The Duocarmycins: Synthetic and Mechanistic Studies

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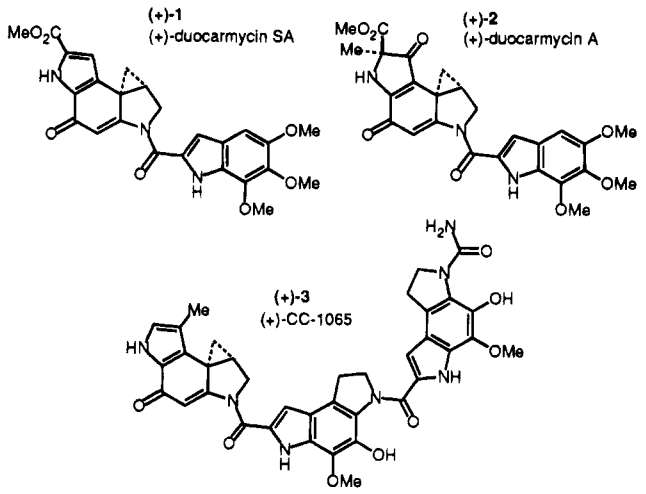
Two recent efforts have described the isolation and structure determination of the initial members of a new class of exceptionally potent antitumor antibiotics including duocarmycins SA (**1**),[1,2] A (**2**),[3–5] B_1–B_2,[6] and C_1–C_2.[4,5,7,8] Given their remarkable potency and structural similarity to (+)-CC-1065 (**3**)[9–16] and its related analogs,[13–22] substantial efforts have been devoted to defining their properties.[22–48] In these



studies, the agents have been shown to exert their biological effects through a sequence selective alkylation of DNA.[26–31] The reversible,[30] stereoelectronically-controlled[34] adenine N3 addition to the least substituted cyclopropane carbon has been found to occur within selected AT-rich sites in the minor groove of DNA,[27,31] and extensive efforts have been devoted to determining the origin of the DNA alkylation selectivity, to establishing the link between DNA alkylation and the ensuing biological properties,[32,40] and to defining the fundamental principles underlying the relationships between structure, chemical reactivity, and biological activity. Herein, we provide a summary of studies conducted with the intention of addressing these important questions of functional reactivity and molecular recognition.

Total Synthesis of Duocarmycin SA (1) and Duocarmycin A (2). Despite the interest in the duocarmycins and their remarkable properties, successful efforts on their synthesis[42–48] and the extension of the technology to related agents have been limited.[21,26,27,31,33–35] This is especially surprising with duocarmycin SA (**1**) since it, unlike **2**, was isolated in insufficient quantity to permit a detailed evaluation of its properties. Moreover, the limited studies conducted with naturally derived **1** revealed a combination of chemical and biological properties that make it the most exciting of the natural products identified to date.[2] In addition to lacking the fatal toxicity characteristic of (+)-CC-1065,[49] **1** is the most stable and most potent member of this class of agents. As a

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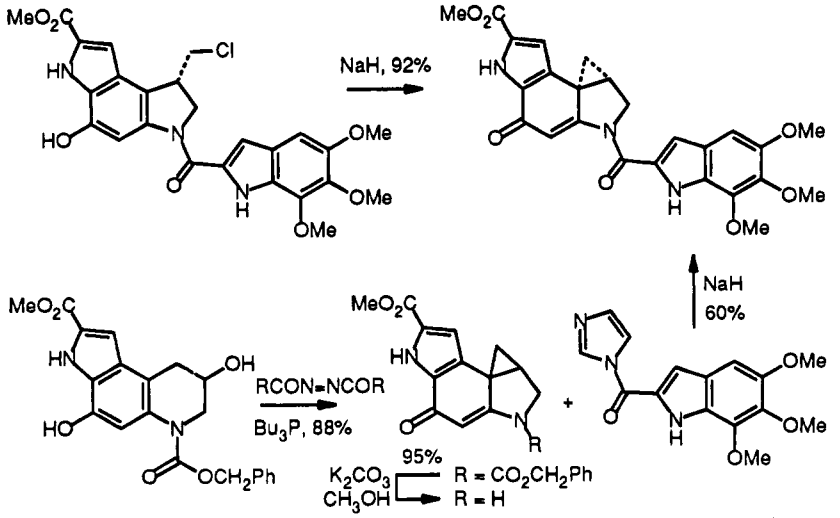
Scheme 1. Spirocyclization Reactions Employed in the Boger (Top) Synthesis of (+)- and *ent*-(−)-Duocarmycin SA (1992) and in the Natsume (Bottom) Synthesis of Racemic Duocarmycin SA (1994)



result of our prior studies,[13,15,20,22] we could anticipate that this combination of properties is not fortuitous, but rather the enhanced chemical stability is directly responsible for this increased biological potency.

The initial total synthesis of **1**[42] not only provided sufficient material to fully assess its properties[31,43] but also provided its unnatural enantiomer and key optically active partial structures. Their examination resulted in the unusual observation that the unnatural enantiomer also constitutes an effective DNA alkylating agent and potent antitumor antibiotic and has led to the emergence of a detailed model of the duocarmycin structural and functional features responsible for their selective alkylation of DNA.[31]

In addition to the strategic distinctions inherent in the approaches to **1** and **2**,[42–48] the two reported syntheses of duocarmycin SA (Scheme 1) and duocarmycin A (Scheme 2) employ the complementary external and internal Winstein Ar-3′ alkylations[50] for introduction of the activated cyclopropane. Moreover, each of these efforts has served to advance the field in complementary ways. The initial nondiastereoselective synthesis of **2** provided both *d,l*- and *d,l*-6-*epi*-duocarmycin A,[47] which through diastereomeric derivatization and chromatographic resolution later provided both enantiomers of the two diastereomers. The comparative examination of these four agents,[39] like that of (+)- and *ent*-(−)-duocarmycin SA (**1**),[31] proved important. The recent synthesis of duocarmycin A (**2**)[45] addressed the distereoselective introduction of the remote stereocenters with control of their absolute configuration and represents the first example of a useful asymmetric synthesis generally applicable to **1–3**.

DNA Alkylation Selectivity. Shortly following the disclosure of (+)-duocarmycins A, B_1–B_2, and C_1–

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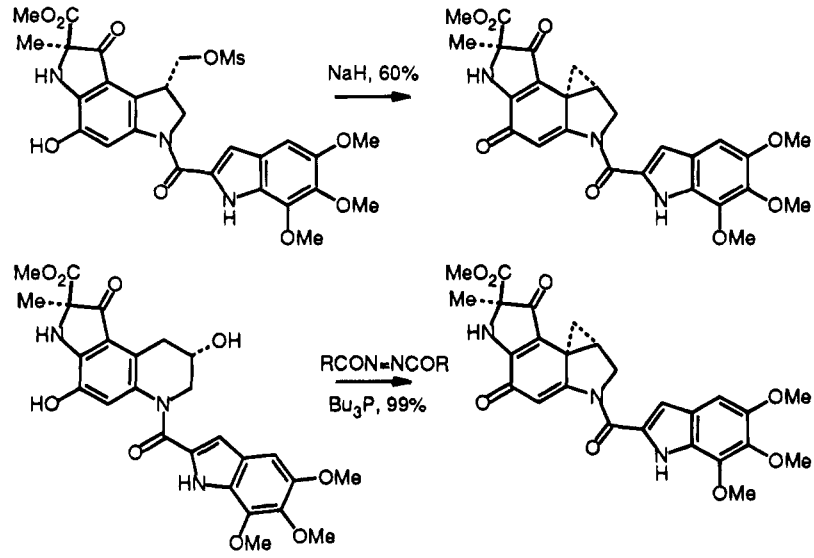
Scheme 2. Spirocyclization Reactions Employed in the Terashima (Top) Synthesis of Racemic and 6-*epi*-Duocarmycin A (1990) Resolved into the Individual Enantiomers (1992) and in the Boger (Bottom) Asymmetric Synthesis of (+)-Duocarmycin A and Its Isomers



C_2, the event,[26,36] sequence selectivity,[27] quantitation,[28] reversibility,[30] and structure determination[28] of their predominant DNA alkylation reaction were defined using natural material. Similarly, a full study of the (+)-duocarmycin SA DNA alkylation reaction[30,31] and its comparison with that of (+)-**2** was detailed along with that of key partial structures and their unnatural enantiomers with use of synthetic material.[43]

The alkylation site identification and the assessment of the relative selectivity were derived through thermally-induced depurination and strand cleavage of labeled DNA after exposure to the agents (Scheme 3).[18] The alkylation selectivity for (+)-duocarmycin A (**2**)[27] and (+)-duocarmycin SA (**1**)[31] proved nearly indistinguishable. Each alkylation site detected was found to be adenine flanked by two 5′ A or T bases, and there proved to be a preference for this three-base sequence: 5′-AA<u>A</u> > 5′-TT<u>A</u> > 5′-TA<u>A</u> > 5′-AT<u>A</u>. In addition, a strong preference but not absolute requirement for the fourth 5′ base to be A or T versus G or C was detected and proved to distinguish the high- versus low-affinity sites. An additional weak preference for a purine versus pyrimidine at the 3′ base preceding the adenine alkylation site was detected and was prominent only in the low- versus high-affinity sites. Table 1 summarizes the consensus sequence derived from the evaluation of (+)-**1** and (+)-**2**.[27,31] Although each agent alkylated the same sites in DNA, two important distinctions were defined. The least reactive of the agents, duocarmycin SA (**1**), was found to alkylate DNA with both a greater efficiency (10–100×) and a greater selectivity among the available alkylation sites: (+)-duocarmycin SA (**1**) > (+)-duocarmycin A (**2**).[31]

ent-(−)-Duocarmycin SA (**1**) was found to alkylate DNA but only at concentrations approximately 10× that required for (+)-**1**. Consequently, the natural enantiomer of **1** proved to be 10× more effective than the unnatural enantiomer. Table 2 summarizes the consensus alkylation sequence for *ent*-(−)-duocarmycin SA.[31] Each alkylation site detected was determined to be adenine, and essentially each alkylation site was flanked by a 5′ and 3′ A or T base which exhibited the following sequence preference: 5′-A<u>A</u>A > 5′-A<u>A</u>T > 5′-T<u>A</u>A > 5′-T<u>A</u>T. An additional strong preference for the second 3′ base from the alkylation site to be A or T versus G or C was detected. In this regard, the *ent*-(−)-duocarmycin SA alkylation proved analogous to that of the natural enantiomer with the exception that the binding orientation is reversed (5′ → 3′) over an AT-rich 3.5 base pair site. However, while the bound conformation of the natural enantiomer covers an AT-rich 3.5 base pair site extending from the adenine N3 alkylation site in the 3′ → 5′ direction across the adjacent two to three 5′ bases (*i.e.*, 5′-AAA<u>A</u>), the AT-rich 3.5 base pair site for the unnatural enantiomer extends in the reverse 5′ → 3′ direction starting at the first 5′ base site preceding the adenine N3 alkylation site and extending across the alkylation site to the first and second adjacent 3′ bases (*i.e.*, 5′-<u>A</u>AAA). The reversed binding orientation is required to permit adenine alkylation at the least substituted carbon of the cyclopropane, and the offset AT-rich alkylation selectivity is the natural consequence of the diastereomeric relationship of the adducts.

DNA Alkylation Reaction: Adenine N3 Alkylation. The initial studies established that the duocarmycins alkylate adenine within the minor groove. Since the thermally-induced cleavage of DNA employed to identify the alkylation sites only detects adducts susceptible to thermal glycosidic bond cleavage (adenine N3 and guanine N3 or N7 alkylation), the occurrence of additional alkylation reactions would not be detected under such conditions. Thus, the quantitation of the adenine N3 alkylation, confirmation of its structure through isolation and characterization of the thermally-released adduct, and the search for undetected alkylation sites were conducted.[28,31] In these studies, the adenine N3 alkylation illustrated in Scheme 3 by (+)-duocarmycins A and SA has been found to quantitatively account for 86–92% and >90% (90–100%), respectively, of their consumption in the presence of excess DNA and to constitute the near exclusive alkylation event under such conditions. Similarly, the thermally-released adenine adduct of (−)-duocarmycin SA was found to account for 92% of its consumption in the presence of excess DNA.[31] The characterization of the adducts led

Scheme 3

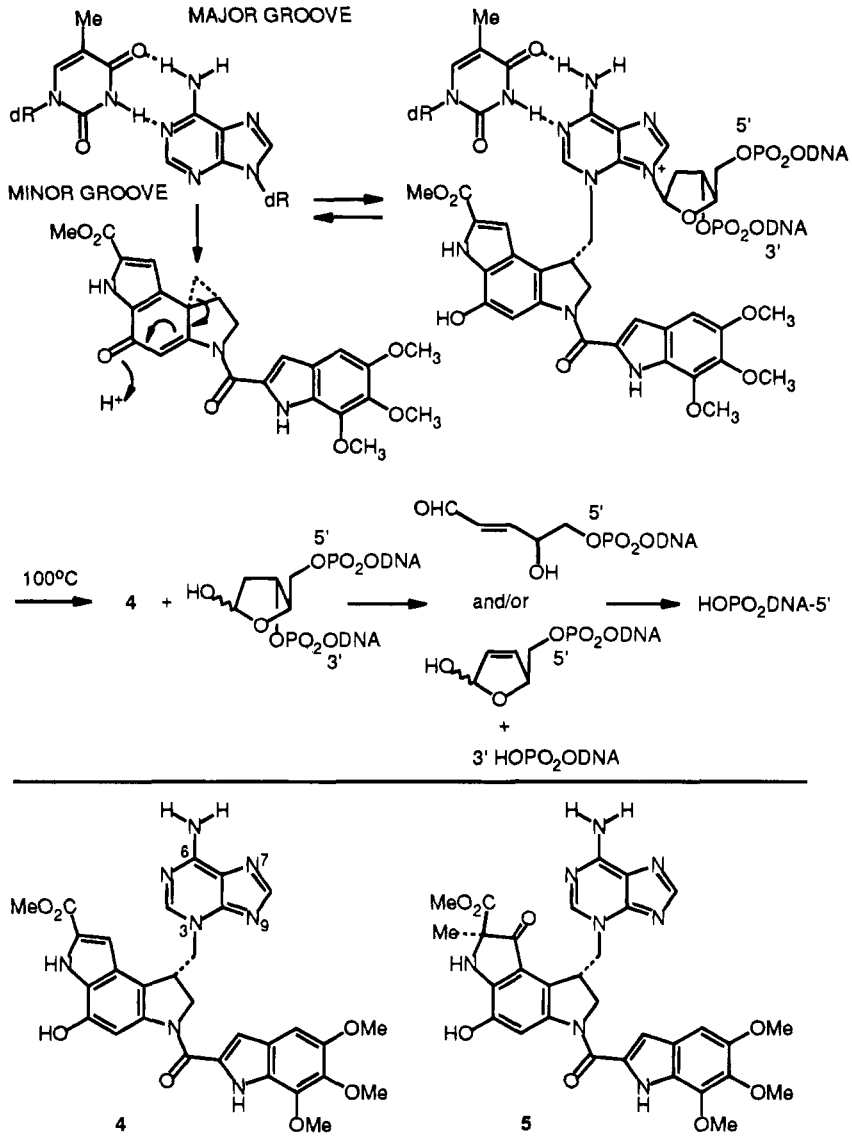


Table 1. Consensus Sequence for the (+)-Duocarmycin A and SA DNA Alkylation

base[a]	3	2	1	0	−1	3′
A (30)[b]	57	67	71	100	51	
T (26)	22	33	29	00	18	
G (21)	13	00	00	00	20	
C (23)	08	00	00	00	11	
A/T (56)	79	100	100	100	69	
Pu (51)					71	
consensus	A/T > G/C	A/T	A/T	A	Pu	

[a] Percentage of the indicated base located at the designated position relative to the adenine N3 alkylation site. [b] Percentage composition within the DNA examined.

Table 2. Consensus Sequence for the *ent*-(−)-Duocarmycin SA DNA Alkylation

base[a]	1	0	−1	−2	−3	3′
A (30)[b]	70	100	77	53	35	
T (26)	23	00	19	20	21	
G (21)	00	00	00	17	26	
C (23)	07	00	04	10	18	
A/T (56)	93	100	96	73	56	
consensus	A/T	A	A/T	A/T > G/C	N	

[a] Percentage of the indicated base located at the designated position relative to the adenine N3 alkylation site. [b] Percentage composition within the DNA examined.

to the unambiguous assignment of the structures **4**[31] and **5**[28] in which adenine N3 addition to the unsubstituted cyclopropane carbon of duocarmycin SA and A was established.

Adenine N3 versus Trace Guanine N3 Alkylation. Under conditions of limiting agent, both duocarmycins SA and A provide predominantly or exclusively adenine N3 adducts (≥90%).[28,31] However, a minor guanine N3 alkylation has been detected with duocarmycin A but only upon isolation of thermally-released adduct following treatment of DNA with excess agent,[38] within oligonucleotides lacking a high-affinity adenine N3 alkylation site,[38] or when the adenine alkylation sites within AT-rich regions of DNA were protected from alkylation with high-affinity AT-rich minor groove binding agents including distamycin.[39] In contrast, the less reactive duocarmycin SA showed no evidence of guanine N3 alkylation when subjected to similar or more forcing conditions.[31] Even under vigorous conditions, unreacted (+)-**1** was recovered and the selectivity for adenine:guanine N3 alkylation was determined to be >25:1. This enhanced selectivity of **1** may be attributed to its decreased reactivity. Notably, under the relevant conditions of limiting agent even duocarmycin A provides near

exclusive adenine N3 alkylation, and since duocarmycin SA is much more potent than duocarmycin A, the minor guanine N3 alkylation cannot be uniquely relevant to the expression of the biological properties and may represent a nonproductive competitive event.

Alkylation Site Models That Accommodate the Reversed Binding Orientation and Offset AT-Rich Selectivity of the Enantiomeric Agents. The characterization of **4** and **5**, the absolute configuration of the agents, and the identification of the consensus sequences for both enantiomers of **1** provided the necessary information for the construction of accurate models of the adenine alkylation reaction. Figure 1 illustrates models of the (+)- and *ent*-(−)-duocarmycin SA alkylation at a common site in w794 DNA, 5′-(CTAATT), which constitutes a high-affinity site for the unnatural enantiomer and a minor site for the natural enantiomer.[31] In both instances, the hydrophobic face of the agent is embedded deeply in the minor groove, the polar functionality lies on the outer face of the complex, and the helical bound conformation of the agent complements the topological curvature and pitch of the minor groove spanning an AT-rich 3.5 base pair site. For (+)-duocarmycin SA, the binding spans 3.5 base pairs starting with the 3′ adenine alkylation site and extends in the 3′ → 5′ direction over the two to three adjacent 5′ base pairs (5′-CTAA). For *ent*-(−)-duocarmycin SA, the binding similarly spans a 3.5 base pair AT-rich site which, however, necessarily starts at the 5′ base adjacent to the alkylation site and extends in the 5′ → 3′ direction over the alkylation site and the one to two adjacent 3′ base pairs (5′-AATT). This reversed and offset alkylation selectivity within an AT-rich site is the natural consequence of the diastereomeric relationship of the adducts, and the reversed binding orientation in the minor groove with respect to the alkylation site is required to permit adenine N3 access to the least substituted carbon of electrophilic cyclopropane. Presumably the relative importance of the fourth base (A/T > G/C) in the binding sequences is the reason this site constitutes a high-affinity site for the unnatural enantiomer but only a minor site for the natural enantiomer.

***N*-BOC-DSA: A DNA Alkylating Agent Whose Selectivity and Efficiency are Independent of Absolute Stereochemistry.** The accuracy of the models was revealed when they were found to explain the unusual observation that both enantiomers of simple derivatives of the alkylation subunit, *i.e.*, *N*-BOC-DSA (**6**), alkylate the same sites in DNA. In addition to illustrating that the DNA alkylation reactions of (+)- and *ent*-(−)-**6** are substantially less efficient (*ca.* $10^4\times$), less selective (selectivity, 5′-AA > 5′-TA), and proceed with an altered profile than either (+)- or (−)-duocarmycin SA, the studies have shown that both enantiomers of **6** alkylate the same sites with essentially the same efficiency independent of the absolute stereochemistry. Although these observations appear unusual, they are the natural consequence of the diastereomeric relationship of the adducts. The natural enantiomer binds in the 3′ → 5′ direction from the site of alkylation extending over the adjacent 5′ base. The unnatural enantiomer binds in the reverse 5′ → 3′ orientation but with binding that also covers the same adjacent 5′ base. These alkylation site models, which are illustrated in Figure 1 for (+)- and *ent*-(−)-**6**, nicely accommodate the observed selectivity of 5′-AA > 5′-TA for *both* enantiomers. This preference of 5′-AA over 5′-TA (*ca.* 2:1)[31] is statistical rather than structural in nature since the complementary DNA strand of a mixed AT sequence contains an identical competitive alkylation site (AT-5′ versus TT-5′). The simple factor controlling alkylation is simply the depth of minor groove penetration accessible to the agent which is required to permit alkylation of the electrophilic cyclopropane. For simple agents such as (+)- and (−)-**6**, this is possible only when the adjacent 5′ base is A or T, and models of a hypothetical but unobserved 5′-GA alkylation support this proposal.[31] For (+)- and *ent*-(−)-**1**, a larger appropriate 3.5 base pair AT-rich site surrounding the alkylation site is required to permit sufficient groove penetration for alkylation and is further enhanced by the preferential noncovalent binding of the agents within the narrower, deeper AT-rich minor groove.[12,19,52]

Reversibility of the DNA Alkylation Reaction: Binding-Driven-Bonding. Although the (+)-duocarmycin SA and A DNA alkylations have proven similar to that of (+)-CC-1065,[16] one important feature of the reactions distinguishes the agents. Unlike (+)-CC-1065, which irreversibly alkylates DNA, (+)-duocarmycins SA and A were found to reversibly alkylate DNA.[30,31] The rate or ease of reversibility proved dependent upon the relative reactivity of the agent and the stability of the adduct as well as the extent of the agent noncovalent binding interactions. Consistent with the relative reactivity of the agents (A > SA)[43] and the expected stability of the adducts, the (+)-duocarmycin A retroalkylation reaction is much slower than that of (+)-duocarmycin SA. In addition, (+)-CC-1065 is less reactive than (+)-duocarmycin A but more reactive than (+)-duocarmycin SA.[43] The lack of detection of a reversible (+)-CC-1065 DNA alkylation reaction indicates that the rate of reversibility is also dependent on the extent of the noncovalent binding stabilization, and consistent with this interpretation, analogs of CC-1065 possessing the same alkylation subunit but simpler, smaller structures do reversibly alkylate DNA.[51]

In the course of early studies and prior to an experimental verification, computational and quantitative molecular modeling studies in conjunction with an intuitive appreciation of the chemical properties of the agents led us to propose that the adenine N3 alkylation reaction by such agents could be expected to be a reversible, near thermal neutral reaction stabilized by extensive noncovalent binding.[12,16] The fact that the adenine addition reaction is not observed with free adenine further suggested that the dominant force driving (stabilizing) the DNA alkylation reaction is not the covalent bond but the noncovalent binding stabilization derived from hydrophobic binding and van der Waals contacts,[17] a process we continue to refer to as hydrophobic binding-driven-bonding.[12] That is, the near thermal neutral and reversible nature of the alkylation reaction is rendered less reversible or irreversible by dominant, noncovalent binding stabilization.

The importance of these proposals become clear in

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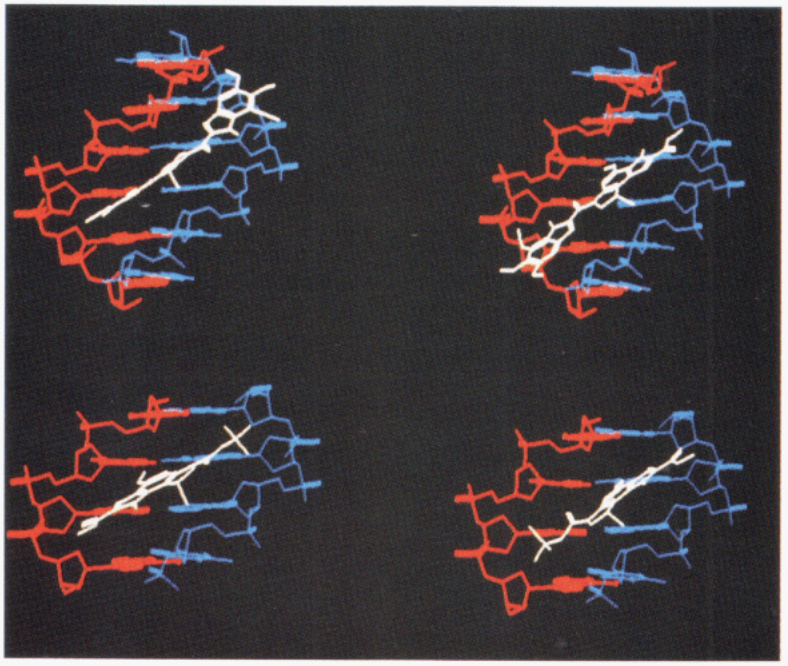


Figure 1. Top: Comparison stick models of the (+)-duocarmycin SA (left) and *ent*-(−)-duocarmycin SA (right) alkylation of a common site within w794 DNA, duplex 5′-(CTAATT). The natural enantiomer extends in the 3′ → 5′ direction from the adenine N3 alkylation site across the minor site 5′-CTAA. The unnatural enantiomer extends in the reverse 5′ → 3′ direction across the high-affinity site 5′-AATT. Bottom: Comparison stick models of the (+)-*N*-BOC-DSA (left) and *ent*-(−)-*N*-BOC-DSA (right) alkylation of the same common w794 DNA site, duplex 5′-(TAAT). The natural enantiomer binds in the 3′ → 5′ direction from the adenine N3 alkylation site across the 5′-AA site while the unnatural enantiomer binds in the reverse 5′ → 3′ direction but also covers the same 5′-AA site.

the comparison of the properties of the agents. Like the comparisons of (+)-CC-1065, (+)-CPI-PDE$_1$, and (+)-CPI-CDPI$_n$ (n = 1−3) with (+)-*N*-BOC-CPI (**8**),[16] the exceptionally potent cytotoxic activity of (+)-duocarmycin SA (**1**) versus the nonpotent activity of (+)-*N*-BOC-DSA (**6**, 1000×) may be attributed to the simple event of noncovalent binding stabilization of the inherently reversible DNA alkylation reaction.[43,53] Consistent with these expectations, a much faster rate of retroalkylation and much lower degree of adduct stability was observed with (+)-**6** versus (+)-**1** and may be attributed to the lack of the trimethoxyindole binding stabilization.[31]

Modified Alkylation Subunits: Definition of a Fundamental Relationship between Functional Reactivity and Biological Activity. The acid-catalyzed activation of the DNA alkylation reaction led to the intuitive proposal that there may exist a direct relationship between the reactivity and cytotoxic activity of the agents and established the expectation that the biological potency may be enhanced as the electrophilic reactivity is increased.[11] However, studies conducted with the agents **6**−**11**[16,19−21] and **1**, **2**, **12**, and **13**,[27,34,35,43,45,46] which were prepared by chemical synthesis employing technology developed in the course of the natural product total syntheses, strongly suggested the reverse relationship and that the agents within a given class possessing the greatest stability may be expected to exhibit the most potent cytotoxic activity.[19,20,34,35,43] Moreover, a near linear relationship between solvolytic stability (pH 3) and biological potency (IC_{50} L1210) was observed and proved to be general with both simple (Table 3)[34,43] and advanced analogs of the natural products (Figure 2).[35]

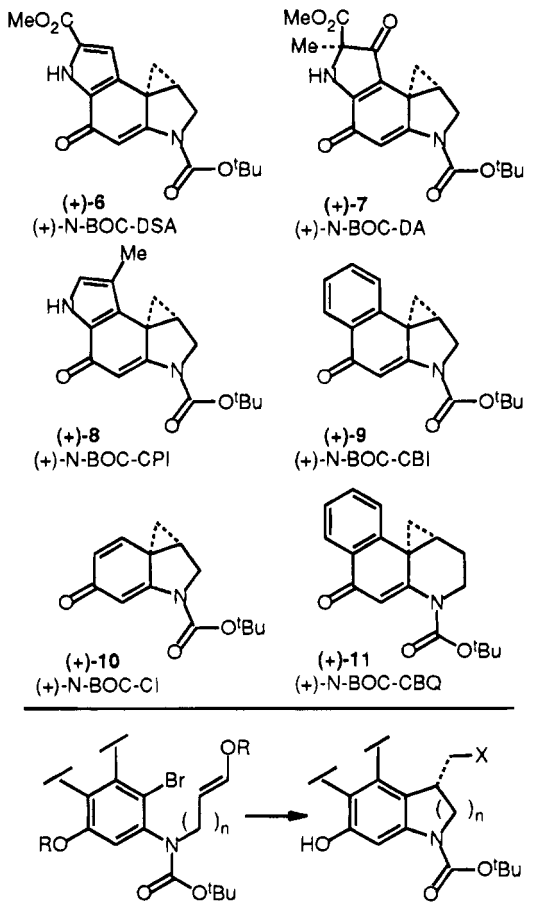
(53) As we have suggested elsewhere, the delayed fatal toxicity of (+)-CC-1065 absent with **1** and **2** may be the consequence of the extent of the noncovalent binding stabilization which renders the DNA alkylation reaction irreversible.

N^2 Substituent Role: Verification of a Fundamental Relationship between Functional Reactivity and Biological Activity. Examination of the properties of **14**−**17**,[33] simple derivatives of CBI,[20] led to validation of this fundamental and direct relationship between solvolysis stability and cytotoxic potency. The examination of **14**−**17** revealed a direct, linear relationship between the cytotoxic potency (L1210, log $1/IC_{50}$) and the solvolytic stability (−log k_{solv}, pH 3) of the agents (Figure 3). Thus, identical to the trend observed with **6**−**11**, the more stable derivatives proved to be the most potent. Similarly, a fundamental linear relationship was found between the electron-withdrawing properties of the N^2 substituents (Hammett σ_p constant) and the solvolysis reactivity (−log k_{solv}, pH 3) with the strongest electron-withdrawing substituents providing the most stable agents (Figure 3). This latter relationship reflects the influence of the N^2 substituent on the ease of C4 carbonyl protonation required for catalysis of solvolysis with the stronger electron-withdrawing substituents exhibiting slower solvolysis rates. Less obvious but more fundamental, the observations follow a predictable linear relationship between the cytotoxic potency (L1210, log $1/IC_{50}$) and the electron-withdrawing properties of the N^2 substituent (σ_p) with the strongest electron-withdrawing substituents providing the most potent agents (Figure 3).

These fundamental correlations between the electron-withdrawing properties of the N^2 substituent, the functional reactivity of the agents, and their biological potency should prove useful in the predictable design of new analogs. For agents that possess sufficient reactivity to effectively alkylate duplex DNA, the chemically more stable agents may be expected to constitute the biologically more potent agents. Pre-

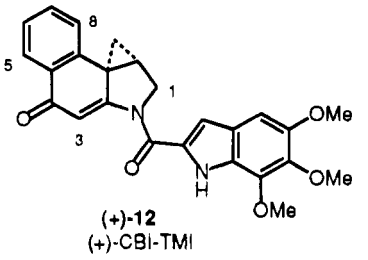
Table 3

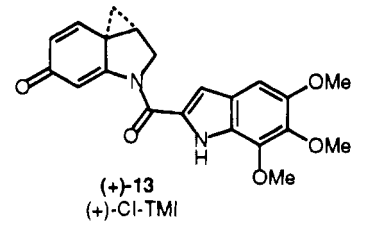
	k (s^{-1}, pH 3)	$t_{1/2}$	IC_{50} (L1210)
6	1.1×10^{-6}	177 h	6 nM
9	1.5×10^{-6}	133 h	80 nM
8	5.3×10^{-6}	37 h	330 nM
7	1.7×10^{-5}	11 h	1000 nM
11	9.1×10^{-5}	2 h	4000 nM
10	2.0×10^{-2}	0.01 h	18000 nM



sumably, this may be attributed to the more effective delivery of the more stable agents to their intracellular target.

Structure versus Reactivity: Stereoelectronic Control and Structural Origin of an Unappreciated but Productive Stability of the Duocarmycin Alkylation Subunits. Two characteristics of the alkylation subunits **6**–**10** have proven important. The first is the stereoelectronically-controlled acid-catalyzed ring opening of the cyclopropane which dictates addition of a nucleophile to the least substituted carbon. The second is the rate of acid-catalyzed solvolysis and the demonstration of a linear relationship between solvolysis stability and cytotoxic potency. A recent comparison of the chemical and structural properties of *N*-BOC-CBQ (**11**)[34,54] and *N*-BOC-CBI (**9**)[20] confirmed the stereoelectronic control of the solvolysis or alkylation reactions of **6**–**10** and revealed an unappreciated but fundamentally important stability for the duocarmycin alkylation subunits and its structural origin. The solvolysis reactivity of **11** and **6**–**10** is summarized in Table 3. In addition to the





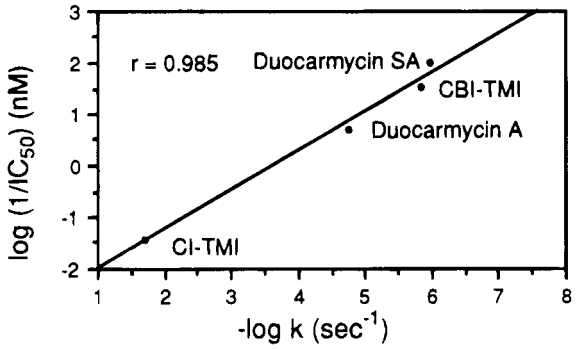


Figure 2.

increased reactivity of **11** (63×) versus *N*-BOC-CBI (**9**), the solvolysis of **11** occurs with nucleophilic addition to both C10 and C10a while that of **6**–**10**, including **9**, occurs nearly exclusively at the least substituted cyclopropane carbon (Scheme 4).[54]

The X-ray structure of *N*-BOC-CBQ (**11**)[34] provided the structural insights into this difference in solvolysis reactivity and regioselectivity. It is clear from the X-ray structure of CBI[20] that the bent orbital[55] of the cyclopropane bond extending to the least substituted carbon is nearly perpendicular to the plane of the cyclohexadienone and consequently overlaps[56] nicely with the developing π-system of the solvolysis product phenol (Figure 4). In contrast, the cyclopropane bond extending to the tertiary carbon is nearly in the plane of the cyclohexadienone, and its orbital is nearly orthogonal to the π-system of the product phenol. Thus, opening of the cyclopropane occurs with addition of a nucleophile to the least substituted carbon, and the stereoelectronic control responsible for this addition selectivity overrides the intrinsic electronic preference for ring expansion. In contrast, the *N*-BOC-CBQ X-ray structure exhibits different characteristics. The cyclopropane is ideally conjugated[56] with the cyclohexadienone π-system, and the plane defined by the cyclohexadienone ideally bisects the cyclopropane with the bonds extending to the secondary and tertiary cyclopropane carbons equally aligned with the π-system. Thus, the two available cyclopropane bonds are equally aligned for cleavage and addition to both are observed to occur.[54]

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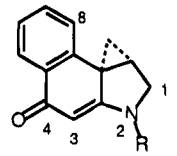


	R	k (s^{-1}, pH 3)	$t_{1/2}$	IC_{50} (L1210)	sigma
14	SO_2Et	0.5×10^{-6}	383 h	24 nM	0.72
15	COEt	2.0×10^{-6}	96 h	110 nM	0.48
16	CO_2CH_3	3.4×10^{-6}	57 h	140 nM	0.45
17	CONHMe	5.4×10^{-6}	36 h	200 nM	0.36

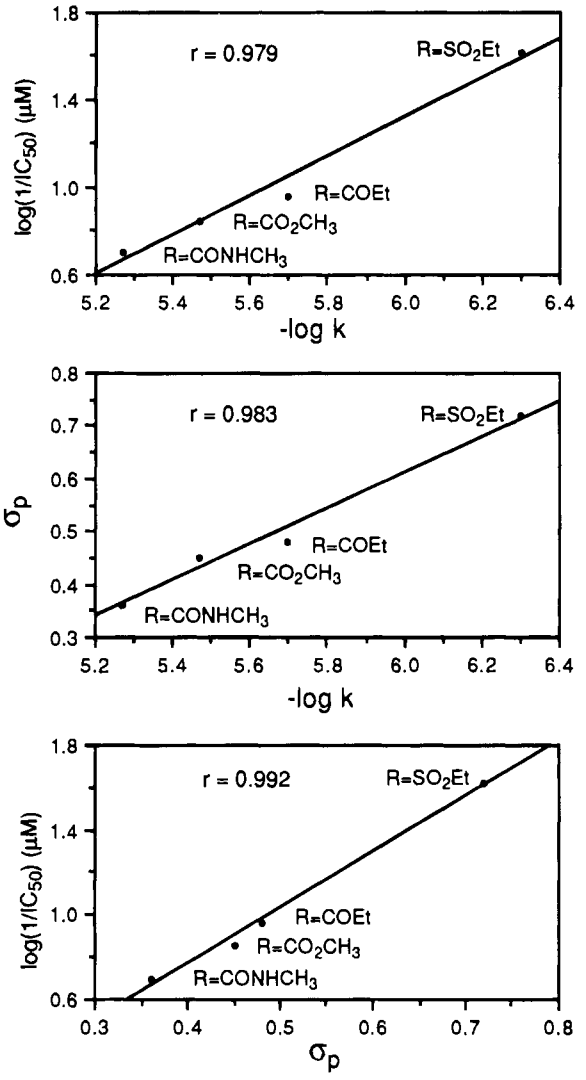
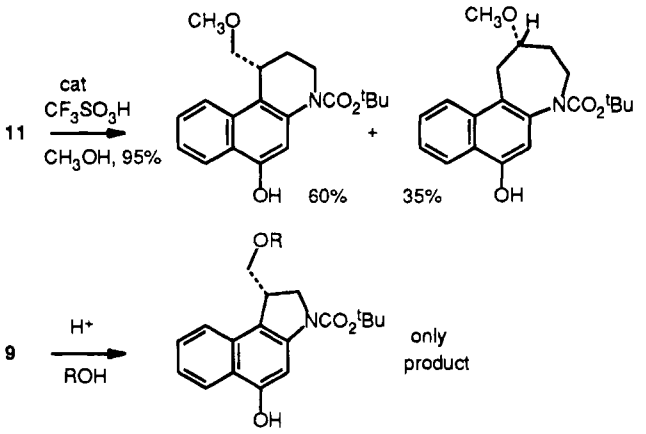


Figure 3.

More surprising was the rapid solvolysis of *N*-BOC-CBQ, and important insights into this enhanced reactivity are clear from the X-ray structures. Although they reside in a cyclopropane fused to a six- versus five-membered ring, the bond lengths of the CBQ C9b–C10a (1.528 Å) and C9b–C10 (1.543 Å) bonds are longer than those found in CBI (1.508 and 1.532 Å, respectively) and nicely reflect the enhanced cleavage reactivity[57] (Figure 4). This lengthening of the cyclopropane bonds may be attributed to the idealized conjugation or π-delocalization of both the

Scheme 4



C9b–C10a and C9b–C10 cyclopropane bonds with the cyclohexadienone π-system.[56] Contributing to this enhanced conjugation is the ideal geometrical alignment of C10 and C10a with C9b, C5, and the carbonyl oxygen. For CBI, the constraints of the fused five-membered ring place its C9 and C9a at a 20° angle offset from this plane and prevent ideal alignment and overlap of either the C8b–C9a or C8b–C9 bond with the cyclohexadienone π-system. This idealized cyclopropane conjugation[56] of CBQ with the cyclohexadienone π-system results in the observed longer bond lengths,[57] weaker bond strengths, and higher solvolysis reactivity.[58]

Thus, the geometrical constraints of the fused five-membered ring found in the duocarmycin alkylation subunits impose the stereoelectronic control on the nucleophilic cleavage of the cyclopropane directing addition to the least substituted carbon. In addition, the nonideal alignment and overlap of the cyclopropane with the cyclohexadienone π-system found in CBI or DSA result in productively diminished electrophilic reactivity. The fundamental insight derived from these comparisons is not the solvolysis reactivity of *N*-BOC-CBQ, but rather the surprising stability of the CBI and DSA alkylation subunits. In spite of structural features that intuitively suggest high reactivity, the latter agents are uncharacteristically stable. This unusual stability is imposed by fusion of the cyclopropane to the five-membered ring which constrains it to a nonideal alignment and overlap with the cyclohexadienone π-system.

Structure versus Reactivity: Additional Key Features. There are a number of additional unique structural features of **6**–**11** that also contribute to their stability. The enhanced stability of **6**, **8**, and **9** > **7** > **10** can be attributed to the diminished gain in delocalization energy that accompanies aromatization in a system that bears a fused aromatic ring.[43] The increased stability of **6** > **8** and **7** > **10** can be attributed to the conjugated electron-withdrawing group which diminishes C4 carbonyl protonation required of solvolysis and alkylation.[33] The increased stability of **9** > **8** can be attributed in part to the release of strain that accompanies the substitution of a fused six-membered for a fused five-membered aromatic ring.[20] Finally, the vinylogous amide stabilization of the cyclohexadienone structure which is lost

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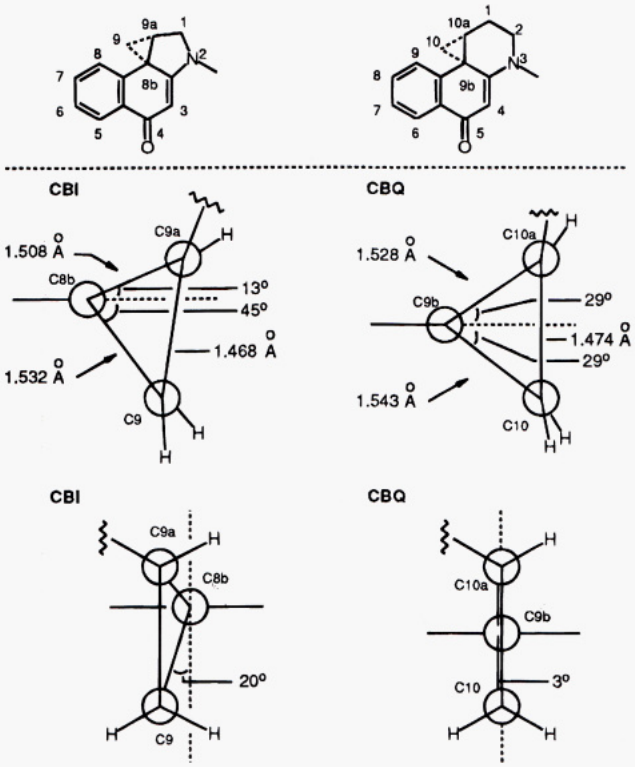


Figure 4. Stick models of the side and rear views of the activated cyclopropane of CBI and CBQ illustrating data taken from the X-ray crystal structures and highlighting the idealized overlap and alignment of the CBQ cyclopropane with the cyclohexadienone π-system.

upon aromatization contributes significantly to the stability of **6**–**11**.[59]

Structural Origin of the Enantiomer Distinctions: Sensitivity to C7 Steric Interactions. In preceding studies, the efficiency of DNA alkylation by the natural enantiomers was found to diminish with both increased reactivity and increased steric bulk surrounding the C7 center,[19,20,31] and the additional comparisons with the unnatural enantiomers of **1** and **2** and of **12** and **13** were especially revealing.[31,35] While *ent*-(−)-duocarmycin A (**2**) does not detectably alkylate DNA,[29] the remaining three unnatural enantiomers were found to alkylate the same sites in DNA

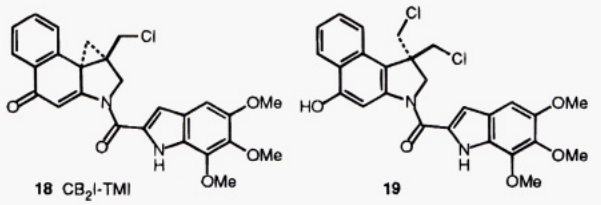
Table 4. Enantiomer Comparisons

agent	re IC_{50}[a]	rel DNA alkylation[b]
13, CI-TMI	1.0	0.5–2.0
1, duocarmycin SA	10	10
12, CBI-TMI	100	100
2, duocarmycin A	≥110	>100

[a] IC_{50} (L1210) of unnatural (−)/natural (+) enantiomer. [b] Concentrations of unnatural (−)/natural (+) enantiomer required to detect DNA alkylation.

(Table 2), and the distinctions lie in their relative efficiency of DNA alkylation and their relative selectivity among the available sites: *ent*-(−)-duocarmycin SA (**1**) > (−)-CBI-TMI (**12**) > (−)-CI-TMI (**13**) ≫ (−)-duocarmycin A (**2**).[35] More importantly, the apparently confusing distinctions in the relative efficiency of the natural versus unnatural enantiomer DNA alkylation reactions (Table 4) were found to correlate with the inherent steric bulk surrounding the C7 center for which the unnatural enantiomers are especially sensitive, consistent with expectations based on the Figure 1 models.

C_2BI-TMI: An Exquisitely Designed Adenine–Adenine Cross-Linking Agent That Benefits from the Reversed and Offset AT-Rich Selectivity of the Enantiomeric Duocarmycins. C_2BI-TMI (**18**) and its achiral precursor **19** were designed on the basis of the CBI-based agents,[20,33] which are more stable than duocarmycin A and of comparable stability to duocarmaycin SA (Table 3, Figure 2) but which are synthetically more accessible and simpler than **1** or **2**. In addition, the acyclic agent **19** lacking the



preformed cyclopropane ring could be expected[15,20] to display properties identical to those of C_2BI-TMI (**18**) but is achiral. This simple feature of **19** not only provides an attractive synthetic candidate which does not require resolution or asymmetric synthesis but

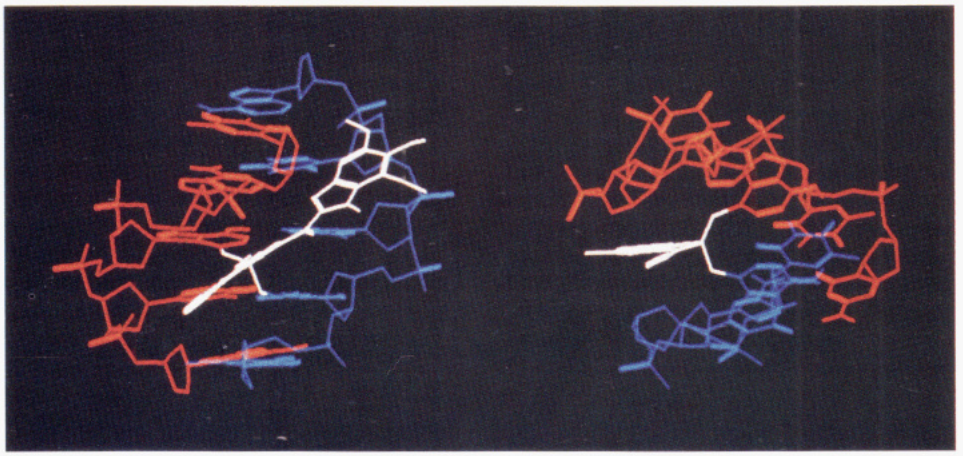


Figure 5. Front and groove views of the cross-linking reaction of C_2BI-TMI in the w794 DNA high-affinity site, 5′-ATTAG. The natural enantiomer alkylation occurs with agent binding in the 3′ → 5′ direction from its site of alkylation across the 5′-ATTA-3′ site while the unnatural enantiomer alkylation occurs one base pair removed on the complementary strand at 3′-TAAT-5′ with agent binding in the reverse 5′ → 3′ direction relative to the alkylated strand but at a 3.5 base pair AT-rich high-affinity site which coincides exactly with that of the natural enantiomer site.

also provides a single agent for biological evaluation free of the requirements for documenting the behavior of both enantiomers. Since both enantiomers of **1** and **12** display effective DNA alkylation properties and potent cytotoxic activity, the in vivo closure of **19** to both enantiomers of **18** was expected to be inconsequential. This is especially true since the bis-alkylating capabilities of **18** and **19** provided the opportunity for DNA cross-linking with the two alkylating groups of **19** serving to act independently as the individual enantiomers of **12**. When this occurs within a sequence such as

3′-TAAT-5′

5′-ATTA-3′

the natural enantiomer adenine N3 alkylation occurs at its high-affinity site of 5′-ATTA-3′ with agent binding in the 3′ → 5′ direction *relative to the alkylated strand* across a 3.5 base AT-rich site while that of the unnatural enantiomer is directed to the complementary strand leading to cross-linking with adenine N3 alkylation at its high-affinity site of 3′-TAAT-5′ with agent binding in the reversed 5′ → 3′ direction *relative to the alkylated strand* across a 3.5 base AT-rich site (Figure 5). Consistent with the fact that the reversed binding orientation relative to the alkylated strand and the offset AT-rich binding sites relative to the alkylated adenine of both enantiomers are beautifully accommodated into a single binding and cross-linking site, **18** and **19** were found to be efficient cross-linking agents.[21]

Apoptosis: A Potential Link between DNA Alkylation, Cytotoxic Activity, and Antitumor Properties. The first step in defining the link between DNA alkylation and effective antitumor activity was detailed in studies which demonstrated that *sensitive* tumor cell lines including L1210 and Molt-4 are triggered to undergo apoptotic cell death at relevant agent concentrations (100 pM) below that which is required for typical cell death by necrosis observed in nonsensitive cell lines.[32]

Conclusions. The continued examination of the basis for the initiation and signaling of apoptosis as well as future studies with additional structural analogs of the duocarmycins will provide further insights into the origin of their properties. No doubt this will lead to rationally designed synthetic agents whose properties overcome inherent limitations of the natural products themselves and further define subtle relationships between their structure, functional reactivity, and biological properties.

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