CC-1065 Functional Analogues Possessing Different Electron-Withdrawing Substituents and Leaving Groups: Synthesis, Kinetics, and Sequence Specificity of Reaction with DNA and Biological Evaluation

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Antitumor agent CC-1065 functional analogues possessing different electron-withdrawing substituents and leaving groups have been synthesized. The extent and the relative rates of DNA cleavage following alkylation by these CPI structures and thermal treatment were determined independently by an ethidium binding assay and by agarose gel electrophoresis experiments. The anticipated preferential covalent binding to adenine sites within the minor groove was confirmed by sequencing determination of selected agents on high-resolution gels. Certain of the synthetic agents, unlike CC-1065, also bind covalently to G sites with weaker intensity. The cytotoxicities of these compounds were also determined against KB cells *in vitro*. Compounds bearing a bromo or nitro group in the benzene ring and a methylsulfonyl as a leaving group are 10 and 5 times more potent than their unsubstituted counterparts, respectively. Compounds bearing a methylsulfonyl as a leaving group are more potent than those bearing a chlorine.

Introduction

CC-1065 (Figure 1), an antibiotic isolated from *Streptomyces zelensis*,[1] is extremely potent against a variety of tumor cell lines both *in vitro* and *in vivo*.[2-3] The chemical synthesis of CC-1065 and its structural analogues CBI and CI derivatives (Figure 1) as well as their mechanism(s) of cytotoxic action have been extensively investigated in recent years.[4-12] CC-1065 has been found to bind to double-stranded B-DNA within the minor groove with the sequence preference of 5′-d(A/GNTTA)-3′ and 5′-d(AAAAA)-3′ and to alkylate the N-3 position of the 3′-adenine by its activated cyclopropane ring containing unit (CPI) present in the molecule.[4] Compounds possessing the pharmacophore (CI) of CC-1065 and its uncyclized *seco* precursor have been synthesized and their cytotoxic activity and DNA-binding properties investigated.[13,14] It was found that the cyclopropane ring is not obligatory for observation of the DNA alkylation characteristic of CC-1065, and the cytotoxic activities of compounds possessing the cyclopropane ring and its *seco* precursor against L1210 cells were comparable.[13] To further understand the mechanism of DNA-binding properties of CC-1065 and its analogues and antitumor activity and to search for more effective antitumor agents, compounds bearing different electron-withdrawing substituents in the benzene ring and different leaving groups on the C3 methylene carbon have been designed, synthesized, and tested. We herein report the chemical synthesis, the kinetics, and sequence specificities of DNA reaction and DNA-cleavage abilities and antitumor cytotoxicity against KB cells *in vitro* of the new substituted CI derivatives.

Synthesis. Compound **3** was synthesized according to the procedure by Warpehoski *et al.*[15] The syntheses of compounds **4-9** are shown in Scheme I. Compound **4** was made by heating lithium chloride with **3** in dimethylformamide. Since the *N*-methylsulfonyl group is much more stable than the methylsulfonyloxy group, the latter was replaced by chloride but the former was not affected under the reaction conditions. The benzyloxy group was reductively removed by hydrogenolysis using ammonium formate as a hydrogen donor.[16] Compound **5** was brominated using *N*-bromosuccinimide and nitrated using nitric acid to afford **6** and **7**, respectively. Compound **4** was brominated using *N*-bromosuccinimide to give **8**. Attempts to make **6** from **8** by hydrogenolysis using ammonium formate and palladium were unsuccessful because the bromo group was also reductively removed and the product was **5** instead of **6**.

Different methods[17] (NaH, THF–DMF; Et_3N; Et_3N, THF; $NaHCO_3$, THF–H_2O) have been used in attempts to cyclize **5**, **6**, and **7**, but all failed. Intermediate **5** was then allowed to react with *tert*-butyldimethylsilyl chloride[18,19] to generate **9** which was then treated with tetrabutylammonium fluoride in tetrahydrofuran.[18] Two products were separated from the reaction mixture in about equal amounts by TLC. One is the cyclized structure **18** and the other is **5**. This result is plausible because chloride is not as good a leaving group as methylsulfonyloxy. Compound **3** was brominated or nitrated as in **6** and **7** to afford **19** and **20**, respectively.

Scheme II shows the synthesis of compounds **10-13**. Compound **3** was treated with dimethylformamide and phosphorus oxychloride to formylate the compound at the 5-position. However under these conditions the compound was not only formylated, the methylsulfonyloxy group was simultaneously replaced by chloride to generate **10**. The formyl group of **10** was sequentially oxidized and esterified by manganese dioxide in methanol catalyzed by potassium cyanide to afford **11**.[20] The benzyloxy groups of **10** and **11** were reductively removed by hydrogenolysis to afford **12** and **13**, respectively. The formyl and ester groups were not affected under these conditions. The NMR spectrum of **11** was different from those of other compounds of this type. In acetone-d_6, the two protons from C2 appeared as two triplets at 5.54–5.52 and 5.13–5.11 ppm, respectively, and the two chloromethyl protons appeared as one triplet at 4.76–4.74 ppm. However the C3 proton signal was still not evident. Surprisingly, in chloroform-*d*, the protons of C2, C3 and the chloromethyl

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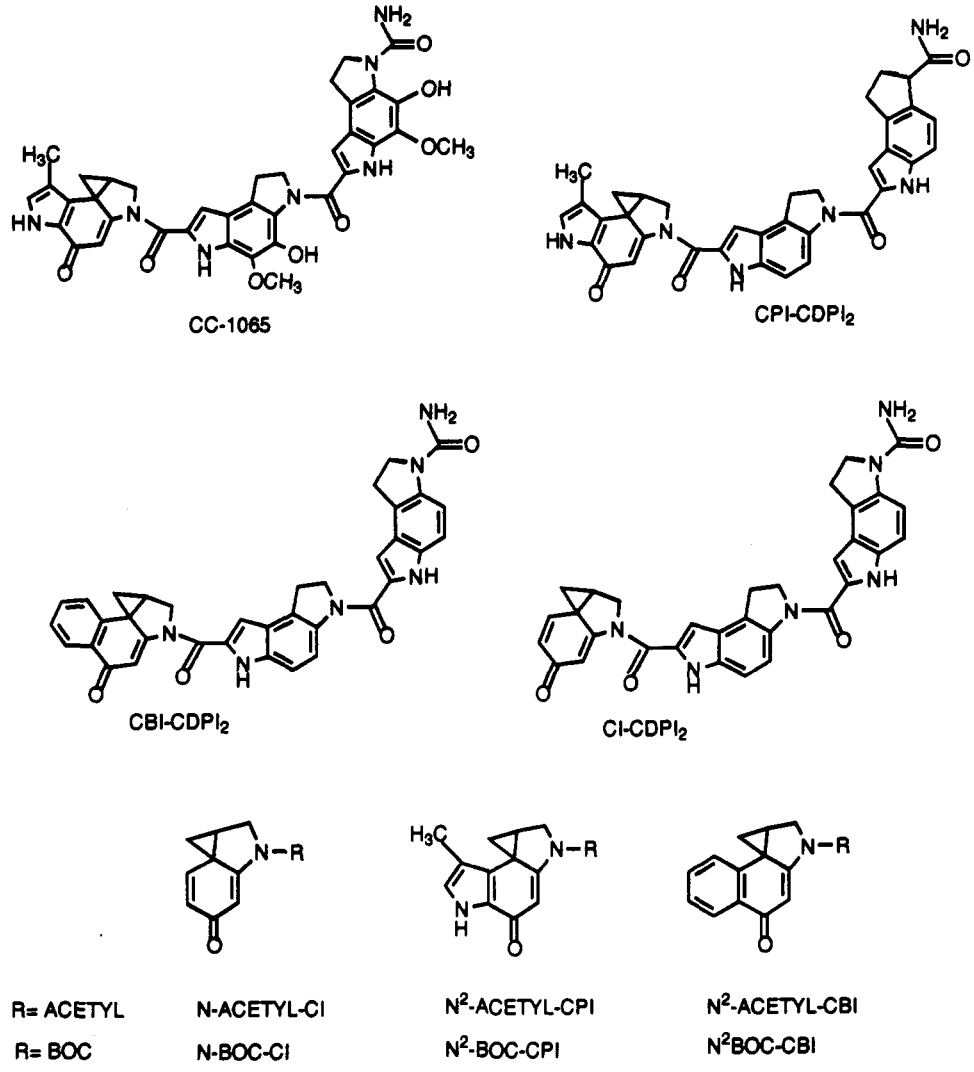


Figure 1. Structures of the antitumor antibiotic CC-1065 and synthetic analogues.

of 13, which was directly derived from 11 by removing the benzyloxy group, all appeared in the NMR spectrum in the pattern characteristic of this type of compound. Attempts to cyclize 12 and 13 using different methods as previously described did not produce the desired products.

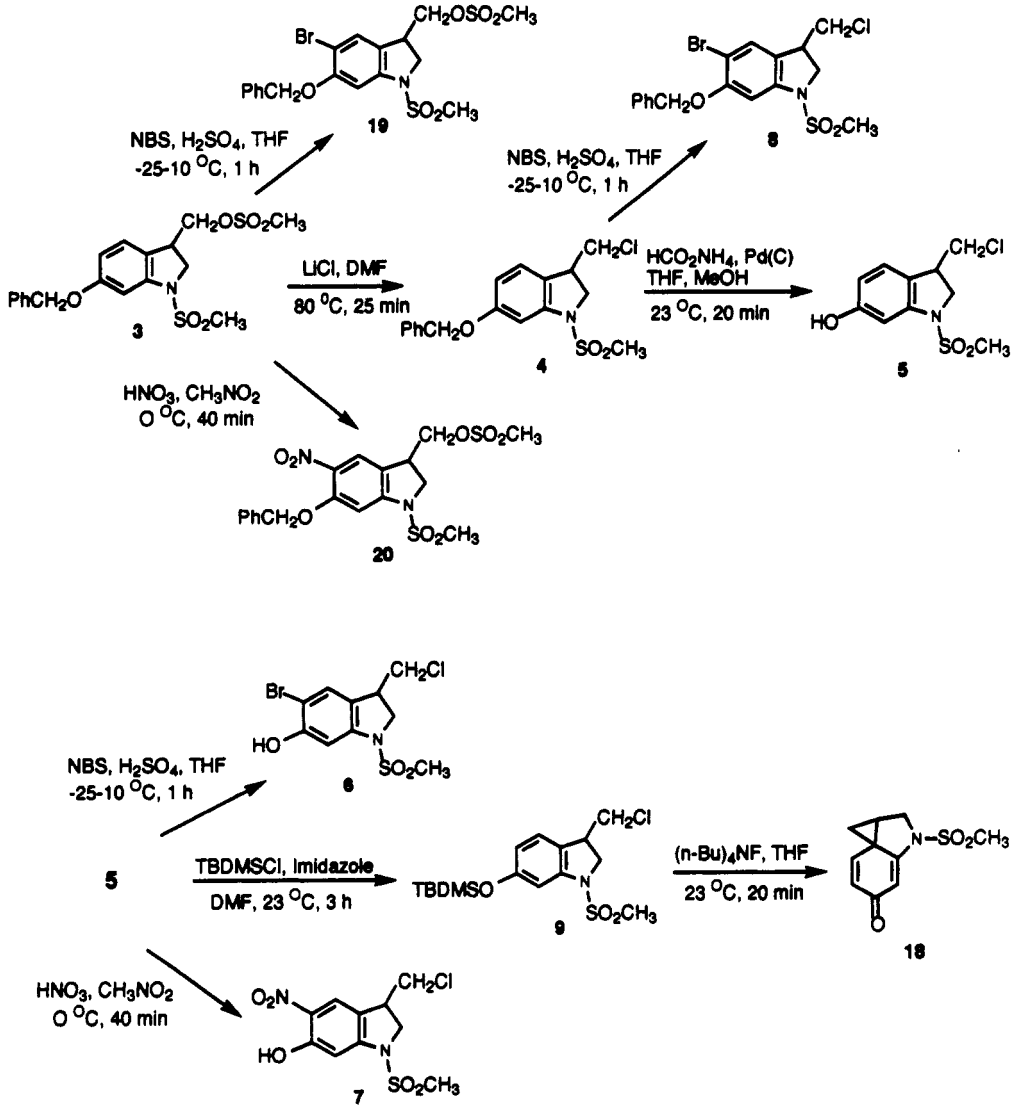
As shown in Scheme III, the benzyloxy group of 3 was removed by treating with trimethylsilyl chloride and sodium iodide.[21] Compound 14 was brominated or nitrated to yield 15 and 16 respectively. Attempts to cyclize 15 and 16 with sodium hydride were unsuccessful. The reaction of 14 with *tert*-butyldimethylsilyl chloride afforded 17 which was then cyclized in the presence of tetrabutylammonium fluoride in tetrahydrofuran to afford 18 in a yield of more than 90%. Compound 17 was converted to 21 by bromination which was then treated with tetrabutylammonium fluoride in tetrahydrofuran in an attempt at cyclization. However, no cyclization was observed, and compound 15 was produced.

Results

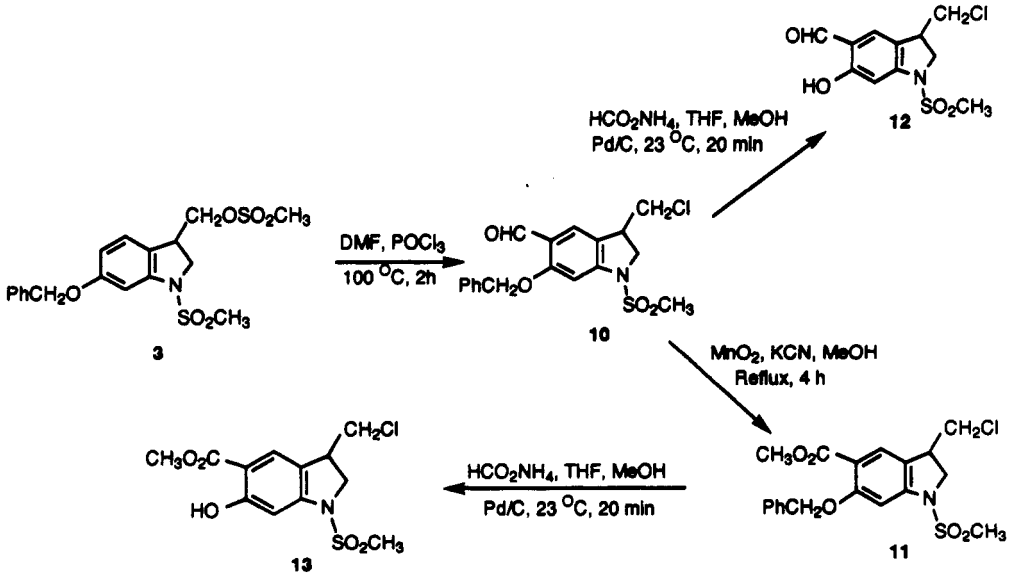
Kinetics of Drug–DNA Reaction and Thermally Induced DNA-Cleavage Studies. A study of the kinetics and sequence preference of drug–DNA reaction of the selected compounds and their thermoinduced cleavage of DNA was conducted by ethidium binding assay, agarose gel electrophoresis, and sequence specificity by polyacrylamide gel electrophoresis.

Ethidium bromide binding assay is convenient for studies of the kinetics of reactions of supercoiled DNA with drugs. Supercoiled covalently closed circular (CCC) and nicked open circular (OC) DNAs permit intercalation of ethidium to different extents, which is revealed by the characteristic fluorescence intensity of bound ethidium. The difference of the fluorescence between unheated and heated assay solution can give information about the damaged DNA since thermal cleavage of alkylated sites on the DNA results in conversion of CCC to OC DNA. In the case of CPI compounds, heating causes thermoinduced cleavage of the drug–DNA adducts and subsequently relaxes the damaged DNA, resulting in a decrease of fluorescence. The results are shown in Figure 2. The relative rates of the DNA cleavage by the CI analogues are 15 > 14 > 16. Since the sequence preferences of the synthetic agents proved to be similar and to differ, at least in the secondary binding sites from CC-1065, relative rate comparisons are restricted to the CI analogues. Independent evidence of the DNA alkylation and thermally-induced cleavage was obtained by agarose gel electrophoresis experiments. After incubation of the DNA with the test drug, the reaction mixtures were heated and loaded on the gel. The results are shown in Figure 3. Under the experimental conditions of progressively increasing concentrations of drugs, CC PM2 DNA was converted to OC DNA by treatment with all compounds. At very high drug

Scheme I. Synthesis of 3-(Chloromethyl)-6-hydroxy-1-(methylsulfonyl)-5-nitroindoline (7) and 6-(Benzyloxy)-1-(methylsulfonyl)-3-[(methylsulfonyl)oxy]methyl]-5-nitroindoline (20)



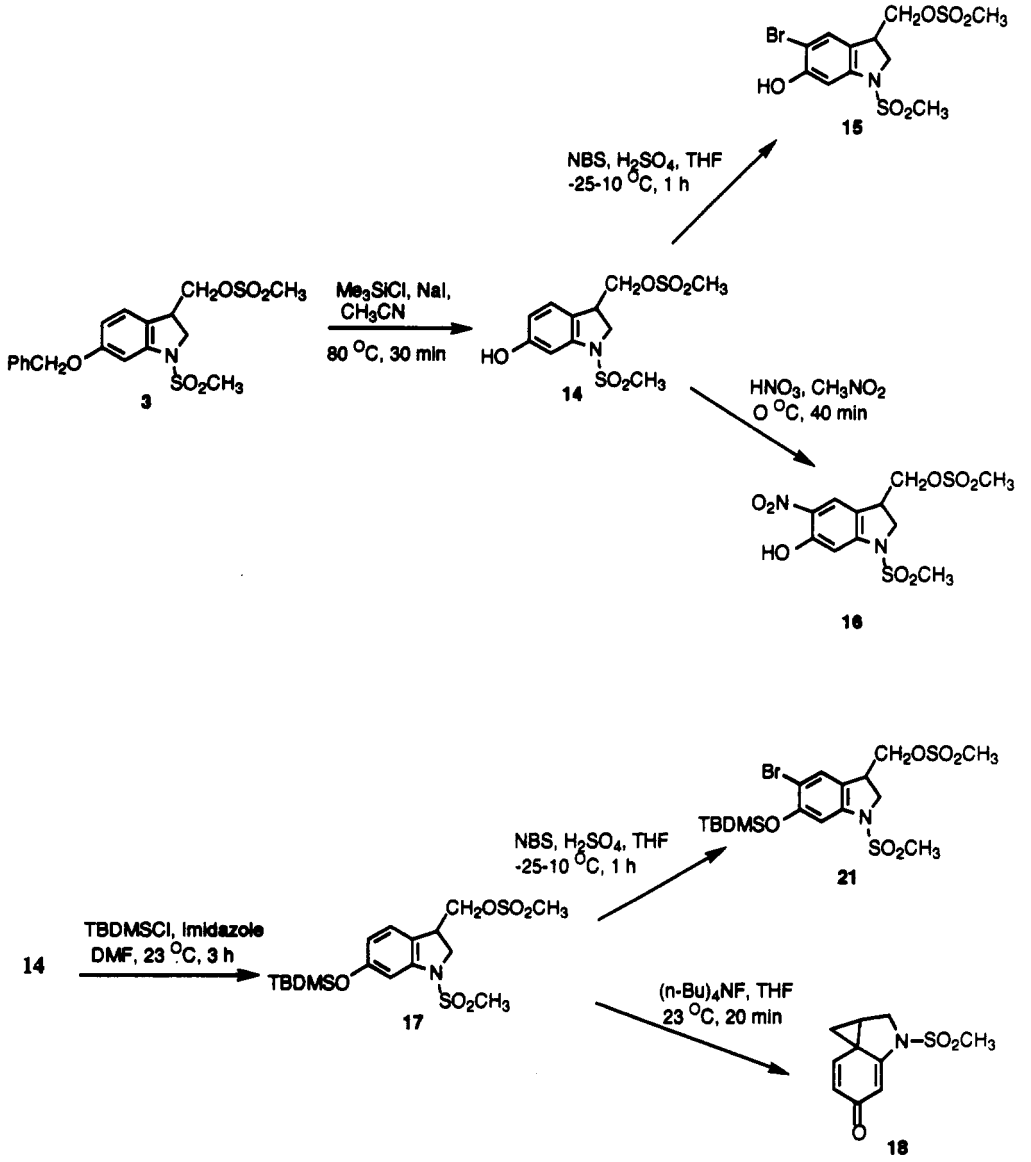
Scheme II. Synthesis of 6-(Benzyloxy)-3-(chloromethyl)-5-(methoxycarbonyl)-1-(methylsulfonyl)indoline (11)



concentrations (80 μmol), the very extensive DNA damage resulted in its complete degradation.

Comparison of DNA Binding and Sequence Specificity. The sites of covalent modifications within the 4260–4325 region of pBR322 DNA by CC-1065 and the synthetic agents are shown in Figure 4. In these studies, the profile of DNA alkylation by CC-1065 and related agents proved distinguishable. The synthetic agents were

Scheme III. Synthesis of *N*-(Methylsulfonyl)-1,2,7,7a-tetrahydrocycloprop[*c*]indol-4-one (18)



clearly less reactive at the common preferred sites than CC-1065 in that higher concentrations are required to produce detectable cleavage. A summary of the results of the densitometric examination of the CC-1065 or related agents—DNA alkylation selectivity within the double stranded DNA fragment—is given in Table I. For each instance of the covalent alkylation sites detected through the thermally induced strand breakage, the site of alkylation proved to be stronger for CC-1065 than other ligands (Table I). Five major alkylating sites were observed for CC-1065, namely 5′-(TTTA)-3′, 5′-(GTTA)-3′, 5′-(ATAA)-3′, 5′-(AATA)-3′, and 5′-(ATAA)-3′. In addition, CC-1065 exhibited the site of alkylation to be exclusively adenine, flanked only by two 5′-A or T bases. In the higher sequence selective region, C or G may follow the alkylation sites, 5′-(GTCA)-3′ and 5′-(GGCA)-3′, but with decreased intensity. AT preference for the first three base pairs may represent a combination of the initial adenine alkylation site and the required stabilization and its binding selectivity.

The prominent sites of alkylation for compounds 14, 15, and 16 proved to be identical, *i.e.* 5′-(TATA)-3′, 5′-(GATA)-3′, and 5′-(AATA)-3′, though, like the parent molecule CC-1065, A-T residues flanked the site of alkylation. In addition to the strong binding sites, there is lower overall preference for adenines by compounds 14, 15, and 16 as indicated by the acceptance of G-residues (Table I). Of all the alkylation sites identified by these analogues, nearly 40–50% occur at G residues, which may or may not contain the preferred consensus sequence 5′-(A/TA)-3′. Interestingly, while there is no absolute specificity for adenines, there is strong preference for G at the next positions as well (Table I).

Since relative band intensity is not sensitive to the thermally induced strand scission under the conditions employed, this intensity is considered as proportional to the rate of alkylation.[16] Under a given set of drug–DNA reaction conditions, the cleavage bands produced at the lowest concentration of a drug identify the most reactive sites for that drug. Figure 4 shows the relative band intensity patterns at the lowest concentrations (lanes 2, 5, 8, and 11) for CC-1065 and the synthetic analogues. The high intensity of CC-1065 is a feature of highly reactive alkylation sites at adenine (Figure 5). At moderate binding sites, alkylation involving G-residues was more pronounced for the synthetic analogues than for CC-1065. As expected, CC-1065 did not show any reactive profile toward G-residues. It must be emphasized that sequence context is

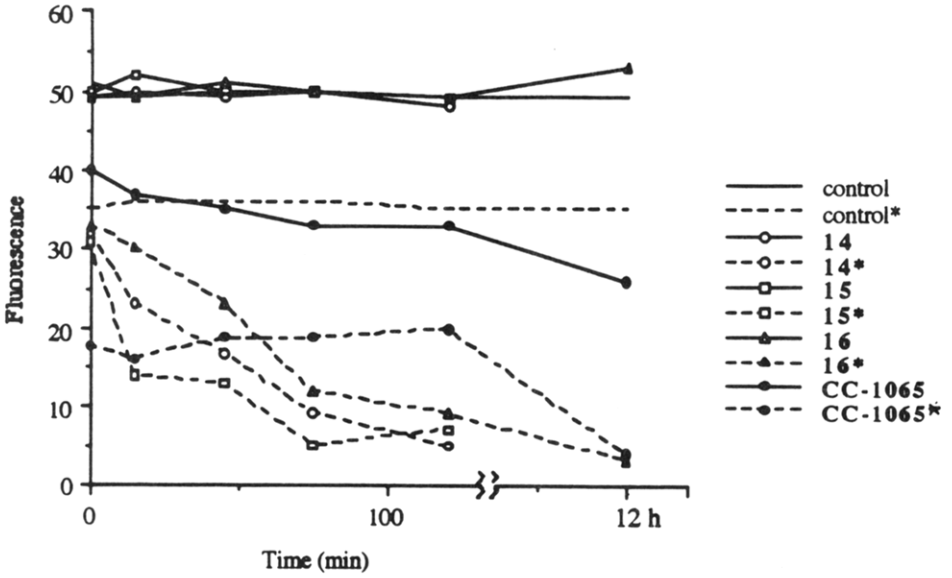


Figure 2. Time dependence of the effects of individual drugs on conversion of PM2 CC-DNA to OC form reflected in the change in fluorescence of bound ethidium. A 100-μL sample of DNA at 1 A_{260} was reacted with the drugs at 80 μM in buffer of pH 8.0. 10 μL of samples were then pipetted into 2 mL of the pH 12 assay solution in the indicated intervals. The fluorescence was first read at room temperature and the solution then heated at 95 °C for 5 min before the second reading (* marked).

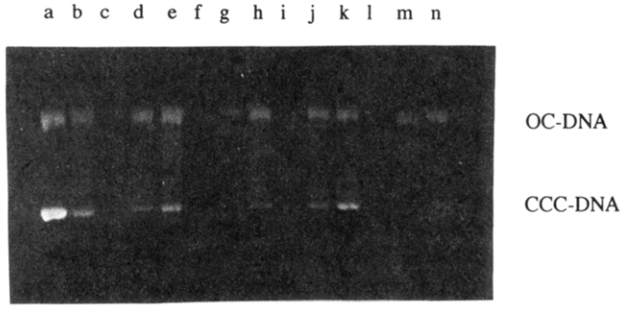


Figure 3. Agarose gel electrophoresis of the reaction mixture of PM2 DNA 0.7 A_{260} with drugs in varying concentrations. The drugs were incubated with DNA for 20 h at room temperature, and the solutions were then heated at 95 °C for 5 min before loading on gels. (a) control (without drug, before heating); (b) control (without drug, after heating); (c–e) 80 μM, 8 μM, 0.8 μM of **14**; (f–h) 80 μM, 8 mM, 0.8 μM of **15**; (i–k) 80 μM, 8 μM, 0.8 μM of **16**; (l–n) 80 μM, 8 μM, 0.8 μM of CC-1065.

different for CC-1065 and the synthetic analogues, and hence, while the specific adenine that is alkylated by all the compounds is the same, the alkylation sites themselves are not identical. On the basis of the alkylation intensities (Figure 1) and analysis of the densitometric data (Table I, Figure 5), the overall bonding intensity is in the order CC-1065 > **15** > **16** > **14**.

Cytotoxicity Studies. Table II summarizes the *in vitro* inhibitory concentration of these compounds against KB cancer cell lines (ATCC CCL, 17). The synthetic agents are significantly less active biologically than CC-1065. Expression of biological activity seems to be, in part, related to the alkylation potential of these compounds, although other pharmacological factors such as cellular uptake and intracellular stability undoubtedly contribute.

Discussion

From the above experiments, it appears that there are two factors which are critical for the formation of the cyclopropane ring by cyclization. One is the electron density of the benzyl moiety. An electron-withdrawing group retards the cyclization, whereas an electron-donating group would be expected to promote the cyclization. Another factor is the leaving ability of the group on the C3 methylene carbon. A better leaving group will favor cyclization. These factors are understandable from the mechanism shown in Scheme IV. For the intramolecular nucleophilic substitution reaction to occur, C9 must be nucleophilic enough that it can effectively attack the electrophilic site. An electron-withdrawing group will reduce the nucleophilicity of C9 and, correspondingly, the possibility of cyclization. An electron-donating group would be expected to have the opposite effect. A better leaving group will increase the electrophilicity of the methylene carbon on C3, thereby increasing the probability of the cyclization. The reactions of **9**, possessing a chloride as a leaving group which is only partially cyclized with tetrabutylammonium fluoride, and **17**, possessing a methylsulfonyloxy which is cyclized in high yield, clearly show that a good leaving group is essential for the formation of the cyclopropane ring. The reactions of **17**, possessing no substituent, which cyclized in good yield, and **21**, possessing a bromo group, which does not permit cyclization at all, with tetrabutylammonium fluoride demonstrates that the nature of the substituent on the benzene ring is also critical for the cyclization.

The independent evidence from the gel electrophoresis showed that when supercoiled PM2 DNA is incubated with **14**, **15**, **16**, or CC-1065 at 80 μmol for 20 h the CCC form was completely converted into OC DNA. This suggests that, like CC-1065, **14**, **15**, and **16** also exert their cytotoxicity through covalent binding to DNA. Further confirmatory evidence is provided by the sequence specificity studies. The bands indicated that for (+)-CC-1065, the site of alkylation is exclusively at adenine flanked by two 5′-A or T bases. Earlier studies have indicated the minor groove binding property of (+)-CC-1065 at AT-rich sequences.[22] Further, it has been postulated that only adenines in the sequences provide sufficiently low energy barriers for the alkylation process with (+)-CC-1065.[23] This may account for the absolute requirement for adenine for alkylation with (+)-CC-1065. The synthetic CI agents examined, **14**, **15**, and **16**, exhibit identical sequence specificity in their strong sites to CC-1065. However

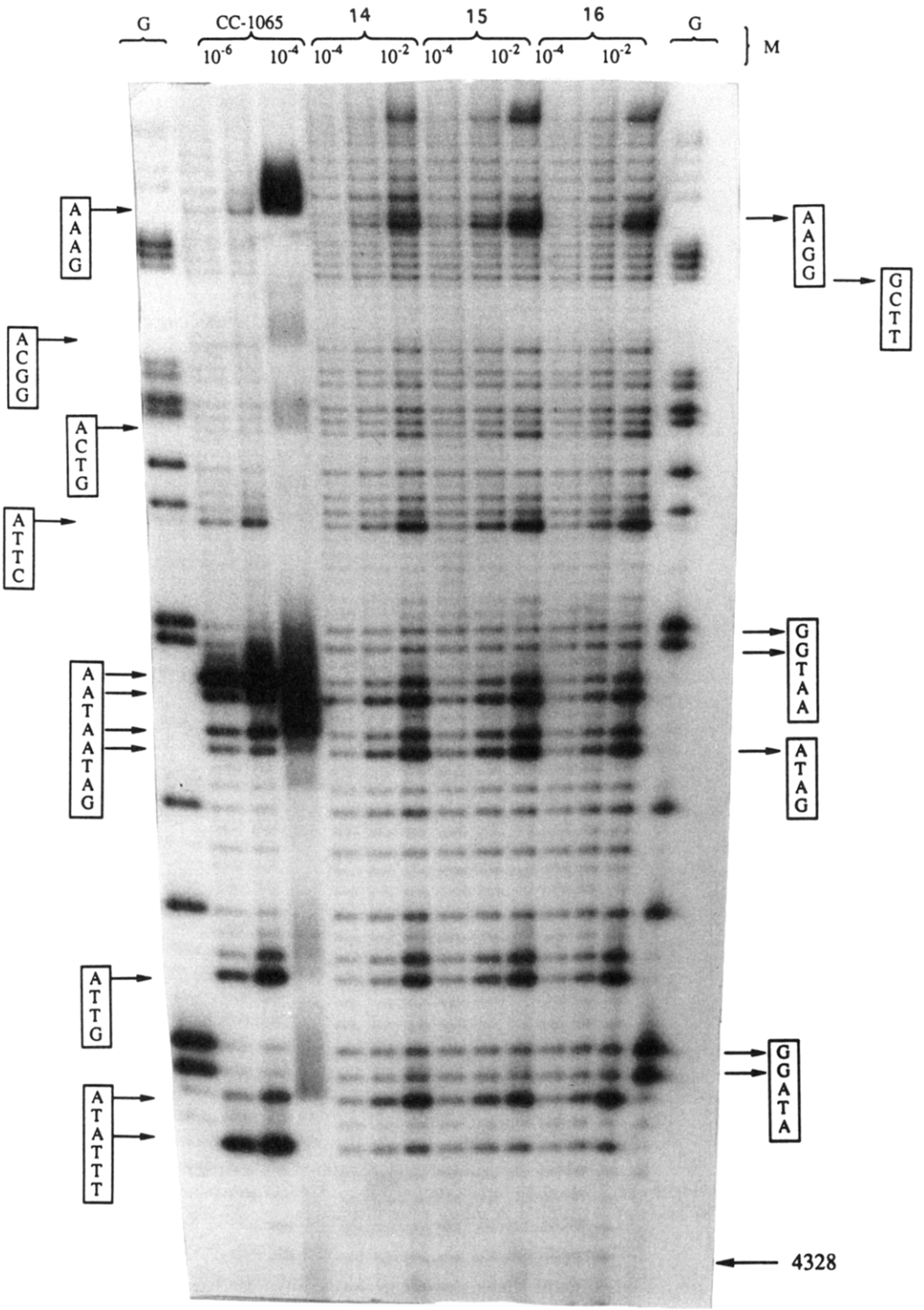


Figure 4. Autoradiogram of thermally induced cleavage pattern induced on a portion of the 5′-labeled pBR322 restriction fragment by CC-1065 and the synthetic CI analogues **14**, **15**, and **16** at the concentrations indicated. G represents the Maxam-Gilbert G cleavage.

additional binding at the G-residues by these synthetic analogues may represent an alternative or additional mechanism for this sequence recognition of DNA.[24,25] In this connection, the DNA sequence specificity of the pyrrolo[1,4]benzodiazepine antitumor antibiotics,[26] which bind through N-2 of guanine, has been supported by theoretical calculations,[27] in which the relative distortion energies of different sequences are predictive of the experimentally proved DNA sequence selectivity. However, the bias for AT regions of the minor groove is strong, and synthetic analogues designed to recognize GC bases may simply be more tolerant of those bases than the noncovalent, groove-binding agents designed by nature.[27]

The influence of electron-withdrawing groups of **15** (bromide) and **16** (nitro) results in 10 and 5 times more cytotoxic potency than their unsubstituted counterpart **14**, respectively. While left-handed subunit (CPI) of CC-1065 is primarily responsible for sequence-specific binding of DNA, the recognition of alternative or additional sequences by the synthetic analogues described herein may assist in our ongoing efforts to exploit them for therapeutic purposes.

Table I. Summary of Covalent Alkylation Sites

compound	alkylation sites[a] (5′–3′) strong	moderate	weak
CC-1065	TTTA, GTTA, ATTA, AATA, ATAA	TATA, TTAA, GATA, CTTA	GTCA, GGCA, GAAA
14	TATA, GATA, AATA	TTTA, TTAA, GTTA, TTAA, AATG, ATAA, ATG, ATGG, CTTA, GGAA	TTAG, GACG, TCAG, CAGG, GGtG, GTGG
15	TATA, GTTA, GATA, AATA, CTTA	TTTA, ATAG, TAGG, TTAA, AATG, CATG, ATAA, GGAA	GTCA, ATGA, AATG, ATGG, TTAG, TAGA, ACGT, TTCG, TCGG, CGGG
16	TATA, GTTA, GATA, AATA, CTTA	TTTA, ATAG, TAGG, TTAA, AATG, CATG, ATAA	GTCA, ATGA, AATG, ATGG, TTAG, TAGA, ACGT, TTCG, TCGG, CGGG, GGAA

[a] Underline denotes the site of alkylation.

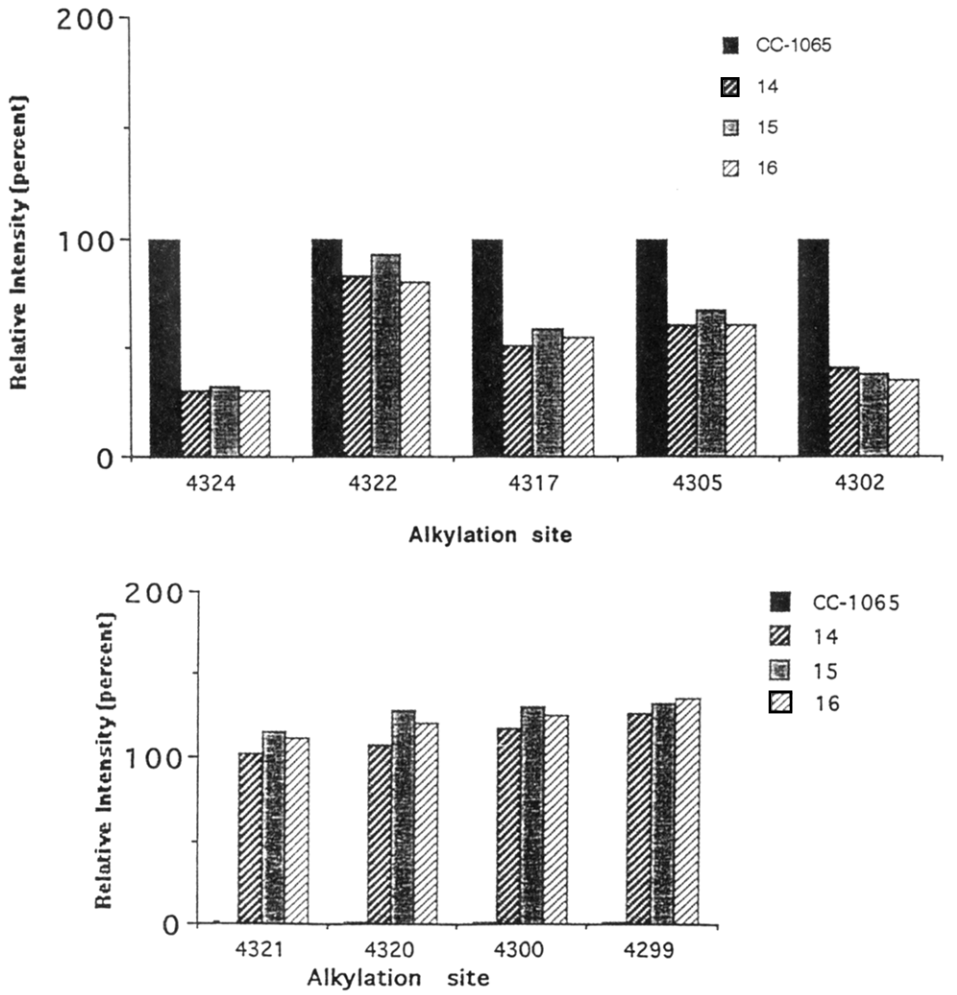


Figure 5. (Upper panel) A comparison in the form of a histogram of the relative extents of alkylation at A residues at major recognition sites by CC-1065 and the synthetic analogues **14**, **15**, and **16** in the region 4302–4324. (Lower panel) A comparison in the form of a histogram of the relative extents of alkylation at G residues at minor recognition sites by CC-1065 and **14**, **15**, and **16** in the region 4299–4321.

In summary, these studies have identified some of the factors, particularly CI substituent effects and leaving-group abilities, that contribute to overall DNA sequence recognition and cytotoxic potency of CC-1065 analogues and which may assist in drug design.

Experimental Section

Chemistry. Melting points were determined with an Electrohome apparatus and are uncorrected. ^{1}H-NMR spectra were recorded at ambient temperature on a Bruker WH-300 spectrometer. High-resolution mass spectra (HRMSFAB) were recorded on a modified MS-50 mass spectrometer equipped with a VG 11-250J data system. Accurate masses were calculated interactively with the data system using a reference (such as CsI in glycerol) peak. Analytical thin-layer chromatography was performed on silica-coated plastic plates (silica gel 60 F-254, Merck) and visualized under UV light. Preparative separations were performed by flash chromatography on silica gel (Merck, 70–230 or 230–400 mesh). Acetone was obtained by distillation from P_2O_5. Tetrahydrofuran was dried by distillation from sodium benzophenone ketyl. Dimethylformamide and triethylamine were dried over molecular sieves (4 Å) before use. The above solvents were stored over molecular sieves (4 Å). All other solvents were used as received and were reagent grade where available.

6-(Benzyloxy)-1-(methylsulfonyl)-3-[[(methylsulfonyl)oxy]methyl]indoline (3). This compound was synthesized according to the methods by Warpehoski *et al.*,[15] and the ^{1}H-NMR and MS spectra were in agreement with its chemical structure.

6-(Benzyloxy)-3-(chloromethyl)-1-(methylsulfonyl)indoline (4). To 300 mg (0.73 mmol) of **3** dissolved in dimethylformamide (3 mL) was added lithium chloride (114 mg, 2.71 mmol) under nitrogen with stirring. The reaction mixture was heated to 80 °C under nitrogen for 25 min. The reaction mixture was

Table II. Cytotoxicities to KB Cells in Culture

compound	indolinyl leaving group	benzene ring 5-substituent	preformed cyclopropane	IC_{50}[a] (μg/mL)
3	OSO_2CH_3	H	-	4.39
4	Cl	H	-	6.32
5	Cl	H	-	4.24
6	Cl	Br	-	3.8
7	Cl	NO_2	-	8.85
8	Cl	Br	-	6.18
10	Cl	CHO	-	3.24
11	Cl	CO_2CH_3	-	7.14
12	Cl	CHO	-	4.83
13	Cl	CO_2CH_3	-	8.38
14	OSO_2CH_3	H	-	3.69
15	OSO_2CH_3	Br	-	0.308
16	OSO_2CH_3	NO_2	-	0.764
18		H	+	8.64
19	OSO_2CH_3	Br	-	5.21
20	OSO_2CH_3	NO_2	-	7.909
adriamycin				0.02

[a] The drug concentration that reduced the viability of KB cells by 50%.

cooled to room temperature, and a 5% $NaHCO_3$ solution (20 mL) was added. The product was extracted by ethyl acetate (40 mL × 3), the organic phase was dried over Na_2SO_4, and the solvent was removed *in vacuo*. The product was recrystallized in ether and washed with hexane. A white solid product (176 mg, 0.50 mmol) was obtained (69% yield): mp 104–106 °C; ^{1}H-NMR ($CDCl_3$, ppm) 7.45–7.29 (m, 5H, C_6H_5), 7.15–7.12 (d, 1H, J = 8.5 Hz, C4-H), 7.11–7.10 (d, 1H, J = 2.5 Hz, C7-H), 6.69–6.66 (dd, 1H, J = 8.5, 2.5 Hz, C5-H), 5.17 (s, 2H, $OCH_2C_6H_5$), 4.12–4.05 (m, 1H, NC*H*H), 3.99–3.94 (dd, 1H, J = 10.5, 4.5 Hz, NCH*H*), 3.76–3.59 (m, 3H, C*H*CH_2Cl, CH_2Cl), 2.87 (s, 3H, SO_2CH_3); EIHRMS calcd for $C_{17}H_{18}ClNO_3S$ 351.0697, found 351.0695.

3-(Chloromethyl)-6-hydroxy-1-(methylsulfonyl)indoline (5). To a solution of 4 (20 mg, 57 μmol) in tetrahydrofuran and methanol (1 mL, 1/1, v/v) were added 10% Pd/C (20 mg) and ammonium formate (20 mg) sequentially. The mixture was stirred at room temperature for 20 min. The reaction mixture was then filtered, and the solid was washed with methanol. The solvent was removed *in vacuo*, and water (5 mL) was added. The product was extracted with ethyl acetate (10 mL × 4). The organic phase was dried over Na_2SO_4 and concentrated to 14 mg (54 μmol, 94% yield) of a colorless oil: ^{1}H-NMR ($CDCl_3$, ppm) 7.11–7.08 (d, 1H, J = 8.5 Hz, C4-H), 6.99–6.97 (d, 1H, J = 2.0 Hz, C7-H), 6.58–6.55 (dd, 1H, J = 8.0, 2.0 Hz, C5-H), 5.95 (s, 1H, OH), 4.18–4.06 (m, 1H, NC*H*H), 4.01–3.95 (dd, 1H, J = 10.5, 4.5 Hz, NCH*H*), 3.76–3.57 (m, 3H, C*H*CH_2Cl, CH_2Cl), 2.95 (s, 3H, SO_2CH_3); EIHRMS calcd for $C_{10}H_{12}ClNO_3S$ 261.0288, found 261.0226.

5-Bromo-3-(chloromethyl)-6-hydroxy-1-(methylsulfonyl)-indoline (6). To a solution of 5 (13.98 mg, 54 μmol) in tetrahydrofuran (1 mL) cooled to −25 °C under nitrogen were added *N*-bromosuccinimide (11 mg, 62 μmol) and concentrated sulfuric acid (1 drop) sequentially. The reaction mixture was stirred under nitrogen for 1 h while the temperature gradually changed from −25 °C to 10 °C. $NaHCO_3$ (10 mg), saturated $Na_2S_2O_3$ (2 mL), and water (3 mL) were added to the reaction mixture sequentially. The reaction mixture was extracted with ethyl acetate (10 mL × 4), the organic phase was dried over Na_2SO_4, and the solvent was removed *in vacuo*. The product was purified by thin-layer chromatography, eluting with ethyl acetate and hexane (v/v, 1/1). The product was recrystallized in dichloromethane. A white solid (11.1 mg, 33 μmol, 61% yield) was obtained: mp 135 °C dec; ^{1}H-NMR ($CDCl_3$, ppm) 7.32 (d, 1H, J = 0.8 Hz, C4-H), 7.13 (s, 1H, C7-H), 5.54 (s, 1H, OH), 4.14–4.08 (m, 1H, NC*H*H), 3.99–3.94 (dd, 1H, J = 10.5, 4.5 Hz, NCH*H*), 3.74–3.61 (m, 3H, C*H*CH_2Cl, CH_2Cl), 2.95 (s, 3H, SO_2CH_3); EIHRMS calcd for $C_{10}H_{11}BrClNO_3S$ 338.9332, found 338.9332.

3-(Chloromethyl)-6-hydroxy-1-(methylsulfonyl)-5-nitroindoline (7). To a solution of 5 (13.98 mg, 54 μmol) in nitromethane (1 mL) cooled to 0 °C under nitrogen was added nitric acid (3 μL, 75 μmol). The reaction mixture was stirred at 0 °C under nitrogen for 40 min. $NaHCO_3$ (10 mg) and water (5 mL) were added to the reaction mixture sequentially. The reaction mixture was extracted with ethyl acetate (10 mL × 4), the organic phase was dried over Na_2SO_4, and the solvent was removed *in vacuo*. The product was purified by thin-layer chromatography eluting with ethyl acetate and hexane (v/v, 1/1). The product was recrystallized in dichloromethane. 7 (9.3 mg, 30 μmol, 57% yield) as yellow needles was obtained: mp 133–135 °C; ^{1}H-NMR ($CDCl_3$, ppm) 11.18 (s, 1H, OH), 8.00 (d, 1H, J = 0.8 Hz, C4-H), 7.13 (s, 1H, C7-H), 4.29–4.22 (dd, 1H, J = 10.5, 9.5 Hz, NC*H*H), 4.10–4.05 (dd, 1H, J = 10.5, 4.5 Hz, NCH*H*), 3.84–3.68 (m, 3H, C*H*CH_2-Cl, CH_2Cl), 3.15 (s, 3H, SO_2CH_3); EIHRMS calcd for $C_{10}H_{11}ClN_2O_5S$ 306.0078, found 306.0055.

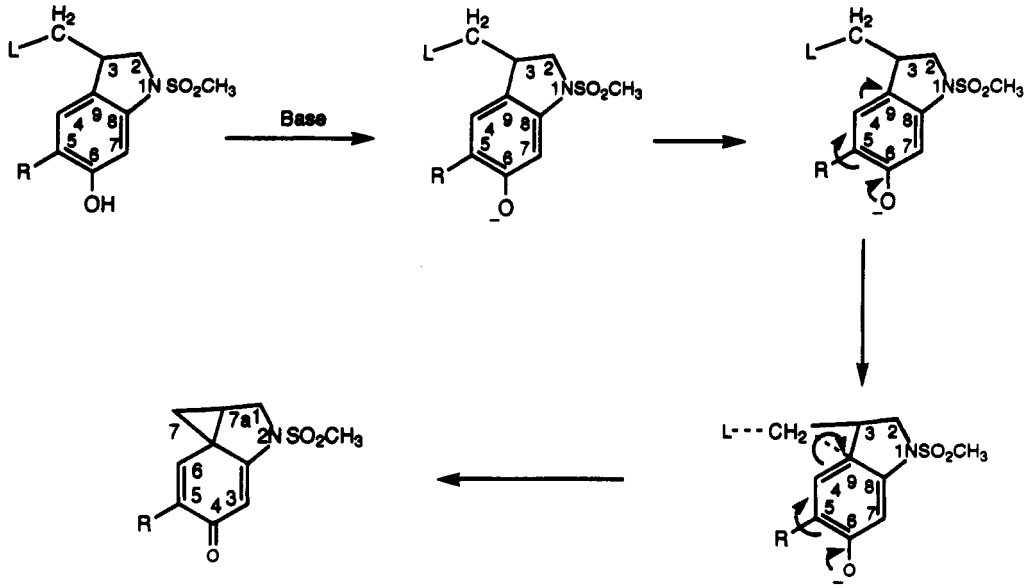
6-(Benzyloxy)-5-bromo-3-(chloromethyl)-1-(methylsulfonyl)indoline (8). To a solution of 4 (40 mg, 114 μmol) in tetrahydrofuran (2 mL) cooled to −25 °C under nitrogen were added *N*-bromosuccinimide (22.3 mg, 125 μmol) and concentrated sulfuric acid (1 drop) sequentially. The rest of the procedure is similar to that described for 6. The product was recrystallized from ethyl acetate. A white powder (49 mg, 114 μmol, 100% yield) was obtained: mp 165–166 °C; ^{1}H-NMR ($CDCl_3$, ppm) 7.50–7.30 (m, 6H, C4-H, C_6H_5), 7.11 (s, 1H, C7-H), 5.20 (s, 2H, $OCH_2C_6H_5$), 4.11–4.04 (dd, 1H, J = 10.5, 9.5 Hz, NC*H*H), 3.97–3.91 (dd, 1H, J = 10.5, 4.5 Hz, NCH*H*), 3.74–3.56 (m, 3H, C*H*CH_2-Cl, CH_2Cl), 2.78 (s, 3H, SO_2CH_3); EIHRMS calcd for $C_{17}H_{17}BrClNO_3S$ 428.9802, found 428.9802.

6-(*tert*-Butyldimethylsiloxy)-3-(chloromethyl)-1-(methylsulfonyl)indoline (9). *tert*-Butyldimethylsilyl chloride (10 mg, 66 μmol) was added to a solution of 5 (11.5 mg, 44 μmol) and imidazole (18 mg, 264 μmol) in dimethylformamide (1 mL). The reaction mixture was stirred at room temperature for 3 h. The reaction was quenched with water (5 mL) and extracted with ethyl acetate (10 mL × 3). The organic phase was dried over Na_2SO_4 and concentrated. The residue was purified by thin-layer chromatography using ethyl acetate and hexane (1/5, v/v) as an eluent affording 9. Product (11.6 mg, 31 μmol, 70% yield) was obtained as a colorless oil: ^{1}H-NMR ($CDCl_3$, ppm) 7.15–7.00 (d, 1H, J = 8.5 Hz, C4-H), 6.89–6.87 (d, 1H, J = 2.5 Hz, C7-H), 6.48–6.44 (dd, 1H, J = 8.0, 2.0 Hz, C5-H), 4.08–3.98 (m, 1H, NC*H*H), 3.91–3.86 (dd, 1H, J = 10.5, 4.5 Hz, NCH*H*), 3.70–3.49 (m, 3H, C*H*CH_2Cl, CH_2Cl), 2.86 (s, 3H, SO_2CH_3), 0.93 (s, 9H, $SiC(CH_3)_3$), 0.17 (s, 6H, $Si(CH_3)_2$); EIHRMS calcd for $C_{16}H_{28}ClNO_3SSi$ 375.1091, found 375.1084.

6-(Benzyloxy)-3-(chloromethyl)-5-formyl-1-(methylsulfonyl)indoline (10). To dimethylformamide (1 mL) cooled to 0 °C under nitrogen were added phosphorus oxychloride (68 μL, 730 μmol) and 3 sequentially. The reaction mixture was heated to 100 °C for 2 h. Ice was added after the reaction mixture was cooled to room temperature, and the solution was neutralized by adding $NaHCO_3$. The product was extracted with ethyl acetate (40 mL × 3), and the organic phase was dried over Na_2SO_4. The product was recrystallized from ethyl acetate, and the solid was washed with ether to give 10 (115 mg, 303 μmol, 62% yield) as a brown solid: mp 153–155 °C; ^{1}H-NMR ($CDCl_3$, ppm) 10.41 (s, 1H, CHO), 7.74 (s, 1H, C4-H), 7.47–7.31 (m, 5H, C_6H_5), 7.15 (s, 1H, C7-H), 5.24 (s, 2H, $OCH_2C_6H_5$), 4.20–4.13 (dd, 1H, J = 11.0, 9.5 Hz, NC*H*H), 4.05–3.99 (dd, 1H, J = 11.0, 5.0 Hz, NCH*H*), 3.80–3.64 (m, 3H, C*H*CH_2Cl, CH_2Cl), 2.88 (s, 3H, SO_2CH_3); EIHRMS calcd for $C_{18}H_{18}ClNO_4S$ 379.0646, found 379.0644.

6-(Benzyloxy)-3-(chloromethyl)-5-(methoxycarbonyl)-1-(methylsulfonyl)indoline (11). Methanol (10 mL) was added to 10 (50 mg, 132 μmol), and the mixture was heated until 10 was completely dissolved. Potassium cyanide (100 mg) and manganese(IV) oxide (250 mg) were then added, respectively. The reaction mixture was heated to reflux for 4 h. The reaction mixture was cooled to room temperature and filtered through Celite washed with methanol. Solvent was removed *in vacuo*, and water (5 mL) was added. The mixture was extracted with ethyl acetate (20 mL × 3). The mixture was purified by thin-layer chromatography eluted with ethyl acetate and hexane (1/3, v/v). The product was recrystallized from ethyl acetate, and the solid was washed with ether affording 11 (19 mg, 47 μmol, 35% yield) as a yellow powder: mp 123–125 °C; ^{1}H-NMR ($CDCl_3$, ppm) 7.95 (s, 1H, C4-H), 7.60–7.29 (m, 5H, C_6H_5), 7.24 (s, 1H, C7-H), 5.54–5.52 (t, 1H, J = 2.5 Hz, NC*H*H), 5.28 (s, 2H, $OCH_2C_6H_5$), 5.13–5.11 (t, 1H, J = 2.5 Hz, NCH*H*), 4.76–4.74 (t,

Scheme IV. Depiction of the Sequence of Events during Closure of the Cyclopropane Ring in CI Structures



2H, J = 2.5 Hz, CH_2Cl), 3.84 (s, 3H, OCH_3), 3.05 (s, 3H, SO_2CH_3); EIHRMS calcd for $C_{19}H_{20}ClNO_5S$ 409.0752, found 409.0758.

3-(Chloromethyl)-5-formyl-6-hydroxy-1-(methylsulfonyl)-indoline (12). To a solution of **10** (9.7 mg, 26 μmol) in tetrahydrofuran and methanol (1 mL, 1/1, v/v) were added 10% Pd/C (10 mg) and ammonium formate (10 mg) sequentially. The rest of the procedure is similar to that described for **5** affording **12** (7.3 mg, 26 μmol, 99% yield) as a white foam: ^{1}H-NMR ($CDCl_3$, ppm) 11.64 (s, 1H, OH), 9.74 (s, 1H, CHO), 7.40 (s, 1H, C4-H), 7.12 (s, 1H, C7-H), 4.26–4.18 (dd, 1H, J = 11.0, 9.5 Hz, NC*H*H), 4.08–4.02 (dd, 1H, J = 11.0, 5.0 Hz, NCH*H*), 3.80–3.68 (m, 3H, C*H*CH_2Cl, CH_2Cl), 3.04 (s, 3H, SO_2CH_3); EIHRMS calcd for $C_{11}H_{12}ClNO_4S$ 289.0177, found 289.0167.

3-(Chloromethyl)-5-(methoxycarbonyl)-6-hydroxy-1-(methylsulfonyl)indoline (13). To a solution of **11** (8 mg, 20 μmol) in tetrahydrofuran and methanol (1 mL, 1/1, v/v) were added 10% Pd/C (10 mg) and ammonium formate (10 mg) sequentially. The rest of the procedure is similar to that described for **5** affording **13** (6 mg, 19 μmol, 96% yield) as a white foam: ^{1}H-NMR ($CDCl_3$, ppm) 1.50 (s, 1H, OH), 7.60 (s, 1H, C4-H), 6.99 (s, 1H, C7-H), 4.19–4.13 (dd, 2H, J = 9.5, 9.0 Hz, NCH_2), 3.93 (s, 3H, OCH_3), 3.55–3.48 (dd, 2H, J = 10.0, 7.0 Hz, CH_2Cl), 3.45–3.33 (m, 1H, C*H*CH_2Cl), 2.94 (s, 3H, SO_2CH_3); EIHRMS calcd for $C_{12}H_{14}ClNO_5S$ 319.0282, found 319.0246.

6-Hydroxy-1-(methylsulfonyl)-3-[[(methylsulfonyl)oxy]-methyl]indoline (14). Trimethylsilyl chloride (310 μL, 2.43 mmol) was added to a mixture of **3** (200 mg, 487 μmol) and sodium iodide (365 mg, 2.43 mmol) stirred under nitrogen in acetonitrile (7 mL). The reaction mixture was heated to 80 °C for 30 min under nitrogen with stirring. The reaction mixture was quenched with saturated sodium thiosulfate solution (10 mL) and then cooled to room temperature. The product was extracted with ethyl acetate (20 mL × 4). The organic phase was dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography using ethyl acetate and hexane (1/1, v/v) as an eluent affording **14** (117 mg, 366 μmol, 75% yield) as a brown oil: ^{1}H-NMR (acetone-d_6) 7.22–7.18 (dd, 1H, J = 8.5, 0.8 Hz, C4-H), 6.93–6.92 (d, 1H, J = 2.0 Hz, C7-H), 6.56–6.52 (dd, 1H, J = 8.0, 2.0 Hz, C5-H), 4.44–4.39 (dd, 1H, J = 10.0, 5.0 Hz, NC*H*H), 4.34–4.28 (dd, 1H, J = 10.0, 7.0 Hz, NCH*H*), 4.17–4.10 (dd, 1H, J = 11.0, 9.5 Hz, C*H*HHO), 3.92–3.87 (dd, 1H, J = 11.0, 5.0 Hz, CH*H*O), 3.81–3.72 (m, 1H, OCH_2CHCH_2N), 3.10 (s, 3H, OSO_2CH_3), 2.97 (s, 3H, NSO_2CH_3); EIHRMS calcd for $C_{11}H_{15}NO_6S_2$ 321.0341, found 321.0341.

5-Bromo-6-hydroxy-1-(methylsulfonyl)-3-[[(methylsulfonyl)oxy]methyl]indoline (15). To a solution of **14** (29.3 mg, 91 μmol) in tetrahydrofuran (1 mL) cooled to −25 °C under nitrogen were added *N*-bromosuccinimide (17 mg, 96 μmol) and concentrated sulfuric acid (1 drop) sequentially. The rest of the procedure is similar to that described for **6**. The product was recrystallized from dichloromethane affording **15** (16.8 mg, 42 μmol, 46% yield) as a light yellow powder: mp 163–165 °C; ^{1}H-NMR (acetone-d_6) 7.51 (d, 1H, J = 1.0 Hz, C4-H), 7.08 (s, 1H, C7-H), 4.50–4.45 (dd, 1H, J = 10.0, 5.0 Hz, NC*H*H), 4.40–4.34 (dd, 1H, J = 10.0, 7.0 Hz, NCH*H*), 4.21–4.14 (dd, 1H, J = 11.0, 9.5 Hz, C*H*HO), 3.95–3.90 (dd, 1H, J = 11.0, 5.0 Hz, CH*H*O), 3.87–3.79 (m, 1H, OCH_2CHCH_2N), 3.13 (s, 3H, OSO_2CH_3), 3.01 (s, 3H, NSO_2CH_3); EIHRMS calcd for $C_{11}H_{14}BrNO_6S_2$ 398.9446, found 398.9449.

6-Hydroxy-1-(methylsulfonyl)-3-[[(methylsulfonyl)oxy]-methyl]-5-nitroindoline (16). To a solution of **14** (29.3 mg, 91 μmol) in nitromethane (1 mL) cooled to 0 °C under nitrogen was added nitric acid (5 μL, 125 μmol). The rest of the procedure is similar to that described for **7**. The product was recrystallized from dichloromethane affording **16** (7.7 mg, 21 μmol, 23% yield) as yellow needles: mp 174–176 °C; ^{1}H-NMR (DMF-d_7) 8.18 (d, 1H, J = 1.0 Hz, C4-H), 7.07 (s, 1H, C7-H), 4.65–4.60 (dd, 1H, J = 10.0, 5.0 Hz, NC*H*H), 4.57–4.51 (dd, 1H, J = 10.0, 6.5 Hz, NCH*H*), 4.39–4.33 (dd, 1H, J = 10.5, 9.5 Hz, C*H*HO), 4.09–4.04 (dd, 1H, J = 10.5, 5.0 Hz, CH*H*O), 4.00–3.91 (m, 1H, OCH_2-C*H*CH_2N), 3.32 (s, 3H, OSO_2CH_3), 3.28 (s, 3H, NSO_2CH_3); EIHRMS calcd for $C_{11}H_{14}N_2O_8S_2$ 366.0192, found 366.0188.

6-(*tert*-Butyldimethylsiloxy)-1-(methylsulfonyl)-3-[[(methylsulfonyl)oxy]methyl]indoline (17). *tert*-Butyldimethylsilyl chloride (25 mg, 166 μmol) was added to a solution of **14** (58.5 mg, 182 μmol) and imidazole (50 mg, 735 μmol) in dimethylformamide (1 mL). The rest of the procedure is similar to that described for **9**. The product was purified by flash chromatography using ethyl acetate and hexane (1/2, v/v) as an eluent affording **17** (68.2 mg, 157 μmol, 86% yield) as a colorless oil: ^{1}H-NMR ($CDCl_3$, ppm) 7.10–7.07 (d, 1H, J = 8.5 Hz, C4-H), 6.96–6.95 (d, 1H, J = 2.0 Hz, C7-H), 6.56–6.52 (dd, 1H, J = 8.0, 2.0 Hz, C5-H), 4.37–4.33 (dd, 1H, J = 10.0, 5.0 Hz, NC*H*H), 4.23–4.17 (d, 1H, J = 10.0, 8.0 Hz, NCH*H*), 4.09–4.02 (dd, 1H, J = 11.0, 9.0 Hz, C*H*HO), 3.98–3.93 (dd, 1H, J = 11.0, 4.5 Hz, CH*H*O), 3.75–3.68 (m, 1H, OCH_2CHCH_2N), 2.98 (s, 3H, OSO_2CH_3), 2.91 (s, 3H, NSO_2CH_3), 0.98 (s, 9H, $SiC(CH_3)_3$), 0.20 (s, 6H, $Si(CH_3)_2$); EIHRMS calcd for $C_{17}H_{29}NO_6S_2Si$ 435.1207, found 435.1208.

***N*-(Methylsulfonyl)-1,2,7,7a-tetrahydrocycloprop[*c*]indol-4-one (18). Method A.** To a solution of **17** (17 mg, 39 μmol) in tetrahydrofuran (7 mL) was added a 1 M solution of tetrabutylammonium fluoride (35 μL, 35 mmol) in tetrahydrofuran, and the reaction mixture was stirred at room temperature for 20 min. The solvent was removed at room temperature. The product is not stable to chromatographic purification (SiO_2). Therefore, the residue was extracted with hexane (2 mL × 2) and then with ethyl ether (2 mL × 10). Most of the desired product has been extracted by ether. The solvent was removed by blowing nitrogen into the flask. An 8.1-mg (36 mmol, 92%) yield of pure white foam was left in the flask.

Method B. To a solution of **9** (11.6 mg, 31 μmol) in tetrahydrofuran (6 mL) was added 1 M solution of tetrabutylammonium fluoride (30 μL, 30 mmol) in tetrahydrofuran, and the reaction mixture was stirred at room temperature for 20 min. The solvent was removed at room temperature. Two products were found from the reaction by TLC, one is the desired **18** and another is **5**. This is confirmed by TLC with authentic **18** and **5** as well as by mass spectroscopy. The ratio of **18** to **5** is approximately 3 to 2 on TLC. These two compounds cannot be separated by simple ether extraction: ^{1}H-NMR ($CDCl_3$, ppm) 6.55–6.52 (d, 1H, J = 10.0 Hz, C6-H), 6.43–6.39 (dd, 1H, J = 10.0, 1.5 Hz, C5-H), 6.32 (d, 1H, J = 1.5 Hz, C3-H), 4.08–4.05 (d, 1H, J = 10.0 Hz, C1-*H*H), 3.97–3.92 (dd, 1H, J = 10.0, 5.0 Hz, C1-H*H*), 3.05 (s, 3H, SO_2CH_3), 2.66–2.60 (dt, 1H, J = 7.7, 5.0 Hz, C7a-H), 1.80–1.76 (dd, 1H, J = 8.0, 4.5 Hz, C7-*H*H), 1.53–1.50 (t, 1H, J = 5.0 Hz, C7-H*H*); EIHRMS calcd for $C_{10}H_{11}NO_3S$ 225.0460, found 225.0457.

6-(Benzyloxy)-5-bromo-1-(methylsulfonyl)-3-[[(methylsulfonyl)oxy]methyl]indoline (19). To a solution of **3** (300 mg, 730 μmol) in tetrahydrofuran (10 mL) cooled to −25 °C under nitrogen were added *N*-bromosuccinimide (143 mg, 803 μmol) and concentrated sulfuric acid (1 drop) sequentially. The rest of the procedure is similar to that described for **6**. The product was recrystallized from ethyl acetate affording **19** (356 mg, 730 μmol, 100% yield) as a brown powder: mp 151–153 °C; ^{1}H-NMR ($CDCl_3$, ppm) 7.50–7.32 (m, 6H, C4-H, C_6H_5), 7.13 (s, 1H, C7-H), 5.20 (s, 2H, $OCH_2C_6H_5$), 4.35–4.31 (dd, 1H, J = 10.0, 5.0 Hz, NC*H*H), 4.22–4.16 (dd, 1H, J = 10.0, 7.5 Hz, NCH*H*), 4.09–4.02 (dd, 1H, J = 11.0, 9.0 Hz, C*H*HO), 3.97–3.92 (dd, 1H, J = 11.0, 4.5 Hz, CH*H*O), 3.76–3.67 (m 1H, OCH_2CHCH_2N), 3.00 (s, 3H, OSO_2CH_3), 2.78 (s, 3H, NSO_2CH_3); EIHRMS calcd for $C_{18}H_{20}BrNO_6S_2$ 488.9916, found 488.9904.

6-(Benzyloxy)-1-(methylsulfonyl)-3-[[(methylsulfonyl)oxy]methyl]-5-nitroindoline (20). To a solution of **3** (100 mg, 243 μmol) in nitromethane (3 mL) cooled to 0 °C under nitrogen was added nitric acid (14 μL, 350 μmol). The rest of the procedure is similar to that described for **7**. The product was recrystallized from ethyl acetate affording **20** (82 mg, 180 μmol, 74% yield) as a yellow powder: mp 154–155 °C; ^{1}H-NMR (acetone-d_6) 8.08 (d, 1H, J = 1 Hz, C4-H), 7.55–7.32 (m, 5H, C_6H_5), 7.31 (s, 1H, C7-H), 5.36 (s, 2H, $OCH_2C_6H_5$), 4.57–4.52 (dd, 1H, J = 10.0, 5.0 Hz, NC*H*H), 4.50–4.45 (dd, 1H, J = 10.0, 6.5 Hz, NCH*H*), 4.35–4.28 (dd, 1H, J = 10.5, 10.0 Hz, C*H*HO), 4.08–4.03 (dd, 1H, J = 11.0, 5.0 Hz, CH*H*O), 3.99–39.92 (m, 1H, OCH_2CHCH_2N), 3.13 (s, 3H, OSO_2CH_3), 3.08 (s, 3H, NSO_2CH_3); EIHRMS calcd for $C_{18}H_{20}N_2O_8S_2$ 456.0662, found 456.0663.

6-(*tert*-Butyldimethylsiloxy)-5-bromo-1-(methylsulfonyl)-3-[[(methylsulfonyl)oxy]methyl]indoline (21). To a solution of **17** (24 mg, 55 μmol) in tetrahydrofuran (1 mL) cooled to −25 °C under nitrogen were added *N*-bromosuccinimide (10 mg, 56 μmol) and concentrated sulfuric acid (1 drop) sequentially. The rest of the procedure is similar to that described for **6** affording **21** (28 mg, 54 μmol, 98% yield) as a brown oil: ^{1}H-NMR ($CDCl_3$, ppm) 7.48 (s, 1H, C4-H), 7.03 (s, 1H, C7-H), 4.35–4.30 (dd, 1H, J = 10.0, 5.0 Hz, NC*H*H), 4.22–4.16 (dd, 1H, J = 10.0, 7.5 Hz, NCH*H*), 4.09–4.03 (dd, 1H, J = 11.0, 9.5 Hz, C*H*HO), 3.95–3.91 (dd, 1H, J = 11.0, 4.5 Hz, CH*H*O), 3.75–3.67 (m, 1H, OCH_2CHCH_2N), 3.00 (s, 3H, OSO_2CH_3), 2.90 (s, 3H, NSO_2CH_3), 1.01 (s, 9H, $SiC(CH_3)_3$), 0.26 (s, 6H, $Si(CH_3)_2$); EIHRMS calcd for $C_{17}H_{28}BrNO_6S_2Si$ 513.0311, found 513.0315.

The Kinetics of Drug–DNA Reaction and DNA-Cleavage Studies.[28] Buffers used and their abbreviations are as follows. TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. F_{12} (fluorescence assay solution, pH 12): 0.02 M K_3PO_4, 0.5 mM EDTA and 0.15 μg/mL ethidium bromide). TBE: 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA. PM2 DNA was purchased from Boehringer Mannheim, which was used in the experiment without further purification (ethidium bromide assay showed that it contained 80% of the covalent closed circular DNA). Fluorescence was recorded on a Turner Model 430 spectrofluorometer.

Drugs were dissolved in DMF and the stock solutions were stored at −20 °C. The stock solution was diluted to the required concentrations using TE buffer immediately before use. For the kinetics study, 1 A_{260} of DNA and the drugs were incubated at 80 μmol concentration in 100 μL of TE solution at room temperature, and 10 μL of the reaction mixture was pipetted into 2 mL of F_{12} solution at 0, 15, 45, 75, and 120 min and 12 h, respectively. The fluorescence was first recorded at room temperature, and the solution was then heated at 95 °C for 5 min to effect thermoinduced cleavage of drug-bonded DNA and cooled to room temperature for the second reading. For the gel analysis, 0.7 A_{260} of DNA was incubated with drugs at varying concentrations in 15 mL of TE solution for 20 h at room temperature. The reaction mixture was then heated at 95 °C for 5 min to induce the DNA cleavage. The resultant reaction mixture was examined by electrophoretic mobility shift assay through 5.6-mm 0.8% agarose gel with TBE as the running buffer. The gels were run at room temperature under the voltage of 3.33 V/cm for 18 h. The resulting gels were stained with ethidium bromide in water at a concentration of 0.5 μg/mL. Bands were visualized under 300-nm UV transillumination and photographed on Polaroid 667 film.

DNA Sequence Specificity Determination. EcoRI digested pBR322 DNA was labeled at the 5′-end using γ-^{32}P-ATP, calf intestinal alkaline phosphatase, and T_4 polynucleotidase. The resultant fragment was digested with Hind III. The resulting 4332 and 31 base pair fragments were not separated prior to the alkylation reactions. Various dilutions of (+)-CC-1065 and analogues were added to aliquots of 5′-^{32}P-labeled DNA fragments and incubated at 37 °C for 24 h, followed by ethanol precipitation to terminate the reaction. After drying the samples, the tubes were heated to 100 °C for 30 min to induce cleavage at alkylation sites.[29]

After drying the samples again, the drug–DNA adducts were resuspended in 100 μL of 10 M piperidine and heated in a sealed tube at 90 °C for 30 min. The sample was lyophilized and resuspended in formamide loading buffer.[30] Control lanes of DNA fragments were treated for G-residues.[31] Samples were preheated to 90 °C before loading on thin denaturing polyacrylamide gels and electrophoresed at 1650 V. The gel was dried (BioRad Model 483 slab dryer) and autoradiographed at −70 °C using Kodak X-Omat AR film. The resulting autoradiogram was scanned on an LKB Ultroscan XL laser densitometer.

Cytotoxicity Assay. The cytotoxicities of the compounds to KB cells were determined by a modified crystal violet assay developed by Gillies *et al.*[32] KB cells were cultivated in Eagles minimum essential medium supplemented with 10% calf serum and incubated at 37 °C in a humidified 5% CO_2 atmosphere to prepare a cell stock. Cells were counted using a Neubauer hemocytometer and seeded in 96-well plates at 100 μL of 3×10^3 cells/mL and cultured for 1 day. Test compounds were added in 100 μL of solution with increasing concentrations in triplicate wells. Control wells were identical except that the test compound was absent. The cells were cultured for 3 days and then fixed by addition of 20 μL of 25% glutaraldehyde for 15 min, washed with water, and dried. The cells were then stained with 100 μL of 0.05% crystal violet for 15 min, washed with water, and dried. The wells were eluted with 100 μL of 0.05 M NaH_2PO_4/ethanol (1:1, v/v) and read on a multiscan spectrophotometer at a wavelength of 450 nm. The IC_{50} values were determined by plotting the drug concentration *versus* the cell viability.

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