin A (1 x 10^{-4}M to 1 x 10^{-7}M); lanes 5-8, *epi*-(+)-duocarmycin A (1 x 10^{-4}M to 1 x 10^{-7}M); lanes 9-12, Sanger G, C, A and T reactions; lane 13, control DNA; lanes 14-17, *ent*-(-)-duocarmycin A (1 x 10^{-3}M to 1 x 10^{-6}M); lanes 18-21, *epi,ent*-(-)-duocarmycin A (1 x 10^{-3}M to 1 x 10^{-6}M).

Figure 3 Duocarmycin DNA Alkylation Properties



Unnatural Enantiomers: *ent*-(-)-Duocarmycin A and *epi,ent*-(-)-Duocarmycin A. The two agents possessing the unnatural absolute configuration of the cyclopropane, *ent*-(-)-duocarmycin (**3**) and *epi,ent*-(-)-duocarmycin (**4**), failed to display effective DNA alkylation properties. Only at concentrations of approximately 100x that of the natural enantiomers did observable DNA alkylation become detectable and did so with a profile of selectivity that is not distinguishable from that of the natural enantiomers. Thus, this apparent behavior of the unnatural enantiomers most likely may be attributed to the trace contaminant natural enantiomer (≤1%) necessarily present in the resolution samples of **3** and **4**. Consistent with this assumption was the observation that the intensity of the alkylation by the unnatural enantiomers did not increase upon longer or more vigorous incubation conditions (*i.e.* 25°C, 48 h) confirming that the relative efficiencies of DNA alkylation observed between the natural and unnatural enantiomers are not due to simple differences in the inherent rate of DNA alkylation.

The lack of effective DNA alkylation properties of the duocarmycin unnatural enantiomers is comparable to the observations made with CPI-CDPI$_1$ and CPI-PDE-I[12] and related aborted analogs[13] of (+)-CC-1065 in which it is only the natural enantiomers of the agents which exhibit efficient DNA alkylation properties and potent cytotoxic activity. Similarly, the unnatural enantiomer of additional related agents containing a short DNA binding subunit composed of a single indole-based segment generally have been found to exhibit a significantly less efficient DNA alkylation than the corresponding natural enantiomer (*i.e.* CBI-CDPI$_1$, *ca.* 10-100x)[14] and to proceed with a profile of alkylation selectivity that is readily distinguishable from that of the natural enantiomers (*i.e.* CBI-CDPI$_1$, CI-CDPI$_1$).[14,15] However, this is in contrast to the observations made with agents that possess longer or more effective DNA binding subunits (*i.e.* CC-1065, CPI-CDPI$_2$,[12,13] CI-CDPI$_2$,[15] and CBI-CDPI$_2$[14]) in which both enantiomers of the agents have been found to exhibit effective and comparably efficient DNA alkylation properties and potent cytotoxic activity.

In Vitro Cytotoxic Assay. Consistent with the quantitative trends observed in the relative DNA alkylation efficiency, (+)-duocarmycin A proved to be 3-8x more potent than *epi*-(+)-duocarmycin A in a range of cytotoxic assays, Table I. Both the natural enantiomers **1-2** were significantly more potent that the two unnatural enantiomers **3-4** and the cytotoxic activity of the unnatural enantiomers was not distinguishable from that which may be attributed to the trace contaminant natural enantiomers potentially present in the resolution samples of **3-4**.

Table I Cytotoxic Activity (IC_{50}, ng/mL)

	Agent	L1210	P388[10]	B16
1	(+)-Duocarmycin A	0.1	0.002	0.2
2	epi-(+)-Duocarmycin A	0.8	0.007	0.8
3	ent-(-)-Duocarmycin A	> 11	≥ 0.3	> 3
4	epi,ent-(-)-Duocarmycin A	> 12	≥ 0.3	> 3

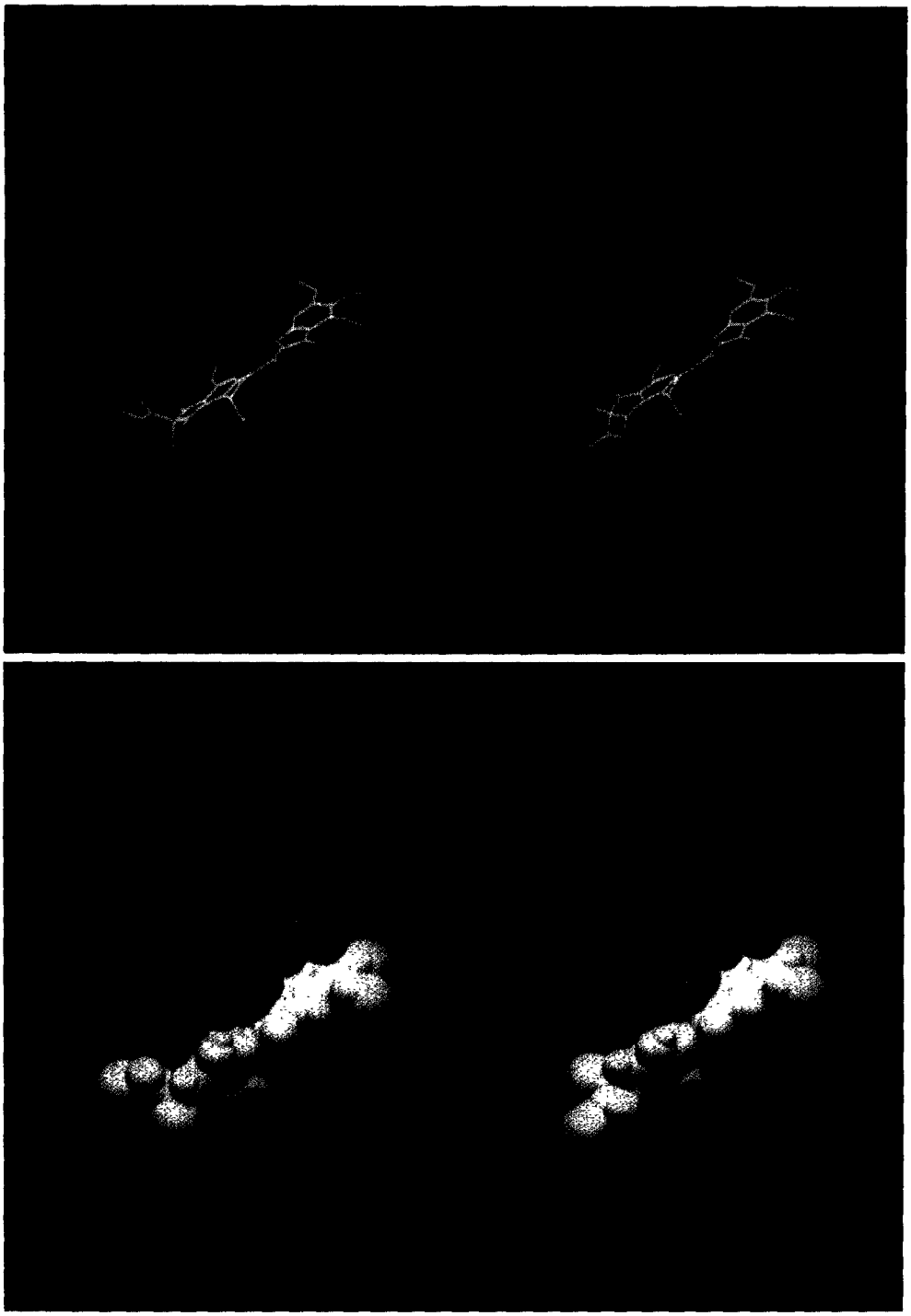


Figure 4. Comparison stick and space filling models of (+)-duocarmycin A and *epi*-(+)-duocarmycin binding within the high affinity site of w794 duplex DNA; left = 5'd(CTCAATTAGTC):(+)-duocarmycin A, right = 5'd(CTCAATTAGTC):*epi*-(+)-duocarmycin A. The complexes were generated with MacroModel (Version 2.5, AMBER force field) supplemented with parameters for the agents derived from the X-ray crystal structure of duocarmycin C_2, see reference 9.

Model of the Duocarmycin DNA Alkylation. In earlier studies, we detailed a model for the duocarmycin DNA alkylation that was based on the observed sequence preference that clearly extends in the 5′ direction from the minor groove adenine N3 alkylation site, the unambiguously established absolute configuration of the agents, and the confirmed structure of the duocarmycin-adenine N3 adduct. In this model, the hydrophobic concave face of the agent is tucked deeply in the minor groove, the polar functionality of the agent lies on the outer face of the complex, the bound conformation of the agent complements the topological curvature and pitch of the minor groove, and the agent binding spans 3.5 base-pairs. Notably, the 3′ adenine alkylation site and the 3′→5′ binding directionality of the agents in the minor groove is consistent with the established absolute configuration of the agents. As detailed elsewhere,[5,8-9] we attribute the alkylation selectivity in part to the noncovalent binding selectivity of the agents preferentially within the narrow, sterically more accessible A-T rich DNA minor groove and the 3.5 base-pair binding site size required to permit full agent binding in the minor groove. This nicely accommodates the experimentally observed strict A-T selectivity required of the first three base-pairs that extends less rigidly to include the fourth base-pair. Illustrated in Figure 4 are the comparative models of the (+)-duocarmycin A and *epi*-(+)-duocarmycin A alkylation binding within the single high affinity alkylation site of w794 duplex DNA based on this model. Apparent from the model and consistent with the observations, the bound alkylation subunit of *epi*-(+)-duocarmycin A required the adoption of a slightly less stable conformation due to a small destabilizing steric interaction of the C2 α-carbomethoxy group with minor groove deoxyribose backbone of the alkylated strand of the duplex DNA.

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