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Catalysis of the CC-1065 and Duocarmycin DNA Alkylation Reaction: DNA Binding Induced Conformational Change in the Agent Results in Activation

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Abstract—A number of indirect observations are summarized that suggest the rate acceleration for the CC-1065 and duocarmycin DNA alkylation reaction is derived in part from a DNA binding-induced conformational change in the agents which substantially increases their inherent reactivity. This ground-state destabilization of the agent, which we suggest results from a binding-induced twist in the linking N2 amide and requires a rigid extended N2 amide substituent, disrupts the vinylogous amide stabilization and activates the agents for DNA alkylation.

CC-1065 and the duocarmycins are the parent members of a class of exceptionally potent antitumor–antibiotics (Fig. 1).[1–4] Since their disclosure and the demonstration that they derive their biological properties through a sequence selective alkylation of duplex DNA, extensive efforts have been devoted to defining the fundamental principles underlying the relationships between structure, chemical reactivity, and biological properties.[5–7] In these efforts, a number of deep-seated modifications in the agents have been made and their subsequent evaluations have provided important insights into the structural origin of their properties.

Despite the efforts that have defined the details of the DNA alkylation reaction which has been shown to proceed by a reversible, stereoelectronically-controlled adenine N3 addition to the least substituted carbon of the activated cyclopropane within selected AT-rich minor groove regions of duplex DNA (Fig. 2),[5–7] the origin of catalysis for the reaction has remained largely unaddressed.[7]

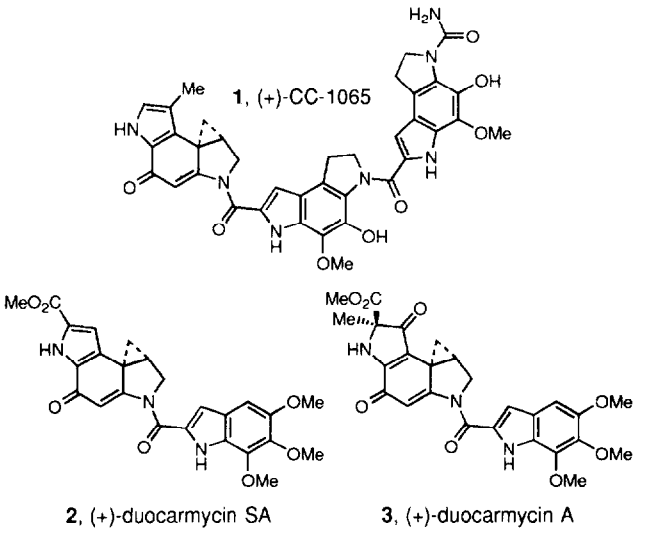


Figure 1.

Three proposals have been advanced to account for the DNA alkylation sequence selectivity and these have been discussed in detail elsewhere (Fig. 3).[5–7] Two of these are based on the premise that **1** and **4** exhibit identical alkylation selectivities and advocate a sequence-dependent DNA reactivity in which catalysis is intimately built into the alkylated sites.[7] One proposes a sequence-dependent phosphate protonation of the C4 carbonyl which activates the agent for DNA alkylation[7–11] and the other invokes a conformationally trapped DNA reactivity[12] (e.g. bent DNA) equivalently expressed for both **1** and **4** without addressing the source of catalysis. While attractive, little experimental evidence has been marshalled to support either of these proposals. In contrast, the proposal that the DNA alkylation selectivity is controlled by the AT-rich noncovalent binding selectivity of the agents and their steric accessibility to the adenine N3 alkylation site is fully consistent with a growing set of experimental observations.[5,13–24]

Most notably, it explains the reverse and offset 5- or 3.5-base-pair AT-rich adenine N-3 alkylation selectivities of the natural and unnatural enantiomers of **1**[13] and **2–3**,[14,15] respectively, and requires that **4–6** exhibit alkylation selectivities distinct from the natural products.[13–15] Moreover, it offers a beautiful explanation for the identical alkylation selectivities (5′-AA>5′-TA) of both enantiomers of the simple derivatives (**4–6**) of the alkylation subunits and this AT-rich selectivity corresponds nicely to the length of the agent and the size of the required binding region surrounding the alkylation site. Further support of this model includes the demonstrated AT-rich noncovalent binding of the agents,[21] their preferential noncovalent binding coincidental with DNA alkylation,[22] the demonstration that the characteristic DNA alkylation is

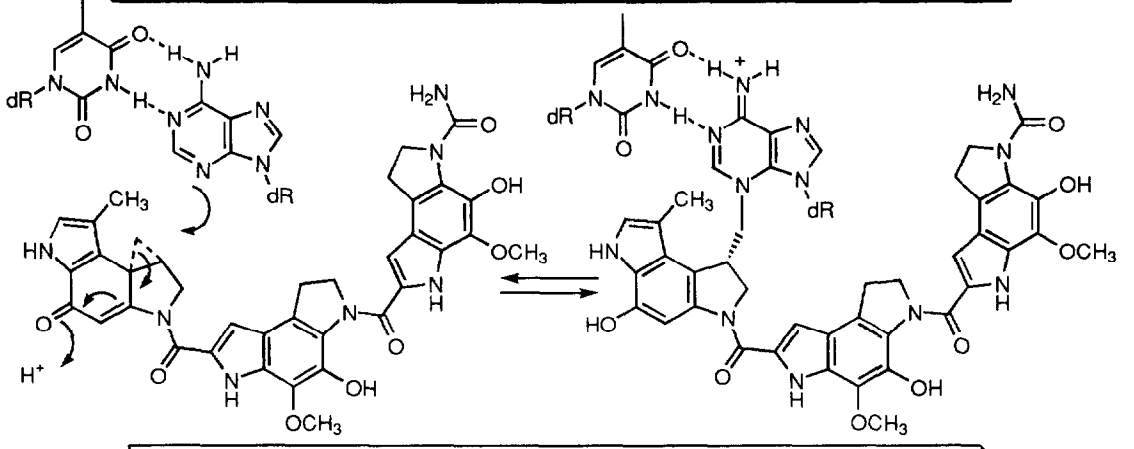
also observed with isomeric alkylation subunits (e.g., *iso*-CI and *iso*-CBI)[23] and does not require the presence of the C4 carbonyl or even the activated cyclopropane,[16] and the reversal of the inherent enantiomeric alkylation selectivity of the agents by the simple reversal of the orientation of the DNA binding subunits.[24] In our assessment, this latter study is unambiguous in establishing the origin of the DNA alkylation selectivity. Nonetheless, the model does not address the issue of catalysis except to require that it not be uniquely site dependent.

The remarkable chemical stability of **1–2** and the requirement of acid-catalysis for addition of typical nucleophiles to the activated cyclopropane has lead to the assumption that the DNA alkylation must similarly be an acid-catalyzed reaction. Although efforts have gone into supporting the extent and role of this acid catalysis,[7–12,25,26] it remains largely undocumented for the DNA alkylation reaction. This analysis is further complicated by potential Lewis acid catalysis by metal cations associated with the DNA phosphate backbone. At pH 7.4, one can estimate that the DNA phosphate backbone ($pK_a = 1–1.5$) is fully ionized with less than 1 in 2,500,000–800,000 phosphates bearing a proton (0.0001–0.00004% protonated). Consequently, it is unlikely that the catalysis for DNA alkylation is derived from a phosphate backbone delivery of a proton to the C4 carbonyl. Although increases in the local hydronium ion concentrations surrounding 'acidic domains' of DNA have been invoked to explain DNA mediated acid-catalysis,[27] nucleotide reactivity,[27] and even extrapolated to alkylation site catalysis,[25,26] the remarkable stability of **1–3** even at pH 5 suggests to us that it would more likely be derived from C4 carbonyl complexation with a metal cation associated with the phosphate backbone. As such, efforts to compare acid-catalyzed reactivity (solvolysis) with DNA alkylation rates as a measure of establishing the extent of catalysis may not be productive.[26]

In addition, we have made a number of observations that are inconsistent with this simple role of acid or Lewis acid catalysis for accelerating the CC-1065 and duocarmycin DNA alkylation reaction and wish to propose an additional mechanism for achieving the rate enhancement for their reaction with DNA. Herein, we summarize a number of indirect observations that suggest this rate acceleration is derived from a DNA binding-induced conformational change in the agent which substantially alters its inherent reactivity (Fig. 4).[28,29] This ground state destabilization of the substrate, which we suggest results from a binding-induced twist in the linking N2 amide, disrupts the

Adenine N-3 Alkylation

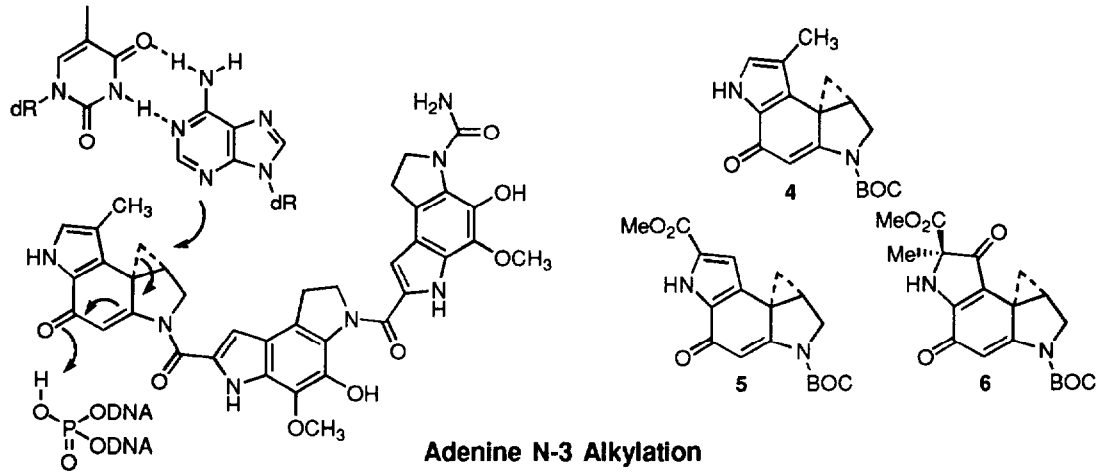
- Minor Groove
- Adenine N-3 Alkylation
- S_N2 not S_N1 Addition
- Stereoelectronic Control
- Inherently Reversible Reaction
- Direct Relationship of Functional Stability - Potency
- Partial Activation of Cyclopropane Partial Conjugation of Cyclopropane Unexpectedly Stable
- Both Enantiomers Effective



Consensus Alkylation Sequences

Agent	base	4	3	2	1	0	-1	-2	-3	-4	3'
(+)-CC-1065	% A/T	67	78	94	98	100	55	-	-	-	
Consensus		A/T>G/C	A/T	A/T	A/T	A	Pu>Py				
ent-(-)-CC-1065	% A/T	-	-	-	88	100	93	82	73	56	
Consensus					A/T	A	A/T	A/T	A/T	N	

Figure 2.



Alkylation Site Model	Noncovalent Binding Model
• (+)-CPI and (+)-CC-1065 selectivities identical selectivity inherent in CPI subunit and nature of alkylation reaction	• (+)-CPI and (+)-CC-1065 selectivities different modest electrophile superimposed on 5 base-pair AT-rich DNA binding agent
• Alkylation select. independent of binding select.	• Alkylation select. is controlled by binding select.
• Different features control *ent*-(-)-CC-1065	• Same features control *ent*-(-)-CC-1065
• Alkylation at junctions of bent DNA or	• Alkylation within AT-rich binding sequences
• Sequence dependent phosphate activation C4 carbonyl required	• Phosphate protonation does not control select. C4 carbonyl not required

Figure 3.

vinylogous amide stabilization responsible for their unusual stability and increases their inherent reactivity toward nucleophiles. We further suggest that this activation is not uniquely alkylation site dependent, but rather a general consequence of the forced adoption of a helical conformation upon AT-rich minor groove binding. As such, the DNA minor groove binding cocks the pistol or activates the warhead but does not pull the trigger for further reaction.

Two possibilities may arise as a consequence of a twisted N2 amide (Fig. 5). The N2 lone pair remains conjugated with the cyclohexadienone and the amide resonance is diminished. This would release the N2 lone pair into the enamide structure and should decrease its pK_a leading to C4 carbonyl protonation at a higher pH or more effective metal cation complexation. The pK_a estimates of protonated enaminones range from 2.5 to 3.1 while that of an N^2-acyl CPI derivative is 1.2,[25] suggesting that a twist of the N2 amide that would break the linking amide conjugation and release the full vinylogous amide would result in an increase in the pK_a by 1–2 units. Alternatively, the N2 lone pair remains conjugated with the amide and the cyclohexadienone structure loses the benefit of its vinylogous amide stabilization. This would provide a much more reactive intermediate prone to nucleophilic addition. Although we do not wish to distinguish between the two possibilities at the present time, one would suspect that an increase in reactivity would be derived from the latter process and N2 would prefer to relinquish the vinylogous amide conjugation in a manner analogous to the reaction product. Similarly, one might expect the former process to provide increased stability, albeit on an intermediate more prone to C4 carbonyl protonation or metal complexation.

Rates and mechanism of acid-catalyzed nucleophilic addition

In our efforts, simple derivatives of the authentic alkylation subunits and a wide range of analogues have been carefully studied (Fig. 6).[5] Except for the most reactive analogues, they are stable at pH 7 exhibiting no measurable rate of reaction with nucleophiles. Only at pH 2–3 do they exhibit easily monitored rates of acid-catalyzed nucleophilic addition and the rates become too slow to monitor in the higher pH ranges of 5–7.[30] In two different studies,[25,29] the rates have been shown to exhibit a first-order dependence on both the acid and nucleophile concentration consistent with a mechanism of rapid and reversible C4 carbonyl protonation followed by rate-determining nucleophilic attack on the cyclopropane in a reaction that exhibits S_N2 characteristics [eq. (1)].[28,29,31] The proposal that the minor abnormal ring expansion solvolysis of CPI occurs by a change in reaction mechanism (S_N1 versus S_N2)[25] with the generation of a free carbocation has been shown to not be operative with CBQ,[28] duocarmycin A,[31] and duocarmycin SA[31] and suggests that the ambiguous experimental basis for the CPI proposal

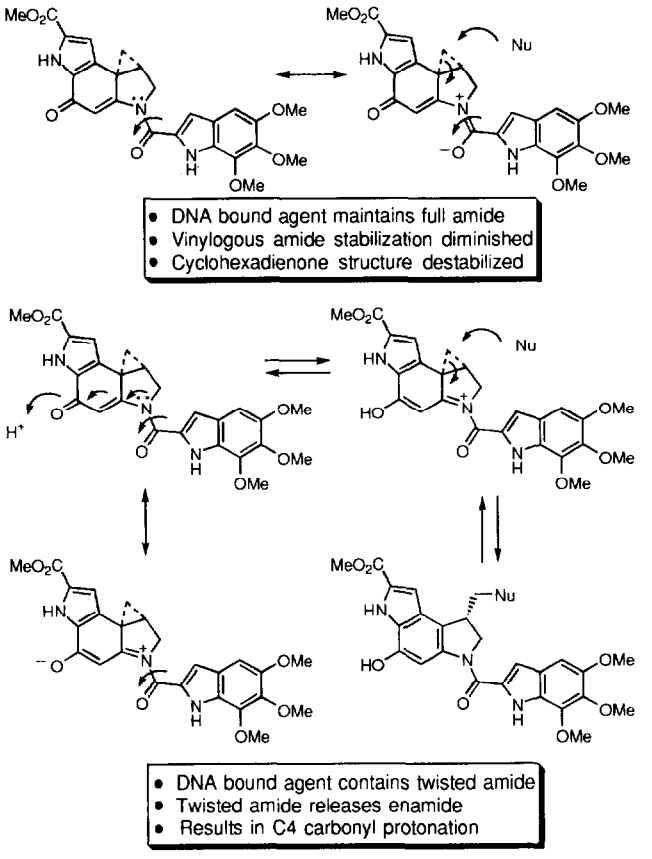


Figure 4.

should be reexamined. In one study, the pK_a of the protonated substrate (CPI) was derived from a detailed analysis and estimated to be 1.2.[25] As the estimated pK_a would suggest, little protonation of this species would be expected to be observed at pH 7.4 regardless of intramolecular delivery from phosphate or intermolecular proton delivery and its equilibration with bulk solvent might prove faster than the rate-determining nucleophilic addition. Moreover, under even mildly basic conditions (2–5% aq $NaHCO_3$–THF), the agents no longer undergo the characteristic cyclopropane addition reaction. Rather, the labile N2 linking amide is hydrolytically cleaved, illustrating that the N2 lone pair is normally conjugated with the vinylogous amide stabilizing the cyclohexadienone structure. Nonetheless, since the acid-catalyzed nucleophilic addition reactions exhibit a first-order dependence on acid concentration, extrapolated rates of reaction at pH 7.2 have been used to derive a rate acceleration of 10^{10}–$10^{12}\times$ for a pseudo first-order, acid-catalyzed DNA alkylation reaction.[26]

Rates of DNA alkylation

Although there are few sequence-dependent alkylation rates which have been accurately quantitated, it is sufficient to qualitatively state that the rates of DNA alkylation are much faster than first-order acid-catalyzed nucleophilic addition conducted even at pH 2–3. They are sufficiently rapid that complete reaction occurs within 2–4 h at pH 7.5 even at 4 °C under pseudo first-order conditions conducted with excess DNA and they may be much faster at selected alkylation sites. The observed rate of DNA alkylation under pseudo first-order conditions have been measured for duocarmycin A within a single site oligonucleotide, d(CGTATACG)$_2$: $k_{obs}=6.8\times10^{-5}$ s^{-1} (0 °C, pH 7.0) and 2.8×10^{-4} s^{-1} (37 °C, pH 7.0),[32] and for a CC-1065 analogue within poly[d(A-T)]·poly[d(A-T)], $k_{obs}=1.1\times10^{-3}$ s^{-1}, and poly[dA]·poly[dT], $k_{obs}=2\times10^{-2}$

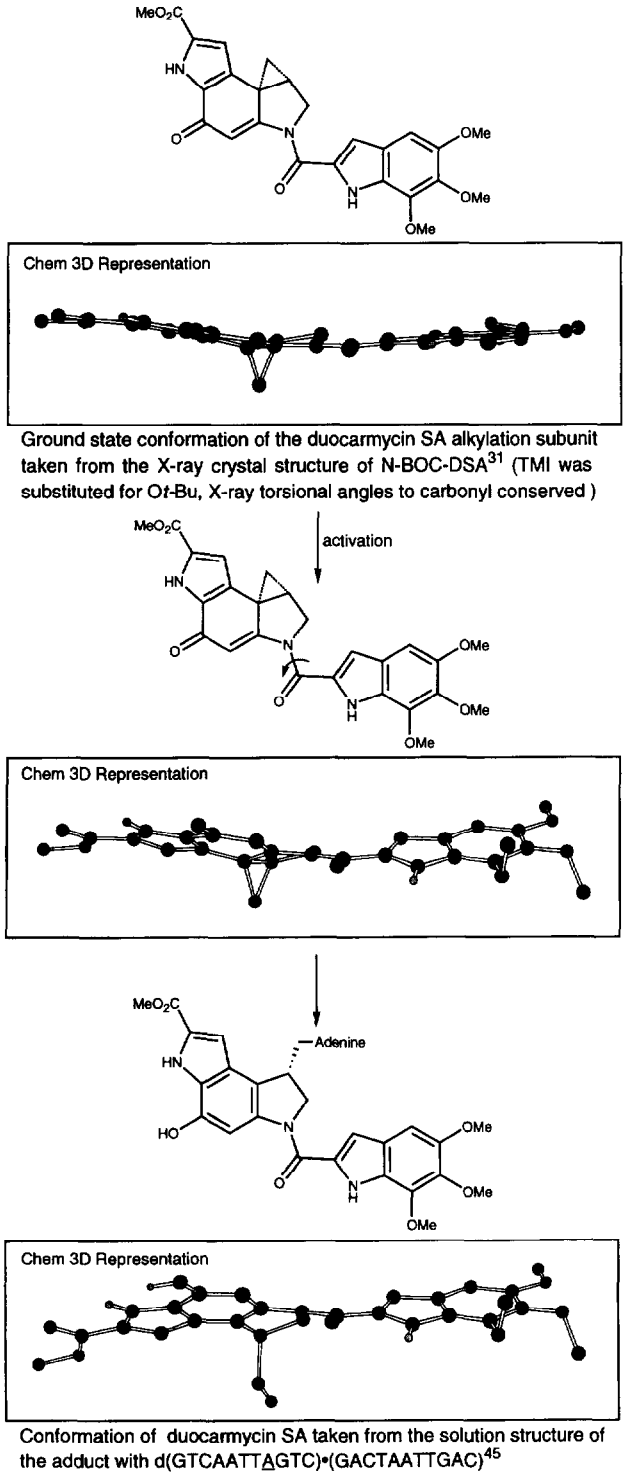
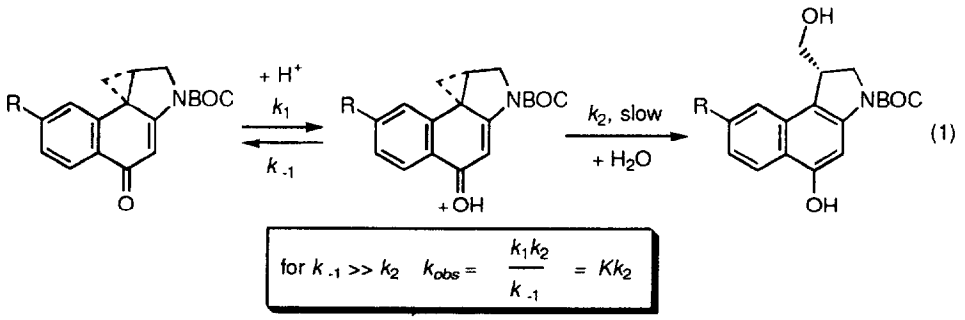


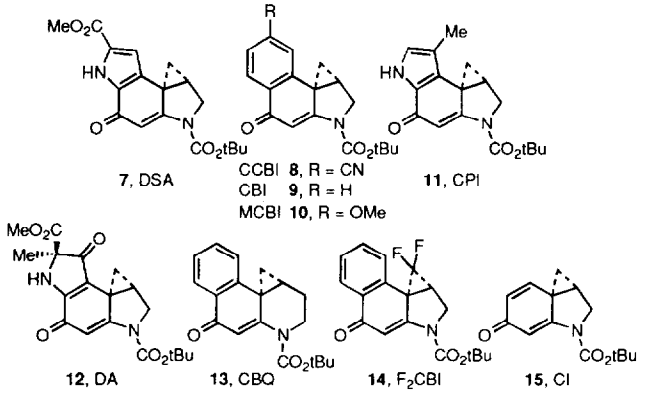
Figure 5.



s^{-1} (pH 7.0–7.2). In the latter case, the data fit the Michaelis–Menton equation with $K_m = 0.4$ mM and $k_u = 4 \times 10^{-3}\ s^{-1}$ ($k_u = k$ alkylation).[26] At pH < 7, k_{obs} was stated to increase linearly with acid concentration with a slope dependent on ionic strength, the DNA polymer, and the agent, although no details were provided.

Anomalous relative rates of DNA alkylation

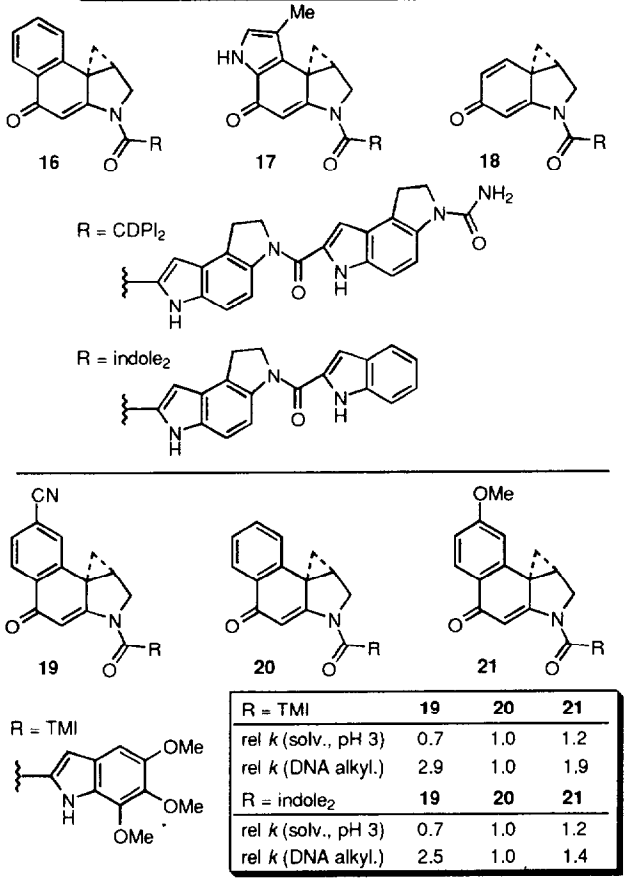
More interestingly, in the instances where this has been carefully examined, the relative rates of DNA alkylation do not follow the relative rates of acid-catalyzed solvolysis (Fig. 7).[18,29,33–35] Although this was first disclosed with a series of agents (**16–18**) that possessed sufficiently different structures that the origin of the effects was unclear, the latter series (**19–21**) are so closely related that the subtle structural differences are unlikely to be contributing to an alteration of the expected order of reactivity. Rather, the unexpected order of DNA alkylation rates observed with the latter agents may be influenced by a previously unrecognized effect intimately linked to catalysis. In this latter series, the impact of the C7 substituent (**19–21**, R = CN ≥ OCH_3 > H) seems to be related to its simple presence rather than its electronic nature (R = OCH_3 > H > CN). We now suggest this impact is due to the extended length of the alkylation subunit and the corresponding increase in the inherent twist of the linking N2 amide that would accompany DNA minor groove binding.



	k (s^{-1}, pH 3)	$t_{1/2}$ (pH 3)	IC_{50} (L1210)
7	1.08×10^{-6}	177 h	6
8	0.99×10^{-6}	213 h	20 nM
9	1.45×10^{-6}	133 h	80 nM
10	1.75×10^{-6}	110 h	90 nM
11	5.26×10^{-6}	37 h	330 nM
12	1.75×10^{-5}	11 h	1000 nM
13	9.07×10^{-5}	2.1 h	2000 nM
14	7.05×10^{-4}	0.3 h	50000 nM
15	1.98×10^{-2}	0.01 h	18000 nM

Figure 6.

R = $CDPI_2$	16	17	18
rel k (solv., pH 3)	1.0	3.6	13700
rel k (DNA alkyl.)	1.0	0.05	<0.01
R = $indole_2$	16	17	
rel k (solv., pH 3)	1.0	3.6	
rel k (DNA alkyl.)	1.0	0.07-0.05	



R = TMI	19	20	21
rel k (solv., pH 3)	0.7	1.0	1.2
rel k (DNA alkyl.)	2.9	1.0	1.9
R = $indole_2$	19	20	21
rel k (solv., pH 3)	0.7	1.0	1.2
rel k (DNA alkyl.)	2.5	1.0	1.4

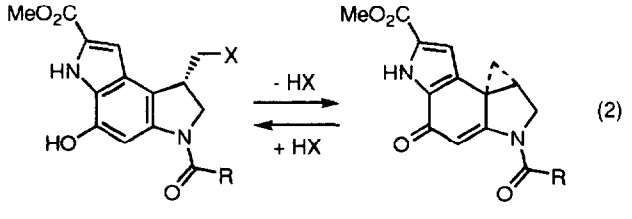
Figure 7.

DNA alkylation rates and efficiency of simple derivatives of the alkylation subunits

The rates of DNA alkylation by simple derivatives of the alkylation subunits (e.g., **4–15**) are much slower than **1–3**. The DNA alkylation requires much higher agent concentrations ($10^4\times$), more vigorous reaction conditions (37 °C), and much longer reaction times (24–72 h) to detect.[5-20,28,29,34-36] To date, several explanations have been advanced to account for these observations including the rate enhancement derived from effective noncovalent minor groove binding of the full agents, positioning (proximity) effects imposed on the DNA bound agents, or the relative degree of apparent reversibility of the adenine N3 alkylation. All invoke a benefit derived from the DNA binding or stabilization provided by the noncovalent contacts of the attached right-hand subunits. While these effects no doubt contribute to the distinctions, we suggest that they fail to account for the full magnitude of the differences. We now suggest that in the absence of the extended right-hand subunit, DNA minor groove binding no longer requires an induced twist in the N2 amide linkage depriving the agent of the additional activation toward DNA alkylation. Importantly, the consequences of this proposal are different from the Hurley proposal of a sequence dependent DNA reactivity that is equivalently expressed for both **1** and **4** and responsible for their purportedly equivalent DNA alkylation selectivities.[12] Rather, any sequence-dependent activation derived from a binding-induced conformational change in **1–3** that might lead to catalysis of the DNA alkylation reaction and influence the sequence selectivity would lead to distinctions, not similarities, in the DNA alkylation profiles of **1** and **4**.

Reversibility

In contrast to intuitive expectations, the cyclopropane ring is very easily introduced through an Ar-3′ spirocyclization [eq. (2)]. This occurs so readily that the precursor agents will often close upon formation or even upon exposure to conventional chromatography supports (e.g., SiO_2). Although the ring closure reactions are usually conducted with stoichiometric strong base (NaH, DBU, Et_3N), the most stable of the agents (i.e., **7–10**) may be prepared by simple exposure to even aq 2–5% $NaHCO_3$ (X = Cl). In early studies, this suggested to us that the adenine N3 adduct in DNA should be formed in a readily reversible manner.[5,13] Ultimately, this proved to be the case although the degree of reversibility was lower and the rate of retroalkylation was slower than the chemical precedent would suggest (Fig. 8).[14,15,37-39] It is perhaps



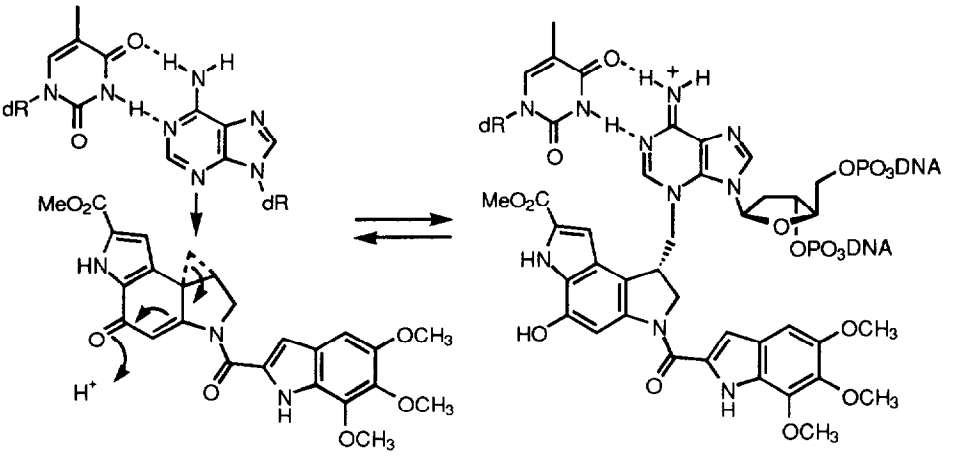
(2)

no accident of design that the agents fail to react with free adenine or simpler heteroaromatics in solution even with acid catalysis and it is reasonable to propose that such charged adducts should undergo retro nucleophilic addition (Ar-3′ spirocyclization)[40] more readily than the conventional synthetic precursors (X = Cl). The unusual stability of the DNA adducts and the slow rate of retro alkylation have been attributed to the noncovalent binding stabilization provided by the right-hand subunits. We now suggest that the adoption of the DNA alkylated conformation no longer facilitates or may even decelerate Ar-3′ spirocyclization with reversal of the DNA alkylation reaction and that this may further contribute to the unusual stability of the DNA adducts. For us, this is most easily conceptualized by viewing the conformational change upon DNA binding as leading to ground state destabilization resulting from diminished vinylogous amide conjugation. Not only would this account for the rate acceleration for formation of the adduct by lowering the apparent activation energy but also contribute to a shift in the thermodynamic equilibrium to favor adduct formation since the product would not be similarly destabilized by adopting a helical conformation.

Vinylogous amide stabilization

Perhaps the best evidence illustrating the impact of the vinylogous amide relationship is found in the mild, base-catalyzed hydrolysis of the N2 amide which can be accomplished under unusually mild conditions with LiOH [25 °C, <1 h, eq. (3)].[40] Although most additional evidence documenting the impact of the vinylogous amide stabilization of the alkylation subunits is circumstantial and predicated on the unusual stability of the agents relative to simple spirocyclopropylcyclohexadienones,[41] one additional direct study has nicely illustrated the extent of its effects. This rests on the isolation and characterization of the *p*-quinomethide **22**.[42] In contrast to typical *p*-napthoquinomethides which are inferred reactive intermediates incapable of isolation because of their reactivity, **22** could not only be isolated and characterized but it was also sufficiently stable to partially survive chromatography purification on SiO_2. We now suggest that disruption of this vinylogous amide stabilization found in **1–3** through adoption of the DNA bound conformation accounts for the increase in reactivity toward DNA alkylation.

Direct structural evidence of vinylogous amide can be found in the available X-ray crystal structures of the alkylation subunits and their cyclopropane ring opened derivatives (Fig. 9). Consistent with expectations, the N^2—C^{3a} bond length shortens with both the availability and relative extent of this vinylogous conjugation. In fact, all bonds in the vinylogous amide exhibit similar perturbations in their expected lengths consistent with the proposal that it plays an important role in the stabilization of the ground state conformation. Moreover, the introduction of a N^2 acyl substituent significantly reduces the extent of the vinylogous conjugation consistent with the relative reactivities of the

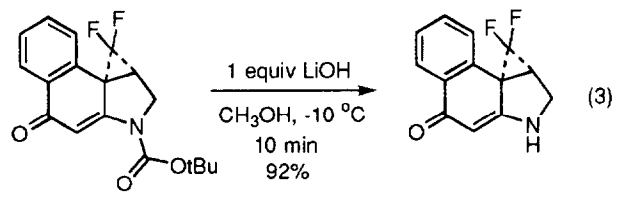


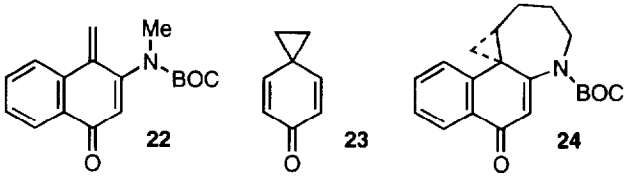
- Reversibilty: increases with pH, temperature, ionic strength
- Duocarmycin SA > Duocarmycin A
  - Reversibility: decreases with increasing adduct stability
  - Reversibility: decreases with increasing agent reactivity
- Duocarmycins > CC-1065
- *N*-BOC-DSA > Duocarmycin SA
  - Reversibility: decreases with increasing agent size or with increasing noncovalent binding stabilization

Figure 8. Characteristics of the reversible DNA reaction.

resulting agents (N2—COR > N2—H). We now suggest that upon adopting the DNA bound conformation, the twisted N2 amide diminishes the extent of the vinylogous amide conjugation and further increases the inherent reactivity of the alkylation subunit.

Two important questions that this proposal raises that have not yet been addressed experimentally are the energetic penalty that is paid in disrupting the vinylogous amide stabilization and the extent of the rate acceleration for a nucleophilic addition that might be gained by its loss. Even with the limiting estimates of 10–15 kcal mol^{-1} for amides that might not be accurate for **1–3**, the magnitude of the energetic barrier that must be overcome to disrupt the vinylogous amide conjugation is capable of being provided by the initial noncovalent DNA minor groove binding. Similarly, although the analogous ring systems of **1–15** lacking the vinylogous amide structure have not yet been prepared for examination, the exceptional chemical reactivity of **23** which does lack the vinylogous amide structure supports the contention that a sufficient increase in reactivity might be obtained simply by its disruption. Although the absence of additional structural features in **23** preclude an accurate extrapolation to **1–3**, **23** exhibits a pseudo first-order rate constant for solvolysis in methanol of 2.4×10^{-2} s^{-1} ($t_{\frac{1}{2}} = 28$ s)[41] even at pH 7.9, suggesting the effect may be sufficient to fully account for the DNA alkylation catalysis. Moreover, recent studies with **24**, which exhibits unusually high reactivities ($t_{\frac{1}{2}} = 2.1$ h, pH 7; $t_{\frac{1}{2}} = 1.7$ min, pH 3),[42] suggest the required DNA alkylation reactivities may be achieved by subtle structural perturbations in **1–3** without invoking acid catalysis.





Substituent effects on reactivity

We have conducted two studies which employ a classical Hammett series to establish the magnitude of substituent electronic effects on reactivity.[29,43] The comparisons made in these two studies provide some of the most convincing evidence that even small perturbations in the vinylogous amide can have a powerful effect on reactivity (Fig. 10). Both studies were conducted on the CBI nucleus and were used to

quantify the magnitude of the N2 and C7 substituent effects on functional reactivity. The C7 substituent effects were established with **25** and were found to be exceptionally small: $\rho = -0.30$.[29] Although the introduction of a strong electron-withdrawing group slowed acid-catalyzed solvolysis and the introduction of a strong electron-donating substituent accelerated solvolysis, the effect was very small and only a two-fold rate difference between *N*-BOC-CCBI (X=CN) and *N*-BOC-MCBI (X=OMe) was observed.

In sharp contrast, the nature of the N2 substituent established with **26** had an exceptionally large effect: $\rho = -3.0$.[43] The large negative value indicates substantial positive charge build up at the substituent site in the transition state of the reaction and proved consistent with a mechanism of rapid and reversible protonation followed by slow S_N2 nucleophilic addition to the activated cyclopropane.[43] Moreover, the solvolysis rate changed by a factor of 10 even with the small change of going from R=—SO_2Et to —CONHMe, both electron-withdrawing substituents. Thus, a large perturbation in the reactivity accompanied a small change in the nature of the vinylogous amide.

Relative rates of DNA alkylation by reversed versus extended and sandwiched analogues of duocarmycin SA

Perhaps the most convincing evidence that the presence and orientation of the extended heteroaryl N2 amide conveys a special DNA alkylation reactivity that is independent of the alkylation sites was observed in the examination of the reversed versus extended and sandwiched analogues of duocarmycin SA (Fig. 11).[24] In these studies, it was shown that the reversal of the orientation of the DNA binding subunits results in the complete reversal of the inherent enantiomeric DNA alkylation selectivity: *ent*-(−)-$CDPI_2$-DSA=(+)-DSA-$CDPI_2$ and (+)-$CDPI_2$-DSA=*ent*-(−)-DSA-$CDPI_2$. In addition, both enantiomers of the sandwiched agents (CDPI-DSA-CDPI) containing a single DNA binding subunit attached to each side of the alkylation subunit alkylated the same sites and did so with a selectivity

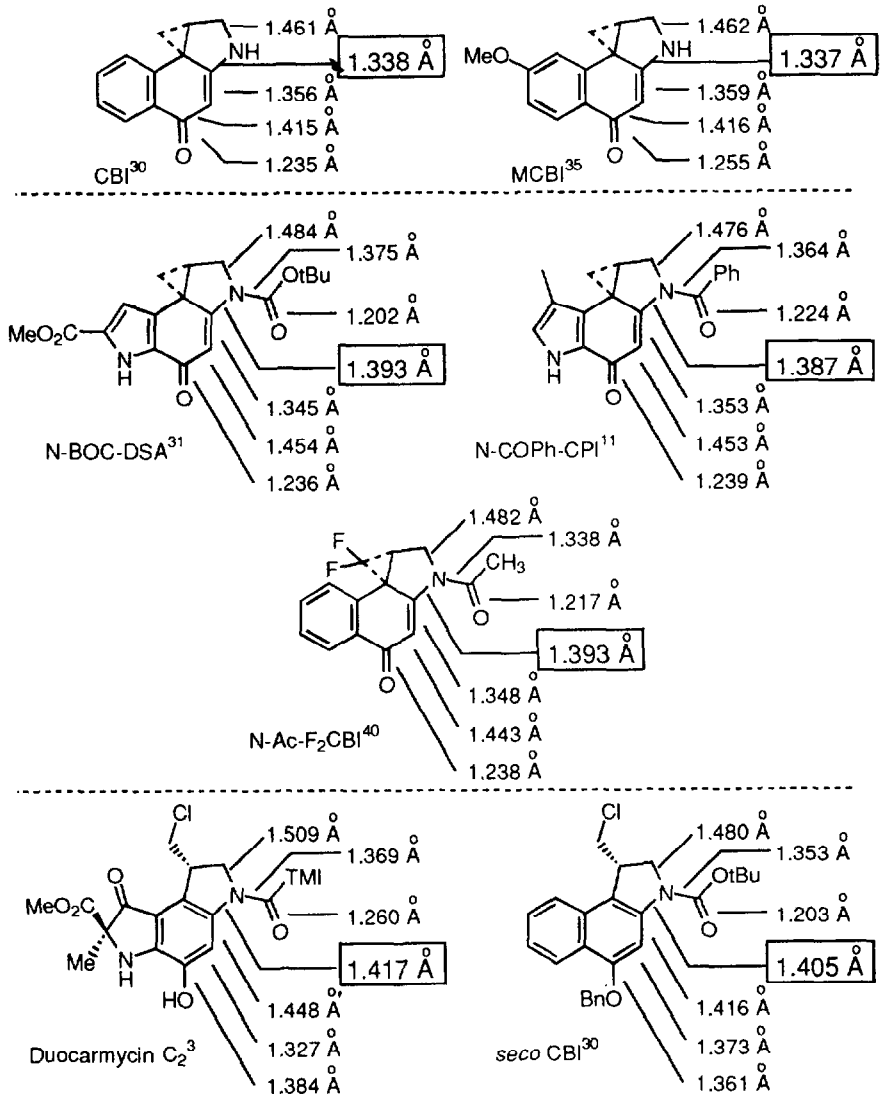
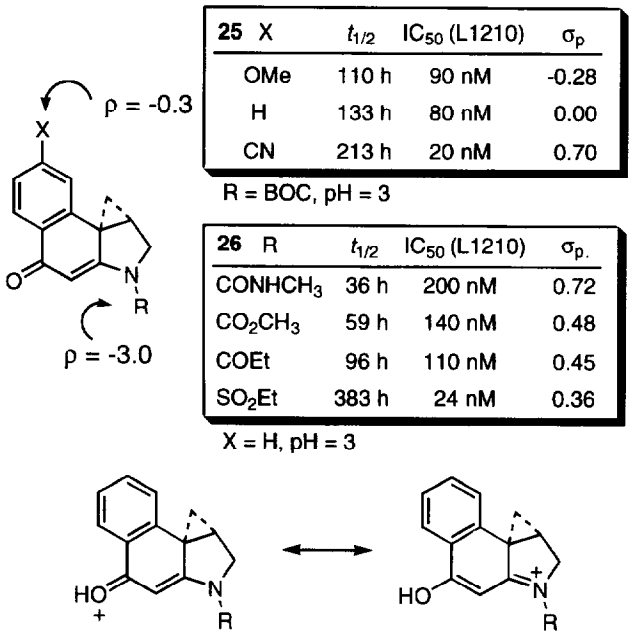


Figure 9.



25 X	$t_{1/2}$	IC_{50} (L1210)	σ_p
OMe	110 h	90 nM	-0.28
H	133 h	80 nM	0.00
CN	213 h	20 nM	0.70

R = BOC, pH = 3

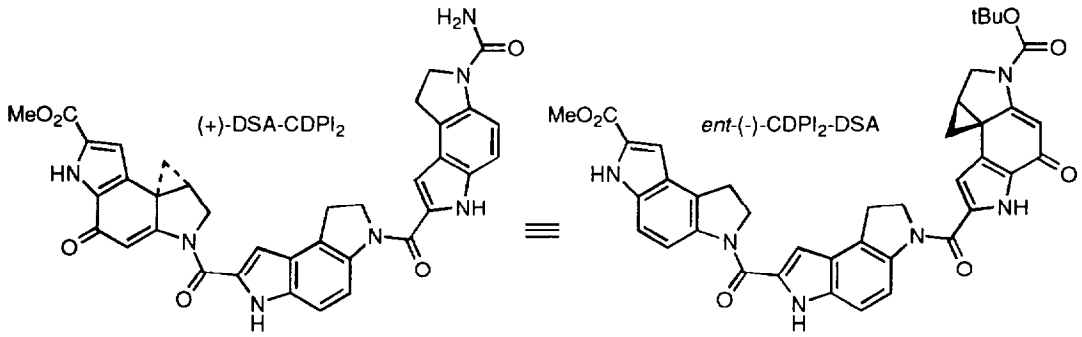
26 R	$t_{1/2}$	IC_{50} (L1210)	σ_p.
$CONHCH_3$	36 h	200 nM	0.72
CO_2CH_3	59 h	140 nM	0.48
COEt	96 h	110 nM	0.45
SO_2Et	383 h	24 nM	0.36

X = H, pH = 3

Figure 10.

distinct from the extended or reversed agents. In our assessment, these studies unambiguously established that the preferential AT-rich noncovalent binding selectivity of the agents controls the DNA alkylation sequence selectivity and that the natural and unnatural enantiomers are subject to the same polynucleotide recognition features.

In addition to the reversal of the inherent enantiomeric alkylation selectivity that was observed with the reversed agents, there was a substantial change in the rate of DNA alkylation. The DNA alkylation rate of the extended as well as the sandwiched agents was exceptionally fast (4 °C, 2–4 h) and typical of this class of agents, while that of the reversed agents was exceptionally slow (37 °C, 24–120 h) proceeding at rates similar to those of the simple agents that lack DNA binding subunits altogether. Although there are many potential explanations for this observation, the inherent enantiomeric DNA alkylation selectivity of the reversed versus extended analogues simply reversed with these agents but no new sites were detected. Thus, in the comparisons of the extended and reversed agents, it was the rates but not the sites that were



Agent	base	4	3	2	1	0	-1	-2	-3	-4	3'
(+)-CC-1065	% A/T	67	78	94	98	100	-	-	-	-	
(+)-DSA-$CDPI_2$		71	85	100	100	100	-	-	-	-	
(+)-$CDPI_2$-DSA		-	-	-	95	100	98	85	70	55	
ent-(-)-CC-1065		-	-	-	88	100	93	82	73	56	
ent-(-)-DSA-$CDPI_2$		-	-	-	100	100	100	90	73	58	
ent-(-)-$CDPI_2$-DSA		69	81	98	98	100	-	-	-	-	
(+)-CDPI-DSA-CDPI		-	-	87	95	100	95	74	-	-	
ent-(-)-CDPI-DSA-CDPI		-	-	86	94	100	94	78	-	-	

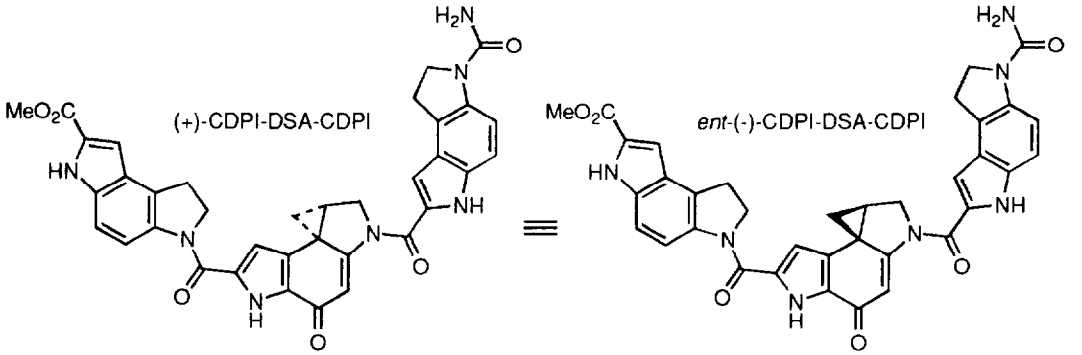


Figure 11.

altered. Although it is tempting to suggest this is due to orientation and positioning effects, it is observed with all sites in DNA and one would expect one or more binding sites to provide an optimal reaction alignment and orientation. Similarly, if the catalysis were attributable to a local increase in hydronium concentration within 'acidic domains' of DNA,[27] it is unlikely this effect would provide selective catalysis for the extended versus reversed analogues. Further supporting this observation, the nature of the simple terminal acyl group ($CO_2{}^tBu$, CO_2Me, $COCH_3$, H) on the reversed agents did not effect this inherent slow rate of DNA alkylation. Rather, this effect would appear more intimately tied to catalysis.

Consistent with this and in fact requiring such considerations, the sandwiched agents were found to alkylate DNA at a rapid rate (4 °C, 2–4 h) indistinguishable from the extended agents and much faster than the reversed agents. Moreover, this occurs with an altered sequence selectivity. Both enantiomers of the sandwiched agents alkylated the same sites and their selectivity was unique from that of either the natural or unnatural enantiomers of the extended or reversed duocarmycin SA analogues. Thus, the rapid rate of DNA alkylation was observed at a new set of alkylation sites independent of the absolute configuration of the agent indicating that the source of catalysis was not uniquely imbedded in the original DNA alkylation sites.

Rather, the distinguishing feature between the extended and sandwiched analogues of duocarmycin SA and the reversed analogues is the presence of the right-hand heteroaryl N2 amide. We suggest that upon binding to DNA with the adoption of a helical bound conformation, the inherent twist of the alkylation subunit N2 amide in the reversed analogues is not altered and, thus, not activated for adenine N3 addition. Consequently, they undergo DNA alkylation at rates comparable to those of the simple derivatives **4–7**, **8–15** themselves which also contain the short N2 amide substituents.

Importance of the duocarmycin SA peripheral substituents

Although not emphasized in studies disclosed to date, two of the duocarmycin SA substituents have been shown to contribute significantly to its properties (Fig. 12).[24,44] Both the left-hand subunit C6 methyl ester[24] and the right-hand subunit C5 methoxy group[44] increase biological potency (ca. 5–10×) and DNA alkylation efficiencies and rates (ca. 5–20×) in a substantial manner. Both substituents are deeply imbedded in the minor groove with the DNA bound agent and both can provide stabilizing noncovalent binding contacts that may account in part for their unique importance. We now suggest their important role may also be derived from their simple presence extending the rigid lengths of the two subunits resulting in a corresponding increase in the inherent twist of the linking N2 amide upon DNA minor groove binding.

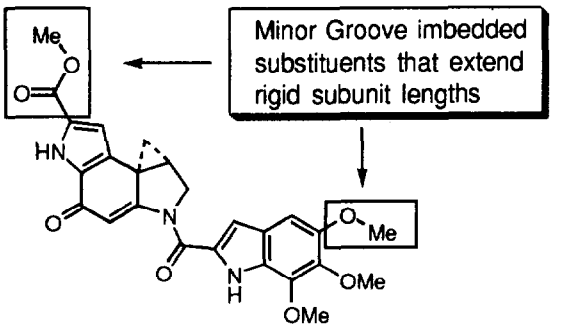


Figure 12.

DNA bound conformation

Although a number of NMR studies have addressed the site and structure of adducts of CC-1065, the duocarmycins, and their analogues with deoxyoligonucleotides,[45–49] for simplicity we will highlight only two[45,46] because of our familiarity with the results and our confidence of the efforts that went into the structure refinements. These are the structures of the single site alkylations by (+)-duocarmycin A and SA within the oligonucleotides d(GAAAAGG)·(CCTTTTC)[46] and d(GTCAATTAGTC)·(GACTAATTGAC),[45] respectively. In the former complex, the left- and right-hand subunits of duocarmycin A adopt a conformation containing a 36° twist[46,50] while the duocarmycin SA subunits are twisted by 43° in the latter complex[50] (Fig. 13).

These two studies establish both the presence and the considerable extent of the inherent twist in the bound helical conformation of the agents. For comparison purposes, the X-ray structure of *N*-BOC-DSA exhibits C3—N2—C=O and C3—C3a—N2—CO torsional angles of 4.5 and 6.9°, respectively, while those of duocarmycin SA in the latter oligonucleotide adduct are estimated to be 10.4 and 22.1°, respectively. While the experimentally derived relative twist in the agents is accurate, the placement of the amide carbonyl and the latter torsional angle values are of course dependent on the force field employed in the structure refinements and thus should only be considered approximations. Nonetheless, it is clear that the C3—C3a—N2—CO torsional angle is substantially perturbed consistent with disruption of the substrate vinylogous amide.

Natural versus unnatural enantiomer rates of DNA alkylation

One confusing observation with the agents examined to date is the relative rate and efficiency of DNA alkylation by enantiomeric pairs. This is most pronounced with the intermediate-sized agents (e.g., duocarmycins A and SA), and is less pronounced with the simple derivatives of the alkylation subunits (e.g., **4–15**) or the larger agents (e.g., CC-1065). Representative of this trend, (+)-duocarmycin SA (**2**) alkylates DNA 50× more rapidly than *ent*-(−)-duocarmycin SA while (+)-*N*-BOC-DSA (**5**) appears to be only 2–4× more

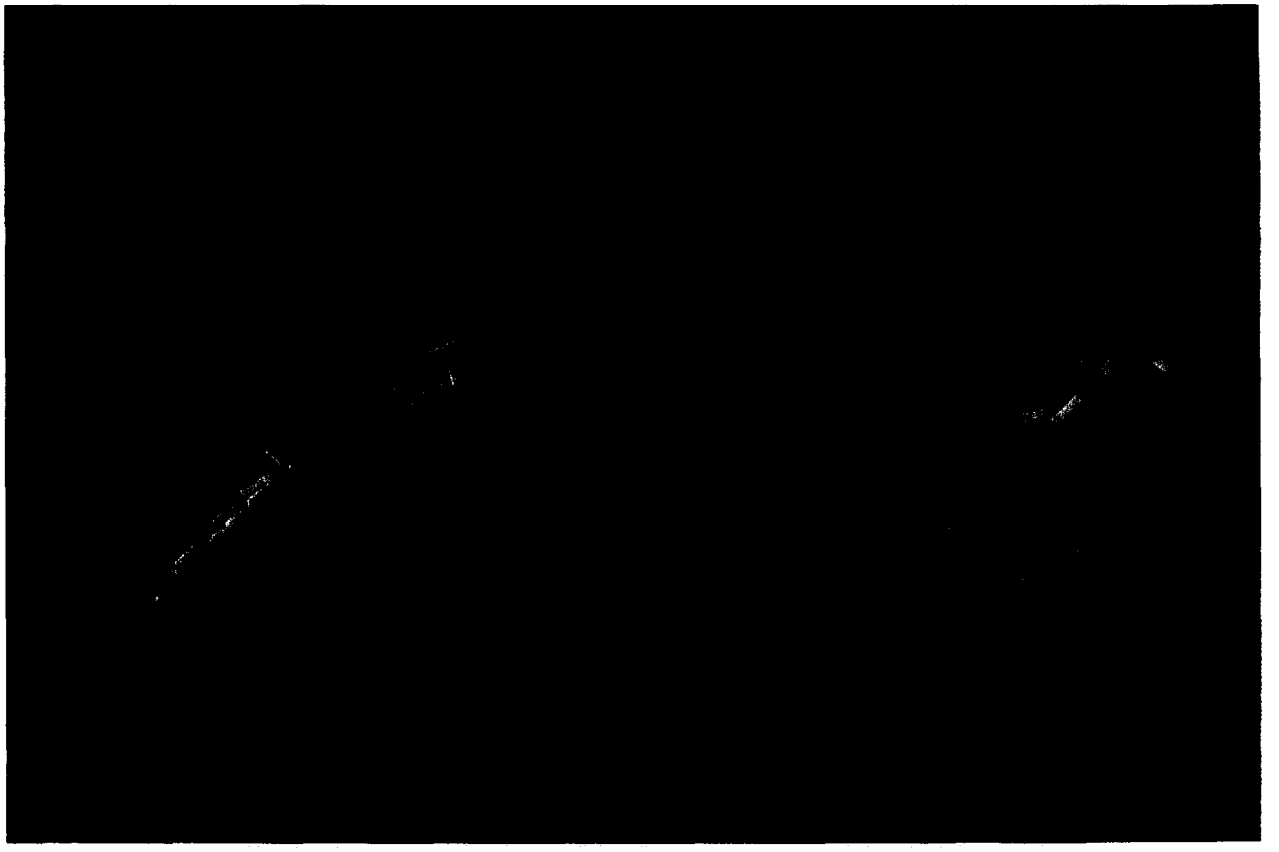


Figure 13. Front and groove views of the covalent adduct of (+)-duocarmycin SA bound to d(ATTAG)·(CTAAT) taken from the solution structure of duocarmycin SA–d(GTCAATTAGTC)·(GACTAATTGAC).[45]

Figure 14. Front and groove views of the comparison convalent adduct of *ent*-(−)-duocarmycin SA bound to d(ATTAG)·(CTAAT) taken from modeled complex with d(GTCAATTAGTC)·(GACTAATTGAC).

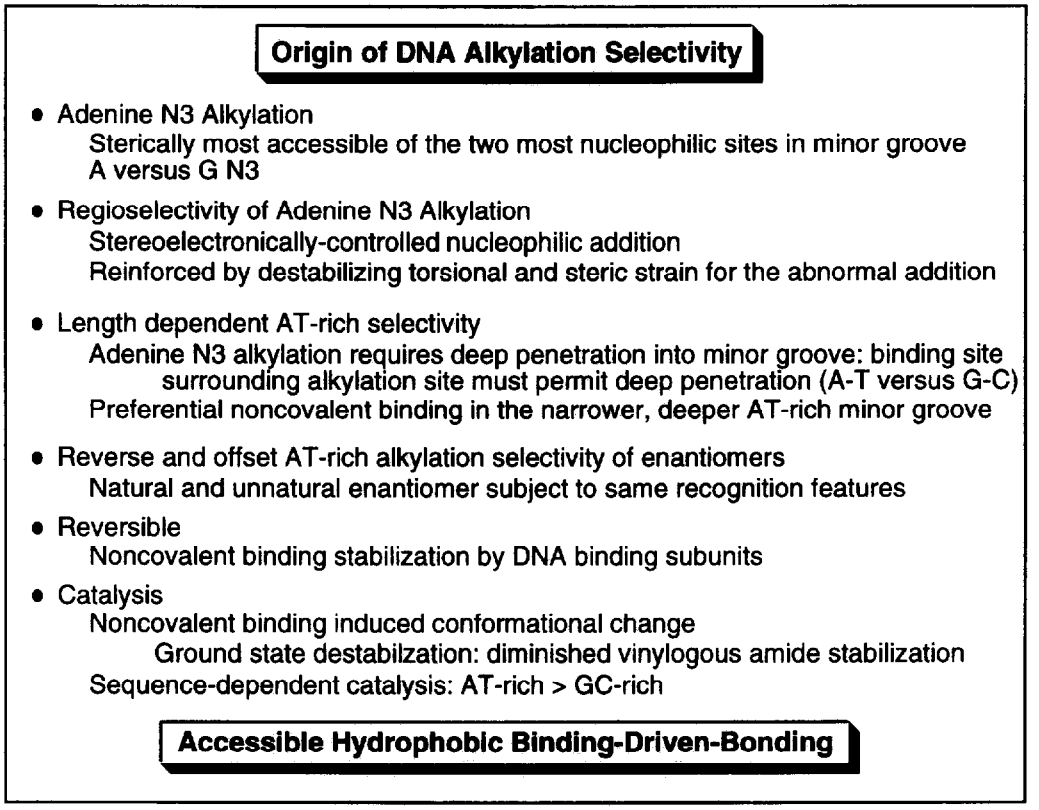


Figure 15.

effective than *ent*-(−)-*N*-BOC-DSA.[15] Contributing significantly to this distinction is the sensitivity of the unnatural enantiomer DNA alkylation reaction to steric bulk at the DSA C7 center. Consistent with the results of qualitative molecular modeling studies, the DNA adducts derived from the unnatural enantiomers suffer a destabilizing steric interaction between the DSA C7 center and the 5′-base preceding the alkylated adenine that is not present or as pronounced in the diastereomeric adducts obtained with the natural enantiomers. Supporting this proposal, the distinguishing features of the behavior of enantiomeric pairs diminish as the steric bulk surrounding C7 are reduced.

We now suggest that further contributing to this distinction in the behavior of enantiomeric pairs is the relative extent of the DNA binding-induced conformational change and the resulting degree of disruption of the vinylogous amide stabilization of the reacting alkylation subunit of the natural versus unnatural enantiomers. Modeling studies on both the natural and unnatural enantiomer alkylations consistently reveal that the natural enantiomers adopt a bound and alkylated conformation incorporating a greater helical twist and rise than the unnatural enantiomers. Although this is consistently observed with all alkylation site models independent of the specific sequence, it is instructive to illustrate the trends with the w794 high affinity site characterized structurally with the natural enantiomer in the Chazin NMR studies summarized above. Both the natural enantiomer, d(GTCAATTAGTC)·(GACTAATTGAC), and unnatural enantiomer, d(GTCAATTAGTC)·(GACTAATTGAC), of duocarmycin SA alkylate a single site extending across the exact same binding site within the deoxyoligonucleotide with alkylation occurring on opposite strands. Remarkably consistent with the results of Chazin's NMR-derived structural model, repetitive model building and simple energy minimization of the alkylated complex of the natural enantiomer provide a model in which the adopted conformation of the agent contains an average 41° twist. In contrast, the unnatural enantiomer within the same binding site adopts a conformation possessing on average a much smaller 28° twist between the right and left-hand subunits (Fig. 14). We suggest that the greater twist angle induced in the natural enantiomer bound conformation results in greater disruption of the vinylogous amide stabilization and preferentially accelerates its alkylation of duplex DNA. NMR experimental verification of the accuracy of the modeling studies are in progress through examination of the solution structure of the unnatural enantiomer adduct with d(GTCAATTAGTC)·(GACTAATTGAC) and the results should prove revealing.

Consistent with this proposal, the distinctions in the relative rates of DNA alkylation by the two enantiomers of simple derivatives (**4–15**) of the alkylation subunits diminish or nearly disappear. These smaller distinctions may accurately reflect the magnitude of the destabilizing C7 steric interactions affecting the unnatural enantiomers with the remainder of the distinctions with **1–3** attributable to the differences in

the extent of the DNA binding-induced conformational change.

Conclusions

Herein, we suggest that DNA binding leads to adoption of a bound conformation of CC-1065 and the duocarmycins that is inherently more reactive than the ground state conformation and that this occurs by inducing a twist in the linking N2 amide. For this to occur, it requires an extended rigid N2 amide substituent. The bound agent is forced to follow the inherent helical twist of the minor groove with the helical rise of the agent adjusted by a twist in the linking amide disrupting the vinylogous amide stabilization.

In addition, we suggest that the sequence-dependent conformational characteristics of DNA may influence the degree of this binding-induced increase in reactivity and effect the magnitude of catalysis. This induced conformational change may be expected to be a more significant characteristic of binding within an AT-rich versus GC-rich minor groove site and may even account for rate distinctions within AT-rich sites. The deeper agent penetration into the narrower AT-rich minor groove would be expected to force the adoption of a conformation containing a greater inherent twist that is adjusted at the site of the linking N2 amide. As such, our original description of the characteristics of the CC-1065 alkylation of duplex DNA as 'Accessible Hydrophobic Binding-Driven-Bonding' may be even more accurate today than when originally detailed (Fig. 15).[5,13] This was originally disclosed to describe preferential AT-rich minor groove binding derived from hydrophobic contacts with subsequent reversible alkylation at the sterically most accessible nucleophilic site (adenine N3) providing adducts stabilized by the dominant noncovalent binding contacts. With the assignment of the additional role of a binding-induced adoption of a reactive conformation (binding-induced ground state destabilization), the use of binding-driven-bonding to describe this process may prove even more accurate than originally recognized.

Acknowledgments

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50. This twist angle was established by measuring the dihedral angle formed by connecting C3—C3a—C2′—C3′.

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