# DNA Alkylation Properties of CC-1065 and Duocarmycin Analogs Incorporating the 2,3,10,10a-Tetrahydrocyclopropa[*d*]benzo[*f*]quinol-5-one Alkylation Subunit: Identification of Subtle Structural Features That Contribute to the Regioselectivity of the Adenine N3 Alkylation Reaction

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**Abstract:** The DNA alkylation properties of (−)- and *ent*-(+)-CBQ-TMI and (−)- and *ent*-(+)-*N*-BOC-CBQ, analogs of the duocarmycins incorporating the reactive 2,3,10,10a-tetrahydrocyclopropa[*d*]benzo[*f*]quinol-5-one (CBQ) alkylation subunit, are detailed. The agents were found to alkylate the same sites as the corresponding enantiomers incorporating the DSA, DA, CPI, CBI, or CI alkylation subunit. Consistent with their reactivity, they exhibited less selectivity among the available alkylation sites and alkylated DNA with a lower efficiency. Importantly, only adducts derived from adenine N3 addition to the least substituted cyclopropane carbon were detected under the relevant conditions of limiting agent. The adenine N3 adduct obtained by thermal depurination was isolated, quantitated, characterized, and shown to be derived from addition to the least substituted cyclopropane carbon of CBQ-TMI. Since the CBQ-based agents exhibit nonselective solvolysis regioselectivity with cleavage of both the normal external C9b−C10 and the abnormal internal C9b−C10a cyclopropane bonds, the observations suggest that the clean regiochemical course of the characteristic adenine N3 DNA alkylation reaction may benefit from not only the stereoelectronic control but additional features that complement the normally observed regioselectivity as well. These potentially include preferential adoption of binding orientations that favor the normal adenine N3 addition (proximity effects), and destabilizing torsional strain and steric interactions that accompany the abnormal adenine N3 nucleophilic addition, as well as a potential binding induced conformational change in CBQ which imposes a stereoelectronic preference for adenine N3 addition to the least substituted cyclopropane carbon.

(+)-CC-1065 (**1**)[1] and the duocarmycins **2**−**3**[2−6] represent the parent agents of a growing class of exceptionally potent antitumor antibiotics that derive their biological properties through a sequence selective alkylation of DNA.[7−17] The characteristic DNA alkylation reaction 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 sites in the minor groove.

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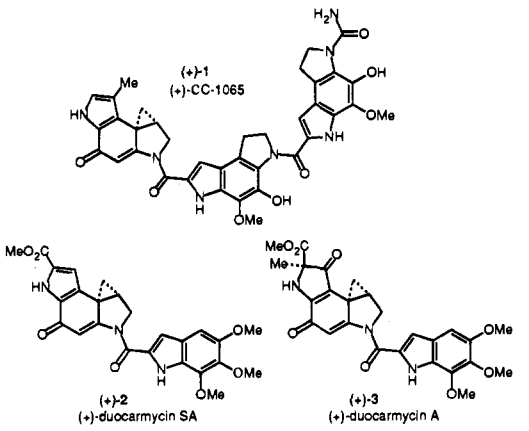
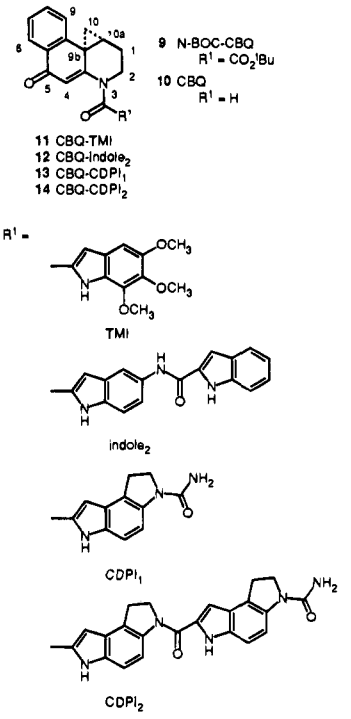



The examination of synthetic samples of the natural and unnatural enantiomers of the scarce natural products[12-14] and synthetic analogs[18-25] containing deep-seated structural changes has contributed significantly to the identification of the features of **1-3** responsible for polynucleotide recognition and functional reactivity. The AT-rich noncovalent binding selectivity of the agents[26] and their steric accessibility to the alkylation site that accompanies deep penetration into the narrow AT-rich minor groove have been shown to control the sequence selectivity.[27] In addition, the electrophilic cyclopropane was found not to be obligatory for observation of the characteristic alkylation selectivity, and alternative electrophiles incorporated into structurally related agents have been found to act similarly.[19] The studies have revealed a direct relationship between solvolysis stability and cytotoxic potency,[20] the structural origin of the distinguishing behavior of the natural and unnatural enantiomers,[12,14,20] the extensive noncovalent binding stabilization of the inherently reversible DNA alkylation reaction,[13,14] and the readily distinguishable alkylation selectivities of **1-3** versus **4-8**,[12,14,19,20] and have led to the introduction of alkylation site models that accommodate the reversed binding orientations and offset AT-rich selectivity of the natural and unnatural enantiomer DNA alkylation reactions.[12,14] Notably, the models offer a beautiful explanation for the unusual observation that the natural and unnatural enantiomers of the simple BOC derivatives of **4-8** alkylate the same sites within DNA (5′-AA > 5′-TA) independent of their absolute configuration.[12,14]

We recently disclosed the preparation and preliminary evaluation of CC-1065 and duocarmycin analogs incorporating the 2,3,10,10a-tetrahydrocyclopropa[*d*]benzo[*f*]quinol-5-one (CBQ) alkylation subunit including **9-11** and the seco precursors to **12-14** which contain the deep-seated structural change of a ring-expanded fused six-membered ring.[24,25] Contrary to expectations, the CBQ-based agents exhibited exceptional solvolysis reactivity. *N*-BOC-CBQ (**9**) was found to be 63× more

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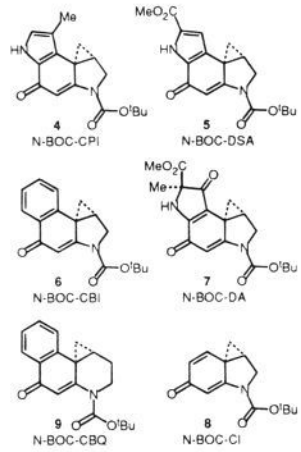


| | k ($s^{-1}$, pH 3) | $t_{1/2}$ | $IC_{50}$ (L1210) |
|---|---|---|---|
| 5 | $1.1 \times 10^{-6}$ | 177 h | 6 nM |
| 6 | $1.5 \times 10^{-6}$ | 133 h | 80 nM |
| 4 | $5.3 \times 10^{-6}$ | 37 h | 330 nM |
| 7 | $1.7 \times 10^{-5}$ | 11 h | 1000 nM |
| 9 | $9.1 \times 10^{-5}$ | 2 h | 4000 nM |
| 8 | $2.0 \times 10^{-2}$ | 0.01 h | 18000 nM |

**Figure 1**

reactive than *N*-BOC-CBI (**6**), its closest analog (Figure 1). In addition, the acid-catalyzed solvolysis was found to proceed nonselectively with cleavage of both the external C9b−C10 and internal C9b−C10a cyclopropane bonds. The latter was shown to occur with exclusive inversion of stereochemistry, illustrating that the solvolysis and alkylation reactions proceed by $S_N2$ versus $S_N1$ cyclopropane ring opening. Both the increased reactivity and the loss of stereoelectronic control for the cyclopropane ring opening were shown to be attributed to the idealized conjugation and alignment of the CBQ cyclopropane with the cyclohexadienone $\pi$-system. The careful X-ray structural comparisons of **9** with **6** revealed that it was not the rapid or mixed solvolysis regioselectivity of the CBQ-based agents that was unusual, but rather that the unexpected observations are the surprising stability of **1**−**8** and related agents. The unusual stability and solvolysis regioselectivity are imposed by fusion of the activated cyclopropane to the five-membered ring which constrains it to a nonideal alignment and overlap with the cyclohexadienone $\pi$-system.[24,25]

Herein, we detail a study of the DNA alkylation properties of both enantiomers of the CBQ-based agents which are consistent with their chemical properties. The agents were found to alkylate the same sites as the corresponding agents containing the DSA, CPI, CBI, or CI alkylation subunit. Consistent with their relative reactivity, they exhibited less selectivity among the available alkylation sites and alkylated DNA with a lower efficiency. Importantly, only DNA adducts derived from adenine N3 addition to the least substituted cyclopropane carbon were detected under the relevant conditions of limiting agent. These observations suggest that the regioselectivity of the DNA alkylation reaction may benefit from not only stereoelectronic control but additional features that complement the normally observed regioselectivity as well. The potential origin of these effects is discussed.

## Results

**DNA Alkylation Selectivity and Efficiency.** The DNA alkylation properties of the agents were examined within two 150 base pair segments of duplex DNA for which comparative results are available for related agents. Two clones of phage M13mp10 were selected for study and contain the SV40 nucleosomal DNA inserts w794 (nucleotide nos. 5238−138) and its complement w836 (nucleotide nos. 5189−91).[28] The alkylation site identification and the assessment of the relative selectivity among the available sites were obtained by thermally-induced strand cleavage of the singly 5′-end-labeled duplex DNA after exposure to the agents. The full details of this procedure have been disclosed and discussed elsewhere.[14,28] The DNA alkylation reaction selectivities observed under the incubation conditions of 25 °C (24 h) for the agents detailed herein have proven identical to the alkylation selectivities observed with shorter or extended reaction periods or when the reactions were conducted at different temperatures (37 or 4 °C, 0.5−7 d). As discussed below, the relative efficiencies of DNA alkylation were altered by changing the reaction temperatures.

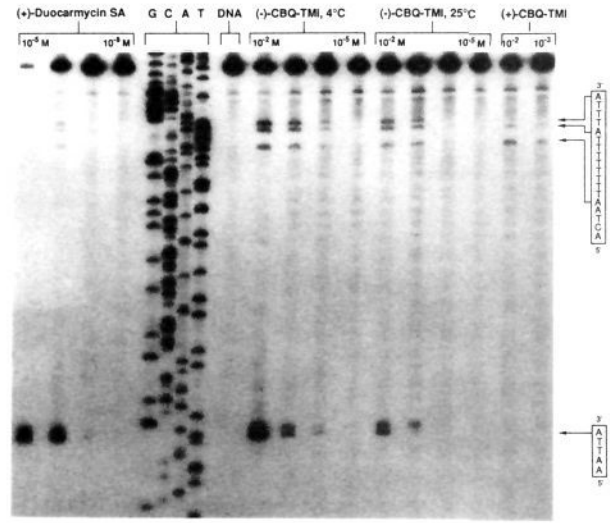


**Figure 2.** Thermally-induced strand cleavage of w794 DNA (SV40 DNA segment, 144 bp, nucleotide nos. 138−5238): DNA-agent incubation for 24 h, removal of unbound agent and 30 min of thermolysis (100 °C), followed by denaturing 8% PAGE and autoradiography; lanes 1−4, (+)-duocarmycin SA ($1 \times 10^{-5}$ to $1 \times 10^{-8}$ M, 25 °C); lanes 5−8, Sanger G, C, A, and T reactions; lane 9, control DNA; lanes 10−13, natural (−)-CBQ-TMI ($1 \times 10^{-2}$ to $1 \times 10^{-5}$ M, 4 °C); lanes 14−17, natural (−)-CBQ-TMI ($1 \times 10^{-2}$ to $1 \times 10^{-5}$ M, 25 °C); lanes 18−19, *ent*-(+)-CBQ-TMI ($1 \times 10^{-2}$ and $1 \times 10^{-3}$ M, 25 °C).

**DNA Alkylation Properties of (−)- and *ent*-(+)-CBQ-TMI.** The comparison of the DNA alkylation by (−)- and *ent*-(+)-CBQ-TMI (**11**) with (+)- and *ent*-(−)-duocarmycin SA (**2**) within w794 DNA is illustrated in Figure 2 and is representative of the full set of comparisons that have been made with **1**−**3** versus **11**−**14**. Table 1 summarizes the consensus alkylation sequences established in our examination of both enantiomers of **11**.

The natural enantiomer of CBQ-TMI alkylated the same sites as (+)-duocarmycin SA but did so with less selectivity among the available sites. Within w794, it alkylated the same major 5′-ATTA site as (+)-duocarmycin SA, but it also alkylated the three minor sites of 5′-CTAA, 5′-TTTA, and 5′-TTTA more prominently. In addition, the (+)-duocarmycin SA DNA alkylation was detectable at $10^{-6}$−$10^{-7}$ M while that of the natural enantiomer of CBQ-TMI was detected at $10^{-4}$ M (4 °C, 24 h). Thus, the natural enantiomer of CBQ-TMI is 100−1000× less efficient than (+)-duocarmycin SA in the adenine N3 alkylation. The efficiency of DNA alkylation by the natural enantiomer of CBQ-TMI increased as the reaction temperature decreased (4 °C versus 25 °C, Figure 2) and may be attributed

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**Table 1.** Consensus Alkylation Sequences Established for (−) and *ent*-(+)-CBQ-TMI[a]

| agent | base | 5′ | 4 | 3 | 2 | 1 | 0 | −1 | −2 | −3 | 3′ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (−)-CBQ-TMI | A/T (56)[b] | | 58 | 79 | 98 | 100 | 10 | 69 | 57 | | |
| | consensus | | N | A/T > G/C | A/T | A/T | A | Pu | N | | |
| *ent*-(+)-CBQ-TMI | A/T (56)[b] | | | | | 93 | 100 | 96 | 73 | 56 | |
| | consensus | | | | | A/T | A | A/T | A/T > G/C | N | |

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

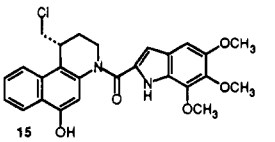
to the nonproductive competitive solvolysis of **11** at 25 °C. Similar observations have been made with the reactive agents (+)-duocarmycin A (**3**)[13,14] and CI-TMI[19] but are not observed with the more stable agents including (+)-duocarmycin SA (**2**). Importantly, no new alkylation sites unique to CBQ-TMI were observed, and no guanine N3 alkylation was detected under these conditions of limiting agent. Only alkylation events attributable to adenine N3 addition to the least substituted carbon of the activated cyclopropane were detected, and no alternative alkylation sites due to ring expansion adenine N3 alkylation were observed.

Similarly, the unnatural enantiomer of CBQ-TMI (Figure 2) was found to alkylate the same sites as *ent*-(−)-duocarmycin SA. For example, it alkylated the same high-affinity 5′-AATT site observed with *ent*-(−)-duocarmycin SA within w794 DNA (Figure 7, supporting information). In addition, the *ent*-(−)-duocarmycin SA alkylation was observed at $10^{-6}$ M (Figure 7, supporting information) while that of the unnatural enantiomer of CBQ-TMI was detected at $10^{-2}$ to $10^{-3}$ M (25 °C, 24 h). Consequently, the unnatural enantiomer of CBQ-TMI is 1000× less efficient than *ent*-(−)-duocarmycin SA and 10× less efficient than the corresponding natural enantiomer of CBQ-TMI. Consistent with past observations, the rate of DNA alkylation by the unnatural enantiomer is qualitatively slower than that of the natural enantiomer, and this may account for the distinctions observed in Figure 2.

Each of the alkylation sites proved to be adenine, and no minor guanine N3 alkylation was observed for CBQ-TMI under the conditions of these assays. Like observations made with **2** and **3**,[13,14] each adenine N3 alkylation site for the natural enantiomer proved to be flanked by two 5′-A or -T bases, and there was a preference for the three base sequence that follows the order of 5′-AAA > 5′-TTA > 5′-TAA > 5′-ATA (Table 1). There was also a preference for the fourth 5′ base to be A or T, and this preference distinguishes the high-versus low-affinity alkylation sites.

The unnatural enantiomer alkylation proved consistent with the model established for *ent*-(−)-duocarmycin SA (**2**) with 5′ adenine alkylation, agent binding in the minor groove in the reverse 5′ → 3′ direction from the alkylation site covering 3.5 base pairs across an AT-rich region. Like observations made with *ent*-(−)-**2**,[14] all alkylation sites detected for *ent*-(+)-**11** proved to be adenine, and nearly all the 3′ and 5′ flanking bases were A or T (Table 1). There was a preference for this three base sequence that follows the order of 5′-AAA > 5′-AAT > 5′-TAA > 5′-TAT. There was also a weaker preference for the second 3′ base to be A or T. This reversed binding orientation of the unnatural enantiomer is required to permit adenine N3 addition to the least substituted cyclopropane carbon, and the offset 3.5 base pair AT-rich binding sites relative to the alkylation site is a natural consequence of the diastereomeric relationship of the adducts. Thus, the unnatural enantiomer CBQ-TMI alkylation occurs with a binding orientation that extends in the reverse 5′ → 3′ direction across a 3.5 base pair AT-rich region (*i.e.*, 5′-AAAA) and is offset from that observed with the natural enantiomer (3′ → 5′, *i.e.*, 5′-AAAA).

Both enantiomers of **15** were found to exhibit the same DNA alkylation selectivity and efficiency as the corresponding enantiomer of **11**, and no distinguishing features were noted.




The only difference detected was that **15** did not show the inverted temperature dependence on the efficiency of DNA alkylation. Rather, the extent of DNA alkylation at 4 or 25 °C (48 or 24 h) was comparable to that exhibited by **11** at 4 °C, and reaction at 25 °C was slightly more rapid. This is especially interesting since **15** does not close to the corresponding cyclopropane-containing agent nearly as readily as the preceding agents studied. Therefore, these observations may represent either an unusually facile closure under the conditions of assay or direct alkylation of DNA by **15** itself but with a selectivity and efficiency not distinguishable from the agent containing the preformed cyclopropane.

One useful way of analyzing the behavior of the CBQ-based agents is to relate the normal and (*i.e.*, **16**) and abnormal (*i.e.*, **17**) adenine N3 alkylations to the natural and unnatural enantiomer duocarmycin SA DNA alkylation reactions. For the natural enantiomer of CBQ-TMI, the normal adenine N3 addition to the least substituted cyclopropane would provide DNA alkylation sites analogous to those observed with the natural enantiomers of duocarmycin SA or A. The abnormal adenine N3 addition to the most substituted cyclopropane carbon with ring expansion must occur on the opposite face of the alkylation subunit chromophore and is analogous to alkylation by the unnatural enantiomer of duocarmycin SA. To the first approximation, one might expect that all alkylation sites observed in the evaluation of the natural enantiomer of **11** that coincide with alkylation sites of the natural enantiomer of duocarmycin SA and A may be attributed to the normal adenine N3 alkylation regioselectivity. Any additional unique sites and especially those unique sites which represent an unnatural enantiomer duocarmycin SA or A alkylation site would constitute candidate abnormal adenine N3 additions proceeding with ring expansion. No such sites were detected in our evaluations. Similarly, any unique duocarmycin SA or A natural enantiomer sites observed in the examination of the unnatural enantiomer of CBQ-TMI would constitute candidate abnormal adenine N3 alkylations proceeding with ring expansion, and no such sites have been detected to date. Some sites constitute alkylation sites for both the natural and unnatural enantiomers. Within w794 DNA this includes the minor natural enantiomer site 5′-CTAA which is the major unnatural enantiomer alkylation site 5′-AATT. As described elsewhere in detail,[14] the natural and unnatural enantiomers adopt reversed and offset bound orientations at such sites covering the 3.5 base pairs indicated. At

such sites established in the examination of **2** and **3**, the distinctions between normal and abnormal adenine N3 addition of **11** could not be made, but their appearance can be expected and explained simply by the normal adenine N3 addition reaction.

**DNA Alkylation Properties of (−)- and *ent*-(+)-*N*-BOC-CBQ.** A similar comparison of the DNA alkylation properties of both enantiomers of *N*-BOC-CBQ (**9**) within w794 and w836 DNA revealed that the agents alkylated the same sites (5′-AA > 5′-TA) and that they coincide with the alkylation sites observed with both enantiomers of **4**–**8**. For the natural enantiomer, this is the result of 3′ adenine N3 alkylation with addition to the least substituted cyclopropane carbon and agent binding in the 3′ → 5′ direction from the site of alkylation across the adjacent 5′ base. For the unnatural enantiomer, this similarly requires adenine N3 alkylation with addition to the least substituted cyclopropane carbon but with the reversed 5′ → 3′ binding orientation required for adenine N3 access to the activated cyclopropane. However, as a consequence of the diastereomeric relationship of the adducts, the binding of the unnatural enantiomer covers the same adjacent 5′ base as the natural enantiomer. Consistent with their relative reactivity, they were substantially less efficient than **4**–**7** and the detection of their DNA alkylation was more limited. Like observations made with **4, 6**, and **7**,[12,13,20] the natural enantiomer of **9** was approximately 5× more effective than the unnatural enantiomer. This is distinct from the observations made with **5** and **8**, where the two enantiomers were indistinguishable (**8**) or comparable (**5**) in their relative efficiencies of DNA alkylation.[14,19] Consistent with past proposals,[13,14] this may be attributed to the destabilizing steric interactions surrounding the CBQ C9 center which are most significant with the unnatural enantiomer adducts and which are reduced or removed with the agents **5** and **8**.

**Quantitation of the Adenine N3 Alkylation and Characterization of the Adenine N3 Adduct 16.** The studies detailed before illustrate that both enantiomers of **11** alkylate the same sites as the corresponding enantiomers of duocarmycin SA but do so with less selectivity among the available sites and with a lower efficiency than **2** and **3**. Importantly, no new alkylation sites were detected. All the alkylation and subsequent thermal cleavage sites detected to date within end-labeled restriction fragments on sequencing gels were found to occur at adenine. In this protocol, high-affinity and minor alkylation sites that lead to thermal cleavage are detected while trace alkylation events are not detectable due to competing multiple alkylations that lead to production of only short segments of DNA. In addition, the thermal cleavage of DNA following alkylation only permits the detection of adducts susceptible to thermal glycosidic bond cleavage (adenine N3 or N7, guanine N3 or N7, cytosine O2), and alternative alkylation events involving additional nucleophilic sites in the minor groove (guanine C2 amine, thymine C2 carbonyl) may not be detected. Consequently, the quantitation of the adenine N3 alkylation reaction was conducted with the intention of determining whether the lower efficiency of DNA alkylation could be attributed to alternative undetected DNA alkylation reactions. This was addressed in a study of the alkylation of calf thymus DNA. For this purpose, the long-wavelength UV absorption of the agent and the adduct outside the UV absorption range of DNA provided a useful quantitative measure of the amount of adenine alkylation through UV quantitation of DNA bound agent, quantitation of the thermally released adduct, and independent quantitation of recovered **11** or its solvolysis products.

Thus, extraction (EtOAc) of aqueous buffer solutions containing calf thymus DNA following alkylation with **11** (25 °C, 5 d, 150 bp equiv) afforded 20% of a mixture of the solvolysis products **18** and **19** (UV quantitation, HPLC identification) from the EtOAc supernatant. Thermal treatment of the alkylated DNA in aqueous 10 mM sodium phosphate buffer (100 °C, 30 min, pH 7.4) followed by EtOAc extraction provided crude **16** in an exceptionally clean reaction (Scheme 1). The only UV active material detected in the organic extract was **16** (60% yield) and a small amount of additional solvolysis products (7–10%) derived from **11**. UV assay of the remaining aqueous DNA solution indicated <5% of the CBQ-TMI chromophore remained attached to DNA. Extending the reaction time did not alter these results, but the use of shorter reaction times, lower temperatures, and lower DNA base pair equivalents led to greater amounts of recovered **11** or its solvolysis products (Table 2). Unambiguous confirmation of the structure of **16** was obtained upon isolation and $^1$H NMR characterization which established clean adenine N3 addition to the least substituted carbon of the activated cyclopropane.

**Scheme 1**

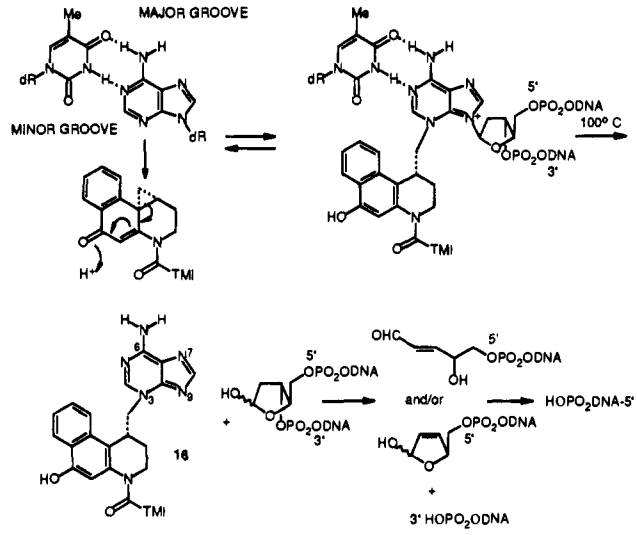


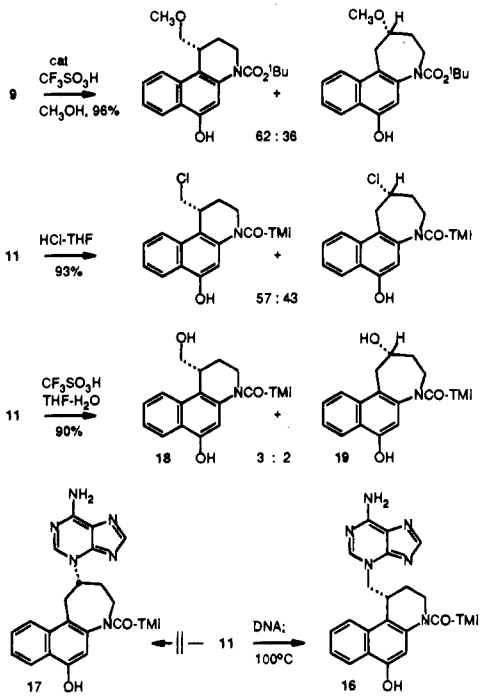

**Table 2.** Calf Thymus Alkylation

| conditions | bp equiv | yield of solvolysis products (1st)[b] (%) | yield of **16** (%) | yield of solvolysis products (2nd)[b] (%) | total yield |
|---|---|---|---|---|---|
| 5 d, 25 °C | 44 | 30 | 50 | 10 | 90 |
| 5 d, 4 °C | 150 | 25 | 65 | 7 | 97 |
| 5 d, 25 °C | 150 | 20 | 60 | 10 | 90[a] |
| 10 d, 25 °C | 150 | <20[c] | 60 | 10 | |

[a] <5% of the CBQ-TMI chromophore remained with DNA. [b] Solvolysis products **18** and **19** isolated prior to (1st) and after (2nd) thermolysis. [c] Multiple minor products detected in the first organic extract, but the major components were the solvolysis products **18** and **19**.

These results indicate that little or no CBQ-TMI alkylates DNA to provide nonthermally labile adducts and that the adenine N3 alkylation with addition to the least substituted cyclopropane carbon accounts for >75% of the consumption of agent in the presence of DNA, with solvolysis accounting for the majority of the remainder. Moreover, no significant amount of a guanine N3 adduct or the alternative adenine N3 adduct **17** was detected under these conditions. The solvolysis products detected following the thermal reaction presumably are derived from unrecovered **11**, **18**, and **19** or reversal of a DNA alkylation reaction and subsequent solvolysis of the regenerated CBQ-TMI. The nature of the DNA adduct that might lead to the regeneration of the small amount of CBQ-TMI was not established but could include either nonthermally labile adducts

**Scheme 2**




incapable of depurination or the observed adenine N3 adduct leading to **16** itself.[13,14]

In comparison, the (+)-duocarmycin SA alkylation proceeds to completion (0% recovered **2**) and provides the single adenine N3 adduct corresponding to **16** in >95% yield (UV and quantitated isolation) under even milder conditions (25 °C, 24 h, 70 bp equiv).[14] Thus, the natural (−)-CBQ-TMI DNA alkylation reaction is considerably less efficient even though the agent itself is considerably more reactive.

The comparisons detailed above were made under the conditions of limiting agent where both duocarmycin SA and A provide predominantly or exclusively adenine N3 adducts. Like observations made with duocarmycin A, additional reactions of the reactive CBQ-based agents including guanine N3 alkylation might be expected to be observed under more vigorous conditions or when employing limiting amounts of DNA and excess agent.[29−31] These less relevant studies were not pursued.

## Discussion

In agreement with models of the origin of the DNA alkylation selectivity,[13,14] the reactive CBQ-based agents were found to alkylate the same sites as the corresponding DSA-, CPI-, DA-, CBI-, and CI-based agents. Consistent with the relative reactivity of the agents,[25] the distinguishing behavior of CBQ-based agents was that they alkylated DNA with a lower efficiency (100−1000×) and with less selectivity among the available alkylation sites. This is consistent with the observation that the agents were also 100−1000× less potent in cytotoxic assays[25] and follow a well-defined relationship between chemical stability and biological potency. However, unlike their chemical solvolysis behavior where nonselective cleavage of both the external C9b−C10 and internal C9b−C10a cyclopropane bonds is observed (Scheme 2),[25] only DNA alkylation derived from

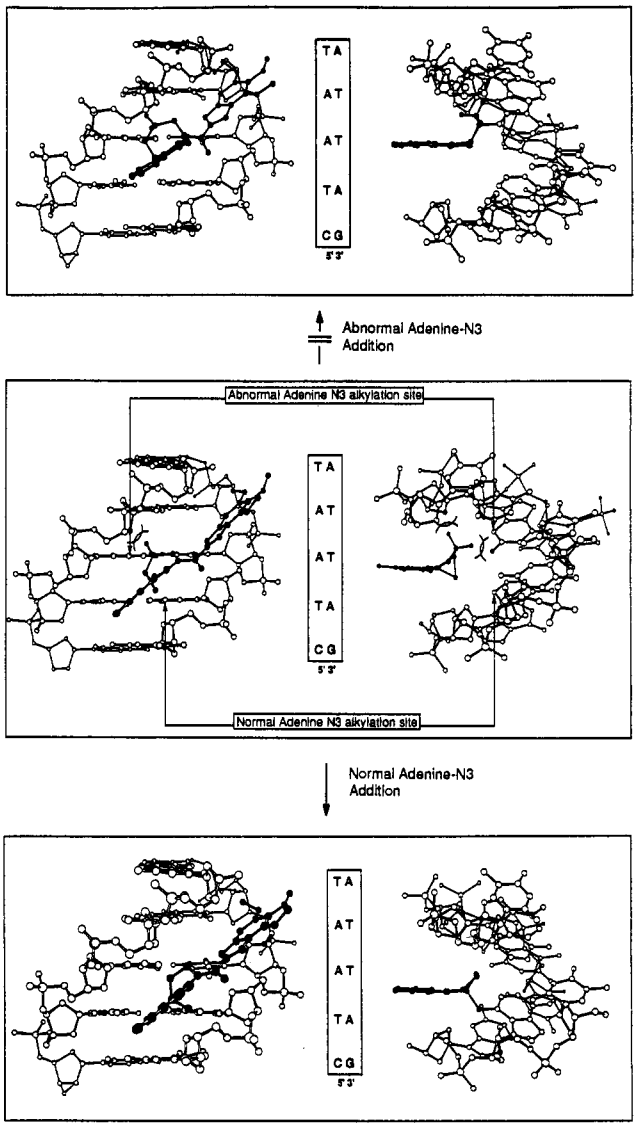



**Figure 3.** Front and groove views of models of the normal and atypical adenine N3 alkylation by the natural enantiomer of CBQ-TMI within the w794 high-affinity alkylation site. The unobserved atypical alkylation suffers destabilizing steric interactions surrounding the CBQ C1-$H_2$ position. For clarity the trimethoxyindole subunit has been removed in the groove views.

adenine N3 addition to the unsubstituted cyclopropane carbon with cleavage of the external C9b−C10 bond was detected.

At least three potential explanations can be advanced to account for the observations. First, the observed regioselectivity may be a consequence of a selectively accessible reaction in which minor groove binding of the agent occurs such that the adenine N3 nucleophilic site is preferentially positioned to attack the least hindered cyclopropane carbon. Thus, the noncovalent binding of the agent may either enhance addition to the observed site or potentially exclude addition to the more substituted cyclopropane carbon. The Figure 3 and 4 models illustrating the natural and unnatural enantiomer alkylations provide such insights into the potential origin of the observations. For the natural enantiomer, three significant destabilizing steric interactions disfavor the abnormal adenine N3 addition (Figure 3). The axial C1-$H_\beta$ adjacent to the more substituted cyclopropane carbon is placed proximal to the 3′ deoxyribose oxygen (1.56 Å) on the abnormal alkylated strand, and the resulting destabilizing steric interaction is sufficient to prevent the atypical addition to the more substituted cyclopropane carbon. In addition, the alkylated adenine C2-H of the abnormal addition

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(31) Yamamoto, K.; Sugiyama, H.; Kawanishi, S. *Biochemistry* **1993**, *32*, 1059.

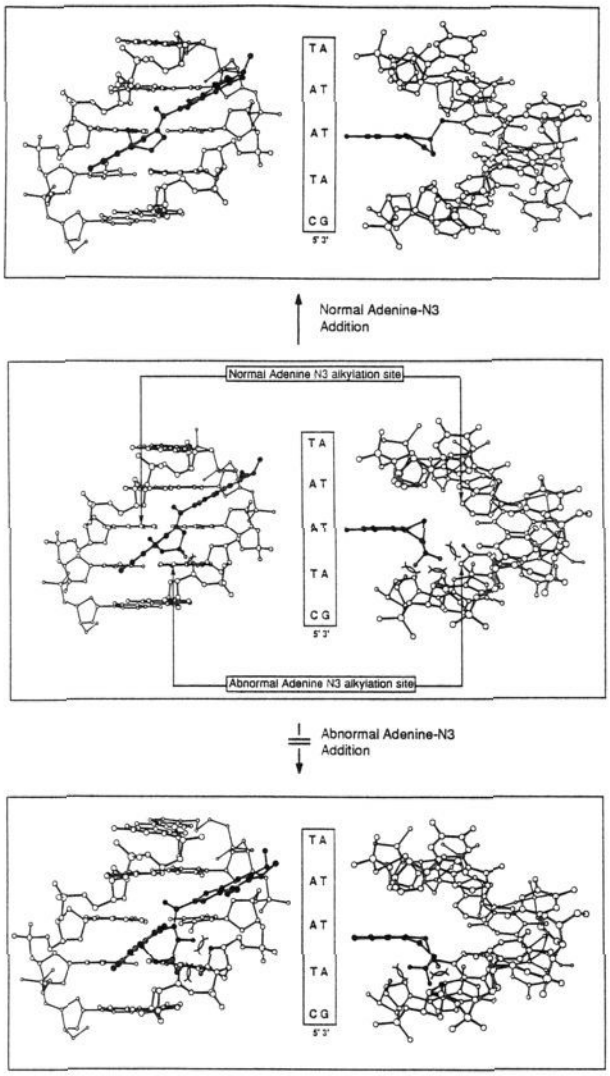


**Figure 4.** Front and groove views of models of the normal and atypical adenine N3 alkylation by the unnatural enantiomer of CBQ-TMI. The unobserved atypical alkylation suffers destabilizing steric interactions surrounding the CBQ C1-$H_2$ position. For clarity the trimethoxyindole subunit has been removed in the groove views.

suffers a destabilizing steric interaction with the C1-$H_\alpha$ (1.64 Å), and this is discussed in more detail below. Finally, like the distinctions between the natural and unnatural enantiomers of **2**, the atypical adenine N3 alkylation is destabilized by steric interactions surrounding the C9 center and the adjacent 5′ base which is located on the alkylated strand. In Figure 3, the 5′ thymine C2 carbonyl is located proximal to the C9-H. In contrast, the C9 center of the normal adduct as well as C1-$H_\beta$ does not suffer comparable destabilizing steric interactions and benefits from its more distal placement from the complementary strand containing the interacting sites, and the C1-$H_\alpha$ no longer suffers the eclipsing interaction.

Similarly the atypical adenine N3 addition for the unnatural enantiomer suffers substantial destabilizing steric interactions between C1-$H_2$ and the alkylated strand deoxyribose backbone as well as between the alkylated adenine C2-H and the CBQ C1−$H_\alpha$ (Figure 4). Importantly, the destabilizing steric interactions between C9 and the alkylated strand 5′ base can be expected to destabilize the normal adduct formation and favor the atypical adenine N3 addition. The fact that the atypical adducts are not observed suggests that the steric effects surrounding the C1-$H_2$ rather than C9 may be predominating.

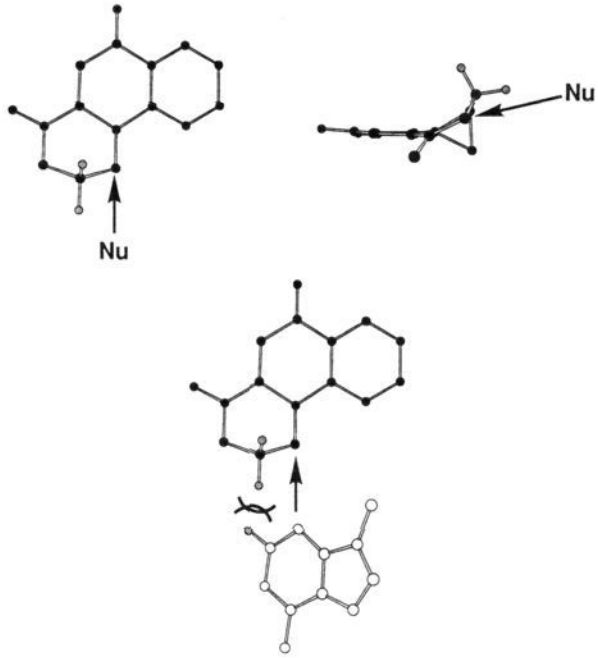


**Figure 5.** Top and side views of the atypical nucleophilic addition to the most substituted cyclopropane carbon of CBQ: top, trajectory approach of a nucleophile that illustrates the nucleophile/C1-$H_\alpha$ eclipsing interaction and resulting torsional strain; bottom, adenine N3 addition taken from the central panel of Figure 3 illustrating the approximate location and relative orientation of the reactants when constrained in the minor groove highlighting the additional adenine C2-H/C1-$H_\alpha$ destabilizing steric interaction. The trimethoxyindole subunit has been removed for clarity.

Agents **1**−**8** and their related analogs lack this C1-$H_2$ methylene group, and the fused five-membered ring adopts a more planar conformation. Consequently, the destabilizing C1-$H_2$ steric interactions are removed with these agents.

Perhaps more importantly, the nucleophile undergoing $S_N2$ addition[25] to the more substituted cyclopropane carbon of CBQ suffers an eclipsing interaction with the adjacent C1-$H_\alpha$ which occupies an equatorial position on the boat conformation of the six-membered ring. Notably, this is analogous to the torsional strain commonly accepted as the structural origin of the Felkin−Ahn model for nucleophilic addition to electrophilic $\pi$-systems. This destabilizing torsional strain which was first suggested to contribute to the subtle differences in the solvolysis regioselectivity of **9**[25] is absent in the addition to the least substituted cyclopropane carbon and may be especially significant in the case of the adenine N3 addition. The larger size of the nucleophile and the parallel versus orthoganol approach of adenine when constrained in the minor groove exaggerates the inherent torsional strain through introduction of an additional significant destabilizing steric interaction between adenine C2-H and C1-$H_\alpha$ (Figure 5). This would be expected to decelerate the abnormal adenine N3 addition reaction relative to the normal alkylation. Importantly, this feature would also favor the normal adenine N3 addition for **1**−**8** and can be expected to further reinforce their inherent stereoelectronic preference for reaction at the least substituted cyclopropane carbon. Consequently, this may also account for the single regiospecific adenine addition to duocarmycin A and CC-1065 which similarly undergo solvolysis to provide minor amounts of the abnormal ring-expanded products. To date, efforts to promote the addition of adenine or related heteroaromatic nucleophiles to such agents have not been successful. Consequently, it is not presently possible to distinguish whether such observations are unique

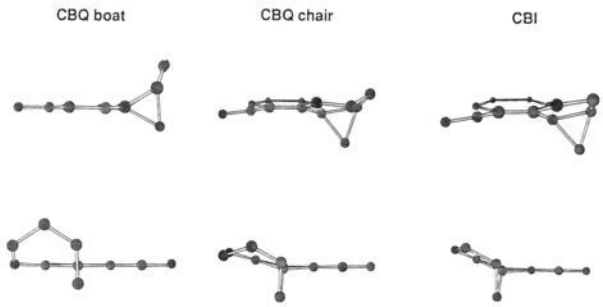


**Figure 6.** Side and rear views of the CBQ boat (left, X-ray[25]), CBQ half-chair (middle), and CBI (**6**; right, X-ray[20]) conformations illustrating the relative orientations of the cyclopropane. Like CBI, the higher energy half-chair conformation of CBQ ($\Delta E$ = 1.0−1.5 kcal/mol) would impose stereoelectronic control dictating addition of the least substituted cyclopropane carbon. Hydrogen atoms have been removed for clarity.

to the DNA alkylation reaction and its imposed relative adenine orientations or generalizable to even a standard solution phase reaction.

Finally, a conformational change of the CBQ alkylation subunit may precede DNA alkylation, and the reactive conformation may structurally impose the stereoelectronic preference for addition to the least substituted cyclopropane carbon. Consistent with this possibility, a conformational search of *N*-acetyl-CBQ and CBQ revealed two accessible low-energy conformations (≤5 kcal/mol). The lowest energy conformation was found to be identical to the X-ray crystal structure[25] of *N*-BOC-CBQ. It possesses a boat conformation for the fused six-membered ring with the cyclopropane perfectly conjugated[32] and aligned with the $\pi$-system of the cyclohexadienone. The plane of the cyclohexadienone perfectly bisects the cyclopropane, and both the C9b−C10 and C9b−C10a bonds are equally aligned for cleavage consistent with the experimental solvolysis results (Figure 6).[33] A second conformation was located that was 1.0−1.5 kcal/mol higher in energy [$\Delta E$ = 1.0 (MM2), 1.5 (AMBER), 1.0 (AM1) kcal/mol] and thus constitutes an accessible and potentially more reactive conformation. The fused six-membered ring adopts a half-chair conformation, and the relative orientation of the cyclopropane closely approximates that found in **4**−**8**. While it remains perfectly aligned with the $\pi$-system of the cyclohexadienone,[34] only the C9b−C10 bond is stereoelectronically positioned for cleavage. In this conformation, the bent orbitals[35] of the C9b−C10 bond are perpendicular to the plane of the cyclohexadienone and overlap nicely with the developing $\pi$-system of the product phenol. In contrast, the C9b−C10a bond lies nearly in the plane of the cyclohexadienone and its orbitals are orthogonal to the developing $\pi$-system of the product phenol. DNA alkylation through this more reactive but less accessible conformation could account for the exclusive adenine N3 addition to the least substituted cyclopropane carbon under the constraints of stereoelectronic control.

## Conclusions

The CBQ-based agents were found to alkylate DNA with the same selectivity as the corresponding enantiomers of the DSA-, DA-, CPI-, or CI-based agents. Consistent with their reactivity, CBQ-TMI exhibited less selectivity among the available sites and alkylated DNA with a lower efficiency than duocarmycin SA or A. Importantly, only adducts derived from adenine N3 addition to the least substituted cyclopropane carbon were detected under the relevant conditions of limiting agent. Since the CBQ-based agents exhibit nonselective solvolysis regioselectivity, the observations suggest that the clean regiochemical course of the characteristic adenine N3 alkylation may benefit from additional features that complement the normally imposed stereoelectronic control. Prominent among such features is the destabilizing torsional strain and steric interactions illustrated in Figure 5 that would accompany the abnormal adenine N3 nucleophilic addition.

## Experimental Section

**DNA Alkylation Studies: Selectivity and Efficiency.** Eppendorf tubes containing singly $^{32}$P 5′-end-labeled double-stranded DNA[28] (9 μL) in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) were treated with the agents in DMSO (1 μL, at the specified concentrations). The solutions were mixed by vortexing and brief centrifugation and subsequently incubated at 25, 37, or 4 °C for 24−48 h. The covalently modified DNA was separated from unbound agent by EtOH precipitation of the DNA. The EtOH precipitations were carried out by adding tRNA as a carrier (1 μL, 10 μg/μL), a buffer solution containing salt (0.1 volume, 3 M NaOAc in TE), and −20 °C EtOH (2.5 volumes). The solutions were mixed and chilled at −78 °C in a REVCO freezer for 1 h or longer. The DNA was reduced to a pellet by centrifugation at 4 °C for 15 min, washed with −20 °C 70% EtOH (in TE containing 0.2 M NaCl), and recentrifuged briefly. The pellets were dried in a Savant Speed Vac concentrator and resuspended in TE buffer (10 μL). The solutions of alkylated DNA were warmed at 100 °C for 30 min to induce cleavage at the adenine N3 alkylation sites. After brief centrifugation, formamide dye solution (5 μL) was added. Prior to electrophoresis, the samples were denatured by warming at 100 °C for 5 min, placed in an ice bath, and centrifuged briefly, and the supernatant (2.8 μL) was loaded onto a gel. Sanger dideoxynucleotide sequencing reactions[36] were run as standards adjacent to the agent-treated DNA reaction samples. Polyacrylamide gel electrophoresis (PAGE) was run on an 8% sequencing gel under denaturing conditions (19:1 acrylamide/*N*,*N*′-methylenebisacrylamide, 8 M urea) in TBE buffer (100 mM Tris, 100 mM boric acid, 0.2 mM $Na_2EDTA$). PAGE was prerun for 30 min with formamide dye solution prior to loading the samples. Autoradiography of dried gels was carried out at −78 °C using Kodak X-Omat AR film and a Picker Spectra intensifying screen.

**DNA Alkylation Reaction: Quantitation and Characterization of the Adenine N3 Adduct 16.** A solution of calf thymus DNA (20 mg, 150 bp equiv) in 10 mM sodium phosphate buffer (pH 7.4, 1.25 mL) was treated with a solution of natural (−)-CBQ-TMI[25] (100 μg) in DMSO (50 μL), and the mixture was stirred slowly for 5 d at 25 °C in the dark. The solution was extracted with EtOAc (3 × 0.2 mL), and the extracts were monitored by UV to assure that the last extract contained no UV active material. The organic layers were combined, the solvent was evaporated, and the residue was dissolved in $CH_3OH$ (1.0 mL). Quantitative UV assay and subsequent HPLC analysis of the solution (Vydac C18, 0.46 × 25 cm analytical column, 0.8 mL/min, 25−50% $CH_3CN/H_2O$, 15 min gradient elution) showed mainly the solvolysis products **18** and **19**[37] derived from (−)-CBQ-TMI (85−90% purity, $t_R$ = 19.45 (**18**), 20.24 (**19**), and 21.8 (**11**) min) corresponding to 20% recovery of the material.

The aqueous solution was further extracted with BuOH (200 μL) and phenol (200 μL), and then warmed for 30 min at 100 °C. The resulting solution was cooled to 25 °C and extracted with EtOAc (3 × 200 μL). The organic extracts were combined and concentrated, and the residue was dissolved in $CH_3OH$ (1.0 mL). Quantitative UV assay and subsequent HPLC analysis (Vydac C18, 0.46 × 25 cm analytical column, 25−50% $CH_3CN/H_2O$, gradient elution) revealed a mixture containing two components (6:1): the adenine N3 adduct **16** ($t_R$ = 24.37

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min) isolated in 60% yield and 10% *seco*-CBQ-TMI (**18** and **19**) constituting the solvolysis products. The total recovery of material was 90%. Data for **16**: $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.11−8.09 (overlapping d and s, 2H, adenine C2-H and C7-H), 7.84−7.82 (m, 1H, adenine C8-H), 7.37−7.35 (m, 1H, C10-H), 7.28 (t, 1H, $J$ = 8 Hz, C9-H), 7.15 (br t, 1H, $J$ = 8 Hz, C8-H), 6.86 (s, 1H, C9′-H), 6.70 (s, 1H, C3′-H), 6.52 (s, 1H, C5-H), 4.77 (dd, 1H, $J$ = 4.4, 12.8 Hz, CH*H*-adenine), 4.61−4.46 (m, 3H, C1-H, C3-H, and C*H*H-adenine), 3.98 (s, 3H, $OCH_3$), 3.87 (s, 3H, $OCH_3$), 3.85−3.79 (m, 1H, C3-H), 3.83 (s, 3H, $OCH_3$), 2.41 (m, 1H, C2-H), 2.20 (m, 1H, C2-H); $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 11.62 (s, 1H, NH), 10.08 (s, 1H, OH), 8.72 (d, 1H, $J$ = 8.6 Hz, C7-H), 8.42 (s, 1H, adenine C2-H), 8.09 (d, 1H, $J$ = 8.2 Hz, C10-H), 8.03−7.85 (m, 2H, $NH_2$), 7.94 (s, 1H, adenine C8-H), 7.58 (m, 1H, C9-H), 7.46 (t, 1H, $J$ = 7.8 Hz, C8-H), 6.83 (s, 1H, C9′-H), 6.73 (s, 1H, C3′-H), 6.45 (s, 1H, C5-H), 4.74 (dd, $J$ = 4.4, 12.8 Hz, CH*H*-adenine), 4.53 (m, 1H, C1-H), 4.40 (m, 1H, C*H*H-adenine), 4.32 (ddd, 1H, $J$ = 8.8, 8.4, 5.2 Hz, C3-*H*H), 3.93 (s, 3H, $OCh_3$), 3.81 (m, 1H, partially obscured C3-H*H*), 3.79 (s, 3H, $OCH_3$), 3.75 (s, 3H, $OCH_3$), 1.90 (m, 2H, C2−$H_2$); UV $\lambda_{max}$ 325 ($\epsilon$ 21 200), 259 (sh), 207 nm; FABHRMS (NBA/CsI) *m/e* 580.2285 ($C_{31}H_{29}N_7O_5$, $M^+$ + H requires 580.2308).

The remaining aqueous DNA solution was examined by UV against a DNA solution of the same concentration in the same buffer. UV absorption was checked outside of the DNA absorption range (356 mm) and exhibited only trace absorption corresponding to less than 5% CBQ-based material. The UV $\lambda_{max}$ ($\epsilon$) values employed were for **11** 323 (20 600), 243 (sh, 26 800), and 210 (44 600)[25] nm and for *seco*-CBQ-TMI (**18** and **19**) 303 (21 200), and 207 (44 700) nm. For **16** the absorption at 303 nm was employed ($\epsilon$ 21 200) analogous to the measured comparisons of the duocarmycin A and SA adenine adducts versus their seco derivatives.[13,14] For detection of **11** bound to DNA, the absorbance at 360 nm ($\epsilon$ 10 600) was employed.

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**Supporting Information Available:** Figures 7 and 8 showing the thermally-induced strand cleavage of w794 DNA (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(37) Authentic samples of the solvolysis products **18** and **19** were prepared by treatment of **11** with $CF_3SO_3H$ (0.12 equiv) in THF/$H_2O$ (9:1) at 0 °C for 1 h. Data for **18**: $^1$H NMR (acetone-$d_6$, 400 MHz) $\delta$ 8.34 (d, 1H, $J$ = 8.6 Hz, C7-H), 8.27 (d, 1H, $J$ = 8.7 Hz, C10-H), 7.65 (t, 1H, $J$ = 7 Hz, C9-H), 6.68 (s, 1H, C4′-H), 6.49 (s, 1H, C5-H), 5.43 (s, 1H, C3′-H), 4.86−4.32 (m, 2H, C3-$H_2$), 3.98 (s, 3H, $OCH_3$), 3.77 (s, 3H, $OCH_3$), 3.74−3.68 (m, 1H, C1-H), 3.68 (s, 3H, $OCH_3$), 3.30−2.80 (m, 2H, C$H_2$OH), 2.81 (br s, 2H, OH), 2.18−2.00 (m, partially obscured by acetone-$d_6$, C2-$H_2$); FABHRMS (NBA/CsI) *m/e* 595.0851 (M + $Cs^+$, $C_{26}H_{26}N_2O_6$ requires 595.0842). Data for **19**: $^1$H NMR ($CD_3OD$, 400 MHz) of two conformers, $\delta$ 8.2−8.15 (m, 1H, C8-H), 8.1−8.08 (m, 1H, C11-H), 7.55−7.40 (m, 2H, C9 and C10-H), 6.68 and 6.63 (two s, 1H, C6-H), 6.61 and 6.59 (two s, 1H, C4′-H), 4.30−4.20 and 4.10−4.00 (two m, 1H, C4-H), 3.98 (s, 3H, $OCH_3$), 3.9−3.8 and 3.7−3.65 (2 m, 1H, C2-H), 3.75 (s, 3H, $OCH_3$), 3.65 (s, 3H, $OCH_3$), 3.4 (m, 1H, C4-H), 3.1 (m, 1H, C1-H), 2.76 (m, 1H, C1-H), 1.9 (m, 1H, C3-H), 1.8 (m, 1H, C3-H); FABHRMS (NBA/CsI) *m/e* 595.0857 (M + $Cs^+$, $C_{26}H_{26}N_2O_6$ requires 595.0842).