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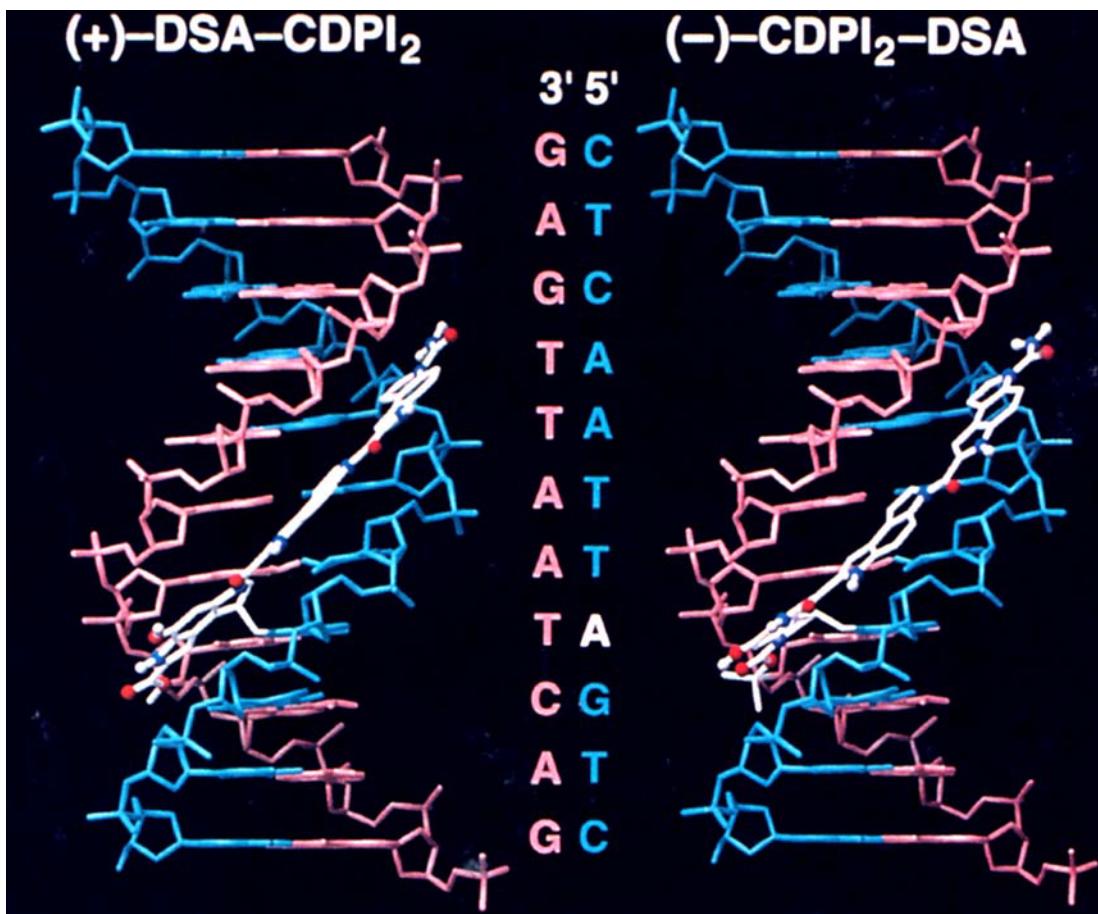


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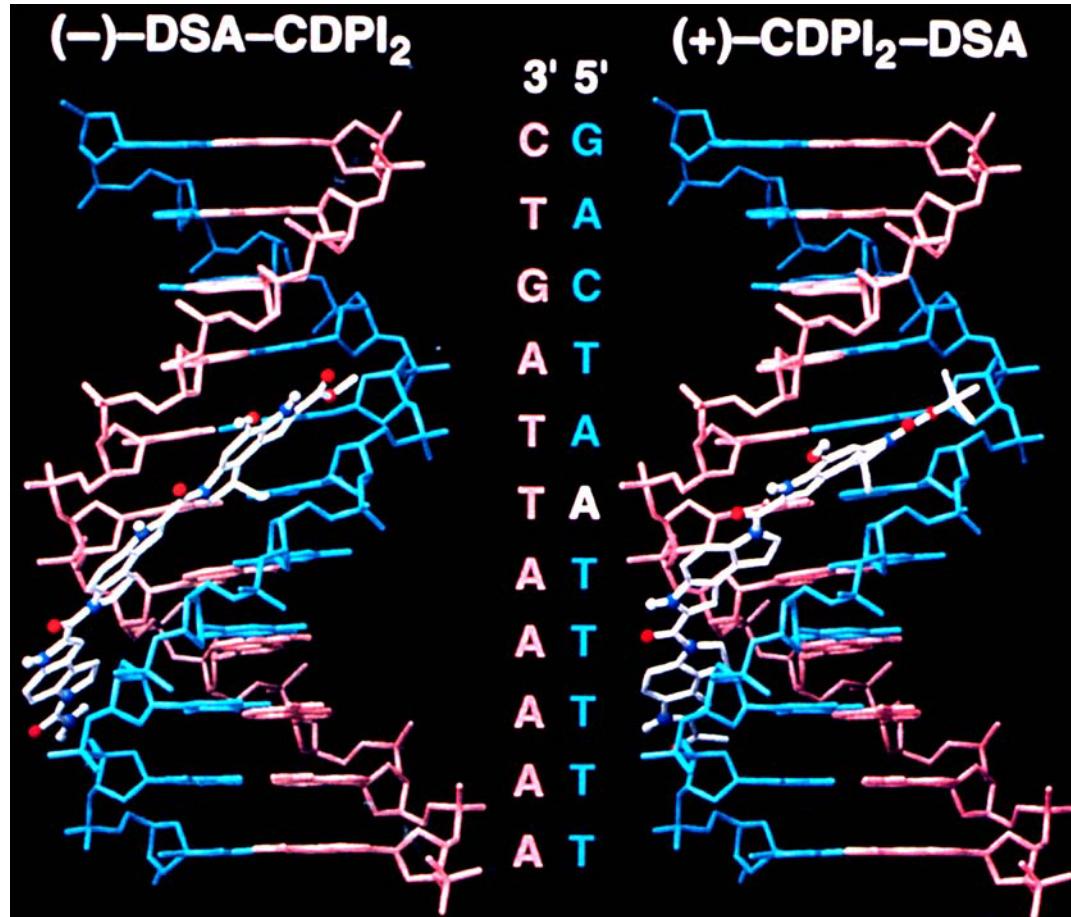
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Merely reversing the arrangement of the DNA-binding structural elements ($\text{DSA-CDPI}_2 \rightarrow \text{CDPI}_2\text{-DSA}$) causes a DNA alkylating agent to behave like the enantiomer of the natural product, although the alkylation unit has the configuration found in nature. Model studies on the binding of the enantiomers to DNA provide an explanation.



CC-1065 and the Duocarmycins: Understanding their Biological Function through Mechanistic Studies**

Dale L. Boger* and Douglas S. Johnson

We review here studies defining the DNA alkylation properties of a class of potent antitumor antibiotics that includes CC-1065 and the duocarmycins, as well as investigations that delineate fundamental relationships between their structure, functional reactivity, and biological properties. In conjunction with the study of the natural products themselves, the examination of synthetic agents containing deep-seated structural changes, the unnatural enantiomers of the natural products, and related analogs has defined the structural basis for the sequence selective alkylation of duplex DNA and proved to be the key to understanding the fundamental relationships between chemical structure,

functional reactivity, and biological properties. The characteristic DNA alkylation proceeds by reversible, stereoelectronically controlled adenine-N3 addition to the least substituted carbon of the activated cyclopropane within AT-rich minor groove sites. Both the natural and unnatural enantiomers alkylate DNA, and the sequence selectivity of both is controlled and dominated by the preferential noncovalent binding of the agents within the narrower, deeper, AT-rich minor groove and the steric accessibility to the alkylation site on penetration of this groove. Among the fundamental relationships between structure and properties, the most striking is the direct relationship between

chemical or functional stability and biological potency. Within a short time, simplified and readily accessible synthetic agents rationally based on the natural product leads have been developed, which exhibit comparable and exceptional biological potency ($IC_{50} = 50$ to 51 pm) and improved efficacy. The fundamental relationships that have been defined to date can be expected to provide the foundation for future developments and advances.

Keywords: antibiotics • antitumor agents • DNA alkylation • duocarmycins

1. Introduction

Substantial progress has been made toward understanding the origin of the sequence-selective recognition of duplex DNA by small organic molecules^[1–5] including a wide range of naturally occurring antitumor antibiotics. Major factors contributing to the interest include the desire to define the basis for the therapeutic properties of the naturally occurring antitumor antibiotics that have been shown to bind DNA, to define the nature of DNA lesions induced by toxic, mutagenic, or carcinogenic materials,^[6] and to alter transcription, translation, or protein expression and exploit the ramifications for therapeutic purposes.

Three fundamental issues to be addressed in the examination of DNA binding agents are the origin of binding affinity, binding selectivity, and reaction selectivity including DNA alkylation or cleavage. Each can independently exert control on the apparent sequence-selective recognition of DNA, and the rela-

tive importance and origin of these effects remain an important objective of most investigations. The emergence of experimental techniques for the rapid sequencing of DNA,^[7–10] for the determination of DNA binding selectivity and affinity including footprinting and affinity cleavage techniques,^[11,12] and for the determination of sites of DNA modification^[13] coupled with the structural characterization of DNA complexes at defined sites by X-ray crystallography,^[14,15] NMR spectroscopy,^[16,17] and molecular modeling^[18,19] has advanced the understanding of the molecular interactions responsible for the sequence-selective recognition of DNA.

A powerful complement to such tools in the examination of naturally derived DNA binding agents is the preparation and subsequent examination of key partial structures, agents containing deep-seated structural modifications in the natural product, and their corresponding unnatural enantiomers. Well-conceived, deep-seated structural modifications may be used to directly address and define both the structural basis for the sequence-selective recognition of DNA and fundamental relationships between structure, functional reactivity, and biological properties. This is especially important in the case of natural products that are identified by directed screening efforts. In contrast to the study of the biological role of primary metabo-

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lites, the presence of secondary metabolites and the perceived origin of the identified lead is often misrepresented. Nature, with its vast array of secondary metabolites, provides a diverse library of small organic molecules for random screening. Not always, and perhaps only rarely, is the role of the identified natural products related to the biological basis of the screening efforts responsible for their detection and identification. Consequently, it may be a mistake to assume that nature has optimized or even devised the agent for purposes related to the biological process for which the screening assay was set up. Rather, it is more accurate to recognize that nature has only provided a novel lead or solution that has not yet been optimized. A challenging problem remains: to understand the beautiful solutions and subtle design elements that nature has provided in the form of a natural product and to extend this solution through rational design elements to provide more selective, more efficacious, or more potent agents designed specifically for the problem or target under investigation.

One of the newest class of agents shown to alkylate DNA includes (+)-CC-1065 (**1**),^[20–23] (+)-duocarmycin A (**2**),^[24–29] and (+)-duocarmycin SA (**3**)^[30–33] (Fig. 1). Because of their remarkable cytotoxic potency, substantial efforts have been devoted to defining their properties.^[34–50] These studies show that the agents exert their biological effects through a sequence-selective alkylation of DNA. The reversible, stereoelectronically controlled adenine-N3 addition to the least substituted cyclopropane carbon has been found to occur within selected AT-rich sites in the minor groove, 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,^[51] and to defining the fundamental principles underlying the relationships between structure, chemical reactivity, and biological activity. The initial limited studies conducted with naturally derived duocar-

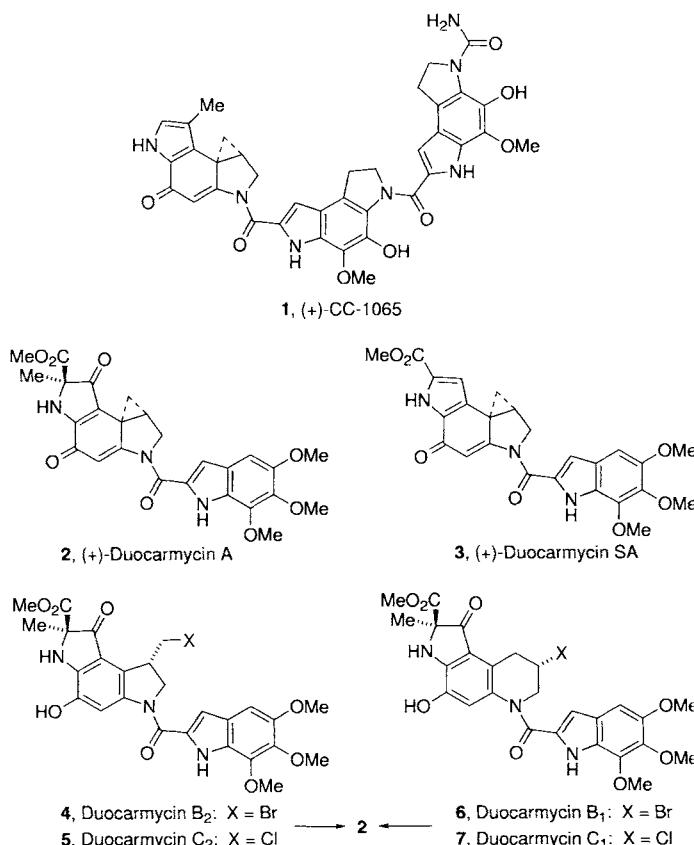
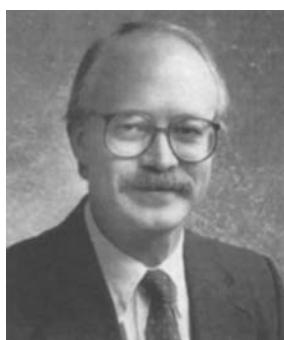


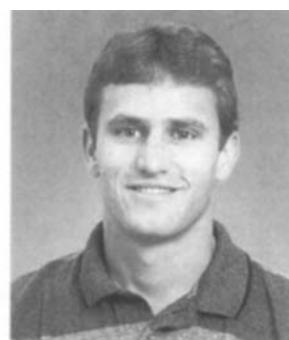
Fig. 1. Structures of CC-1065 and the duocarmycins.

carmycin SA (**3**), the most recent addition to this class of agents, revealed a combination of properties that make it the most exciting of the natural products identified to date. In addition to lacking the characteristic of delayed toxicity of (+)-CC-

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1065,^[52] it is the most stable and most potent member of this class of agents. In fact, the name duocarmycin SA is an abbreviation of its original name, duocarmycin Stable A, which was introduced before its structure was established. As detailed herein, this combination of properties is not fortuitous, but rather the enhanced functional stability of duocarmycin SA is directly responsible for its increased biological potency. The studies complement those conducted on other DNA alkylating agents including the nitrogen mustards, the mitomycins, aflatoxin, epoxides of polyaromatic hydrocarbon diols, azinomycin, saframycin, anthramycin and related agents, and dichloroplatinum and related agents.^[1–6]

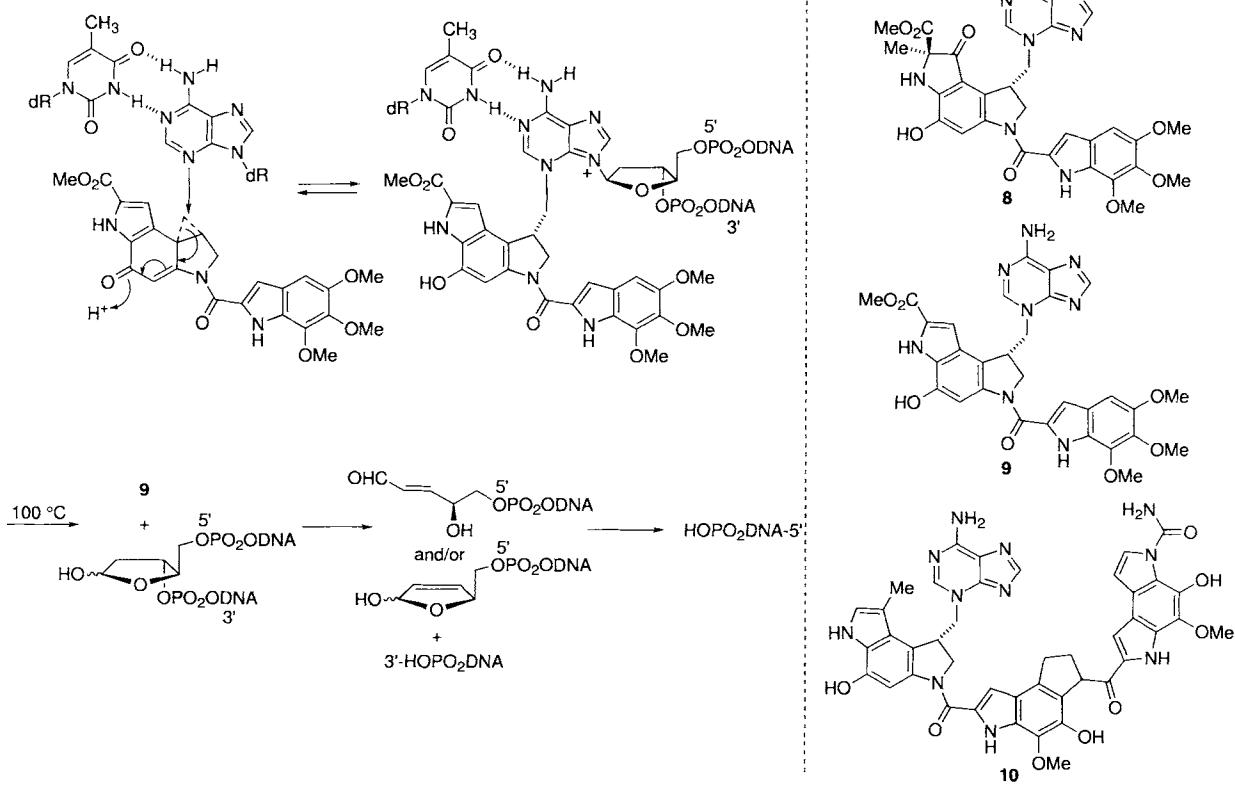
2. DNA Alkylation Properties

2.1. DNA Alkylation Selectivity of the Natural Products

The event, sequence selectivity, quantitation, reversibility, and structure determination of the predominant DNA alkylation by **1–3** have been defined.^[53–72] The identification of the alkylation site and the assessment of relative selectivity were achieved by thermally induced depurination and strand cleavage of labeled DNA after exposure to the agents (Scheme 1). The selectivity in alkylations by (+)-duocarmycin A (**2**) and (+)-duocarmycin SA (**3**) proved nearly indistinguishable.^[54, 56] In each case alkylation took place at an adenine residue that was flanked by two 5'-A or -T bases, and the preferred order of these three-base sequences proved to be 5'-AAA>5'-TTA>5'-TAA>5'-ATA. In addition, A or T dominated over G or C as

a fourth 5' base, and this feature distinguished high-affinity sites from low-affinity sites. An additional weak preference for a purine (Pu) over pyrimidine (Py) at the 3' base preceding the adenine alkylation site was detected, which was more prominent in the low-affinity sites. Table 1 summarizes the consensus sequence derived from the evaluation of alkylations with **2**^[56] and **3**.^[54] Although these agents alkylated the same sites, two important distinctions were defined. The least reactive of the agents, (+)-duocarmycin SA (**3**), alkylated DNA with a greater efficiency (10–100×) and a greater selectivity among the available sites. In addition, the selectivity of the related naturally occurring agents **4–7** (see Fig. 1) was indistinguishable from that of **2**, which may be attributed to their facile cyclopropane ring closure under the conditions of assay.^[54, 56] This behavior of **6–7** requires that they first close to form **2** prior to alkylating DNA, and that this is the biologically relevant and more potent form of the agent. These observations with **4–7** are general throughout the set of agents examined to date. In fact, not only are the selectivities in DNA alkylation of such seco agents indistinguishable, but often the DNA alkylation efficiencies and biological potencies are also the same, which indicates that the cyclopropane ring closure is not a limiting factor. Only in the cases of the more difficult or slower spirocyclizations have distinguishing features in DNA alkylation efficiency or biological potency been observed.^[53, 56]

The preceding studies of (+)-CC-1065 (**1**) revealed a selectivity for a similar, but more extended five-base-pair, AT-rich alkylation site.^[53, 64–72] Table 1 summarizes the consensus sequence derived from our evaluation of (+)-CC-1065.^[53, 71–72] Each site of alkylation proved to be an adenine residue flanked by two



Scheme 1. Course of the DNA alkylation by **3** and structures of the characterized adenine adducts of **1** (**10**), **2** (**8**), and **3** (**9**).

Table 1. Consensus sequences for the DNA alkylation reactions of the natural products and their unnatural enantiomers [a].

Agent	Base [b]	5'	4	3	2	1	0	-1	-2	-3	-4	3'
1	A/T (56)		67	78	94	98	100	55	-	-	-	-
	consensus		A/T ≥ G/C	A/T > G/C	A/T	A/T	A	Pu ≥ Py	-	-	-	-
2 and 3	A/T (56)		58	79	100	100	100	69	-	-	-	-
	consensus	N		A/T > G/C	A/T	A/T	A	Pu > Py	-	-	-	-
<i>ent</i> - 3	A/T (56)	-	-	-	-	93	100	96	73	56	-	-
	consensus	-	-	-	-	A/T	A	A/T	A/T > G/C	N	-	-
<i>ent</i> - 1	A/T (56)	-	-	-	-	88	100	93	82	73	56	-
	consensus	-	-	-	-	A/T	A	A/T	A/T > G/C	A/T > G/C	N	-

[a] The numbers listed in the table give the percentage of the indicated base located at the designated position relative to the adenine-N3 alkylation site. [b] The numbers in parentheses give the percentage composition within the DNA examined.

5'-A or -T bases. The sequence preference for the three-base, AT-rich alkylation site followed a similar order: 5'-AAA = 5'-TTA > 5'-TAA > 5'-ATA. In addition, the agent exhibited a strong preference for the fourth 5' base to be A or T, a weaker preference for the fifth 5' base to be A or T, and a weak preference for the 3' base preceding the alkylation site to be a purine. These preferences for the fourth and fifth 5' bases distinguished the high- from the low-affinity sites, and the preference for a 3' purine over pyrimidine was more prominent in the lower affinity sites. The strict AT requirement within the first three base pairs represents a combination of the initial 3' adenine-N3 alkylation site and an adjacent 5' two-base AT site required to accommodate the central subunit binding. The weaker preferences for A or T as the fourth and fifth 5' bases reflect, as observed with

the high-affinity sites, a preferential AT-rich site for the third subunit also bound in the minor groove. A study of shorter and more extended analogs of CC-1065 revealed that this selectivity for AT-rich sites corresponds nicely to the length of the agent and, hence, the size of binding site required to accommodate the agent.^[53, 71]

Figure 2 illustrates the similarities of **2** and **3** and their subtle distinctions from **1** in their behavior toward DNA. According to the concentrations required to detect alkylation, (+)-duocarmycin SA (**3**) is approximately ten times more efficient at alkylating the DNA than (+)-duocarmycin A (**2**) and subtly more efficient than (+)-CC-1065 (**1**). Although **3** and **2** alkylate the same sites, **3** exhibits a greater degree of discrimination among the available sites. This is evident from the relative concentrations required to detect the minor and major alkylation sites for the two agents. Similarly, while CC-1065 and the duocarmycins both alkylate the same high-affinity site in this segment of DNA, distinctions in the alkylation of the minor sites are apparent, including the lack of alkylation by (+)-CC-1065 at the shorter three-base AT-rich site 5'-GCAA.

The sequence selectivity observed with intracellular SV40 DNA is essentially identical to that observed with purified SV40 DNA except that higher concentrations of **1** are required.^[74–76] Thus, *in vitro* analyses with purified DNA reflect the sequence-selective reactivity of **1** with intracellular DNA.

2.2. DNA Alkylation Selectivity of the Unnatural Enantiomers

One of the more revealing discoveries that was made possible through use of synthetic materials was that the unnatural enantiomers may also constitute effective DNA alkylating agents and potent cytotoxic agents.^[53, 54, 68, 71] Moreover, the ability to compare the behavior of the two enantiomers proved to be the key to understanding the structural origin of the sequence selectivity in DNA alkylations. Consistent with its relative cytotoxic potency, *ent*-(-)-duocarmycin SA was found to alkylate DNA at concentrations approximately ten times that required for the natural enantiomer.^[54] Table 1 summarizes its consensus alkylation sequence. Each alkylation site detected was an adenine residue, and essentially each alkylation site was flanked by a 5'- and 3'-A or -T base and exhibited the following sequence preference: 5'-AAA > 5'-AAT > 5'-TAA > 5'-TAT. An additional strong preference for A or T as the second 3' base from the alkylation site was observed. In this regard, the (-)-duocarmycin SA alkylation was analogous to the natural enanti-

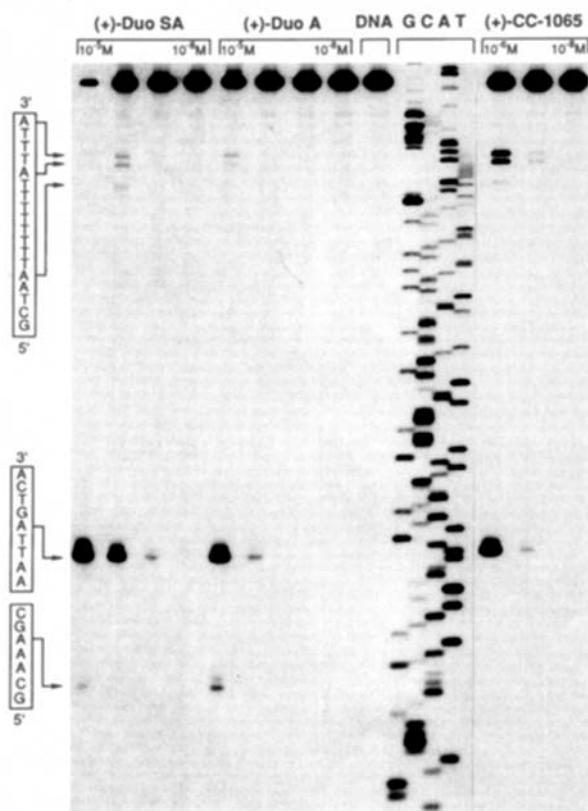


Fig. 2. Thermally induced strand cleavage (by heating to 100 °C for 30 min) of double-stranded DNA (SV40 DNA fragment, 144 base pairs, nucleotide no. 5238–138, clone w794) after 24 h incubation of DNA with the agent at 25 °C and removal of unbound agent by EtOH precipitation followed by denaturing PAGE (8%) and autoradiography. Lanes 1–4: **3** (1×10^{-5} M– 1×10^{-8} M); lanes 5–8: **2** (1×10^{-5} M– 1×10^{-8} M); lane 9: control DNA; lanes 10–13: Sanger G, C, A, and T reactions; lanes 14–16: **1** (1×10^{-6} M– 1×10^{-8} M).

tiomer with the exception that the binding orientation is reversed ($5' \rightarrow 3'$) over an AT-rich, 3.5-base-pair site. However, whereas the bound natural (+)-enantiomer covers an AT-rich, 3.5-base-pair site extending from the adenine-N3 alkylation site in the $3' \rightarrow 5'$ direction across the adjacent two to three 5' bases (that is, 5'-AAAA), the AT-rich, 3.5-base-pair site for the unnatural (-)-enantiomer extends in the reverse $5' \rightarrow 3'$ 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 (that is, 5'-AA~~A~~AA). The reversed binding orientation is required to permit adenine addition to the least substituted cyclopropane carbon, and the alkylation selectivity for an offset AT-rich site is the natural consequence of the diastereomeric relationship of the adducts.

Similarly, preceding studies had shown that (-)-CC-1065 alkylates DNA with an efficiency equivalent to that of the natural enantiomer, but the alkylation rate is slower.^[68, 71] Consistent with these observations, the unnatural and natural enantiomers of CC-1065 have been found to exhibit indistinguishable cytotoxic potency and *in vivo* antitumor activity.^[68, 71] Table 1 summarizes the consensus alkylation sequence for (-)-CC-1065, which proved similar to (-)-duocarmycin SA but which exhibits an AT selectivity that extends across five base pairs.^[53, 68] Each alkylation site proved consistent with 5'-adenine-N3 alkylation and agent binding in the minor groove in the $5' \rightarrow 3'$ direction from the alkylation site covering five base pairs across an AT-rich region. All alkylation sites detected in the sequencing studies proved to be adenine, and nearly all of the 3' and 5' bases flanking the adenine-N3 alkylation site proved to be A or T. The preferred order for the sequence of these bases is as follows: 5'-AAA > 5'-TAA > 5'-AAT, 5'-TAT. There also proved to be a substantial preference for A or T as the second and third 3' base.

Unlike duocarmycin SA, the unnatural enantiomer of duocarmycin A proved to be 100 or more times less effective than the natural enantiomer at alkylating DNA and 100 or more times less potent in cytotoxic assays.^[59] Although these observations initially appeared confusing, they could be attributed to well-defined differences in the diastereomeric adducts derived from the two enantiomers, for which duocarmycin A is especially sensitive.^[54, 73] Inversion of the configuration at C6 of **2**, which leads to **11** (Fig. 3), had no effect on the DNA alkylation

Table 2. Cytotoxic potencies of **1–3** and their isomers and the relative DNA alkylation efficiencies.

Agent	IC_{50} , L1210 [pm]	Rel. Alkylation Efficiency
(+)- 3 , (+)-duocarmycin SA	10	1
(-)- 3 , <i>ent</i> -(+)-duocarmycin SA	100	0.1
(+)- 2 , (+)-duocarmycin A	200	0.05
(-)- 2 , <i>ent</i> -(-)-duocarmycin A	>22 000	<0.0005
(+)- 11 , <i>epi</i> -(+)-duocarmycin A	1 600	0.01
(-)- 11 , <i>ent, epi</i> -(+)-duocarmycin A	>24 000	<0.0005
(+)- 1 , (+)-CC-1065	20	0.5
(-)- 1 , <i>ent</i> -(+)-CC-1065	20	0.5

sequence selectivity and only a small impact on the DNA alkylation efficiency and cytotoxic potency of the agent (Table 2).

As detailed in the following sections, the DNA alkylation properties and selectivity of the unnatural enantiomers have proven uniquely valuable in establishing the key recognition elements and structural features responsible for polynucleotide recognition. Ultimately their preparation and subsequent evaluation alongside the natural enantiomers provided deeper insights into the behavior of the natural products themselves and led to the evolution of models that clearly delineate the requirements for DNA alkylation and the origin of the selectivity.

2.3. Adenine-N3 Alkylation

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 adducts, and the search for undetected alkylation sites have been conducted. In these studies, the adenine-N3 alkylation of DNA present in excess by (+)-duocarmycin A (**2**), (+)-duocarmycin SA (**3**), (-)-duocarmycin SA, and (+)-CC-1065 (**1**) accounts for 86–92%,^[57] 90–100%,^[54] 86–92%,^[54] and 80–90%,^[66] respectively, of their consumption and constitutes the virtually exclusive alkylation event (Scheme 1, p. 1441). The full characterization of the adducts led to the unambiguous assignment of the structures **8–10** (see Scheme 1) in which addition of the adenine-N3 atom to the unsubstituted cyclopropane carbon of the agents was established. The demonstration that **6** and **7** also provide the adduct **8**^[56, 57] requires that they first convert to **2** prior to alkylating DNA.

2.4. Adenine-N3 and Trace Guanine-N3 Alkylation

All three agents and their corresponding seco precursors provide predominantly or exclusively adenine-N3 adducts (>90%). However, a minor guanine-N3 alkylation has been detected with **2** and to a lesser extent with **1**, but only upon isolation of the thermally released adduct following treatment of DNA with excess agent,^[61, 62] within oligodeoxynucleotides lacking a high-affinity adenine-N3 alkylation site,^[62] or when the adenine alkylation sites within AT-rich regions of DNA were

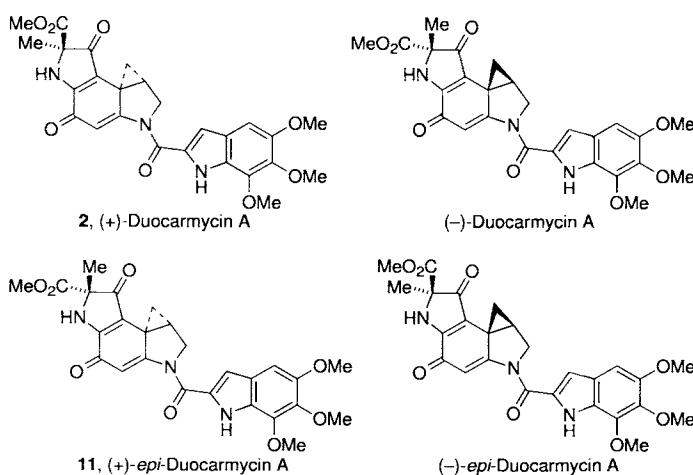


Fig. 3. Stereoisomers of (+)-duocarmycin A (**2**).

protected from alkylation with agents (including distamycin) that bind with high affinity in the AT-rich minor groove.^[63] A study of this minor guanine-N3 alkylation with **2** revealed that as the agent-to-base-pair ratio in the reaction mixture was varied from 1:10 to 1:160, the ratio of adenine-N3 to guanine-N3 alkylation steadily increased from 1.4:1 to 11:1 (92:8),^[61] which clearly indicates that adenine-N3 alkylation is preferred when available.^[57] In contrast, duocarmycin SA showed no evidence of guanine-N3 alkylation when subjected to similar or more forcing reaction conditions.^[54] Even under vigorous conditions, unchanged agent was recovered, and the selectivity (adenine-N3/guanine-N3 alkylation) was greater than 25:1. This enhanced selectivity of **3** may be attributed to its decreased reactivity. Notably, under the relevant conditions of limiting agent even **2** provided nearly exclusive adenine-N3 alkylation.^[57] Since **3** is much more potent than **2**, the minor guanine-N3 alkylation cannot be uniquely important to the biological properties of the agents and may represent a nonproductive competitive event.

Thus, the relative reactivity of the agents may influence the DNA alkylation selectivity. Under the conditions of limiting agent and excess DNA, where little or no guanine-N3 alkylation is detected, the studies have illustrated that the more stable and less reactive agents are both more efficient and more selective among the available alkylation sites. These distinctions would appear to be most important to exploit.

2.5. Reversibility of the DNA Alkylation Reaction: Binding-Driven Bonding

Although the duocarmycin DNA alkylations have proven similar to those of CC-1065, one important feature distinguishes the agents. Unlike **1**, which irreversibly alkylates DNA, duocarmycins **2** and **3** alkylate DNA reversibly.^[54, 58] The ease of reversibility proved dependent upon the relative reactivity of the agent and hence the stability of the adduct, as well as on the extent of the noncovalent binding interactions (Fig. 4). Consistent with the relative reactivity of the agents and the expected stability of the adducts, the (+)-duocarmycin A (**2**) retroalkylation

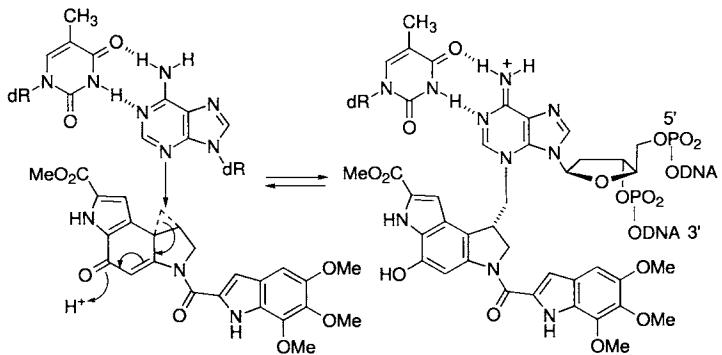


Fig. 4. Reversible DNA alkylation reaction, exemplified with **3**. The characteristics of the reversible reaction are as follows: The rate of the reverse reaction increases with increasing pH, temperature, and ionic strength. It is greater for duocarmycin SA than for duocarmycin A, showing the general decrease with increasing stability of the adduct and increasing agent reactivity. It is greater for the duocarmycins than for CC-1065 and even larger for N-BOC-DSA indicating a general decrease with increasing stabilization provided by the noncovalent binding interactions.

reaction was slower than that of (+)-duocarmycin SA (**3**). In addition, the rate of retroalkylation was faster, and the adduct was much less stable with N-BOC-DSA (**12**) (see Fig. 5) than with **3**, which may be attributed to the lack of stabilization from the trimethoxyindole binding. (+)-CC-1065 (**1**) is less reactive than **2** but more reactive than **3**. The lack of detection of a reversible alkylation by **1** under comparable conditions may be taken as a further indication of the dependence of reversibility on the extent of the noncovalent binding stabilization. Consistent with this interpretation, analogs of CC-1065 possessing the same alkylation subunit but simpler and smaller binding subunits were the first such agents shown to alkylate DNA reversibly.^[70]

Thus, dominant forces stabilizing the DNA alkylation reaction are not only the covalent bond but also the stabilizing noncovalent interactions derived from hydrophobic binding and van der Waals contacts. That is, the alkylation reaction is rendered less reversible or irreversible by noncovalent binding stabilization. The importance of these observations becomes clear when the properties of the agents are compared. The exceptionally potent cytotoxic activity of the natural products relative to that of the simple derivatives of the alkylation subunits themselves (Fig. 5) may be attributed in part to the simple event of noncovalent binding stabilization of the inherently reversible DNA alkylation reaction.

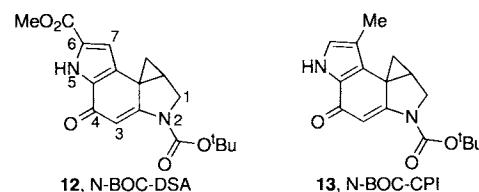
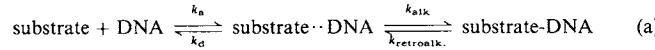


Fig. 5. Structures of derivatives of the alkylation subunits.

This reversible nature of the alkylation reaction itself along with the equilibrium binding requires that four rate constants be addressed in assessing the sequence-selective alkylation [Eq. (a)]. The large differences in the extent of reversibility sug-



gest that treatments to date have neglected two of the more important rate constants,^[43, 46] namely the relative rates of retroalkylation at competing or potentially nonobserved sites and the dissociation rates (off rates) for the available binding sites. The off rates for some noncovalent adducts have been shown qualitatively to be quite slow.^[82]

These observations coupled with studies indicating that the noncovalent binding equilibrium is established through decomplexation, not through procession along the minor groove, suggest that dissociation rates and not simply association rates or $K_b (= k_a/k_d)$ may be especially important.^[96] Defined oligonucleotides that contain sites capable of noncovalent binding but incapable of alkylation have been shown to inhibit the rates of

DNA alkylation. This would suggest that the rates of equilibrium binding may be slow and that the dissociation rate or relative K_b , not the association rate or rate of alkylation, may be rate-determining. Moreover, this inhibition of DNA alkylation was the same whether the competitive site for noncovalent binding was inter- or intramolecular. These studies would seem to demonstrate that the first binding equilibration in Equation (a) may be slow relative to alkylation and that the off rates or relative K_b are significant and should not be neglected.

2.6. Models of the DNA Alkylation for Enantiomeric Agents

The examination of the natural products, their synthetic unnatural enantiomers, and key partial structures not only resulted in the unusual observation that the unnatural enantiomers constitute effective DNA alkylating agents and potent antitumor antibiotics, but has also led to the emergence of a detailed model^[53, 54] of the structural features responsible for their selective alkylation of DNA. The characterization of **8–10**, the unambiguously established absolute configuration of the agents, and the definition of the alkylation consensus sequences for both enantiomers of **1–3** provided the necessary information for the construction of accurate models of the adenine-N3 alkylation. Figure 6 illustrates models of the (+)- and (-)-duocarmycin SA alkylation at a common site in w794 DNA (5'-CTAATT), which is a high-affinity site for the unnatural enantiomer, and a minor site for the natural enantiomer.^[54]

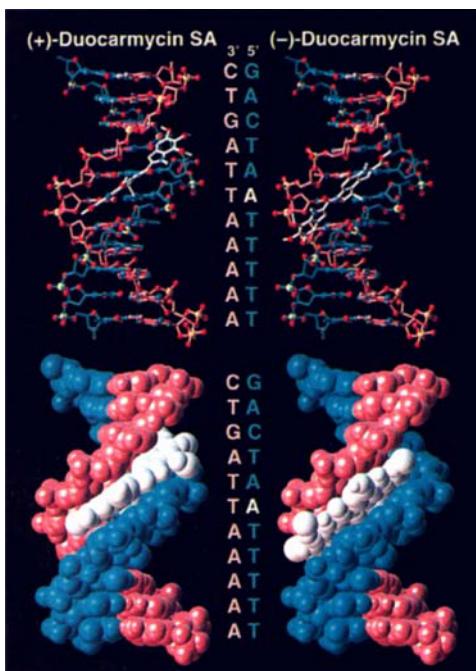


Fig. 6. Stick and space-filling models illustrating the alkylation at the same site within w794 DNA (duplex 5'-d(GACTAATTCTT)) by (+)-duocarmycin SA (left) and (-)-duocarmycin SA (right). The binding of the natural enantiomer extends in the 3' → 5' direction from the adenine-N3 alkylation site across the sequence 5'-CTTAA, that of the unnatural enantiomer in the reverse 5' → 3' direction across the site 5'-AATT.

Similar models of the (+)- and (-)-CC-1065 alkylations may be found in Figure 7.^[53] For both the duocarmycins and CC-1065 the hydrophobic face of the agent is imbedded deeply in the minor groove, the polar functionality lies on the outer face of the complex, and the bound helical conformation of the agent complements the topological curvature and pitch of the minor

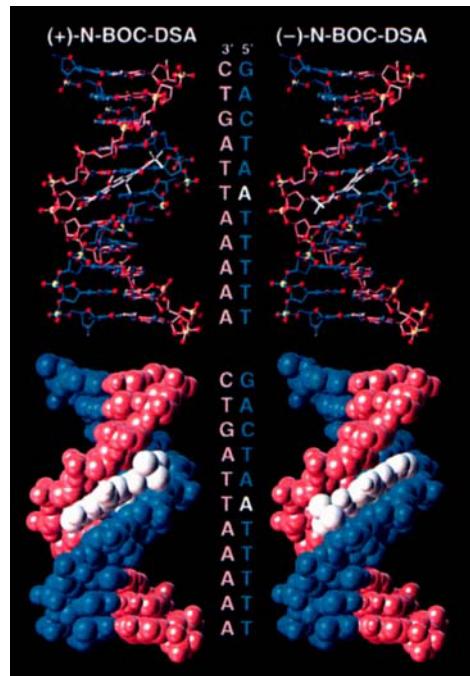


Fig. 7. Stick and space-filling models illustrating the alkylation at the same site within w794 DNA (duplex 5'-d(GACTAATTTTT)) by (+)-CC-1065 (left) and (-)-CC-1065 (right). The binding of the natural enantiomer extends in the 3' → 5' direction from the adenine-N3 alkylation site across the sequence 5'-ACTAA, that of the unnatural enantiomer in the reverse 5' → 3' direction across the site 5'-AA TTT.

groove spanning an AT-rich, 3.5- or 5-base-pair site, respectively. 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 two to three adjacent 5' base pairs (5'-CTAA). For the enantiomer, the binding similarly spans a 3.5-base-pair, AT-rich site, but one which necessarily starts at the 5' base adjacent to the alkylation site and extends in the 5' → 3' direction over the alkylation site and one to two adjacent 3' base pairs (5'-A_nATT). This alkylation selectivity within an offset AT-rich site is the natural consequence of the diastereomeric relationship of the adducts, and the reversed binding orientation (with respect to the alkylation site) in the minor groove is required to permit addition of the adenine-N3 atom to the least substituted carbon of the electrophilic cyclopropane. The importance of the fourth base (A/T > G/C) in the binding sequences is the reason why this site constitutes a high-affinity site for the unnatural enantiomer but only a minor site for the natural enantiomer. Similarly, the CC-1065 models^[53] accommodate the larger size of the more extended five-base-pair, AT-rich binding site of the enantiomeric agents, their reversed binding orientation in the minor groove, and their AT-rich selectivity offset relative to the alkylation site.

The apparently confusing distinctions in the relative efficiency of DNA alkylations by the natural and unnatural enantiomers were found to correlate with the extent of the stabilization of the inherently reversible noncovalent binding in the adducts, as well as with the degree of steric bulk surrounding the C7 center of the agent. Consistent with expectations based on the models, the unnatural enantiomers are especially sensitive to the steric bulk around this C7 center.^[53, 54, 73]

3. Key Partial Structures

3.1. N-BOC-DSA: A DNA Alkylating Agent Whose Selectivity and Efficiency Are Independent of Its Absolute Configuration

The accuracy of the models were revealed when they were found to provide a beautiful explanation for the unusual observation that both enantiomers of simple derivatives of the alkylation subunit, for example N-BOC-DSA (**12**)^[54] or N-BOC-CPI (**13**, see Fig. 5),^[53, 68] alkylate the same sites in DNA. In addition to illustrating that the DNA alkylation reactions of (+)- and (-)-**12** are both substantially less efficient (about $10^4 \times$) and less selective (selectivity: 5'-AAA > 5'-TAA), and proceed with a different profile from that of (+)- or (-)-duocarmycin SA or CC-1065, the studies have shown that both enantiomers of **12** alkylate the same sites with essentially the same efficiency independent of the absolute configuration.^[54] This feature of such simple agents is general, and the *N*-acetyl derivatives have been shown to behave similarly. Figure 8 illustrates these features nicely, and the consensus alkylation selectivity for both (+)- and (-)-N-BOC-DSA and -N-BOC-CPI is summarized in Table 3. Although these observations may appear unusual, they are a 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,^[53, 54] which are illustrated in Figure 9, nicely accommodate the observed selectivity of 5'-AAA > 5'-TAA for both enantiomers. The apparent preference of 5'-AAA over 5'-TAA (about 2:1) observed when examining one strand of single end-labeled DNA at a time is statistical rather than structural in nature. The unlabeled complementary partner strand of a 5'-TA sequence contains an identical competitive site, whose alkylation diminishes the apparent alkylation efficiency of the labeled strand. The factor controlling alkylation is simply the depth of penetration into the minor groove achieved by the agent, which is essential for the adenine-N3 alkylation of the electrophilic cyclopropane. For simple agents such as **12** and **13**, it is sufficient when the adjacent 5' base is A or T, and models of an unobserved 5'-GAA alkylation support this proposal.^[53, 54] For **3** or **1**, a larger 3.5- or 5-base-pair, AT-rich region surrounding the alkylation site is required to permit sufficient groove penetration for alkylation, which is further facilitated by the preferential noncovalent binding of the agents within the narrower, deeper, AT-rich minor groove.^[82]

These distinctions are important but not uniformly accepted in the work conducted to date. Whereas our studies revealed

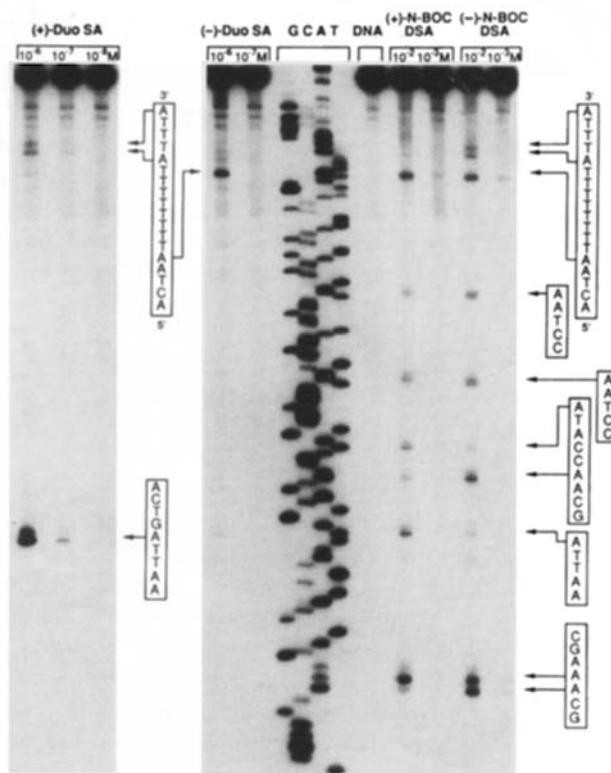


Fig. 8. Thermally induced strand cleavage (by heating to 100 °C for 30 min) of w794 DNA (SV40 DNA segment, 144 base pairs, nucleotide no. 5238-138) after 24 h incubation of DNA with (+)- or (-)-duocarmycin SA at 25 °C and with (+)- or (-)-N-BOC-DSA at 37 °C and removal of unbound agent by EtOH precipitation followed by denaturing PAGE (8%) and autoradiography. Lanes 1–3: **3** (1×10^{-6} – 1×10^{-8} M); lanes 4 and 5: **ent-3** (1×10^{-6} – 1×10^{-7} M); lanes 6–9: Sanger G, C, A, and T reactions; lane 10: control DNA; lanes 11 and 12: (+)-**12** (1×10^{-2} – 1×10^{-3} M); lanes 13 and 14: (-)-**12** (1×10^{-2} – 1×10^{-3} M).

distinctions between the natural products and simple derivatives of the alkylation subunit in the extent of the alkylation selectivity, the collaborative efforts of the Hurley and Upjohn groups with CPI-derivatives report an indistinguishable or subtly distinguishable alkylation selectivity between (+)-CC-1065 and (+)-N-acetyl-CPI.^[43, 46, 47, 67, 68] This premise has led them to the conclusion that all the structural requirements for observation of the alkylation selectivity by (+)-CC-1065 are embodied in the CPI alkylation subunit.

Despite the similarities in the behavior of N-BOC-DSA (**12**) and N-BOC-CPI (**13**), two important differences were observed. First, both the natural and unnatural enantiomers of **12** are more efficient at alkylating DNA and are biologically more potent than the corresponding enantiomers of **13**. These differences may be attributed to the enhanced stability of **12**, which leads to more productive DNA alkylation. Secondly, while little or no distinction was observed in the DNA alkylation efficiencies of the natural and unnatural enantiomers of **12**, the natural enantiomer of **13** was found to be both more efficient and more cytotoxic than its unnatural enantiomer by a factor of between 10 and 100. As detailed later, this apparently confusing behavior of enantiomeric pairs may be attributed to the degree of steric bulk surrounding the C7 center, for which the diastereomeric adducts derived from the unnatural enantiomers are especially sensitive.

Table 3. Consensus sequences for DNA alkylation by key substructures and analogs of CC-1065 and the duocarmycins [a].

Agent	Base [b]	5'	6	5	4	3	2	1	0	-1	-2	-3	-4	-5	3'
<i>natural enantiomers</i>															
(+)-CPI-CDPI ₃	A/T (56)	72		77	69	69	100	100	100	54	-	-	-	-	-
(+)-17	consensus	A/T > G/C	A/T	A/T	A	N	-	-	-	-	-				
(+)-CC-1065	A/T (56)	-		-	67	78	94	98	100	55	-	-	-	-	-
1	consensus				A/T ≥ G/C	A/T > G/C	A/T	A/T	A	Pu ≥ Py	-	-	-	-	-
(+)-CPI-CDPI ₂	A/T (56)	-		-	67	78	94	98	100	55	-	-	-	-	-
(+)-16	consensus				A/T ≥ G/C	A/T > G/C	A/T	A/T	A	Pu ≥ Py	-	-	-	-	-
(+)-CPI-PDE-I ₁	A/T (56)	-		-	-	65	100	100	100	58	-	-	-	-	-
(+)-14	consensus					A/T ≥ G/C	A/T	A/T	A	Pu ≥ Py	-	-	-	-	-
(+)-CPI-CDPI ₁	A/T (56)	-		-	-	65	100	100	100	58	-	-	-	-	-
(+)-15	consensus					A/T ≥ G/C	A/T	A/T	A	Pu ≥ Py	-	-	-	-	-
(+)-duocarmycin SA	A/T (56)	-		-	-	79	100	100	100	69	-	-	-	-	-
3	consensus					A/T > G/C	A/T	A	Pu > Py	-	-	-	-	-	-
(+)-N-BOC-CPI	A/T (56)	-		-	-	-	-	97	100	66	-	-	-	-	-
(+)-13	consensus						A/T	A	Pu > Py	-	-	-	-	-	-
(+)-N-BOC-DSA	A/T (56)	-		-	-	-	-	95	100	65	-	-	-	-	-
(+)-12	consensus						A/T	A	Pu > Py	-	-	-	-	-	-
<i>unnatural enantiomers</i>															
(-)-N-BOC-DSA	A/T (56)	-		-	-	-	-	95	100	65	-	-	-	-	-
(-)-12	consensus						A/T	A	Pu > Py	-	-	-	-	-	-
(-)-N-BOC-CPI	A/T (56)	-		-	-	-	-	97	100	66	-	-	-	-	-
(-)-13	consensus						A/T	A	Pu > Py	-	-	-	-	-	-
(-)-duocarmycin SA	A/T (56)	-		-	-	-	-	93	100	96	73	56	-	-	-
ent-3	consensus						A/T	A	A/T	A/T > G/C	N	-	-	-	-
(-)-CPI-CDPI ₂	A/T (56)	-		-	-	-	-	88	100	93	82	73	56	-	-
(-)-16	consensus						A/T	A	A/T	A/T > G/C	A/T > G/C	N	-	-	-
(-)-CC-1065	A/T (56)	-		-	-	-	-	88	100	93	82	73	56	-	-
ent-1	consensus						A/T	A	A/T	A/T > G/C	A/T > G/C	N	-	-	-
(-)-CPI-CDPI ₃	A/T (56)	-		-	-	-	-	100	100	100	100	86	86	57	-
(-)-17	consensus						A/T	A	A/T	A/T	A/T > G/C	A/T > G/C	N	-	-

[a] The numbers listed in the table give the percentage of the indicated base located at the designated position relative to the adenine-N3 alkylation site. [b] The numbers in parentheses give the percentage composition within the DNA examined.

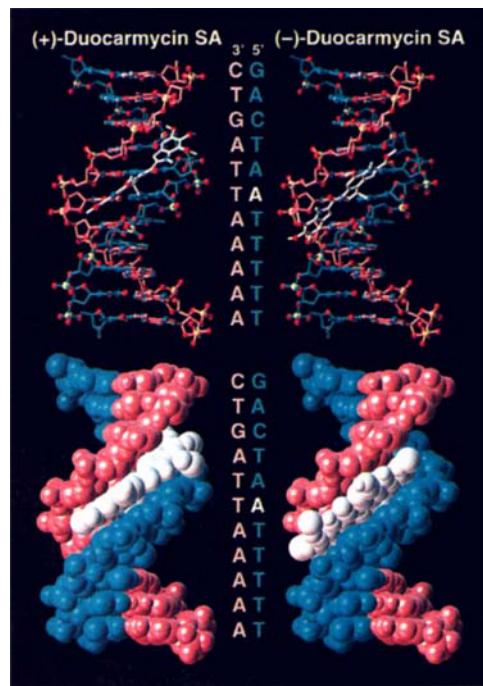


Fig. 9. Stick and space-filling models illustrating the alkylation of the same site within w794 DNA (duplex 5'-d(GACTAATTTT)) by (+)-12 (left) and (-)-12 (right). The binding of the natural enantiomer extends in the 3' → 5' direction from the adenine-N3 alkylation site across the site 5'-AA, that of the unnatural enantiomer in the reverse 5' → 3' direction but across the same 5'-AA site.

3.2. CPI-PDE-I₁: A Key Substructure of CC-1065

In early efforts to establish the role of each of the subunits of CC-1065, both enantiomers of CPI-PDE-I₁ (**14**, Fig. 10) were prepared for evaluation.^[71] Although a number of interesting

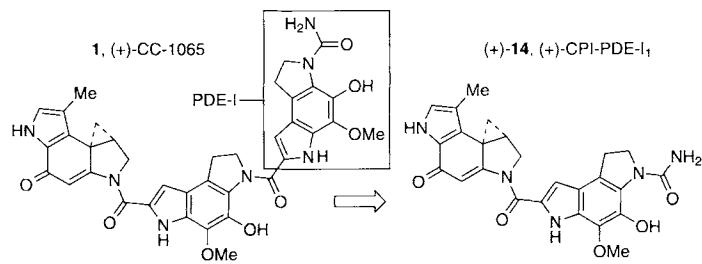


Fig. 10. CPI-PDE-I₁ (**14**), a key substructure of CC-1065.

observations were made in this early study, only two of the more seminal are described below. First, (+)-CPI-PDE-I₁ displayed in vitro cytotoxic activity indistinguishable from (+)-CC-1065, indicating that the entire righthand subunit framed in Figure 10 was unnecessary for observation of the full biological potency of the natural product. The subsequent discovery of the duocarmycins, which also lack this third subunit, provided indepen-

dent confirmation of its relative unimportance to the biological properties of the natural enantiomers. In addition, the natural enantiomer of **14** exhibited a similar, although perceptibly altered DNA alkylation selectivity from that observed with (+)-CC-1065. Table 3 (Section 3.1) summarizes the consensus alkylation sequence found for (+)-**14**. Like (+)-CC-1065, each alkylation site detected proved to be adenine, and each site was flanked by two 5'-A or -T bases. The same order of preference as in the case of **1** was observed for this three-base, AT-rich alkylation region: 5'-AAA \approx 5'-TTA $>$ 5'-TAA $>$ 5'-ATA. The distinctions between (+)-**14** and (+)-CC-1065 were found to lie in the AT preference extended to the fourth and fifth 5' bases. With (+)-**14**, a weaker preference for the fourth 5'-A or -T base was observed, and unlike (+)-CC-1065, no preference for the fifth 5' base was observed. Thus, like the subsequent observations made with duocarmycin A and SA, (+)-**14** exhibits alkylation selectivity for a shorter 3.5-base-pair, AT-rich site. Consequently, the presence of the DNA binding subunits were found to influence the sequence selectivity of DNA alkylation, and a full study of shorter and more extended analogs of CC-1065 revealed that this AT-rich selectivity corresponds nicely to the length of the agent and the size of the region surrounding the alkylation site required to bind the agent (see Table 3).^[53, 71]

The relative cytotoxic potencies of related agents, including those that lack the DNA binding subunits altogether, suggested an additional important functional role for the DNA binding subunits. (+)-N-BOC-CPI and (+)-N-acetyl-CPI, which lack an attached DNA binding subunit, proved to be approximately 1000 to 10 000 times less potent than (+)-CC-1065. In contrast, the natural enantiomers of the agents **14**–**17** (see Figs. 10 and 12), which possess one to three DNA binding subunits, proved equipotent with the natural product (Table 4). Similarly, (+)-N-BOC-CPI and (+)-N-acetyl-CPI were substantially less efficient (1000–10 000 \times) at alkylating DNA, whereas **14**–**17** and **1** exhibited comparable efficiencies but different DNA alkylation rates and subtly distinguishable selectivities for AT-rich alkylation sites, corresponding to their relative sizes. This, in conjunction with computational studies, quantitative molecular modeling studies, and an appreciation of the intrinsic stability of the

agents, suggested to us that the DNA alkylation was inherently reversible but stabilized by the noncovalent interactions provided by the DNA binding subunits. Furthermore, it suggested that a single DNA binding subunit would provide this stabilization for the natural enantiomers and that it was the simple event of the stabilization that provided the full potentiation of the biological properties. At this time the reversible nature of the DNA alkylation was not recognized, and the Hurley and Upjohn groups, in their complementary efforts, proposed that the distinctions were due to the rates of DNA alkylation and the kinetic aspects of the formation of the DNA adducts.^[46] These varying proposals led to the subsequent discovery that the DNA alkylation reactions are indeed reversible and that the adducts are increasingly stabilized by the presence and size of the DNA binding subunits.^[58, 70]

3.3. CPI-CDPI₂: A Precise Functional Analog of CC-1065

Among the first analogs of CC-1065 examined, that which was derived from removal of the methoxy and hydroxy substituents from the central and righthand subunits proved especially revealing (Fig. 11). CPI-CDPI₂ proved particularly im-

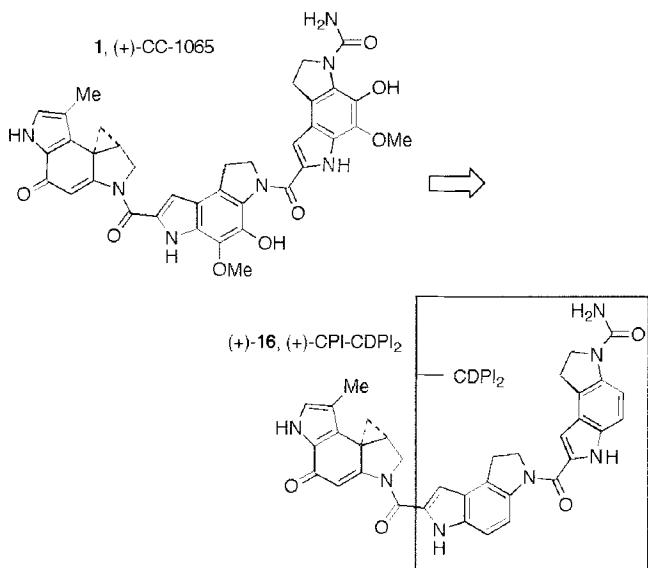


Fig. 11. CPI-CDPI₂ (**16**), a precise functional analog of CC-1065.

Table 4. In vitro cytotoxic activity of key partial structures of CC-1065 and the duocarmycins.

Agent	IC_{50} , L1210 [pm]
<i>natural enantiomers</i>	
(+)- 12 , (+)-N-BOC-DSA	6000
(+)- 13 , (+)-N-BOC-CPI	300 000
3 , (+)-duocarmycin SA	10
1 , (+)-CC-1065	20
(+)- 14 , (+)-CPI-PDE-I ₁	20
(+)- 15 , (+)-CPI-CDPI ₁	40
(+)- 16 , (+)-CPI-CDPI ₂	20
(+)- 17 , (+)-CPI-CDPI ₃	20
<i>unnatural enantiomers</i>	
(-)- 12 , (-)-N-BOC-DSA	60 000
<i>ent</i> - 3 , (-)-duocarmycin SA	100
<i>ent</i> - 1 , (-)-CC-1065	20
(-)- 14 , (-)-CPI-PDE-I ₁	≥ 2400
(-)- 15 , (-)-CPI-CDPI ₁	≥ 6300
(-)- 16 , (-)-CPI-CDPI ₂	20
(-)- 17 , (-)-CPI-CDPI ₃	50

portant to examine because of the growing perception at the time that the polar functionality on CC-1065 was contributing significantly to the properties of the agents. Its examination addressed three important questions. The first was the contribution that the substituents may make to DNA binding, the resulting alkylation properties of the natural product, and their contribution to the biological properties. Although the substituents were initially expected to influence the DNA alkylation properties significantly, extensive molecular modeling studies did not support such a role.^[38, 41] Secondly, the C4 hydroxy group had been suggested to facilitate DNA binding by hydrogen bonding to the carbonyl group of the linking subunit, thereby stabilizing

a curved and helical conformation required for groove binding. However, this intramolecular hydrogen bonding introduced an exaggerated pitch to the helical conformation of CC-1065, and the less pronounced inherent pitch of the helical conformation of (+)-**16** more closely followed that required for minor groove binding.^[38, 77, 78] Finally, oxidation of the central and righthand subunits to an extended *para*-quinone imine was thought potentially to contribute to the delayed toxicity of (+)-CC-1065.^[80]

The preparation of both enantiomers of CPI-CDPI₂^[77] and their comparison with the enantiomers of CC-1065 revealed that the two agents were indistinguishable.^[71] The cytotoxic activity, *in vivo* antitumor activity, and DNA alkylation efficiencies and selectivities of both enantiomers of CPI-CDPI₂ were identical to those of the corresponding CC-1065 enantiomer. Moreover, (+)-CPI-CDPI₂ exhibited the delayed toxicity characteristic of (+)-CC-1065, whereas both the unnatural enantiomers lacked this property.^[79] Thus, the examination of **16** established that the hydroxy and methoxy substituents on the central and righthand subunits of CC-1065 do not contribute to the properties of the natural product. Some of the conclusions drawn from a complementary and concurrent study were initially distinct from this^[79] but later revised.^[46]

The indistinguishable profiles of DNA alkylation proved particularly revealing when compared to those of the series of structurally related agents detailed in the next section. The studies were facilitated through introduction of a useful protocol for rapidly securing substantial quantities of DNA labeled at one end with ³²P.^[72]

3.4. CPI-CDPI_n: Extended and Shortened Analogs of CC-1065

In initial efforts to establish the functional roles of each of the CC-1065 subunits, the full set of shortened and extended agents **15–17** were prepared for comparative examination (Fig. 12).^[71] In these studies, CPI-CDPI₁ and CPI-PDE-I₁ proved indistinguishable, and the natural enantiomers of CPI-CDPI_n ($n = 1–3$) and CPI-PDE-I_n ($n = 1–2$) exhibited the same cytotoxic potency. Pertinent to defining the origin of the DNA alkylation selec-

tivity of (+)-CC-1065 is the observation that the natural enantiomers of (+)-CPI-CDPI₁ and (+)-CPI-PDE-I₁, as well as (+)-CPI-CDPI₃, were distinguishable from natural product and (+)-CPI-CDPI₂. The former exhibited a selectivity for shorter 3.5-base-pair, AT-rich alkylation sites, while the latter favored a more extended, 6.5–7-base-pair, AT-rich site.^[53] Table 3 summarizes the consensus alkylation sequences for both the natural and unnatural enantiomers of these key substructures of CC-1065.

One of the initially confusing observations was that the unnatural enantiomers of CPI-CDPI₁ and CPI-PDE-I₁, unlike (−)-CC-1065, failed to alkylate DNA productively and were relatively nonpotent cytotoxic agents (Table 4). Although this was not easily rationalized at the time the observations were made, it now can be attributed to the extent of stabilization provided by the noncovalent contacts and the degree of steric bulk surrounding the C7 center of the CPI or DSA alkylation subunit (**12** or **13**, respectively). For the unnatural enantiomer, this results from destabilizing steric interactions with the 5' base preceding the alkylated adenine residue on the alkylated strand of DNA, which decelerate the rate of DNA alkylation and destabilize reversible adduct formation. A single DNA binding subunit is necessary to stabilize reversible adduct formation for the natural CPI alkylation subunit, whereas the unnatural CPI enantiomer requires two such DNA binding subunits. In sharp contrast, a single DNA binding subunit is required for both the natural and unnatural enantiomers of DSA, since the destabilizing steric interactions at C7 unique to the unnatural enantiomer adducts have been reduced.

Further simplifications in the DNA binding subunits have been accomplished and studies with agents containing deep-seated modifications in this region have been described. These are discussed in more detail in Section 10.

4. Noncovalent Binding Affinity and Selectivity

4.1 PDE-I_n, CDPI_n, ACDPI_n, and TACDPI_n

Important early insights into the structural origin of the sequence-selective DNA alkylation were derived from the examination of the characteristics of the noncovalent binding to DNA of **18–21**, key partial structures of **1–3** that were not capable of the covalent bond formation (Fig. 13). Although the studies have been conducted with a range of agents and various DNA,^[81–84] only the seminal aspects of the studies are summarized in Figure 13. Both relative and absolute DNA binding constants have been established, and the agents exhibited a substantial selectivity for binding to the AT-rich minor groove (Table 5).^[82, 83] In addition, CDPI₃ was the optimum binding agent within the CDPI_n series (Fig. 13, bottom). The minor groove binding of CDPI₃ spans five base pairs or half a helix turn, which constitutes the largest site accessible for synchronous binding of both ends of the rigid agent. Partially bound forms of the larger agents, for instance CDPI₄ bound through a CDPI₂ subunit only, constitute productive and stable noncovalent complexes, and CDPI₄ and CDPI₅ were not as effective as CDPI₃. The removal of the hydroxy and methoxy substituents (PDE-I_n → CDPI_n) had only a small impact on the

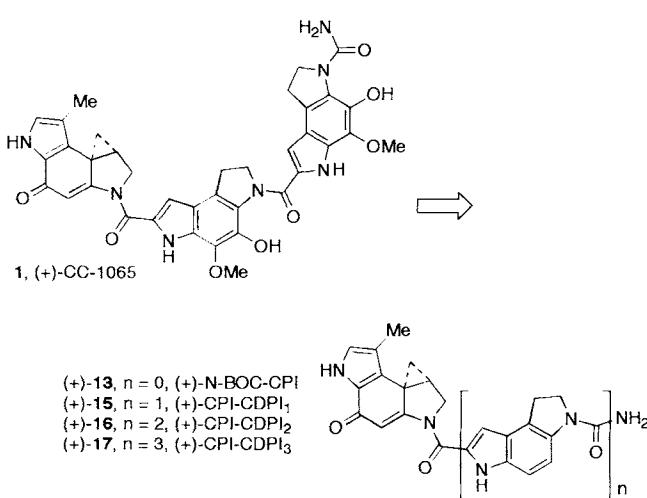


Fig. 12. CPI-CDPI_n, extended and shortened analogs of CC-1065.

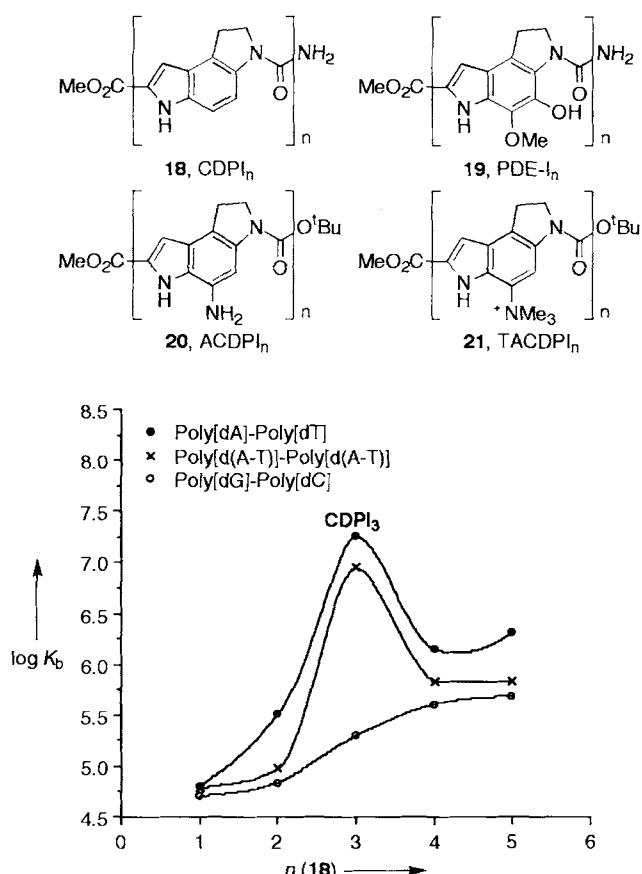


Fig. 13. Structures (top) and binding constants (bottom) of substrates that bind noncovalently to DNA.

binding affinity and no apparent impact on the selectivity for AT-rich binding sites.^[82] Since there is no CDPI_n functionality that would provide hydrogen bonding or electrostatic stabilization, the affinity is derived principally from stabilizing van der Waals contacts. The preference for AT-rich minor-groove binding is probably due to the deeper penetration of the agents into the narrower, AT-rich minor groove, where the stabilizing contacts are maximized.

Consequently, the studies suggested that (+)-CC-1065 is best represented as a selective alkylating group superimposed upon the rigid CDPI_3 skeleton^[82] rather than as a reactive alkylating agent (CPI) attached to a DNA binding agent (PDE-I₂). The agents exhibit a substantial preference for noncovalent binding at AT-rich regions in the minor groove, which is optimal with the rigid trimer. Among features that contribute to minor-groove binding selectivity, at least two structural characteristics of an A-T base pair and of a run of A-T base pairs are important to agents that rely on stabilizing van der Waals contacts and hydrophobic binding. First, the amino substituent of a G-C base pair that extends into the minor groove has no counterpart in an A-T base pair (Fig. 14). This results in a sterically more accessible region adjacent to an A-T base pair that permits the deeper penetration of an agent into the minor groove and enhances the stabilization derived from van der Waals contacts. In addition, X-ray crystallographic studies of oligodeoxynucleotides that contain a run of A-T base pairs in the center of their structure have revealed characteristic features including the constricted width of the AT-rich minor groove.^[14, 15] Illustrated in Figure 14 (bottom) is a plot of the effective width of the minor groove of four such oligodeoxynucleotides that highlights this

Table 5. Binding constants and free energies of binding for the noncovalent binding to three DNA sequences.

Agent	$K_b [\text{M}^{-1}]$	$\Delta G_{298}^{\circ} [\text{kcal mol}^{-1}]$	Agent	$K_b [\text{M}^{-1}]$	$\Delta G_{298}^{\circ} [\text{kcal mol}^{-1}]$	Agent	$K_b [\text{M}^{-1}]$	$\Delta G_{298}^{\circ} [\text{kcal mol}^{-1}]$
<i>Poly[dA]-Poly[dT]</i>								
CDPI ₁	6.4×10^4	-6.6	ACDPI ₁	5.7×10^3	-5.1	TACDPI ₁	3.8×10^5	-7.6
CDPI ₂	3.2×10^5	-7.5	ACDPI ₂	2.8×10^4	-6.1	TACDPI ₂	1.4×10^7	-9.8
CDPI ₃	1.8×10^7	-9.9	ACDPI ₃	9.6×10^5	-8.2	TACDPI ₃	1.3×10^9	-11.1
CDPI ₄	1.4×10^6	-8.4	ACDPI ₄	5.6×10^5	-7.8			
CDPI ₅	2.1×10^6	-8.6						
CDPI ₃ -OtBu	1.5×10^7	-9.8						
PDE-I ₂	1.6×10^7	-9.8						
PDE-I ₃	> 4.8×10^7	-						
distamycin	> 1.1×10^8	(-11.4)						
<i>Poly[d(A-T)]-Poly[d(A-T)]</i>								
CDPI ₁	6.1×10^4	-6.5	ACDPI ₁	4.3×10^3	-5.0	TACDPI ₁	4.5×10^5	-7.7
CDPI ₂	9.6×10^4	-6.8	ACDPI ₂	2.1×10^4	-5.9	TACDPI ₂	2.1×10^7	-10.0
CDPI ₃	8.9×10^6	-9.5	ACDPI ₃	1.4×10^5	-8.4	TACDPI ₃	5.4×10^8	-11.9
CDPI ₄	6.7×10^5	-8.0	ACDPI ₄	2.2×10^5	-7.3			
CDPI ₅	6.7×10^5	-8.0						
CDPI ₃ -OtBu	7.3×10^6	-9.3						
PDE-I ₂	4.6×10^6	-9.1						
PDE-I ₃	> 2.6×10^7	-						
distamycin	> 7.2×10^7	(-12.6)						
<i>Poly[dG]-Poly[dC]</i>								
CDPI ₁	5.1×10^4	-6.4	ACDPI ₁	3.2×10^3	-4.8	TACDPI ₁	2.1×10^4	-5.9
CDPI ₂	6.9×10^4	-6.6	ACDPI ₂	4.3×10^3	-5.0	TACDPI ₂	1.0×10^6	-8.2
CDPI ₃	2.0×10^5	-7.2	ACDPI ₃	1.4×10^5	-7.0	TACDPI ₃	1.6×10^6	-8.4
CDPI ₄	4.1×10^5	-7.7	ACDPI ₄	1.0×10^5	-6.8			
CDPI ₅	4.9×10^5	-7.8						
CDPI ₃ -OtBu	2.1×10^5	-7.3						
PDE-I ₂	-	-						
PDE-I ₃	> 7.7×10^5	-8.0						
distamycin	> 2.9×10^6	-						

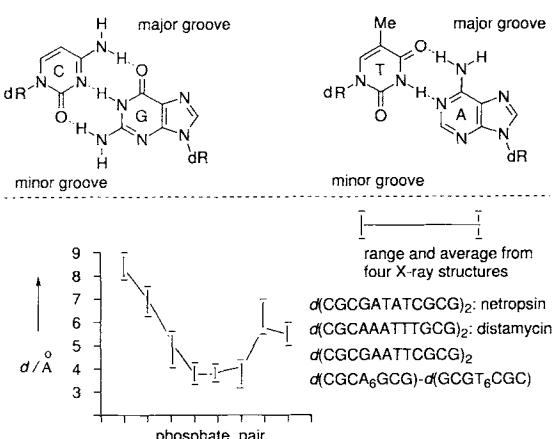


Fig. 14. GC and AT base pairs illustrating the resulting difference in width of the DNA groove. d = effective width of the minor groove.

narrower, AT-rich minor groove. This important conformational feature of DNA possessing runs of A-T base pairs contributes prominently to the selectivity of the agents that depend on stabilizing van der Waals contacts for noncovalent binding. The noncovalent complexes of such agents may be expected to form preferentially within the narrower, sterically more accessible, AT-rich minor groove, where the stabilizing van der Waals contacts are most effectively provided.

More recently the DNA binding properties of ACDPI_n (20, $n = 1-4$) and TACDPI_n (21, $n = 1-3$) have been described.^[83] As with the CDPI_n series, ACDPI₃ proved to be the optimal minor groove binding agent and exhibited a selectivity for AT-rather than GC-rich DNA. Similarly, TACDPI_n exhibited a substantial selectivity for AT-rich DNA binding sites ($n = 3$, $\Delta\Delta G = -2.7$ to $-3.5 \text{ kcal mol}^{-1}$). The comparison of CDPI_n

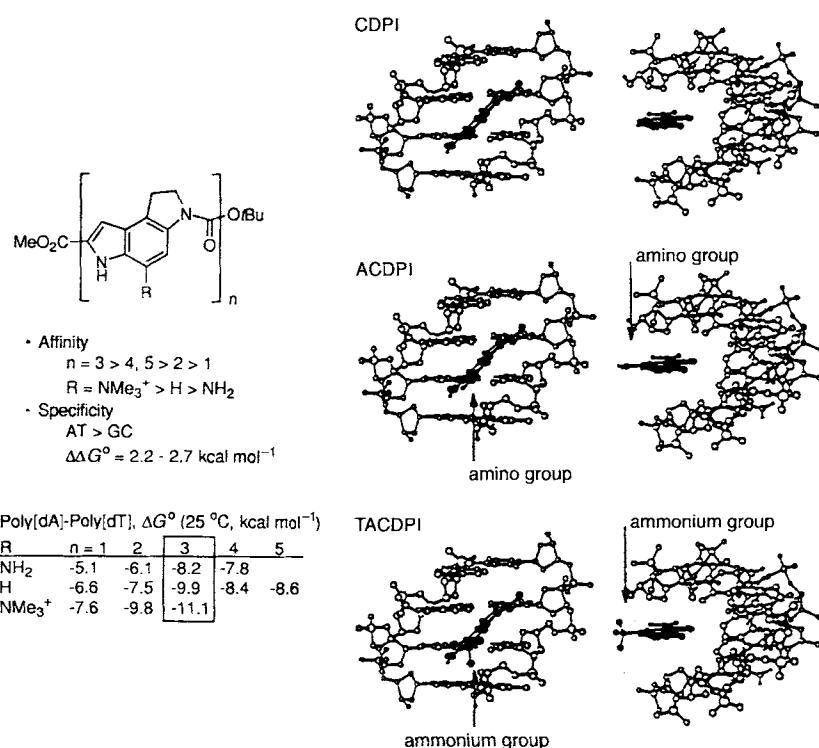


Fig. 15. Influence of the C5 substituent of the agent binding in the minor groove of DNA on binding affinity and selectivity.

substrates with ACDPI_n substrates illustrated that the introduction of a strong electronegative substituent onto the outer face of the agent reduced the binding affinity through introduction of destabilizing electrostatic interactions with the phosphate backbone. In contrast, the behavior of TACDPI_n substrates revealed that the introduction of a C5 quaternary amine substantially enhanced the binding affinity through introduction of stabilizing electrostatic interactions with the phosphate backbone (Fig. 15).

Finally, the terminal urethane of CC-1065 was found not to contribute to the minor-groove binding affinity or selectivity.^[82]

4.2. Affinity Cleavage: The Sites for Noncovalent Binding and for Alkylation Coincide

A study of the efficiency and selectivity of DNA cleavage by the Fe^{III} complex of the affinity cleavage agent 22^[85] (Fig. 16)

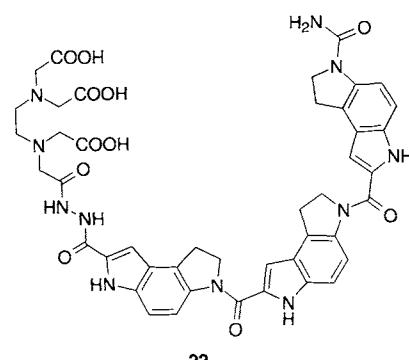


Fig. 16. Structure of the affinity cleavage agent 22.

has provided further direct evidence that for both (+)- and (-)-CC-1065 all DNA alkylation sites coincide with AT-rich regions suitable for noncovalent binding.^[86] Importantly, no DNA alkylation sites were observed to lie outside such preferred noncovalent binding regions. To a first approximation, the relative alkylation efficiencies between sites paralleled the relative cleavage efficiencies of Fe^{III}-22 at the competing sites. As the affinity cleavage agent 22 provided clean cleavage at both ends of a five-base-pair binding site, it must bind in both orientations in the site. The offset H-abstraction sites leading to DNA cleavage confirm minor-groove binding. This relationship of the DNA cleavage sites for 22 surrounding the w794 five-base-pair, AT-rich, high-affinity alkylation site for CC-1065 is illustrated in Figure 17. Notably, (+)-CC-1065 can only alkylate one site and strand in this sequence, since in its reversed bound orientation the activated cyclopropane is placed adjacent to the two 3' T bases on the left strand.

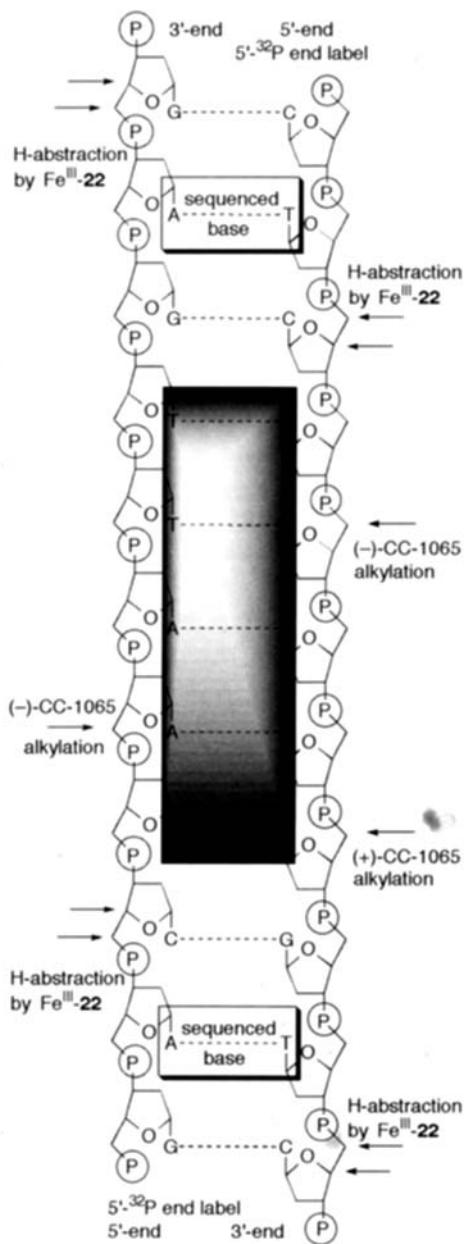


Fig. 17. Model illustrating the relationships between the sequenced cleavage sites for $\text{Fe}^{\text{III}}\text{-22}$, its C4' and C5' H-abstraction sites, the five-base-pair, AT-rich site for noncovalent binding, and the sites of adenine-N3 alkylation by (+)- and (-)-CC-1065 in w794 DNA.

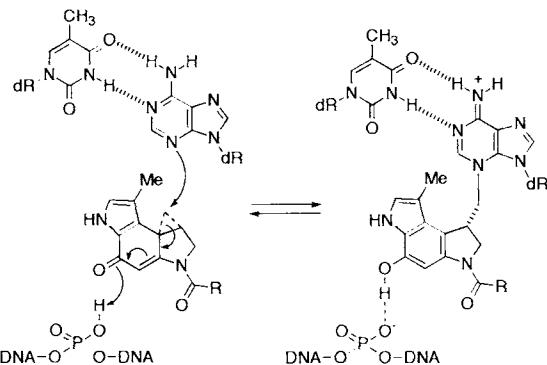
4.3. Circular Dichroism Studies

Difference circular dichroism studies initially demonstrated that CC-1065 binds in the AT-rich regions of the minor groove in double-stranded B DNA.^[87–97] This technique has also shown that (+)-CC-1065 forms a stable noncovalent complex with the Dickerson dodecamer d(CCGGAATT-CGCG)₂.^[91, 92, 96] In fact, all adenine-containing duplex deoxyoligonucleotides initially bind (+)-CC-1065 noncovalently, as evidenced by a transient absorption at 390 nm, and then react to produce a covalent adduct, which shows a maximum ellipticity at 370 nm.^[89–97] In the exceptions, which bind but do not react with CC-1065, the binding sites do not contain an adenine prop-

erly positioned to permit alkylation. For example, the activated cyclopropane of (+)-CC-1065 is placed proximal to the two 3'-T bases of the Dickerson dodecamer in both bound orientations, and thus no accessible nucleophilic site is available for alkylation. Contrary to assertions of the uniqueness of the alkylation reaction, the positioning of this nucleophilic adenine-N3 site need not be exquisite but must be on the right end of the binding site. Consistent with this, the unnatural enantiomer of CC-1065 readily alkylates the Dickerson dodecamer, and its activated cyclopropane is oriented toward the opposite strand proximal to the two 5'-A bases. It is misleading to suggest that such studies illustrate that noncovalent binding cannot dominate or control DNA alkylation, if only a single mismatched binding site is examined.^[46, 96] As the studies with the affinity cleavage agent **22** illustrate, noncovalent binding at an AT-rich site in the minor groove may not guarantee alkylation, but it is a prerequisite to the observation of DNA alkylation.

5. Proposals for the Origin of Polynucleotide Recognition

In the course of studies on CC-1065 and the duocarmycins, the alkylation selectivity of the natural enantiomers has been attributed to a sequence-dependent activation of the reaction through C4 carbonyl protonation by a strategically positioned phosphate in the DNA backbone,^[43–47, 67–69] to the conformational variability of DNA and alkylation at junctions of bent DNA,^[98–103] or to preferential noncovalent binding and subsequent alkylation within the narrower, deeper, AT-rich minor groove of DNA (Scheme 2).^[34–42] Central to the different interpretations are the perceived similarities^[43–48, 64–68] or dis-



Aalkylation Model:

- (+)-CPI and (+)-CC-1065 alkylation selectivities are identical
- selectivity is inherent in the CPI alkylation subunit and the alkylation reaction
- Alkylation selectivity is independent of binding selectivity
- Different features control the (-)-CC-1065 alkylation
- Conformational flexibility of DNA with alkylation at junctions of bent DNA
- Sequence-dependent activation by C4 carbonyl protonation by the phosphate backbone
- C4 carbonyl is required for activation and alkylation selectivity

Noncovalent Binding Model:

- (+)-CPI and (+)-CC-1065 alkylation selectivities are different
- CPI is a modest electrophile superimposed on a five-base-pair, AT-rich binding agent
- Alkylation selectivity is controlled by binding selectivity and steric accessibility to the alkylation site
- Same features control the (-)-CC-1065 alkylation
- Alkylation within appropriate AT-rich binding sequences
- Phosphate activation does not control the alkylation selectivity
- C4 carbonyl is not required

Scheme 2. Summary of the key features of the proposed models.

tinctions^[34–42, 53, 54] in the alkylation selectivity of simple derivatives, including **12** and **13** and the natural products **1–3**. The former two proposals advocated by the Hurley and Upjohn groups are based on the premise that (+)-**13** and (+)-**1** alkylate the same sites and that the alkylation subunit or alkylation reaction controls the selectivity irrespective of noncovalent binding. In contrast, the latter proposal advocated in our studies requires that the selectivity of the agents for AT-rich noncovalent binding sites and their steric accessibility to the alkylation site that accompanies deep penetration into the AT-rich minor groove control the sequence selectivity. This latter model accommodates nicely the reverse and offset 3.5- (for **3**) or 5-base-pair (for **5**), AT-rich, adenine-N3 alkylation selectivities of the natural and unnatural enantiomers of **3** and **1** and requires that **12** and **13** and **1–3** exhibit distinct alkylation selectivities (see Fig. 8 and Table 3).^[53, 54, 71]

All groups have concluded that the DNA alkylation selectivity displayed by the unnatural enantiomer is dominated by the selectivity of the agents for AT-rich noncovalent binding, as advanced in our studies.^[45, 53, 54, 68] However, the Hurley–Upjohn proposal for the natural enantiomers requires that different fundamental polynucleotide recognition elements are operative for the natural and unnatural enantiomers.^[68] Because of such distinct interpretations, we set out to unambiguously test the proposed models with the intention of establishing the origin of the DNA alkylation selectivity. This has proven to be especially important to address at this juncture, since the (+)-CC-1065 alkylation of duplex DNA has been suggested to be, and is often cited as, a prototypical example of sequence-dependent DNA reactivity.^[43, 46, 47] Before such a proposal should be widely accepted, experimental support and verification should be provided.

5.1 Definitive Tests of Proposed Models for the Origin of the CC-1065 and Duocarmycin DNA Alkylation Selectivity

5.1.1. Test of the Importance of C4-Carbonyl Protonation for Sequence-Selective DNA Alkylation

Although the proposal that a sequence-dependent activation through C4 carbonyl protonation by a strategically placed phosphate in the DNA backbone is attractive, a direct test of its viability indicates it cannot be the source of the DNA alkylation sequence selectivity. In the key study^[104, 105] it was shown that **23–26** (Fig. 18) exhibited identical alkylation selectivities (5'-AAA>5'-TAA) irrespective of their absolute configuration, and the selectivities were identical to those of both enantiomers of **12** and **13**. In addition, the natural enantiomers of **27–30** exhibited DNA alkylation selectivities identical to (+)-CC-1065 (Fig. 19)^[104] and were much more selective and efficient than **23–26**. Because **25/26** and **29/30**, which lack the C4 carbonyl, alkylate the same sites as **23** or **27**, respectively, a sequence-dependent phosphate protonation and activation cannot be the event that determines the alkylation selectivity. In addition, the much more selective DNA alkylation by **27–30/1–3** than by **23–26/12–13**—independent of the nature of the electrophile—is not consistent with the assertions that the alkylation reaction

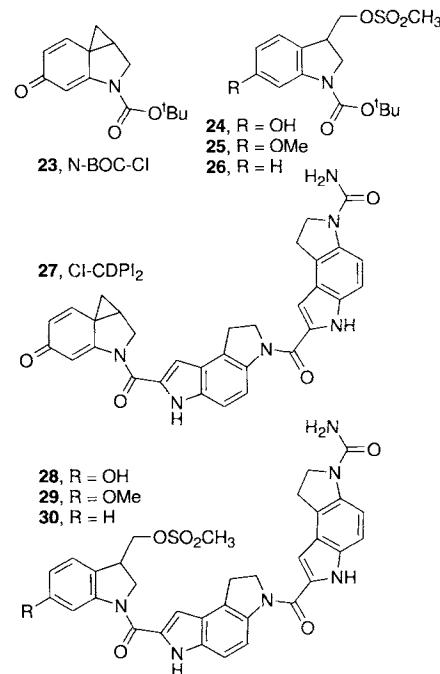
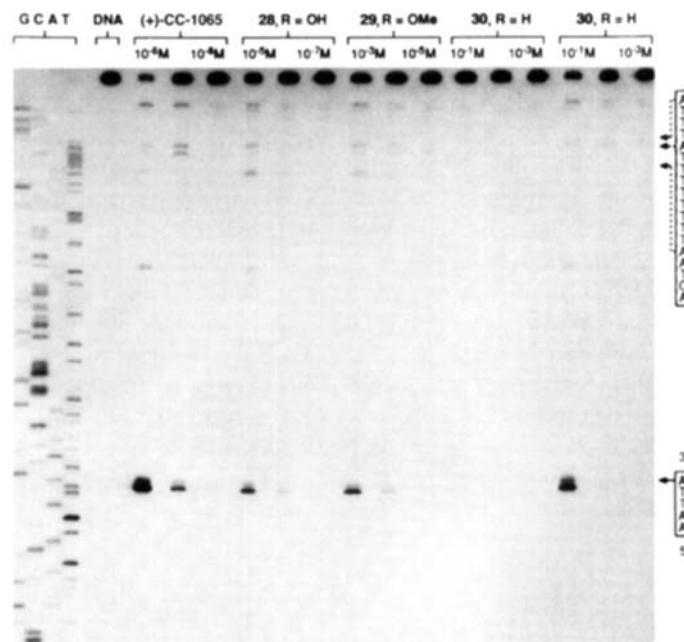


Fig. 18. Structures of the agents **23–30**.



but rather Lewis acid catalyzed by virtue of metal cations associated with the deprotonated phosphate backbone. Attempts to derive rate accelerations for the DNA alkylation reaction must be tempered by this consideration of a Lewis acid or Brønstedt acid catalyzed reaction.

5.1.2. A Switch in the Inherent Alkylation Selectivity of an Enantiomer by Reversal of the Agent Orientation: A Definitive Demonstration of the Origin of the DNA Alkylation Selectivity

Key to the second and definitive study^[108] was the disclosure that both the natural and unnatural enantiomer of duocarmycin SA alkylate DNA efficiently.^[54] One unique feature of **3** is the methyl ester at C6 on the lefthand side of the alkylation subunit that complements the righthand side linking amide. This provides the ability to introduce DNA binding subunits on either side of the duocarmycin SA alkylation subunit. In the studies, both the natural and unnatural enantiomers of the extended and reversed duocarmycin SA analogs **31** and **32** (Fig. 20) were examined. We anticipated that (+)- and (-)-DSA-CDPI₂ (**31**) would, in general, exhibit alkylation selectivity for a more extended (five-base-pair) site than the site favored by **3** and, in particular, behave identically to (+)-CC-1065 (that is, 5'-AAAAA_A selectivity) and (-)-CC-1065 (that is, 5'-A_AAAA selectivity), respectively.^[53] From the noncovalent binding model, the reversed agents (+)- and (-)-CDPI₂-DSA (**32**) were projected to exhibit a similar five-base-pair, AT-rich alkylation selectivity, but one that extends in the atypical reverse direction from an alkylation site. Moreover, the alkylation sites for the natural enantiomer of the reversed agent (+)-**32** should coincide with those of *ent*-**1** and (-)-**31**, and analogously those of the unnatural enantiomer (-)-**32** with (+)-CC-1065 or (+)-DSA-CDPI₂ (**31**). In contrast, the alkylation site model, in which the natural enantiomer alkylation selectivity is embodied in the alkylation subunit and the DNA alkylation reaction is independent of binding selectivity, would require that the two natural enantiomers (+)-**31** and (+)-**32** alkylate the same sites. Thus, the examination of the DNA alkylation selectivities of both enantiomers of **31** and **32** provided an unambiguous test of the models with a definitive resolution.

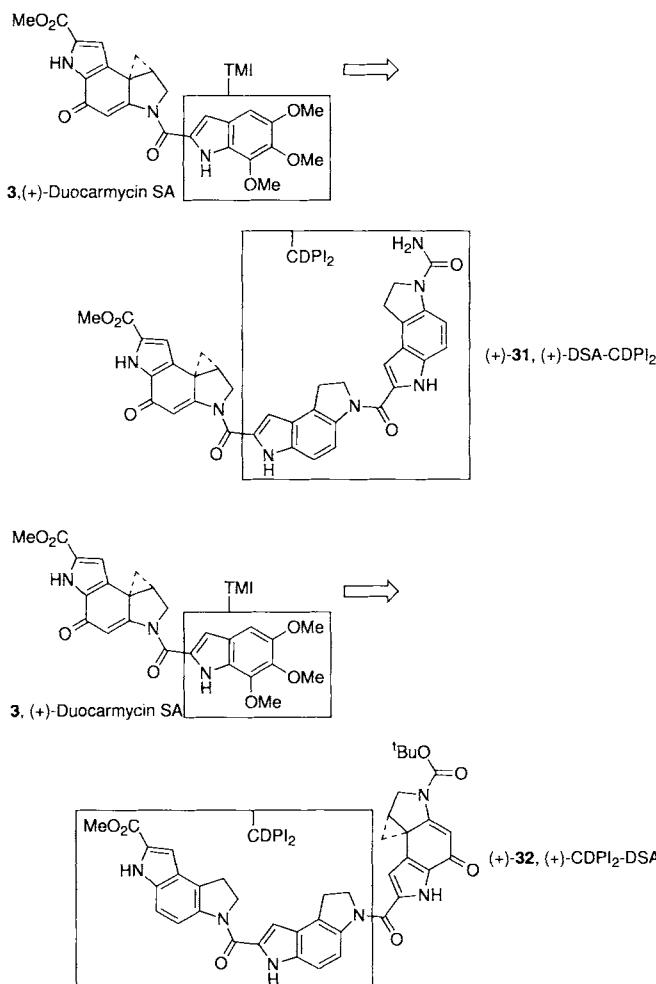


Fig. 20. Extended (top) and reversed (bottom) analogs of (+)-duocarmycin SA (3).

Both (+)- and (-)-**31** were found to alkylate DNA with the same selectivity as (+)- and (-)-**1**, respectively (Table 6).^[109] The natural enantiomer of the reversed agent (+)-**32** alkylated DNA with the same selectivity as (-)-**31** and *ent*-**1**.^[108, 109] This is illustrated beautifully in Figure 21, which highlights the comparisons between the alkylation selectivities not only of the typ-

Table 6. Consensus sequences for the DNA alkylation by extended (**31**) and reversed duocarmycin SA analogs (**32**) [a].

Agent	Base [b] 5'	4	3	2	1	0	-1	-2	-3	-4	3'
<i>natural enantiomers</i>											
(+)-CC-1065 (1)	A/T (56)	67	78	94	98	100	55	—	..	—	—
	consensus	A/T > G/C	A/T > G/C	A/T	A/T	A	Pu ≥ Py	—	..	—	—
(+)-DSA-CDPI ₂ ((+)- 31)	A/T (56)	71	85	100	100	100	63	—	—	—	—
	consensus	A/T > G/C	A/T > G/C	A/T	A/T	A	Pu > Py	—	—	—	—
(+)-CDPI ₂ -DSA ((+)- 32)	A/T (56)	—	—	—	95	100	98	85	70	55	55
	consensus	—	—	—	A/T	A	A/T	A/T > G/C	A/T > G/C	N	—
<i>unnatural enantiomers</i>											
(-)-DSA-CDPI ₂ ((-)- 31)	A/T (56)	—	—	—	100	100	100	90	73	58	—
	consensus	—	—	—	A/T	A	A/T	A/T	A/T	A/T > G/C	N
(-)-CDPI ₂ -DSA ((-)- 32)	A/T (56)	69	81	98	98	100	59	—	—	—	—
	consensus	A/T > G/C	A/T > G/C	A/T	A/T	A	Pu > Py	—	—	—	—
(-)-CC-1065 (<i>ent</i> - 1)	A/T (56)	—	—	—	88	100	93	82	73	56	—
	consensus	—	—	—	A/T	A	A/T	A/T > G/C	A/T > G/C	N	—

[a] The numbers listed in the table give the percentage of the indicated base located at the designated position relative to the adenine-N3 alkylation site. [b] The numbers in parentheses give the percentage composition within the DNA examined.

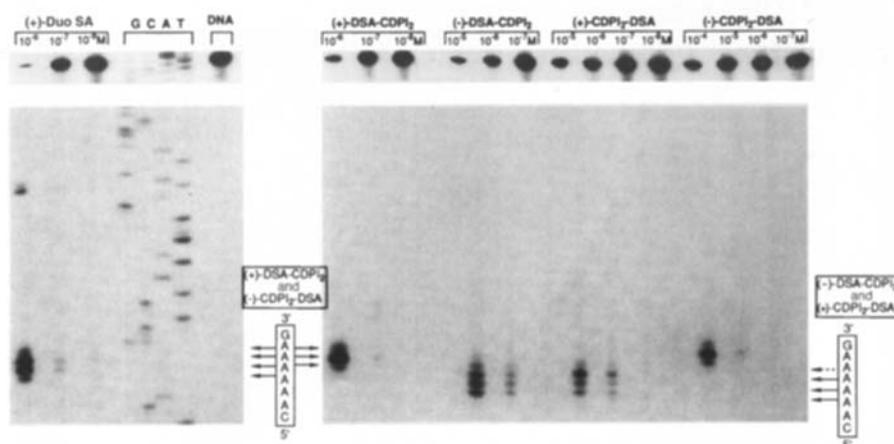


Fig. 21. Thermally induced strand cleavage (by heating to 100 °C for 30 min) of w836 DNA (146 base pairs, nucleotide no. 5189-91) after incubation at 25 °C, removal of unbound agent by EtOH precipitation followed by denaturing 8% PAGE and autoradiography. Lanes 1–3: (+)-3, 1×10^{-6} – 1×10^{-8} M; lanes 4–7: Sanger G, C, A, and T sequencing standards; lane 8: control DNA; lanes 9–11: (+)-31, 1×10^{-6} – 1×10^{-8} M; lanes 12–14: (-)-31, 1×10^{-5} – 1×10^{-7} M; lanes 15–18: (+)-32, 1×10^{-5} – 1×10^{-8} M; lanes 19–22: (-)-32, 1×10^{-4} – 1×10^{-7} M.

ical natural and unnatural enantiomers of **31** but also of the two enantiomers of the reversed agents **32**. This incorporation and conversion of a natural enantiomer of the duocarmycin SA alkylation subunit into an agent that exhibits the DNA alkylation selectivity of a typical unnatural enantiomer by simple reversal of the orientation of the DNA binding subunits is only consistent with a model in which the binding selectivity and the depth of minor groove penetration in the vicinity of the alkylation site is controlling the sites of alkylation. Moreover, the observations are definitely inconsistent with alternative models based on the premise that the natural enantiomer's alkylation subunit controls the alkylation selectivity. Similarly, the unnatural enantiomer of the reversed agent (-)-**32** was found to alkylate the same sites as (+)-**31** and **1**, typical natural enantiomers. Thus, this switch in the inherent enantiomeric alkylation selectivity that accompanies the simple reversal of agent orientation is general and confirms that the same fundamental recognition features are operative for both the natural and unnatural enantiomers (Fig. 22).

Although this behavior of the agents may not be obvious from the two dimensional representations of **31** and **32**, a simple rotation of **32** in the horizontal plane reveals the remarkable structural similarities between the two classes of agents and illustrates that it is the pairs of natural and unnatural enantiomers of different substrates **31** and **32** that orient the cyclopropane in comparable directions relative to the DNA binding subunits. Illustrated in Figure 23 are models of the alkylation by (+)-**31** and (-)-**32** at the high-affinity site 5'-AATTA and in Figure 24, models of the alkylation by (-)-**31** and (+)-**32** at the high-affinity site 5'-ATTTC. The significance of these comparisons should not be underestimated. The results not only require that the same recognition features are affecting both enantiomers, but also represent an unambiguous demonstration that noncovalent binding in an AT-rich region is controlling and determining the sites of DNA alkylation for both the natural and unnatural enantiomers. In addition, since the DNA alkylation selectivities of (+)-**31**/(-)-**32** or (-)-**31**(+)-**32** are identical despite the distinctions in the positioning of the cyclopropane, the positionings need not be precise, just accessible.

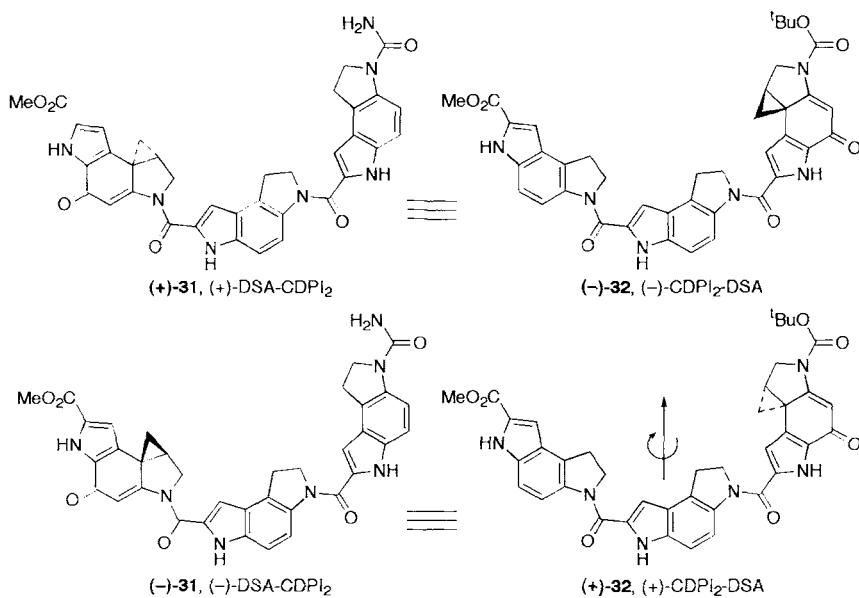


Fig. 22. Representations of the enantiomers of **31** and **32** that highlight the similarities in their experimental behavior. The arrow indicates the rotation of 180° in the horizontal plane (see text).

5.1.3. "Sandwiched" Analogs of Duocarmycin SA: A Class of Agents Whose DNA Alkylation Selectivity Is Independent of Absolute Stereochemistry

In a subsequent and further elegant verification of these observations, the natural and unnatural enantiomers of **33** were prepared by attaching a single DNA binding subunit to both sides of the duocarmycin SA alkylation subunit (Fig. 25) and subjected to examination.^[109] Consistent with the noncovalent binding model, both (+)- and (-)-**33** exhibited identical DNA alkylation selectivities, which were distinct either from the natural or from the unnatural enantiomers of the extended or reversed agents **31** and **32** (Table 7).

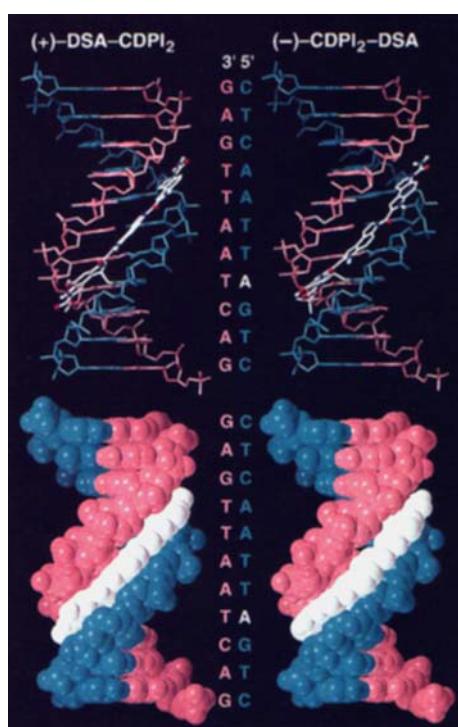


Fig. 23. Stick and space-filling models of the alkylation at a high-affinity site in w794 DNA (5'-CTCAATTAGTC) by (+)-31 (left) and (-)-32 (right). The natural enantiomer extends in the 3' → 5' direction from the adenine N3 alkylation site across the five-base, AT-rich site 5'-AATTA. The unnatural enantiomer of the reversed agent binds in the same 3' → 5' direction across the identical five-base, AT-rich site 5'-ATTTA.

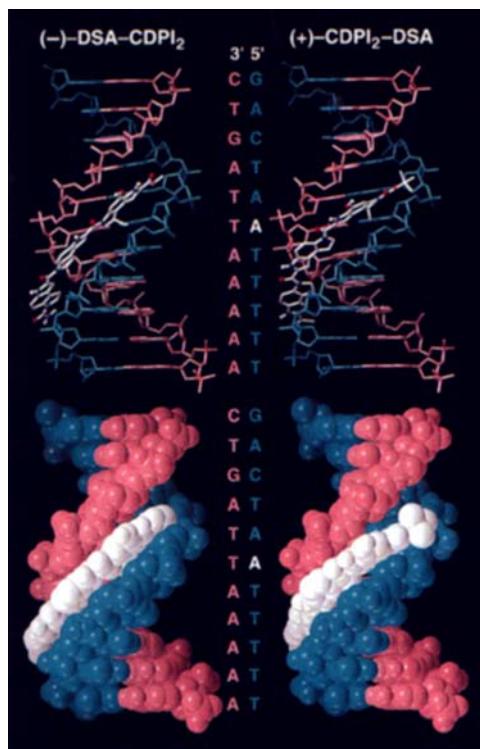


Fig. 24. Stick and space-filling models of the alkylation at a high-affinity site in w794 DNA (5'-GACTAATTTTT-3') by (-)-31 (left) and (+)-32 (right). The unnatural enantiomer binds in the 5' → 3' direction across the five-base, AT-rich site 5'-ATTTA. The natural enantiomer of the reversed agent binds in the same 5' → 3' direction across the identical five-base, AT-rich site 5'-AATTT.

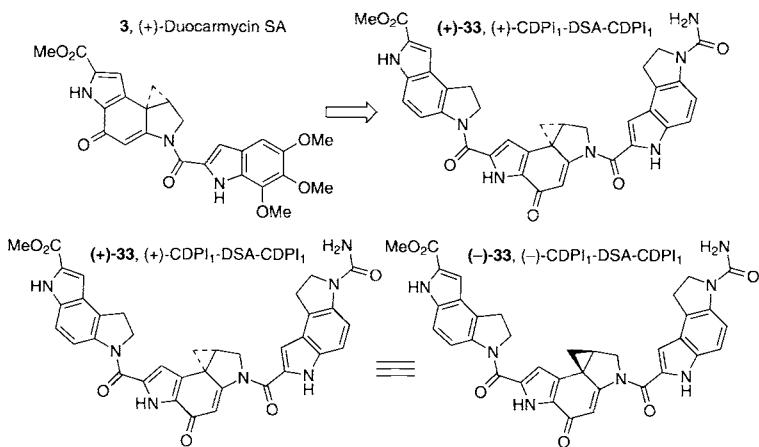


Fig. 25. Sandwich analogs of duocarmycin SA.

6. Structural Characterization of Oligo-Agent Complexes

Spectroscopic studies of complexes of the agents with defined sites of deoxyoligonucleotides have established the accuracy of the models and have begun to reveal subtle elements present in the structures. Most prominent is the confirmation of the binding orientation within the minor groove of the natural and unnatural enantiomers relative to the alkylated strand and the offset nature (relative to the alkylation site) of the binding site occupied by the agent.

A detailed NMR study of the complex formed between the duplex d(GACTAATTGAC) · d(GTCAATTAGTC), which contains the sequence of the high-affinity

Table 7. Consensus sequences for the DNA alkylation by the natural and unnatural enantiomers of sandwich analogs of duocarmycin SA. [a].

Agent	Base [b] 5'	4	3	2	1	0	-1	-2	-3	-4	3'
<i>natural enantiomers</i>											
(+)-CC-1065 (1)	A/T (56) consensus	67 A/T > G/C	78 A/T > G/C	94 A/T	98 A/T	100 A	55 Pu ≥ Py	-	-	-	-
(+)-CDPI ₁ -DSA-CDPI ₁ ((+)-33)	A/T (56) consensus	-- --	68 N	87 A/T > G/C	95 A/T	100 A	95 A/T	74 A/T > G/C	68 N	-	-
<i>unnatural enantiomers</i>											
(-)-CDPI ₁ -DSA-CDPI ₂ ((-)-33)	A/T (56) consensus	- --	64 N	86 A/T > G/C	94 A/T	100 A	94 A/T	78 A/T > G/C	68 N	-	-
(-)-CC-1065 (<i>ent</i> -1)	A/T (56) consensus	- --	- --	- --	88 A/T	100 A	93 A/T	82 A/T > G/C	73 A/T > G/C	56 N	-

[a] The numbers listed in the table give the percentage of the indicated base located at the designated position relative to the adenine-N3 alkylation site. [b] The numbers in parentheses give the percentage composition within the DNA examined.

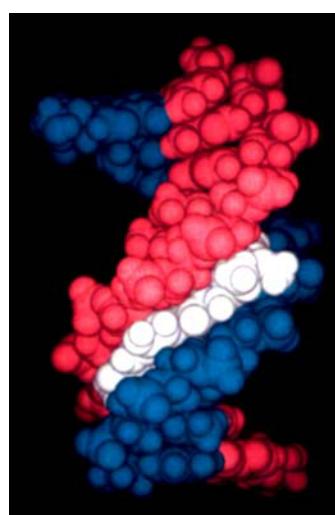


Fig. 26. Space-filling model of the covalent complex between (+)-duocarmycin SA and d(GACTAATTAGC)-d(GTCATTCG) produced by molecular dynamics simulations restrained by NMR-derived distance constraints [110].

CC-1065 with d(GGCGGAGTTAAGG)·d(CCTAACTCCGCC) has also been characterized.^[69, 112] In these studies, the alkylated adenine was shown to exist in the protonated form in which the two hydrogens were located on the exocyclic C6-amino group.^[112] Since no phosphate in the backbone lies close enough to have protonated the C4 carbonyl, it was suggested that an intervening water molecule mediates this protonation by bridging the phenolic proton of CPI and the phosphate between A16 and A17. A very small ¹⁷O (H_2O^{17}) isotope effect on line broadening of this phenolic proton NMR signal was observed and used to support this proposal^[69] and the suggestion that this interaction may provide a molecular basis for the DNA alkylation sequence selectivity of (+)-CC-1065 and its analogs. However, it was later shown that the results of the ¹⁷O experiments cannot be simply attributed to a purported tightly bound water molecule as earlier proposed.^[47] Following adduct formation, the 12-mer duplex becomes bent (17–22°) about a locus lying between T8 and T9, which leads to a narrowing of the minor groove in this region.

A complex of (+)-CPI-CDPI₂ with d(CGCTTAAGCG)₂ has also been studied.^[113] The NMR data support formation of a major adduct at A7 in which the agent binding is oriented toward the 5' end of the alkylated strand.

A site-specific covalent adduct of duocarmycin A with an intramolecular DNA triple helix has been examined.^[114] In this complex, duocarmycin A was found to bind and alkylate in the minor groove at a single adenine in the sequence 5'-AAAAA-3' in a manner analogous to duplex DNA alkylation. The bound duocarmycin A in the minor groove does not expel the third pyrimidine strand from the major groove of the triplex at low

alkylation site found in w794 DNA, and (+)-duocarmycin SA has been conducted.^[110] The studies, which have provided a high-resolution solution structure of the covalent complex, confirm the accuracy of the original models by disclosing the adenine-N3 alkylation and 3' → 5' binding in the minor groove across the 5'-ATTA site (Fig. 26).

A complex of (+)-CC-1065 with d(CGATTAGC)·d(GCTAATCG) has been studied by NMR.^[111] The agent is covalently attached to the N3 atom of the A6 residue and binds in the 3' → 5' direction across five base pairs in the minor groove. A complex of (+)-

temperature and acidic pH. However, it did lower the pK_a for protonation of the third strand cytidines in the major groove by 1.8 pH units.

7. Modified Alkylation Subunits

As distinctions in the importance of the alkylation subunits of **1–3** and their contribution to the DNA alkylation profile surfaced, studies of modified alkylation subunits were initiated to address the problem directly. Agents containing deep-seated modifications in the alkylation subunit not accessible by simple derivatization could be anticipated to define the structural features contributing to functional reactivity and regioselectivity, to define the extent and contributing structural features affecting DNA alkylation selectivity or efficiency, and to delineate key fundamental relationships between structure, functional reactivity, and biological properties.

7.1. CI: Minimum Potent Pharmacophore of the Alkylation Subunits and Common Pharmacophore in the Natural Products

One of the first class of agents examined incorporated the parent alkylation subunit 1,2,7,7a-tetrahydrocyclopropa-[c]indol-4-one (CI, Fig. 27). Consistent with expectations, the CI-based agents proved to be exceptionally reactive electrophiles. In spite of this large change in the structure and relative reactivity of the alkylation subunit, the DNA alkylation selectivities of the agents **27** and **34** were remarkably similar to the natural products **1** and **2/3**, respectively, and their examina-

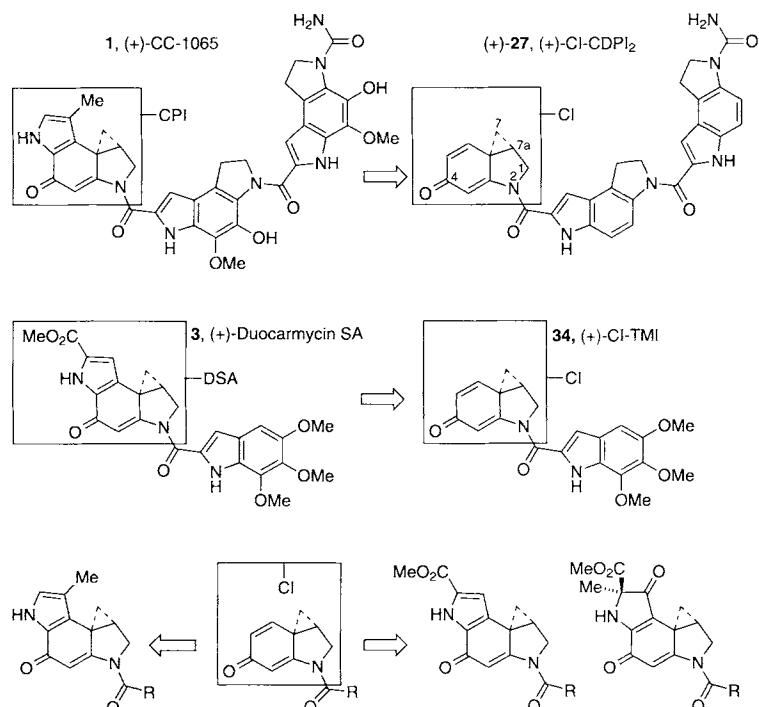


Fig. 27. CI: the minimum potent pharmacophore of the alkylation units of CC-1065 (top, including the atomic numbering for CI) and duocarmycin (center) and thus the common pharmacophore of CC-1065 and the duocarmycins (bottom).

tion provided more insight into the relative importance and role of the alkylation subunit than anticipated.^[55, 56, 72, 115, 116] At the time the studies were conducted, the purported importance of the alkylation subunit was so heavily emphasized that the simple removal of the CPI C7 methyl group, much less the entire pyrrole segment, was arguably considered detrimental to the DNA alkylation selectivity.

The CI alkylation subunit was shown to constitute the minimum potent pharmacophore of the natural products' alkylation subunits and the common pharmacophore of CC-1065 and the duocarmycins. The exceptionally reactive CI-based agents alkylated the same sites as the corresponding CPI- or duocarmycin-based agents, but did so with lower efficiency and less selectivity among the available sites. Importantly, no new adenine-N3 alkylation sites were detected despite a 1000× increase in reactivity. This observation provided the initial indication that the nature of the alkylation reaction was not as critical to DNA alkylation selectivity as suggested by others.^[46] Consistent with their instability the CI agents alkylated DNA less efficiently and exhibited a pronounced decrease in cytotoxic potency.

7.2. CBI: A Simplified and Improved Alkylation Subunit

Similarly, the examination of the class of agents containing the 1,2,9,9a-tetrahydrocyclopropa[c]benz[e]indol-4-one (CBI) alkylation subunit, in which the deep-seated change is the replacement of the fused pyrrole with a fused benzene ring (Fig. 28), proved important and timely.^[115–125] The anticipated release of strain that accompanies the ring expansion of the fused aromatic ring led to decreased reactivity and increased stability relative to the CPI alkylation subunit (4×) and increased cytotoxic potency (4×) without affecting the DNA alkylation selectivity. The CBI-based agents alkylated the same sites as the corresponding CPI or DSA-based agents and with an efficiency and relative selectivity consistent with their reactivity. They were more efficient and more selective among the available alkylation sites than the corresponding CPI-based or DA-based agents and comparable to the DSA-based agents. Despite their diminished reactivity, the CBI-based agents alkylated DNA at a faster rate than the corresponding CPI-based agent^[119, 120] and at a rate comparable to that observed with the corresponding DSA-based agent.^[73] This has suggested that the degree of steric bulk surrounding the C7 center of CPI or DSA may affect both the rate and relative efficiency of DNA alkylation and that the CPI C7 methyl group, once projected as important, may actually diminish the inherent properties of the CPI-based agents.

An examination of the CBI- and CI-based agents led to the proposal that a direct relationship exists between cytotoxic potency and functional stability. At the time these observations were made, the prevailing expectation was that the reverse relationship between functional reactivity (not stability) and cytotoxic potency was important^[46, 159] and that even modest changes in the alkylation subunits would adversely, not productively, influence the DNA alkylation and biological properties of the agents. Consequently, the results from the CBI-based agents led to a shift in the paradigms of the field. Most impor-

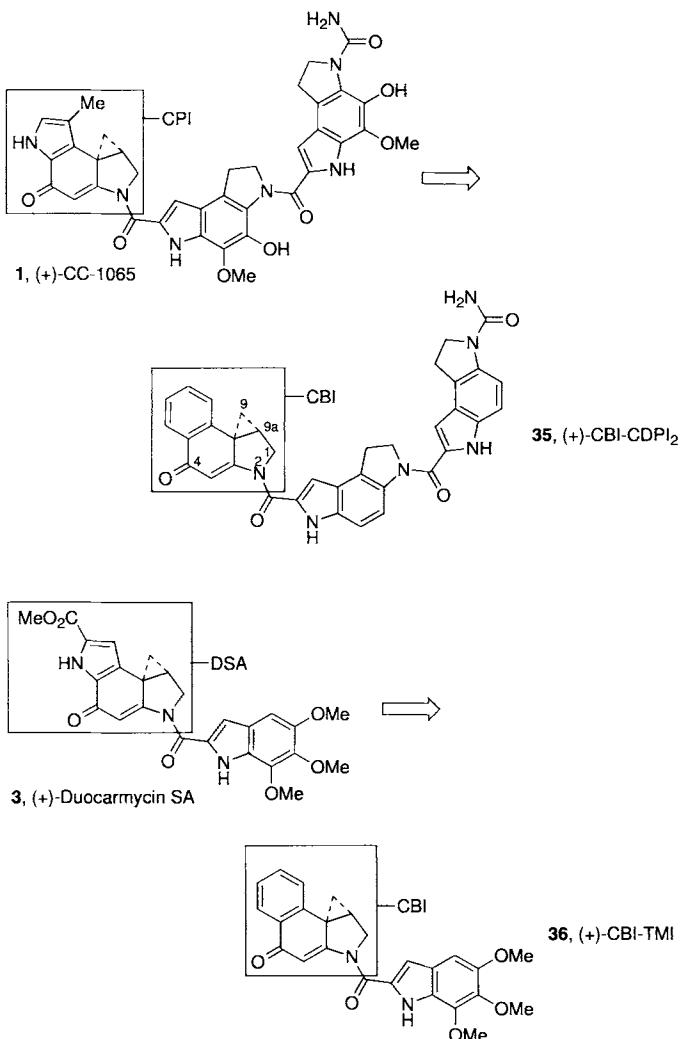


Fig. 28. CBI analogs of CC-1065 (top) and duocarmycin SA (bottom). The atomic numbering for CBI is shown in 35.

tantly, it also revealed that *in vivo* antitumor efficacy^[121, 166] may be observed in agents containing deep-seated and simplifying structural changes in the alkylation subunit and that the potency of the naturally derived materials may be enhanced by simply enhancing chemical stability. Although the sites of DNA alkylation are not altered, the rate and efficiency are increased, and the inherent selectivity among the available sites may be increased by increasing the functional stability of the agent.

7.3. MCBI and CCBI: Electronic Effects on Functional Reactivity

In the continued examination of the structural features that affect reactivity, the substituted CBI derivatives, MCBI (37)^[126] and CCBI (38),^[127] in which a C7 methoxy or cyano substituent has been placed *para* to the C4 carbonyl, have been recently prepared (Fig. 29). Their preliminary and ongoing examination has revealed an important and surprisingly small impact of the C7 substituent on the functional reactivity of the agents. This is discussed in detail in Section 8.

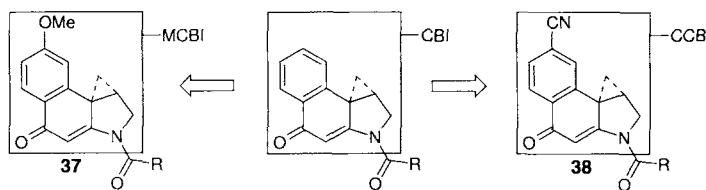


Fig. 29. MCBI and CCBI: structural elements used in the study of the electronic effects on the functional reactivity.

7.4. CBQ: An Exceptionally Reactive Alkylation Subunit

The ring expanded 2,3,10,10a-tetrahydrocyclopropa[*d*]-benzo[*f*]quinol-5-one (CBQ) alkylation subunit was prepared and incorporated into analogs of the natural products (Fig. 30).^[128–130] Their examination revealed a surprising but

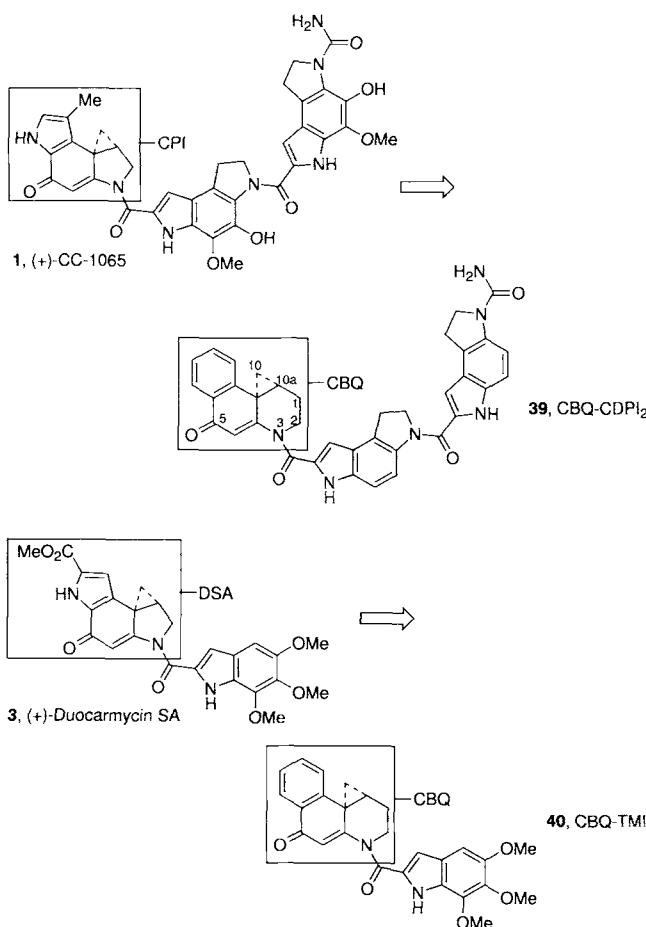


Fig. 30. CBQ-CDPI₂ (39) and CBQ-TMI (40): an alkylation unit showing exceptional reactivity.

normal level of functional reactivity and a number of subtle structural features that impact this reactivity,^[128, 129] reaction regioselectivity,^[129] and mechanism of acid catalyzed solvolysis and nucleophilic addition,^[129] as well as subtle factors contributing to the adenine-N3 alkylation regioselectivity.^[130] These are discussed in detail in Section 8.

7.5. C₂BI-CDPI₂ and C₂BI-TMI: Exquisitely Designed Adenine–Adenine Cross-Linking Agents

The core structure of C₂BI-CDPI₂ and C₂BI-TMI and their achiral precursors **41** and **42** (Fig. 31) was taken from the CBI-based agents, which are exceptionally stable and synthetically more accessible than the core structures of the natural alkylation subunits.^[131, 132] In addition, the acyclic agents **41** and **42** lacking the preformed cyclopropane ring could be expected to

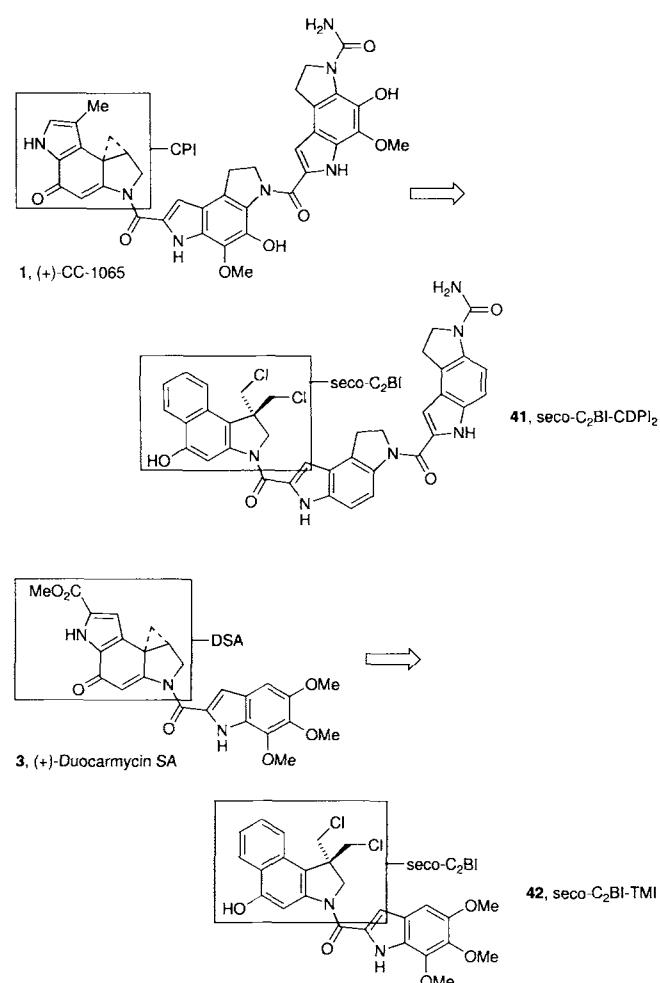


Fig. 31. Achiral precursors of C₂BI-CDPI₂ (**41**) and C₂BI-TMI (**42**): DNA cross-linking agents.

display properties identical to C₂BI-CDPI₂ and C₂BI-TMI, but are achiral. This simple feature of **41** and **42** not only provided attractive synthetic candidates that did not require resolution or asymmetric synthesis, but also a single agent for biological evaluation free of the necessity of documenting the behavior of both enantiomers.

In addition, the bis-alkylating capabilities of **41** and **42** provided the opportunity to cross-link DNA in which their two alkylating groups act independently as the individual enantiomers. When this occurs within a sequence such as d(ATTA)_nd(TAAT), the adenine-N3 alkylation by the natural enantiomer component of **42** occurs at its high-affinity site of 5'-ATTA-3' with agent binding in the 3' → 5' direction relative to

the strand it alkylates, across a 3.5 base, AT-rich site. The alkylation by the unnatural enantiomer component of **42** 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 strand it alkylates, across a 3.5 base AT-rich site (Fig. 32).

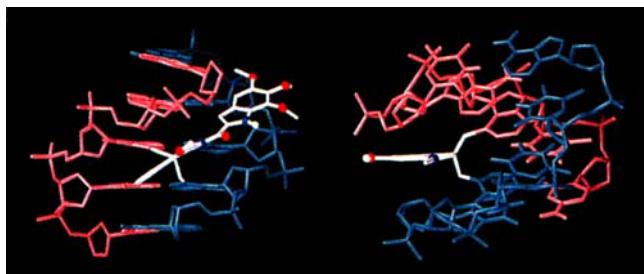


Fig. 32. View from the front and from the groove of the product of the cross-linking reaction of C₂BI-TMI in the w794 DNA high-affinity site (5'-ATTAG-3'-CTAAT). For details, see text.

Consistent with the fact that the reversed binding orientation and the offset AT-rich binding sites relative to the alkylated adenine of both enantiomers coincide in a single binding and cross-linking site, **41** and **42** were found to be efficient cross-linking agents.^[131, 132] Figure 32 gives an alternative depiction of DNA alkylations by the natural and unnatural enantiomers, where complementary strand reactions occur from one binding orientation.

Additional cross-linking agents have been disclosed, including **43**, which is based on the CC-1065 structure and constructed by linking two CPI units together (Fig. 33).^[133–142]

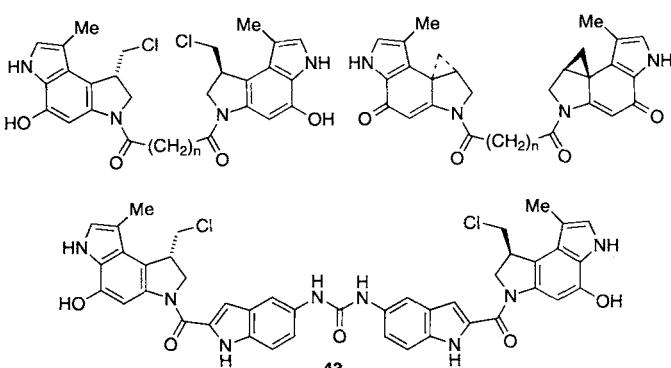


Fig. 33. Structures of additional DNA cross-linking agents.

7.6. Other Modifications in the DNA Alkylation Subunits

A number of agents incorporating additional modifications in the DNA alkylation subunits are shown in Figure 34. These include agents with alteration of the duocarmycin A and B₂ alkylation subunits,^[143] furan analogs of CC-1065^[144] and duocarmycin SA,^[145] substituted CI analogs,^[145] a simplified cyclopropyl derivative,^[146] a gramine analog related to the CPI

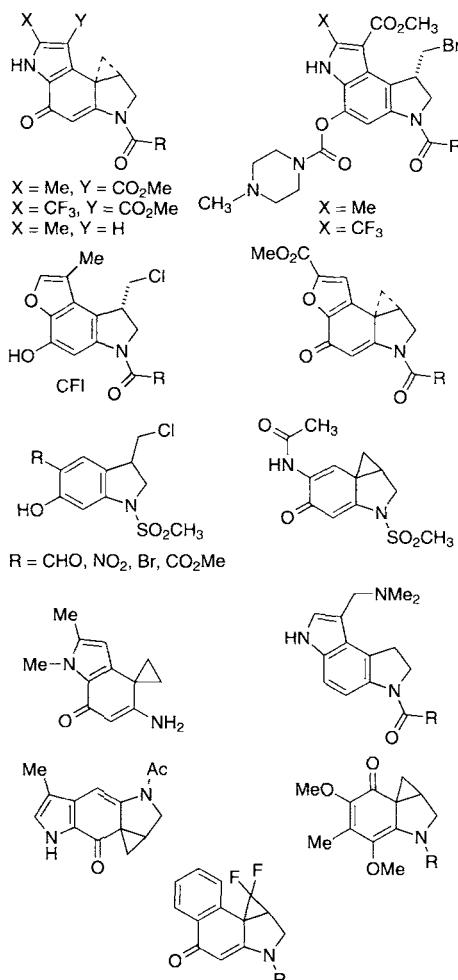


Fig. 34. Other modifications in the DNA alkylation subunits of CC-1065 and the duocarmycins.

unit,^[147] the 9,9-difluoro-CBI derivative,^[148] and an isomeric cyclopropacyclohexadienone that combines the features of both CC-1065 and the mitomycins.^[149]

8. Fundamental Relationships Between Structure, Functional Reactivity, and Biological Properties

In addition to the total synthesis of the natural products (CC-1065 (**1**),^[77, 150–152] duocarmycin A (**2**),^[153, 154] and duocarmycin SA (**3**),^[155–157]) and the extensive number of investigations directed at the authentic subunits of the natural products^[41, 50] and their analogs,^[158–162] studies that have provided agents containing deep-seated structural modifications have proven valuable in defining the relationships between structure, functional reactivity, and biological properties.^[34–38]

8.1. Definition of a Fundamental Relationship Between Functional Reactivity and Biological Activity

The proposed acid catalyzed activation of the DNA alkylation reaction led to the expectation that a direct relationship may exist between the reactivity and cytotoxic activity of the

agents and to the proposal that the biological potency may be enhanced as the electrophilic reactivity is increased.^[159] However, studies conducted with the agents **44–51** revealed the reverse relationship and that the most stable agents may be expected to exhibit the most potent cytotoxic activity (Fig. 35). Moreover, a well-defined, direct relationship between stability toward solvolysis and biological potency (IC_{50} , L1210) has been observed and proved to be general with both simple and full analogs of the natural products.^[118–121, 126, 129, 163, 166]

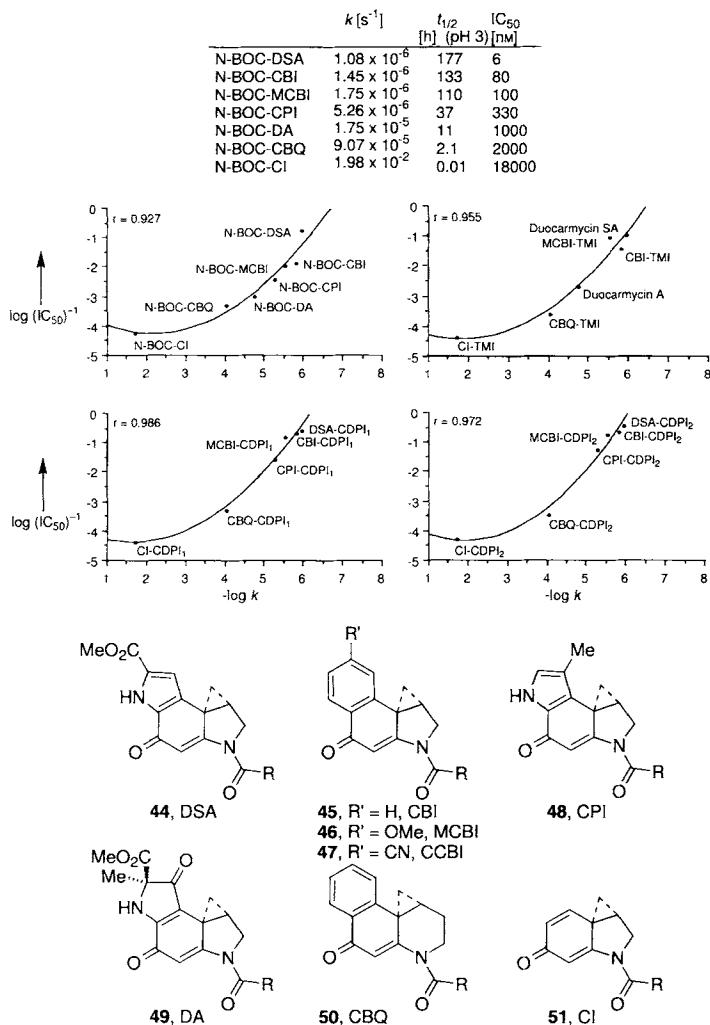


Fig. 35. Direct correlation between chemical or functional stability and cytotoxic potency of analogs of the natural products. R represents BOC, TMI, CDPI, or CDPI₂ shown in the graphs. N-BOC-**44** is identical to **12**. The unit for IC_{50} in the top right plot is nM; in all the other plots it is pm.

8.2. Establishment and Verification of a Fundamental Relationship between Functional Reactivity and Biological Activity

Examination of the properties of **52–55**,^[163] simple derivatives of CBI, led to the validation of this direct relationship between solvolysis stability and cytotoxic potency (Fig. 36). Their examination revealed a direct, linear relationship between

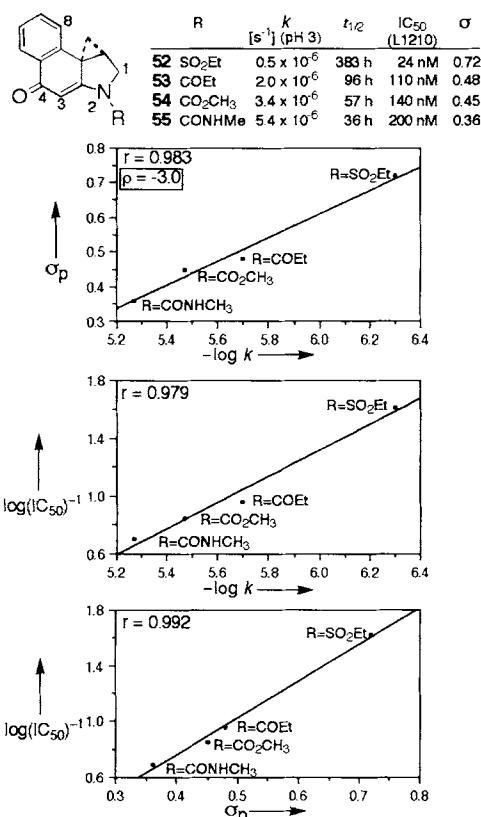


Fig. 36. Effect of the N2 substituent on the cytotoxic activity and the rate of solvolysis.

the cytotoxic potency (L1210, $\log 1/IC_{50}$) and the solvolytic stability ($-\log k$, pH 3) within the reactivity range examined. Similarly, a linear relationship was found between the electron-withdrawing properties of the N2 substituents (Hammett σ_p constant) and the solvolysis reactivity ($-\log k$, pH 3) in which the strongest electron-withdrawing substituents provide the most stable agents. This latter relationship reflects the influence of the N2 substituent on the ease of C4 carbonyl protonation required for catalysis of solvolysis, and those with the stronger electron-withdrawing substituents exhibit the slower solvolysis rates. Moreover, the slope of the plot of σ_p versus $\log k$ revealed a remarkably large ρ value of -3.0 . This large negative ρ value is indicative of the strong vinylogous amide conjugation and suggests that the reactivity may be greatly influenced by the nature of the N2 substituent and its interaction with the cyclopropylcyclohexadienone. 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 N² substituent (σ_p). The agents with the strongest electron-withdrawing substituents proved to be the most potent agents (Fig. 36).

These fundamental correlations between the functional reactivity of the agents and their biological potency should prove useful in designing new analogs. For agents that possess sufficient reactivity to alkylate duplex DNA, the chemically more stable agents are predicted to be the biologically more potent agents. Presumably this may be attributed to the more effective delivery of the more stable agents to their intracellular target.

8.3. Substituted CBI substrates: Magnitude of Electronic Effects on Functional Reactivity and the Verification of the Fundamental Relationship between Functional Reactivity and Biological Activity

In efforts to address the structural features of **1–3** that contribute to their functional reactivity, the substituted CBI agents **46** and **47** (see Fig. 35) and a full set of analogs of the natural products that incorporate these modified alkylation subunits have been prepared.^[126, 127] Against expectations, the nature of the C7 substituent, which is *para* to the C4 carbonyl, had only a very modest effect on the functional reactivity of the agents. On using acid catalyzed solvolysis as a measure of their relative reactivity, **46** was found to be only 1.2 to 1.05 times more reactive than the parent **45**, and **47** less than twice as stable at pH = 3 or 2 (Fig. 37). These studies provided a clear valida-

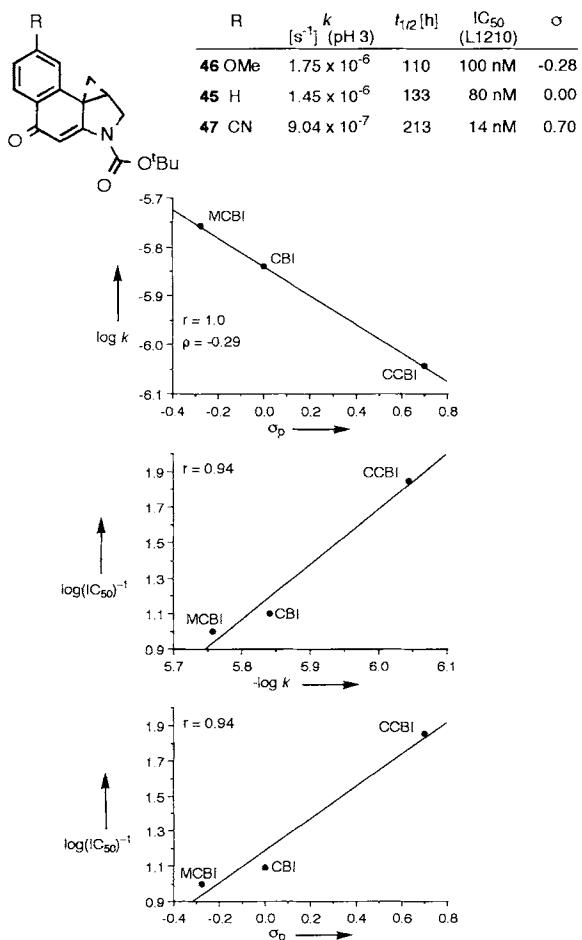


Fig. 37. Electronic effects of the substituted CBI analogs **45–47**.

tion of the direct relationship between functional stability and cytotoxic potency and revealed a predictable direct relationship between the electronic properties of the C7 substituent (σ_p constant) and the functional reactivity ($-\log k$, pH 3), a direct relationship between the cytotoxic potency (L1210, $\log 1/\text{IC}_{50}$) and functional stability ($-\log k$, pH 3), and a fundamental, direct relationship between cytotoxic potency (L1210, $\log 1/\text{IC}_{50}$) and the electronic properties of the C7 substituent (σ_p constant);

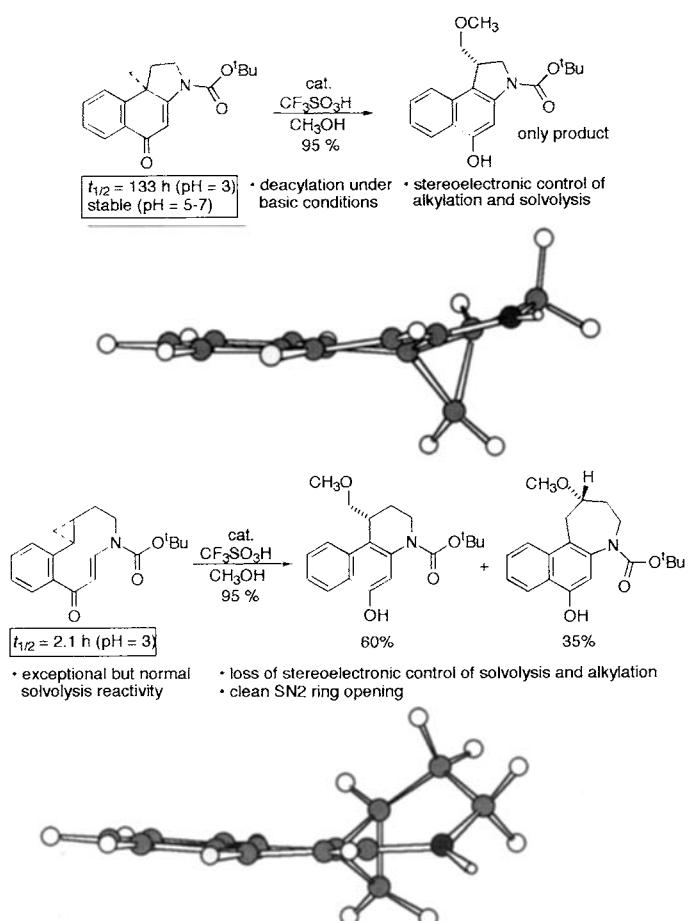
again the strongest electron-withdrawing substituent provided the most potent agent (Fig. 37). Importantly, the very modest effect of the C7 substituent on the functional reactivity was reflected in the determination of the exceptionally small ρ value of -0.3 .

This surprisingly modest effect of the *para* C7 substituent suggests that protonation of the C4 carbonyl may not be the rate-limiting step of solvolysis or acid catalyzed nucleophilic addition and that little charge buildup at the substitution site occurs in the transition state ($\rho = -0.3$). Further study with these agents (Section 8.6) also supports the proposal that the cyclopropane ring opening requires the presence and assistance of a nucleophile (S_N2 mechanism). No doubt this contributes to the DNA alkylation selectivity and is consistent with the expectation that the positioning of the nucleophile (adenine-N3) and not C4–O protonation is the rate-determining event. This remarkably small electronic effect on the solvolysis rate had no impact on the solvolysis regioselectivity, and stereoelectronically controlled nucleophilic addition to the least substituted cyclopropane carbon was observed exclusively.

8.4. Stereoelectronic Control

Two characteristics of the alkylation subunits **44–51** have proven important in the studies to date. The first is the stereoelectronically controlled, acid catalyzed 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 direct relationship between solvolysis stability and cytotoxic potency. A recent comparison of the chemical and structural properties of N-BOC-**50** and N-BOC-**45** confirmed the stereoelectronic control of the solvolysis or alkylation reactions of **44–51** and revealed an unappreciated but fundamentally important stability for the duocarmycin and CC-1065 alkylation subunits and its structural origin.^[128, 129] The solvolysis reactivity of **50** along with that of **44–51** are summarized in Figure 35. In addition to the increased reactivity of **50** ($63 \times$) relative to that of N-BOC-**45**, the solvolysis of **50** occurs with nucleophilic addition to both C10 and C10a, while that of **45** occurs exclusively at the least substituted cyclopropane carbon atom (Scheme 3).^[129]

The X-ray structure of N-BOC-**50**^[128, 129] provided the structural insights into this difference in solvolysis reactivity and regioselectivity (Scheme 3). It is clear from the X-ray structure of CBI^[118, 128] that the bent orbital of the cyclopropane bond extending to the least substituted carbon is nearly perpendicular to the plane of the cyclohexadienone and consequently overlaps nicely with the developing π system of the solvolysis product phenol (Fig. 38), whereas 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 ring opening. In contrast, N-BOC-CBQ exhibits different characteristics. The cyclopropane is ideally conjugated with the cyclohexadienone π system, the plane defined by the



Scheme 3. Comparison of the solvolysis reactivity and X-ray structures of N-BOC-45 (top) and N-BOC-50 (bottom).

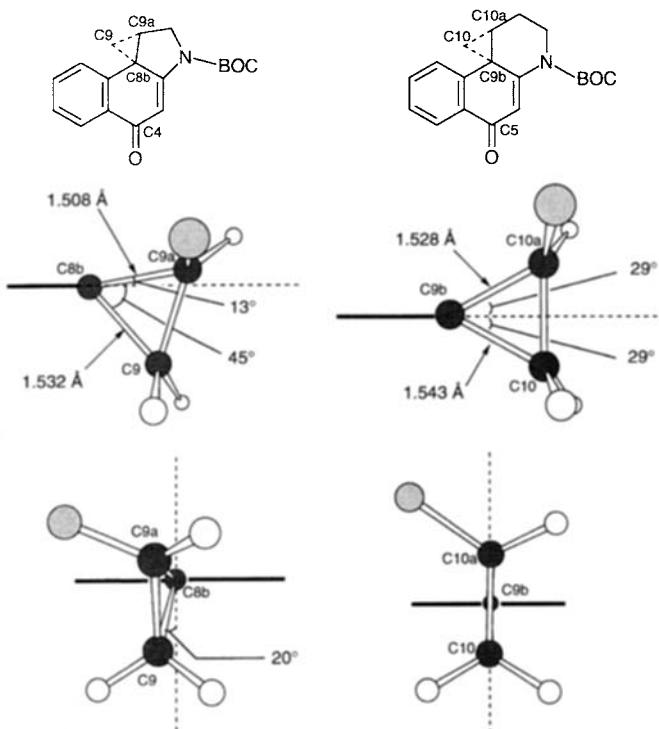


Fig. 38. Side and rear views of the activated cyclopropane rings of 45 and N-BOC-50, illustrating data taken from the X-ray crystal structures and highlighting the idealized overlap and alignment of the cyclopropane ring of N-BOC-50 with the cyclohexadienone π -system.

cyclohexadienone ideally bisects the cyclopropane, and the bonds extending to the secondary and tertiary cyclopropane carbon atoms are equally aligned with the π system. Thus, the two available cyclopropane bonds are equally aligned for cleavage, and addition to both occurs.^[129] The subtle differences in the relative amounts of the ring-expanded solvolysis product may be attributed to destabilizing torsional strain that accompanies nucleophilic attack at the more substituted center.

In contrast to the CBI-based agents, which react regiospecifically to provide exclusively one acid catalyzed addition product under a wide variety of reaction conditions, both N-acyl-CPI and N-acyl-DA agents (including duocarmycin A) have been shown to provide variable amounts of the abnormal ring expansion solvolysis products depending on the reaction conditions. To date, this has not been investigated in detail, and the structural origin of the minor ring expansion reaction with CPI or DA has not been established. It may be related to their increased reactivity or different degrees of stereoelectronic alignment derived from subtly altered cyclopropane conjugation. The delineation of the structural origin of these distinctions may, no doubt, be derived from comparative crystallographic studies of the respective alkylation subunits, and such detailed studies are in progress.

8.5. Structural Origin of an Unappreciated but Productive Stability of the Natural Alkylation Subunits: Extent of Cyclopropane Conjugation and Activation

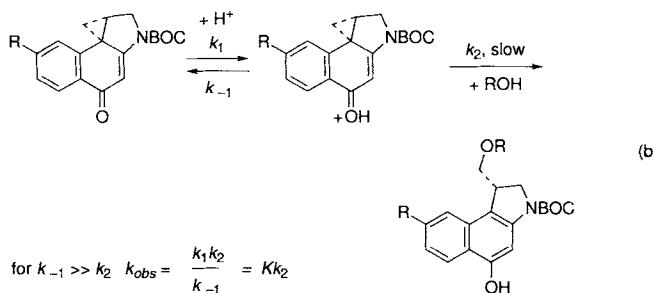
The rapid solvolysis of N-BOC-CBQ was initially surprising; however, important insights into this enhanced reactivity are clear from the X-ray structures. Although they reside in a cyclopropane fused to a six- rather than a five-membered ring, the lengths of the CBQ C9b–C10a (1.528 Å) and C9b–C10 (1.543 Å) bonds are greater than those found in CBI (1.508 and 1.532 Å, respectively) and nicely reflect the enhanced cleavage reactivity^[128] (Fig. 38). This lengthening of the cyclopropane bonds may be attributed to the idealized conjugation or π delocalization of both the C9b–C10a and C9b–C10 cyclopropane bonds with the cyclohexadienone π -system. Contributing to this enhanced conjugation is the ideal geometrical alignment of C10 and C10a with C9b, C5, and the carbonyl oxygen atom. For CBI, the constraints of the fused five-membered ring place its C9 and C9a at a 20° angle off 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 with the cyclohexadienone π system in N-BOC-50 results in the observed longer and weaker bonds and higher solvolysis reactivity.

Thus, the geometrical constraints of the fused five-membered ring found in the CC-1065 or duocarmycin alkylation subunits impose stereoelectronic control on the nucleophilic cleavage of the cyclopropane dictating addition to the least substituted carbon atom. In addition, the nonideal alignment and overlap of the cyclopropane with the cyclohexadienone π system found in the naturally occurring alkylation subunits result in diminished electrophilic reactivity. The fundamental insight derived from these comparisons is not the solvolysis reactivity of N-BOC-50, but rather the surprising stability of the CBI, DSA, and CPI

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.

8.6. Mechanism of Acid Catalyzed Solvolysis and Nucleophilic Addition

Studies with N-BOC-CBQ (**50**) also resulted in an unambiguous determination of the mechanistic course of the acid catalyzed solvolysis reaction. Both the racemate and the pure (+) isomer of **50** were subjected to acid catalyzed methanolysis (0.1 equiv $\text{CF}_3\text{SO}_3\text{H}$, CH_3OH , 25 °C, 1 h, 95%). The abnormal, ring-expanded solvolysis product derived from optically active **50** was formed with exclusive inversion of the configuration at the reacting center, which indicates an S_N2 ring-opening reaction.^[129] The demonstration was unambiguous and contrasts the conclusions of Warpehoski and Harper^[107] in which a free carbocation and an S_N1 mechanism has been invoked to account for the minor CPI ring expansion solvolysis products. These and related studies^[127] with the CBI-based agents have also shown that the acid catalyzed nucleophilic addition displays a first-order dependence on both the acid and nucleophile concentration. Together, these studies illustrate that the slow step is not C4 carbonyl protonation, but rather nucleophilic addition to the activated cyclopropane following rapid and reversible protonation of the C4 carbonyl oxygen atom [Eq. (b)].



These studies have significant implications on the rate-determining step of the DNA alkylation reaction and, thus, the origin of the DNA alkylation sequence selectivity. Even though both of the CBQ cyclopropane bonds are equally aligned for cleavage, the acid catalyzed cyclopropane ring-opening reaction still occurs with a slight preference for normal rather than ring-expansion solvolysis. This latter pathway would be strongly preferred if substantial positive charge were to develop at the reacting cyclopropane carbon atom (secondary rather than primary carbocation character). The fact that it is not preferred and that reaction at the more substituted center occurs with full inversion of configuration by a strict S_N2 reaction mechanism further supports the prior kinetic evidence that the slow step of the reaction is not carbonyl protonation but rather the attack (or positioning) of the nucleophile, which preferentially occurs at the least hindered cyclopropane carbon.

8.7. Additional Subtle Features Contributing to the DNA Alkylation Regioselectivity

The examination of the DNA alkylation properties of CBQ-TMI (**40**) and related agents showed that they alkylated the same sites as the corresponding enantiomers of the agents incorporating the DSA, DA, CPI, CBI, or CI alkylation subunits.^[130] Consistent with their reactivity, they exhibited less selectivity among the available sites and alkylated DNA with a lower efficiency. Importantly, only adducts derived from adenine-N3 addition to the least substituted cyclopropane carbon atom were detected under the relevant conditions of limiting agent concentrations. The adenine-N3 adduct obtained by thermal depurination was isolated, characterized, and shown to be derived only from addition to the least substituted cyclopropane carbon atom of CBQ-TMI (**40**). Since the CBQ-based agents exhibit nonselective solvolysis regioselectivity with cleavage of both the usual external and unusual internal cyclopropane bonds, the observations suggest that the clean regiochemical course of the characteristic adenine-N3 alkylation may benefit not only from stereoelectronic effects, but from additional features that complement the normal addition as well. These include the preferential adoption of binding orientations that favor the normal adenine-N3 addition (proximity effects), destabilizing torsional strain and steric interactions that accompany the abnormal adenine-N3 addition (Fig. 39), and a poten-

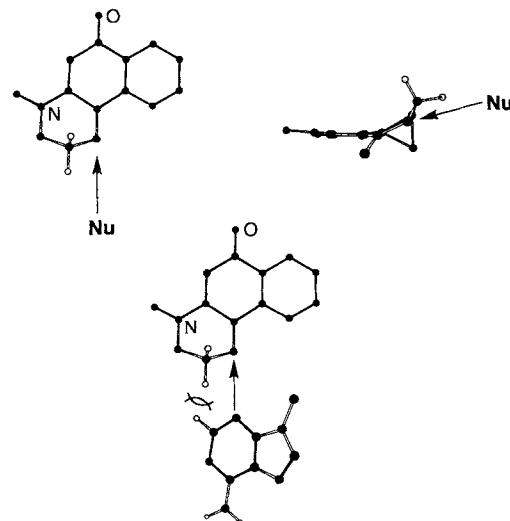


Fig. 39. Top and side views of the atypical nucleophilic addition to the most substituted cyclopropane carbon of CBQ. Top: Illustration of the eclipsing interaction between a nucleophile Nu and the $\text{C}1\text{-H}^*$, and the torsional strain that results from it. Bottom: The relative orientation of the reactants in the adenine-N3 addition taken from a model of a noncovalent complex of DNA and CBQ-TMI^[130] when constrained in the minor groove. The diagram highlights the additional destabilizing steric interaction between $\text{Ad-C}2\text{-H}$ and $\text{C}1\text{-H}^*$. The other hydrogen atoms and the trimethoxyindole subunit have been removed for clarity. In some formulas the cyclopropane ring is obscured.

tial binding-induced conformational change in CBQ that imposes the stereoelectronic preference for adenine-N3 addition to the least substituted cyclopropane carbon (Fig. 40). Of these, the destabilizing torsional and steric interactions that accompany the abnormal adenine-N3 addition might be most significant and contribute similarly to the regioselectivity of the DNA alkylation reactions of **1–3** and related agents.^[130]

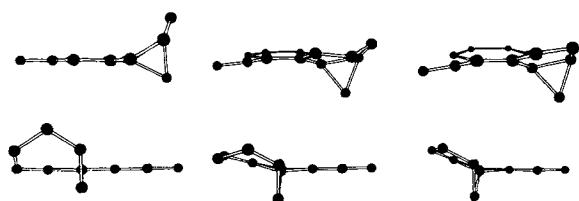


Fig. 40. Side and rear views of the CBQ boat (left, X-ray structure analysis [128,129]), CBQ half chair (middle), and CBI (right, X-ray structure analysis [118]) conformations, illustrating the relative orientations of the cyclopropane ring. Like in the case of CBI, the higher energy half-chair conformation of CBQ ($\Delta E = 1.0 - 1.5 \text{ kcal mol}^{-1}$) would impose stereoelectronic control of the addition to the least substituted cyclopropane carbon atom. (Hydrogen atoms have been removed for clarity.)

8.8. Experimental Verification of Vinylogous Amide Stabilization: A Stable, Isolable *para*-Quinomethide

Because of the significant contribution of the vinylogous amide stabilization of the structures **44–51**, the *para*-quinomethide **56** and its stable precursors were prepared and examined (Fig. 41).^[164] Unlike *para*-naphthoquinomethides, which are

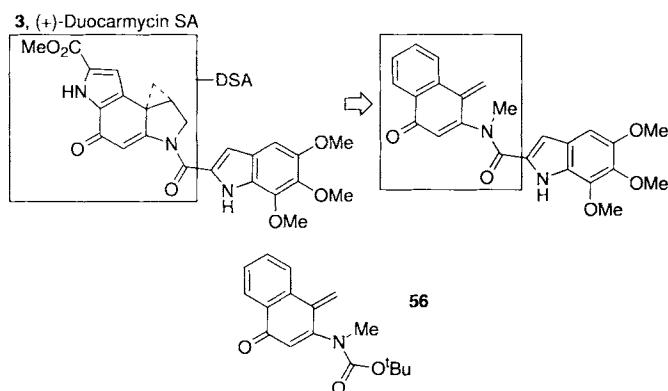


Fig. 41. *para*-Quinomethide (**56**) rendered isolable by vinylogous amide stabilization. For further information on **56**, see text.

not sufficiently stable for isolation, characterization, or detection and are inferred reaction intermediates, the *para*-quinomethide **56** was isolated (with SiO_2 chromatography!) and characterized. Although **56** is still exceptionally reactive and substantially more reactive than **44–51**, the unusual stability that permits its isolation may be attributed to this key vinylogous amide stabilization, which is lost upon aromatization.

8.9. Additional Key Features

A number of additional unique structural features of **44–51** also contribute to their stability. The stability ordering **44–48** > **49** > **51** can be attributed to the diminished gain in delocalization energy that accompanies aromatization in a system that bears a fused aromatic ring.^[116,156] The increased stability ordering **44** > **48**, **47** > **45** and **49** > **51** can be attributed to the conjugated electron-withdrawing group which diminishes C4 carbonyl protonation required of solvolysis and alkylation.^[126,127,156] The increased stability of **45** > **48** can be at-

tributed in part to the release of strain that accompanies the substitution of a fused six-membered for a fused five-membered arene ring.^[118,122] Finally, the vinylogous amide stabilization of the cyclohexadienone structure, which is lost upon aromatization, contributes significantly to the stability of **44–51**.^[164]

8.10. Relationship Between Alkylation Subunit Structure and DNA Alkylation Selectivity

The agents containing the natural (CPI, DSA, or DA) or modified (CI, CBI, CBQ, CFI) alkylation subunits attached to the same DNA binding subunits have been found to alkylate the same sites. Notably, no new or unique sites or alterations in the DNA alkylation sequence selectivity attributable to structural changes in the alkylation sites have been disclosed. In such sequencing studies, the alkylations are conducted with a large excess of DNA and limiting agent concentrations, and consequently, minor or forced additional reactions are not observed (for instance, minor guanine-N3 alkylation). These selectivities, which have been experimentally derived and summarized elsewhere,^[56, 71, 105, 122, 130, 144] can also be extrapolated from the data presented in Tables 1, 3, and 6 for the DSA, DA, or CPI-based agents. The distinctions between the agents lie in the greater selectivity among the available sites observed with the chemically more stable agents ($\text{DSA} \geq \text{CBI} > \text{CPI} > \text{DA} > \text{CBQ} > \text{CI}$). In addition, it is notable that the corresponding precursor agents lacking the cyclopropane (i.e., **4–7**) also alkylate the same sites. Although in most cases this may be attributed to their facile closure to the cyclopropane-containing agents under the conditions of the assay, important and key studies have shown that this is not necessarily required.^[55, 56, 104, 105]

8.11. Relationship Between Functional Reactivity, DNA Alkylation Rate or Efficiency, and Biological Properties

To date, the relative efficiency of DNA alkylation follows closely the relative cytotoxic potencies observed with the agents. As the alkylation subunit reactivity diminishes, the relative efficiency of DNA alkylation increases. This was presumed to be attributable to the nonproductive consumption of the more reactive agents. The assumption was confirmed in the initial^[56] studies with duocarmycin A and reestablished in more recent investigations with duocarmycin A^[61,62] and CBQ-TMI (**40**).^[130] Similarly, as the size of the DNA binding subunits increase, the relative efficiencies of DNA alkylation increase to a maximum and then level off for agents that possess the same alkylation subunit. This is consistent with the expectation that an increase in the noncovalent binding affinity within the minor groove provided by the DNA binding subunits increases both the rate and efficiency of DNA alkylation. The maximum plateau in cytotoxic potency occurs at approximately the same point at which the relative efficiencies in DNA alkylation also reach a plateau, even though the DNA alkylation rates vary widely. Thus, although the relative cytotoxic potencies have been suggested to be directly related to the relative rates of DNA alkylation,^[46] we have suggested that the cytotoxic potencies may more closely follow the trends in relative DNA alkylation

efficiencies.^[71, 120] Since the DNA alkylation reactions are inherently reversible, it is attractive to suggest that the maximum plateau in the DNA alkylation efficiency and cytotoxic activity may be achieved only when the inherently reversible adduct formation is fully stabilized by a sufficiently strong noncovalent binding. This would seem to be validated in the recent studies with the 7-MCBI derivatives.^[126]

Although the careful comparisons are limited in the studies conducted to date, the chemically more stable agents have not only been determined to alkylate DNA more efficiently but also to do so more rapidly. It is not intuitively obvious that this would be expected for a Brønsted acid or Lewis acid catalyzed reaction and may be a consequence of the limited number of agents examined to date. For example, the most reactive of the agents carefully examined to date are the CBQ-based agents, which have been shown qualitatively to alkylate DNA slower than the CPI-, CBI-, or DSA-based agents. Similarly, the CPI-based agents alkylate DNA with both a lower efficiency and lower rate than the CBI-based agents even though they are four times more reactive (Fig. 42). In contrast, the CBI-based agents alkylate DNA with efficiencies and rates that are both only slightly lower than those of the exceptionally stable DSA-based agents (Fig. 42). Thus, the chemically most stable class of agents examined to date also alkylate DNA at the fastest rate. It is also

the class of agents that possess the smallest degree of inherent steric bulk on the inside concave face of the alkylation subunit especially surrounding the DSA C7 position. Thus, it may be that steric bulk surrounding this C7 center diminishes the inherent rate of DNA alkylation, and the agents examined carefully to date follow this trend (DSA > CBI > CPI).

9. Structural Origin of the Distinctions between Enantiomers: Sensitivity to C7 Steric Interactions

The studies have suggested an attractive explanation for the sometimes confusing behavior (relative DNA alkylation efficiency and relative biological potency) of pairs of enantiomeric agents that may be traced to a single structural feature: the degree of steric bulk surrounding the CPI or DSA C7 center within the alkylation subunit for which the unnatural enantiomers are especially sensitive. The distinctions between enantiomers have proven readily detectable with the simple alkylation subunits themselves (that is, N-BOC-DSA (**12**), -CI (**11**–**51**), -CPI (**13**), and -CBI (N-BOC-**45**)), are most prominent with the dimer-based agents (like duocarmycin SA, duocarmycin A, CBI-CDPI₁, CPI-CDPI₁ (**15**), and CPI-PDE-I₁ (**14**), Table 8, Fig. 43), and are less prominent with

the larger trimer- or tetramer-based agents (CC-1065, CPI-CDPI₂ (**16**), CBI-CDPI₂, and CPI-CDPI₃ (**17**)).^[54, 73]

In general, less distinction in the biological potency and relative DNA alkylation efficiency has been observed with the CI and duocarmycin SA enantiomeric pairs, both of which lack substituents or steric bulk at this position within the alkylation subunit. Moreover, the distinctions among enantiomeric CI-based agents, which lack the pyrrole ring altogether, are generally smaller than those observed with the duocarmycin SA-derived agents (DSA > CI). In contrast, the CPI-, CBI-, and duocarmycin A-based agents generally exhibit more pronounced distinctions within enantiomeric pairs (CPI > duocarmycin A > CBI). This suggests that the behavior of the unnatural enan-

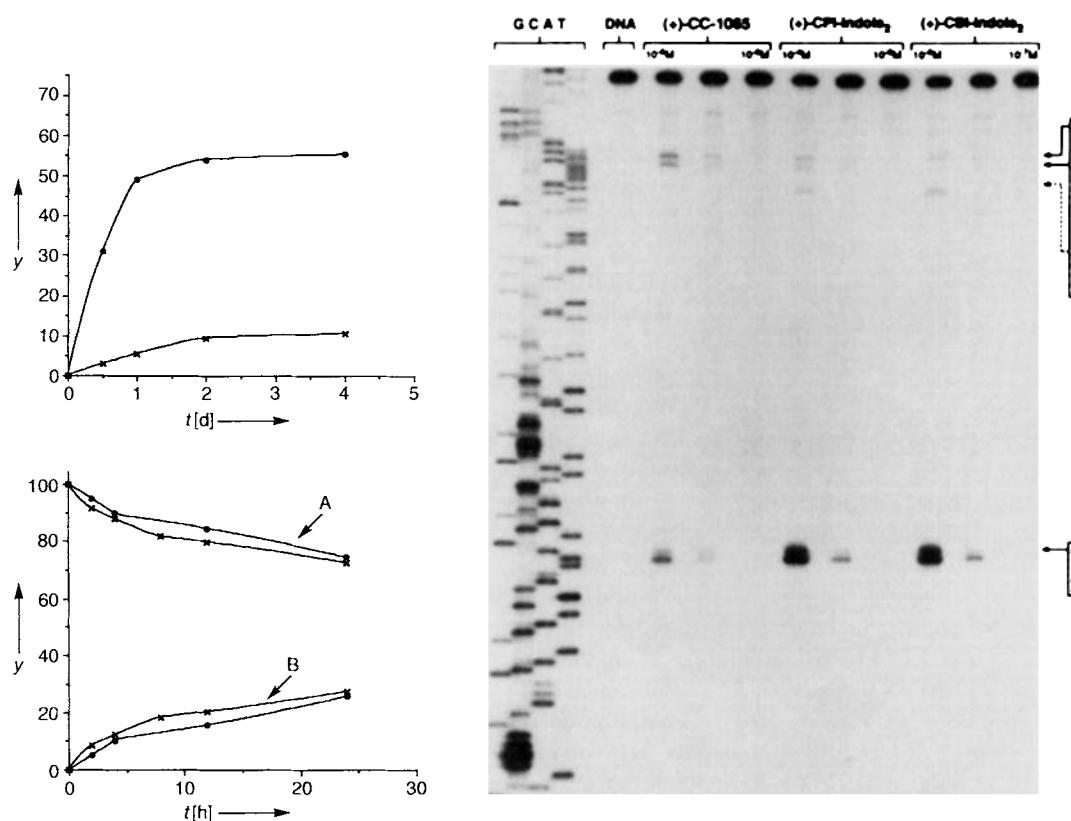


Fig. 42. Top left: Plot of percentage of integrated optical density (*y*) versus time, established through autoradiography of *S*³²P end-labeled DNA and used to monitor the relative rate of w794 alkylation at the 5'-AATTA high-affinity binding site for (+)-CBI(indole)₂ (●) and (+)-CPI(indole)₂ (×); 37 °C, 0–5 days, 1 × 10⁻⁵ M agent. Bottom, left: Plot of percentage of integrated optical density (*y*) versus time, established through autoradiography of *S*³²P end-labeled DNA and used to monitor the relative rate of w794 alkylation at the 5'-AATTA high-affinity binding site for (+)-duocarmycin SA (3, ×) and (+)-CBI-TMI (36, ●); 4 °C, 0–24 h, 1 × 10⁻⁶ M agent. A = unchanged DNA. B = alkylation/cleavage. Right: Thermally induced strand cleavage (by heating to 100 °C for 30 min) of double-stranded DNA (SV40 DNA fragment, 144 base pairs; nucleotide no. 138–5238, clone w794) after 24 h incubation of DNA with the agent at 4 °C, followed by removal of unbound agent with denaturing PAGE (8%) and autoradiography. Lanes 1–4: Sanger G, C, A, and T reactions; lane 5: control DNA; lanes 6–8, I (1 × 10⁻⁶–1 × 10⁻⁸ M); lanes 9–11, (+)-CPI(indole)₂ (1 × 10⁻⁴–1 × 10⁻⁶ M); lanes 12–14, (+)-CBI(indole)₂ (1 × 10⁻⁵–1 × 10⁻⁷ M).

Table 8. Comparison of the natural and unnatural enantiomers of the agents.

Agent	Configuration	IC_{50} , L1210 [ng mL ⁻¹]	rel. IC_{50} [a]	
(+)-CI-TMI	natural	10	1	0.5–2.0
(-)-CI-TMI	unnatural	10	1	
(+)-duocarmycin SA	natural	0.006	1	10
(-)-duocarmycin SA	unnatural	0.06	0.1	
(+)-CBI-TMI	natural	0.01	1	100
(-)-CBI-TMI	unnatural	0.9	0.01	
(+)-duocarmycin A	natural	0.1	1	>100
(-)-duocarmycin A	unnatural	>10	<0.001	
(+)-CI-CDPI ₁	natural	10	1	0.5–2.0
(-)-CI-CDPI ₁	unnatural	20	0.5	
(+)-DSA-CDPI ₁	natural	0.002	1	10–100
(-)-DSA-CDPI ₁	unnatural	0.06	0.03	
(+)-CBI-CDPI ₁	natural	0.002	1	>100
(-)-CBI-CDPI ₁	unnatural	>2	<0.001	
(+)-CPI-CDPI ₁	natural	0.02	1	>100
(-)-CPI-CDPI ₁	unnatural	>3	<0.006	

[a] The relative concentrations of the (unnatural/natural) enantiomers required to detect DNA alkylation.

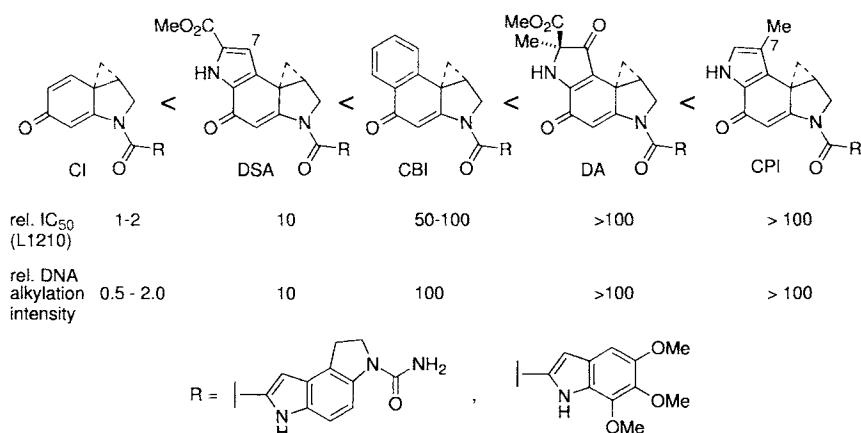


Fig. 43. Origin of the distinctions between the behavior of pairs of enantiomers toward DNA alkylation: steric bulk surrounding the DSA and CPI C7 center.

tiomer of duocarmycin SA as well as that of all agents studied to date is derived from a pronounced steric interaction of the C7 center with the 5' base adjacent to the adenine N3 alkylation site necessarily present with the 5' → 3' binding of the unnatural enantiomer. The 3' → 5' binding model for the natural enantiomers benefits from a less substantial but still potentially significant sensitivity to steric bulk at this same C7 position.

Consistent with the empirical observations to date and in agreement with models of the alkylation adducts, the studies suggest that agents lacking steric bulk surrounding the position occupied by a CPI or duocarmycin SA C7 substituent are predictably more effective at alkylating DNA, that the unnatural enantiomers of such agents are especially sensitive to steric bulk at this position, and that as this steric bulk is reduced, the distinctions between the enantiomers diminish. The only exception to these generalizations was observed with the MCBI-based agents, where the properties of the two enantiomers are more similar than those of the enantiomers of the CBI-based agents.^[126] This has suggested that such distinctions may be reduced or overcome by the introduction of additional noncovalent binding stabilization into the alkylation subunit itself.

10. Modified DNA Binding Subunits

One of the earliest and easiest structural features of the agents subjected to examination was the DNA binding subunits attached by an amide linkage to the naturally occurring alkylation subunits. These studies confirmed that hydrophobic righthand subunits convey binding affinity for the DNA minor groove, confer selectivity for binding at an AT-rich site in the minor groove, stabilize covalent adduct formation, accelerate the rate of DNA alkylation, contribute to and/or dominate the sequence selectivity of DNA alkylation,^[108, 109] and affect the physicochemical properties of the naturally occurring agents. Many of these issues are discussed in the context of the key analogs of CC-1065 and the duocarmycins described in Sections 2–5.

10.1. Systematic Substructure Examination of the CC-1065 DNA Binding Subunits

Early studies defined the degree of simplification and the optimum DNA binding subunits for analogs of CC-1065 that maintain the full potency of the natural product but improve in vivo antitumor efficacy. The PDE-I₂ subunits of CC-1065 could be reduced to (indole)₂ with full maintenance of cytotoxic potency and with significant enhancements in the in vivo antitumor activity (Fig. 44).^[158–162] Further simplifications or reductions in the size of the DNA binding subunits (for example, (indole)₁) resulted in diminished properties, although the attachment of the trimethoxyindole (TMI) subunit of the duocarmycins has provided a potent agent.^[165] This suggests that an appropriately substituted, single DNA binding subunit (for example, TMI or CDPI₁) may provide fully potent cytotoxic agents. Similar studies with the CBI-based agents have been conducted and have extended these observations (Fig. 44).^[73, 117–127, 166]

Notably, this latter class of agents incorporating the synthetic and simplified CBI alkylation subunit and the simplified, synthetic (indole)₂ (57, Fig. 45) or TMI DNA binding subunits constitute synthetic, designed analogs of the natural products that possess potent and efficacious antitumor activity.^[73, 121, 166] Consequently, a large degree of variation may be tolerated in this portion of the structure for optimization of the biological and physicochemical properties of the agents (Fig. 45).

We have advanced the proposal that it is not the kinetic rates of DNA alkylation^[46] that are responsible for the difference in the cytotoxic potencies of the agents bearing a common alkylation subunit, but rather the relative efficiency of DNA alkylation and thermodynamic stability of the adducts.^[38] Consistent with this proposal the cytotoxic potencies plateau for agents bearing a common alkylation subunit when sufficient noncovalent binding provides stable adducts. Moreover, this maximum potency is directly related to the functional stability of the agents. The chemically more stable agents provide the greatest

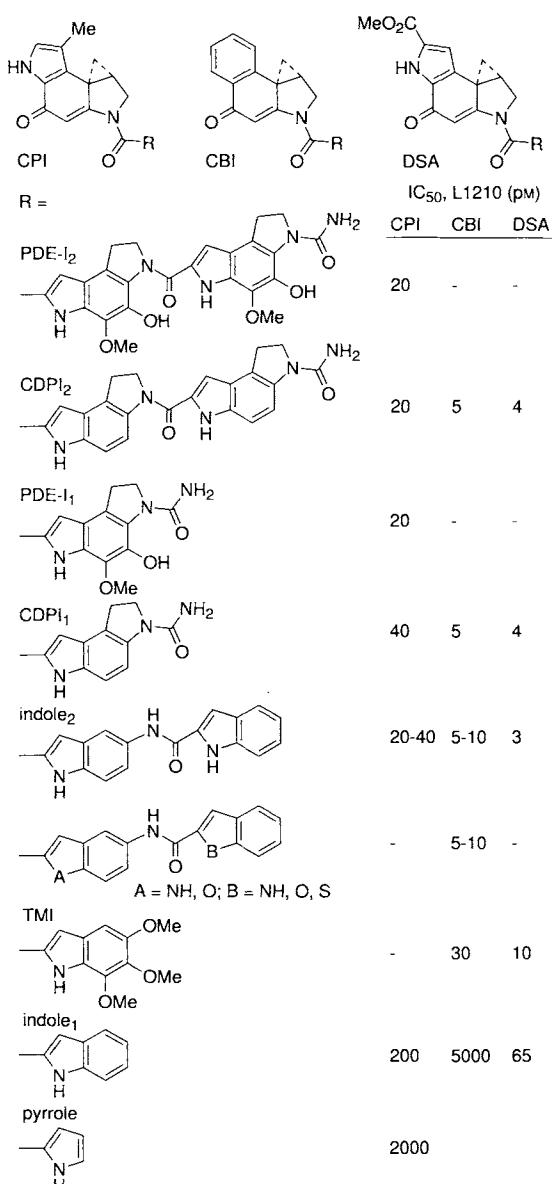


Fig. 44. Influence of the structure of the DNA binding subunits on the cytotoxic activity of CPI, CBI, and DSA analogues. The numbers on the right are the IC₅₀ (L1210) in pm.

potency (CPI: 20 pm, CBI: 5 pm, DSA: 3–4 pm). This is presumably because the chemically more stable agents reach their intracellular target more effectively.

10.2. Systematic Substructure Examination of the Duocarmycin DNA Binding Subunit

More recent studies systematically examined the role of the three methoxy substituents on the trimethoxyindole DNA binding subunit of duocarmycin SA.^[109] The C6 and C7 methoxy substituents, which lie on the outer or peripheral face of the DNA–agent complexes, individually contribute only a small or no enhancement to cytotoxic potency and the relative efficiency of DNA alkylation (C6>C7), while the C5 methoxy substituent, which is deeply imbedded in the minor groove, contributes fully to such properties (Fig. 46). In fact, the agent with

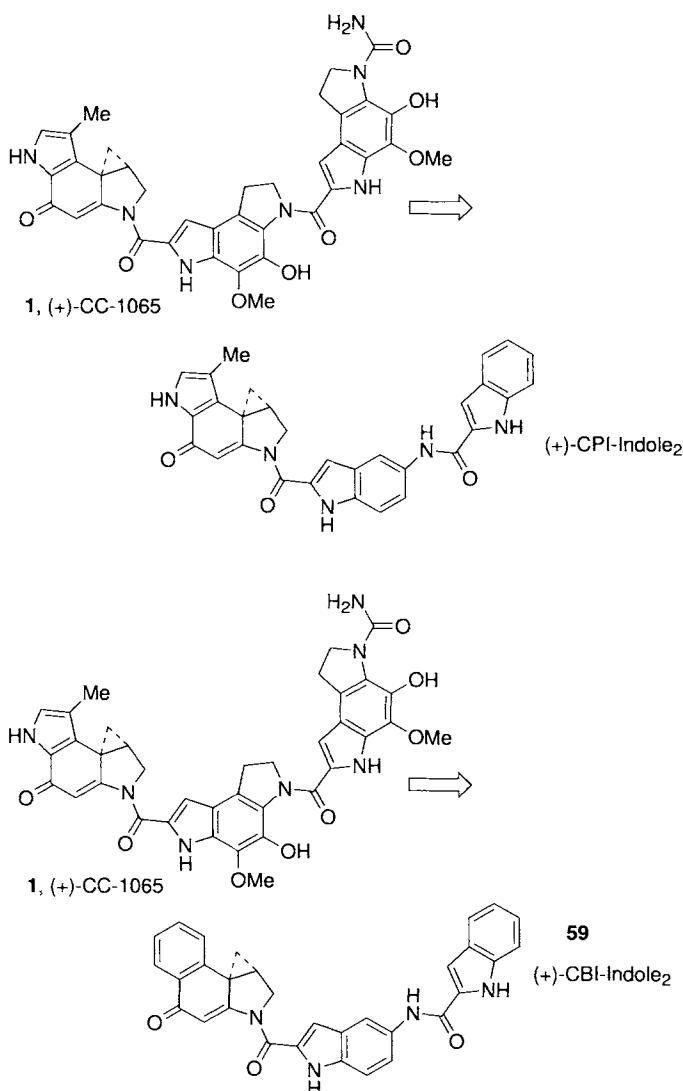


Fig. 45. (+)-CPI(indole)₂ and (+)-CBI(indole)₂: potent antitumor agents.

a single C5 methoxy substituent proved indistinguishable from the natural product indicating that it alone is sufficient for full potentiation of the properties. Consistent with the results of modeling studies detailed in early studies on CC-1065,^[41] this may be attributed to the additional noncovalent binding stabilization provided by the C5 methoxy substituent imbedded in the minor groove.^[82]

Importantly, the studies provided further key evidence supporting the proposal that the full potentiation of the cytotoxic potency of the agents is derived from noncovalent binding stabilization of an inherently reversible DNA alkylation rather than differences in the relative rates of DNA alkylation.^[109]

10.3. Deep-Seated Modifications in the DNA Binding Subunits

Two interesting deep-seated modifications in the DNA binding subunits of the natural products have been detailed. The first was based on the disclosure that electropositive substituents placed on the outer face of the DNA binding subunits substan-

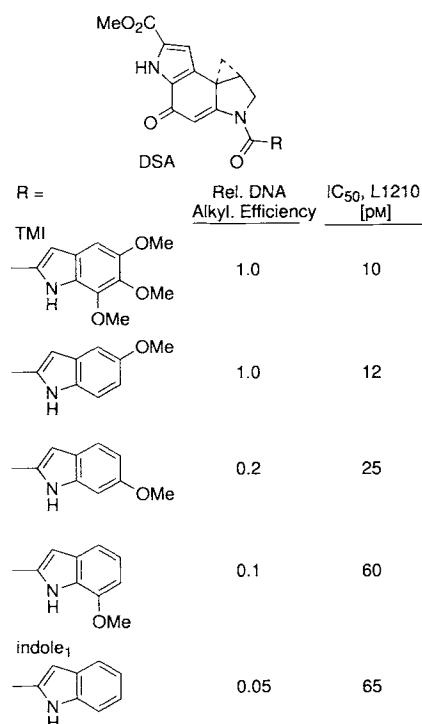


Fig. 46. The effect of methoxy substituents on the activity of duocarmycin SA analogs. Eff_{rel} = relative efficiency of the DNA alkylation.

tially increase DNA binding affinity without affecting DNA binding selectivity and simultaneously increase water solubility. This may be attributed to a spatially well-defined stabilizing electrostatic interaction with the negatively charged DNA phosphate backbone.^[83] These studies have been extended to **59–61** (Fig. 47) containing a quaternary ammonium salt on a single indole DNA binding subunit capable of providing this same strong electrostatic binding stabilization.^[84] Consistent with expectations, the water soluble agents **59–61** alkylated DNA with the same efficiency as **1** or **3** and were one hundred times more effective than **58**, which lacks the positively charged group.

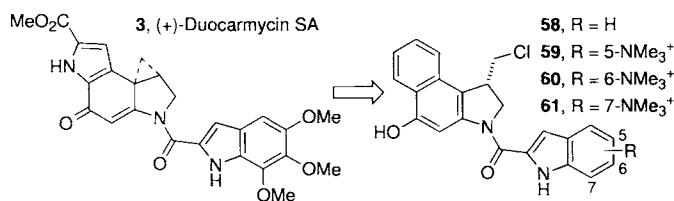


Fig. 47. Structures of **59–61**, the ammonium derivatives of **58**, as analogs of **3**.

The second important modification was based on the observations of Dervan^[12, 167] and Lown^[168] that the AT-rich, non-covalent binding selectivity of distamycin could be altered to accommodate a G-C base pair through introduction of a nitrogen into the backbone structure to serve as a hydrogen bond acceptor from a guanine C2-amine.^[169] In analogy to such studies, the agents **62** and **63** were designed and prepared (Fig. 48).^[170] They contain deep-seated modifications in the

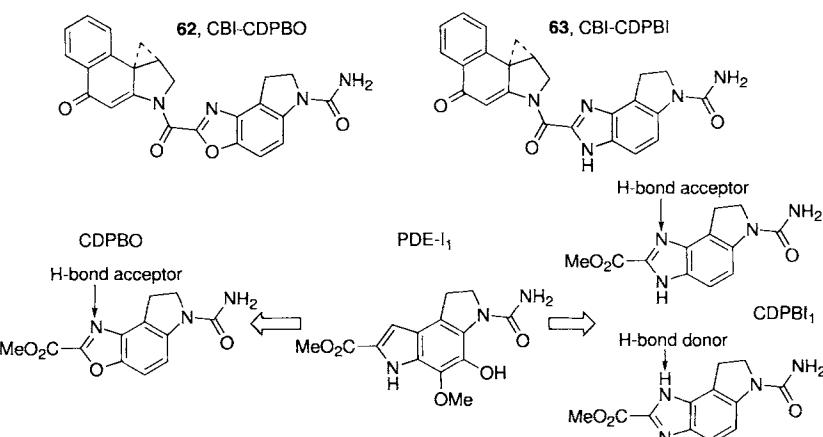


Fig. 48. CBI-CDPBO and CBI-CDPBI: model compounds with modified structural elements for DNA binding.

DNA binding subunits with incorporation of a nitrogen capable of serving as a hydrogen bond acceptor (CDPBO, CDPBI) or hydrogen bond donor (CDPBI) on their inside concave face, which is in intimate contact with the minor groove floor. The DNA alkylation selectivities of **62** and **63** are identical to those of CBI-CDPI₁ but with a substantially diminished efficiency (100×). Thus, the introduction of a single nitrogen atom had a pronounced and detrimental effect on the relative efficiency of DNA alkylation, but no apparent effect on DNA alkylation selectivity.

11. Biochemical Studies: Mechanism of Action

11.1. Apoptosis: A 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 demonstrating that sensitive tumor-cell lines including L1210 and Molt-4 are triggered to undergo apoptotic cell death at agent concentrations (100 pM) below that required for typical cell death by necrosis observed in nonsensitive cell lines.^[151] Apoptosis is a specific mechanism of cell death different from necrosis: cells undergo morphological changes including a characteristic heterochromatin condensation, degradation of genomic DNA, nuclear disintegration, and packaging of cell remains into apoptotic bodies that are removed by macrophages.

11.2. Cell Cycle Effects

The fundamental features of the cell cycle include growth, DNA replication, mitosis, and cell division. The cell cycle is divided into two parts—interphase and mitosis (M). The S phase (DNA synthesis) occurs in the middle of interphase and is preceded by G₁, the gap between mitosis (M) and DNA synthesis (S) and followed by G₂, the gap between replication and mitosis. The extent to which an antitumor agent affects these events has important implications on its mechanism of action. (+)-CC-1065 blocks progression of cells at G₂ and M.^[149, 171–173] No effect was observed in the transitions

from M to G₁ or G₁ to S. At agent concentrations low enough to arrest the cell cycle at G₂ and M, cells were able to pass slowly through S phase even though CC-1065 inhibits DNA synthesis. Similarly, adozelesin (**65**, see Fig. 49) was found to cause G₂ arrest.^[174, 175]

11.3. DNA Processing Enzymes

The alkylation of duplex DNA by CC-1065 and related agents inhibits many DNA processing enzymes^[176] (including the S1 nuclease responsible for digestion of single strand DNA,^[188] T4 ligase,^[177] helicase^[178–181]) and terminates DNA polymerase catalyzed DNA synthesis (replication) at the adduct sites.^[182] The CC-1065 adenine-N3 adduct produces a pronounced inhibition and enhancement patterns of DNA cleavage upon DNase I or MPE-Fe^{II} footprinting.^[183] Attempts to correlate agent structure with the extent of both the mutagenic effects of the adenine-N3 alkylation^[184–191] and resistance^[192–194] have been disclosed.

12. Delayed Toxicity

A serious limitation in the use of agents related to (+)-CC-1065 (**1**) is the characteristic, delayed fatal toxicity of the natural product.^[52] When the toxicity of **1** was examined in non-tumor-bearing mice, an unusual form of lethal, delayed hepatotoxicity was observed at therapeutic antineoplastic doses. At single dose levels of 12.5 µg kg⁻¹ administered intravenously (iv), delayed deaths were observed at 50 days, and at 10 µg kg⁻¹ single dose (ip) administration, delayed deaths were observed at 70 days. The mouse LD₅₀ value (iv) was determined to be 9 µg kg⁻¹ single dose and 0.3 µg kg⁻¹ per day, five daily doses. These delayed deaths were accompanied by dramatic changes in the morphology of hepatic mitochondria. Animal pretreatment with agents that induce cytochrome P-450 metabolism was unsuccessful in preventing the toxicity of **1**. Importantly, the unnatural enantiomer of CC-1065, which is equally potent in vitro and equally efficacious in vivo, does not embody this property suggesting it is not inherent in the structure or a required consequence of the mechanism of action responsible for the productive antitumor activity. Similarly, most but not all CC-1065 analogs do not display this delayed toxicity nor do the duocarmycins and their analogs reported to date. Much speculation on the origin of this unusual property has been detailed.^[47, 97, 176] Among the more intriguing is the suggestion that the delayed toxicity may be related to the degree of DNA alkylation reversibility and consequently the extent of the non-covalent binding stabilization of the reversible adduct.^[37, 58] The agents whose absolute configuration (adducts of the natural enantiomers are more stable than those of unnatural enantiomers) and extent of noncovalent binding render the alkylation irreversible may be prone to this delayed toxicity. This suggests that the optimal agents may be those that form stable but not irreversible adducts. Regardless of whether this impacts on the delayed toxicity, this proposal implies that there may be inherent benefits to the use of the chemically most stable alkylation subunits whose degree of reversibility is greatest and whose

nondiscriminant alkylation reactivity is diminished coupled with their attachment to an appropriately sized DNA binding subunit capable of providing stable but not irreversible adduct formation.

13. Clinical Candidates

Four agents that are analogs of **1** and contain the authentic CPI alkylation subunit or a seco precursor have been put forward as clinical candidates.^[195, 196] The first two, **64** (U-71,184) and **65** (adozelesin), were derived from the first stage of studies on analogs of **1** in which systematic modifications, simplifications, and optimization of the DNA binding subunits were conducted (Fig. 49).^[197–199] Carzelesin (**66**) is a prodrug that after

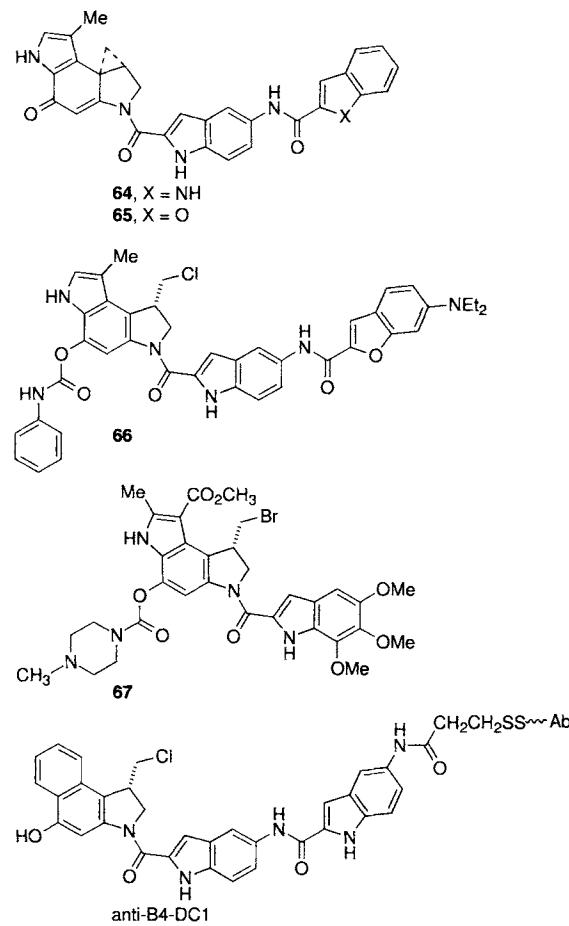


Fig. 49. Candidates for clinical trials.

releasing the free phenol upon carbamate hydrolysis ($t_{1/2} = 18–52$ min in plasma; $t_{1/2} = 40$ min in cell culture) readily cyclizes to the corresponding agent containing the cyclopropane.^[200] Bizelesin (**43**) is a DNA cross-linking agent incorporating two CPI alkylation subunit precursors and is approximately 20 to 30 times more potent than **1** ($IC_{50}(L1210) = 1\text{ pM}$ vs 20 pM).^[201]

From the duocarmycin class of agents, KW-2189 (**67**) has been selected for clinical trials in Japan.^[202–205] It is a semisynthetic duocarmycin B₂ derivative, which possesses improved antitumor activity, water solubility, and stability.

When conjugates of (+)-CBI-(indole)₂ (Fig. 45) that were first prepared with materials from our laboratory were linked by a cleavable disulfide to humanized versions of the antibodies (mAbs) anti-B4 and N901 and directed against the tumor-associated antigens CD19 and CD56, respectively, they proved to be extremely potent and antigen-selective in killing target cells in culture.^[206] The anti-B4 disulfide conjugate with (+)-CBI-(indole)₂ showed antitumor efficacy in an aggressive, *in vivo*, metastatic, human B-cell lymphoma model and completely cured animals bearing large tumor burdens. It was shown to be considerably more effective than doxorubicin, cyclophosphamide, etoposide, or vincristine at their maximum tolerated doses. Such tumor-selective targeting of the potent cytotoxic agents related to **1** and the duocarmycins by reversible linkage to tumor-directed antibodies thus improved their inherent selectivity. Because of the potentially low delivery of the antibody-linked agents, which is limited by the number of cell surface cognate antigens, such approaches require that the linked cytotoxic agent be exceptionally potent. Only a limited number of agents including **1–3** and their synthetic analogs possess such required potencies.

14. Summary and Future Prospects

In a remarkably short period the definition of the DNA alkylation properties and the basis for the sequence-selective recognition and alkylation of DNA by this new class of antitumor antibiotics have been described. This may well represent the best understood and most thoroughly investigated class of sequence-selective DNA alkylating agents examined to date. Major issues in understanding the polynucleotide recognition and functional reactivity of the agents and their potential utility have been detailed. Nonetheless, several fundamental unresolved issues remain, including a consensual agreement on the origin of the DNA alkylation selectivity, the importance of the rates or efficiencies of DNA alkylation relative to the thermodynamic stability of the reversible adducts, the origin of the characteristic but unique delayed toxicity of **1**, the extent and origin of catalysis of the DNA alkylation reaction, and the link between DNA alkylation and selective antineoplastic activity. Importantly, the present understanding of the origin of the properties of the natural products emerged principally through the design and evaluation of synthetic substructures and agents containing deep-seated structural changes (as well as their unnatural enantiomers), rather than through extensive studies conducted solely on the natural products themselves. Speculation on the origin of properties, which may result in misconceptions or misrepresentations, may and should be addressed by direct and often unambiguous tests employing well-devised synthetic agents. Some such agents have also proven much more efficacious than the natural products themselves, which illustrates that their productive properties may be enhanced by well-founded and designed structural changes. The fact that such structural changes may also often simplify the target analog suggest that it will be a synthetically designed rather than naturally derived agent in this class that will ultimately become clinically significant. No doubt, the fundamental principles defined in the studies conducted to date may provide the foundation for such future developments and advances.

Abbreviations

DSA:	duocarmycin SA alkylation subunit
DA:	duocarmycin A alkylation subunit
CPI:	1,2,8,8a-tetrahydro-7-methylcyclopropa[cl]pyrrolo[3,2-e]indol-4-one
CBI:	1,2,9,9a-tetrahydrocyclopropa[c]benz[e]indol-4-one
MCBI:	1,2,9,9a-tetrahydro-7-methoxycyclopropa[c]benz[e]indol-4-one
CCBI:	1,2,9,9a-tetrahydro-7-cyanocyclopropa[c]benz[e]indol-4-one
CI:	1,2,7,7a-tetrahydrocyclopropa[1,2-c]indol-4-one
CBQ:	2,3,10,10a-tetrahydro-1 <i>H</i> -cyclopropa[d]benz[f]quinol-5-one
C ₂ BI:	9a-(chloromethyl)-1,2,9,9a-tetrahydrocyclopropa[c]benz[e]indol-4-one
CDPI:	3-carbamoyl-1,2-dihydro-3 <i>H</i> -pyrrolo[3,2-e]indole-7-carboxylate
TMI:	5,6,7-trimethoxyindole-2-carboxylate
CDPBO:	3-carbamoyl-1,2-dihydro-3 <i>H</i> -pyrrolo[3,2-e]-benzoxazole-7-carboxylate
CDPBI:	3-carbamoyl-1,2-dihydro-3 <i>H</i> -pyrrolo[3,2-e]benzimidazole-7-carboxylate
TACDPI:	5-trimethylammonio-3-carbamoyl-1,2-dihydro-3 <i>H</i> -pyrrolo[3,2-e]indole-7-carboxylate
ACDPI:	5-amino-3-carbamoyl-1,2-dihydro-3 <i>H</i> -pyrrolo[3,2-e]indole-7-carboxylate
IC ₅₀ :	the concentration of agent required to inhibit by 50 % the growth of the indicated cell line (L1210) in a 72 h assay.

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