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# AN ALTERNATIVE AND CONVENIENT STRATEGY FOR GENERATION OF SUBSTANTIAL QUANTITIES OF SINGLY 5′-$^{32}$P-END-LABELED DOUBLE-STRANDED DNA FOR BINDING STUDIES: DEVELOPMENT OF A PROTOCOL FOR EXAMINATION OF FUNCTIONAL FEATURES OF (+)-CC-1065 AND THE DUOCARMYCINS THAT CONTRIBUTE TO THEIR SEQUENCE-SELECTIVE DNA ALKYLATION PROPERTIES

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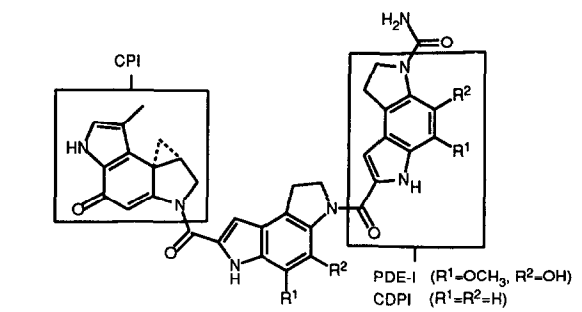
**ABSTRACT.** Development of an alternative strategy for securing substantial quantities of singly 5′-$^{32}$P-end-labeled double-stranded DNA suitable for binding studies is described based on M13 cloning techniques and offers advantages of production of replenishable quantities of singly 5′-$^{32}$P-end-labeled double-stranded DNA of homogeneous length without need for DNA isolation (restriction fragment), dephosphorylation, and lengthy preparative gel electrophoresis procedures. The $^{32}$P label is introduced onto the free 5′-hydroxyl group of a chemically synthesized universal primer [5′-$^{32}$P-d(GTAAAACGACGGCCAGT)-3′] which is used to initiate DNA synthesis on M13-derived single-stranded DNA templates. Following DNA synthesis, a restriction enzyme cleavage reaction produces a uniform length duplex suitable for agent binding studies. The strategy further permits the use of the Sanger dideoxynucleotide sequencing technique for direct and unambiguous identification of cleavage sites introduced by an agent on the end-labeled DNA. The use of the procedure in the examination of the DNA alkylation properties of (+)-CC-1065 (1) and a series of synthetic analogs is reviewed. From these studies a refined definition of the alkylation selectivity of (+)-CC-1065 is detailed. Employing agents possessing the parent 1,2,7,7a-tetrahydrocycloprop[1,2-*c*]indol-4-one (CI) alkylation subunit constituting the minimum pharmacophore of the CC-1065 alkylation subunit (CPI), comparative DNA alkylation studies illustrate that the activated cyclopropane is not obligatory for observation of the CI/CPI characteristic alkylation, highlight the relative nonselectivity of the alkylation event in the absence of noncovalent binding selectivity, illustrate a prominent role for agent binding selectivity for agents that possess such capabilities, and demonstrate that a sequence dependent autocatalytic phosphate activation of the alkylation event may not be uniquely responsible for the nonselective or selective alkylations. The ease with which the procedure may be extended to the rapid and convenient examination of additional agents is illustrated with the demonstration of the strikingly similar DNA alkylation properties of the duocarmycins (3-8) and (+)-CC-1065 (1) which suggest that the agents may be acting by a common mechanism.

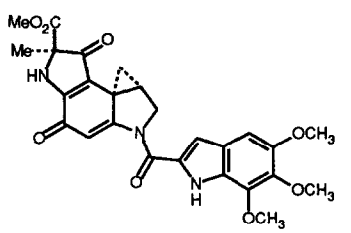
**INTRODUCTION.** (+)-CC-1065 (1, NSC-298223),[1-2] an antitumor antibiotic isolated from cultures of *Streptomyces zelensis*,[3] has been shown to exhibit exceptionally potent in vitro cytotoxic activity, broad spectrum antimicrobial activity, and confirmed in vivo antitumor activity.[4] In a series of extensive investigations the site and mechanism of the (+)-CC-1065 antitumor activity have been related to its selective alkylation of minor groove sites that has been demonstrated to proceed by 3′-adenine *N*-3 alkylation of the activated cyclopropane found in the CC-1065 left-hand subunit (CPI).[4-6] The early demonstration that (+)-*N*-acetyl-CPI exhibits a comparable albeit substantially less intense (ca. 10000x) alkylation of DNA has led to the conclusion that the alkylation subunit plays the dominant role in controlling the properties of the agents.[4-5] However, the recent demonstration that simplified agents including CDPI$_3$ methyl ester exhibit a substantial preference for A-T rich minor groove noncovalent binding (-$\Delta\Delta G^\circ$ poly(dA)·poly(dT) or poly[d(A-T)]·poly[d(A-T)] versus poly(dG)·poly(dC) = 2.6-2.2 kcal)[7] and the results of more recent studies[8-14] have suggested that the extent of the selective noncovalent DNA binding properties of the agents may not have been fully recognized in early studies.[4-6] In these and continued

efforts to address the origin of the sequence-selective DNA alkylation by (+)-CC-1065, we have examined the DNA alkylation properties of a series of agents now available for evaluation[8-16] including the structurally related duocarmycins.[15-16]

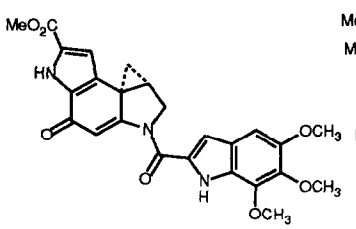
Several techniques have been introduced to study the sequence specificity of DNA binding agents and proteins at high resolution[17] that include the footprinting techniques employing methidiumpropyl-EDTA Fe(II),[18] DNase I,[19] or EDTA·Fe(II),[20] as well as affinity cleavage,[21] exonuclease III digestions,[22] or direct/induced strand cleavage by the agents under study.[23] All such techniques depend on the use of single 3′- or 5′-$^{32}$P-end-labeled DNA for visualization of the initial and modified fragments of DNA through autoradiography after high resolution gel electrophoresis and the current protocols for preparing double-stranded DNA selectively labeled at one end require considerable effort and time. For example, 5′-end-labeling requires preparation of large quantities of plasmids harboring the fragment of interest, plasmid cleavage with an appropriate restriction enzyme, dephosphorylation and subsequent radiolabeled phosphorylation steps, an additional restriction enzyme cleavage reaction, fragment isolation by preparative gel electrophoresis,[19,24-25] and the subsequent generation of sequencing standards requires performing labor intensive chemical cleavage (degradation) reactions.[24] Thus, although the current procedures are powerful for an initial evaluation of the DNA binding properties of an agent, the preparative requirements make them less practical for use in the evaluation of an extensive series of modified agents or when employing less accessible double-stranded DNA. Herein, we provide full details of an alternative strategy for the preparation of singly 5′-end-labeled double-stranded DNA suitable for use in DNA binding studies based on M13 cloning techniques[27] and review its use in a comparative study of the DNA alkylation properties of (+)-CC-1065 (**1**), a series of synthetic analogs of CC-1065,[8-14] and the duocarmycins (**3-8**).[15-16]



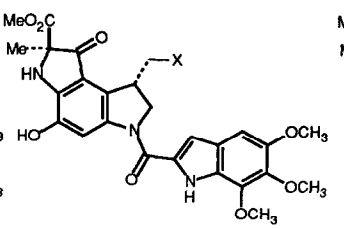

**1** $R^1$=$OCH_3$, $R^2$=OH (+)-CC-1065
**2** $R^1$=$R^2$=H (+)-CPI·$CDPI_2$



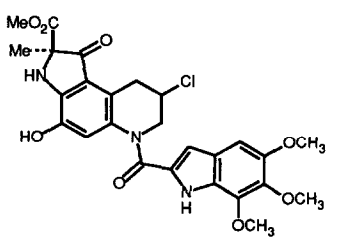
**3** duocarmycin A



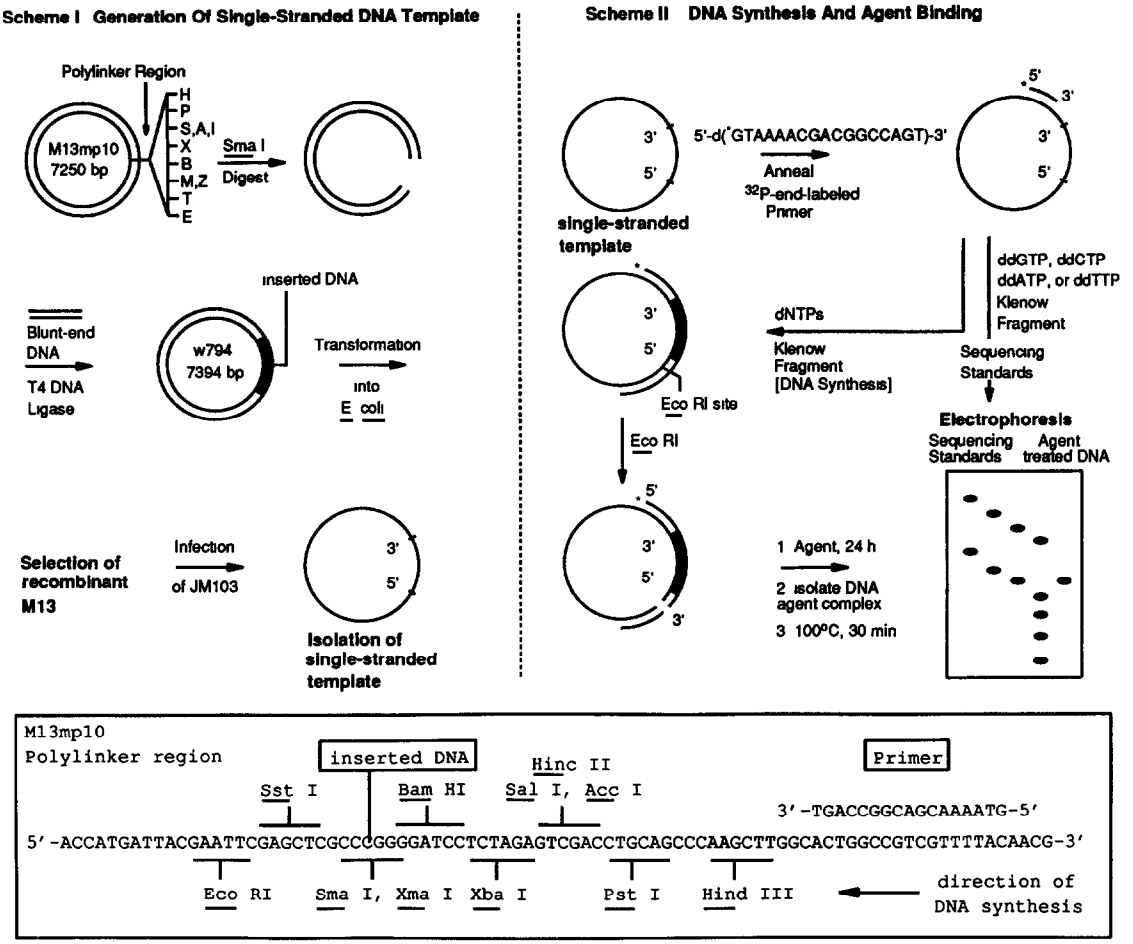
**4** duocarmycin SA



**5** duocarmycin $C_2$ X = Cl
**6** duocarmycin $B_2$ X = Br



**7** duocarmycin $C_1$ X = Cl
**8** duocarmycin $B_1$ X = Br




## RESULTS AND DISCUSSION

**Principal of the Method.** The bacteriophage M13 cloning systems offer a number of technical advantages that make them suitable for cloning desired DNA fragments and generation of singly end-labeled double-stranded DNA. Simian virus 40 (SV40) nucleosomal DNA fragments (140 to 160 base-pairs long) were prepared and cloned into the *Sma* I site of M13mp10 replicative intermediate, Scheme I. Transformation of competent cells yielded a library of phage particles containing various regions of SV40 DNA.[28-30] Such a shotgun approach is not necessary if a specific DNA fragment is needed for binding studies since it can be prepared and introduced directly into the desired M13 derivative vector. In both cases, isolated phage plaques harboring the fragments of interest provide a replenishable source of single-stranded DNA templates for synthesis of double-stranded DNA.

Preparation of singly end-labeled double-stranded DNA for binding studies simply requires annealing 5'-end-labeled, chemically synthesized, universal primer to the template and extending the primer with the Klenow

fragment of DNA polymerase I, Scheme II. One of the major advantages of the chemically synthesized primer is that it contains a single free 5′-hydroxyl group that can be readily phosphorylated with $^{32}P$ using a kinase and $\gamma$-$^{32}P$ ATP. This can be contrasted with restriction fragment 5′-end-labeling which requires a dephosphorylation step prior to the radiolabeled phosphorylation reaction in a process that inherently leads to labeling of both 5′-ends of the restriction fragment requiring a second restriction enzyme cleavage reaction to release a singly 5′-end-labeled duplex fragment. In principle, it is possible to use the extended primer-template duplex in binding studies. However, the extension reactions yield unevenly terminated 3′-ends that produce an uneven pattern in gels for the duplex DNA standard and unreacted duplex DNA and thus interfere with quantitative analysis of the binding reactions. Such problems were eliminated by cleavage of the extended primer-template duplex prior to binding reactions with a restriction enzyme, for example *Eco* RI (Scheme II), at a site immediately following the inserted DNA and produced an end-labeled double-stranded DNA of homogeneous length. Concurrent with primer extension reactions, Sanger's sequencing reactions[31] can be performed on the template, Scheme II. The reactions provide an unambiguous source of standards for sequencing and avoid the necessity of carrying out chemical cleavage reactions for preparation of standards currently required in the use of end-labeled restriction fragments. In addition, it is customary to add excess carrier DNA to end-labeled restriction fragments in order to improve the reproducibility of binding studies and to dilute the relative extent of radiolabeled duplex DNA that is employed. We have found that even moderate care in reproducing the conditions of the primer-template extension reaction with Klenow fragment (temperature and reaction time) provides sufficient excess unlabeled duplex DNA liberated with the final restriction enzyme cleavage to reproducibly serve as carrier duplex DNA.

**Analysis of DNA Binding Reactions.** We elected to examine the DNA alkylation reaction of (+)-CC-1065 (**1**) and related agents[8-16] using SV40 DNA since comparative results were available from prior studies[4-6] From a library of phages harboring SV40 nucleosomal DNA, the clones containing the following inserts were selected for examination: w794 (SV40 nucleotide no. 5238-138) and its complement w836 (nucleotide no. 5189-91); c988 (nucleotide no. 4359-4210) and its complement c820 (nucleotide no. 4201-4356), and c1346 (nucleotide no. 1632-1782).[28] The w clones contain sequences that map to the SV40 regulatory region whereas the c clones map to the SV40 early genes[28-30] and include prototype sequences reactive toward (+)-CC-1065. For the studies detailed herein, demonstration of the site(s) and selectivity (relative intensity) of DNA alkylation by (+)-CC-1065 (**1**) and structurally-related agents was obtained from the thermally-induced strand cleavage of double-stranded DNA after exposure to the agents employing cleavage protocols introduced by the Hurley and Upjohn groups.[5,6] Thus, employing the singly 5′-$^{32}P$-end-labeled SV40 double-stranded DNA fragments derived from clones w794, w836, c988, c820, and c1346 as described in Scheme II, a range of concentrations of agent was incubated with the labeled DNA for 24 h (4°C or 37°C), unbound agent was removed by ethanol precipitation of the DNA, and a solution containing the agent:DNA covalent complex was warmed at 100°C (30 min) to induce strand cleavage at the sites of alkylation. Electrophoresis of the resulting DNA under denaturing conditions alongside the Sanger dideoxynucleotide sequencing reactions followed by autoradiography permitted the identification of the sites of DNA alkylation. Since the thermal treatment of DNA results in depurination and strand cleavage at the site of alkylation while the Sanger sequencing reactions result in base incorporation but halted chain elongation at the sequenced site, cleavage at nucleotide N (sequencing) represents alkylation at nucleotide N + 1. The gel legends included herein identify the alkylation site.

The 3′ end-heterogeneity in the gels (double bands) observed with the use of 5′-end-labeled DNA (not observed with 3′-end-labeled DNA) as detailed herein deserves special comment. The thermal cleavage of 5′-end-labeled DNA often leads to pairs of closely migrating bands in which the major band observed at higher molecular

weight presumably constitutes the thermal cleavage product still containing a modified sugar, presumably a 2,3-dehydro-2,3-dideoxyribose or subsequent derivative. The minor band observed at lower molecular weight has been assumed to constitute a subsequent β-elimination product with complete loss of the 3′ modified sugar and, as such, constitutes DNA containing a 3′-phosphate at the 5′ side of the strand break. Confirmation that these assumptions are at least valid was derived from the observation that this end heterogeneity may be removed by piperidine treatment providing a single cleavage product constituting the minor band at lower molecular weight that comigrates with the Maxam-Gilbert adenine reaction.[33,35] In addition, thermal treatment for longer than 30 min (100°C) has not led to additional detectable cleavage and suggests that the strand cleavage reaction is a simple function of the adenine N-3 alkylation unaffected by the nature of the alkylating agent.

Electrophoresis was carried out under conditions that maximize the resolution of SV40 DNA examined. Not shown in the gels is the remaining 5′-end 55 base-pairs (short fragment of end-labeled DNA) derived from the DNA of the M13mp10 clones. This 55 base-pair region, judiciously chosen [3′-d(CCCCTAGGAGATCTCAGCTGGACGTCGGGTTCGAACCGTGACCGGCAGCAAAATG*)-5′; * denote $^{32}$P end-label], contains a single (+)-CC-1065 alkylation site proximal to the 5′-end-label. The apparent complete consumption of radioactivity on the gels at high agent concentrations constitutes complete cleavage of all 5′-end-labeled double-stranded DNA at this site. The competitive alkylation at this single site does not affect the relative selectivity of the examined sites within the SV40 DNA especially at the relevant low agent concentrations constituting single alkylation events on the DNA but does ensure that the multiple alkylations of a segment of DNA results in cleavage to a single short fragment that is not examined on the gel. This ensures that the gel fade to a single short fragment of 5′-end-labeled DNA at high agent concentrations is not misinterpreted as increasing selectivity.

**(+)-CC-1065 and (+)-CPI-CDPI$_2$.** The autoradiograms comparing the (+)-CC-1065 (**1**) and (+)-CPI-CDPI$_2$ (**2**) sites of covalent modification within the SV40 regulatory region have been presented elsewhere[12] and these results along with those of the full comparative study are illustrated in Figure 1. In these studies, the profile of DNA alkylation by (+)-CC-1065 (**1**) and (+)-CPI-CDPI$_2$ (**2**) proved indistinguishable. A summary of the results of the examination of the (+)-CC-1065 /(+)-CPI-CDPI$_2$ DNA alkylation selectivity within the five segments of double-stranded DNA (750 base-pairs) studied is presented in Table 1. In each instance of the covalent alkylation sites detected through the thermally-induced strand cleavage, the site of alkylation proved to be adenine[5,6] flanked exclusively by two 5′-A or T bases and there proved to be a preference for the sequence of the three base-pairs that follows the order of 5′-(AA<u>A</u>)-3′ = 5′-(TT<u>A</u>) ≥ 5′-(TA<u>A</u>)-3′ > 5′-(AT<u>A</u>). In addition, (+)-CC-1065 and (+)-CPI-CDPI$_2$ exhibited a very strong but not requisite sensitivity to the fourth base-pair preferring an A or T versus G or C base in the fourth 5′-position and they exhibited a weaker sensitivity to the fifth base-pair again preferring an A or T base in the fifth 5′-position.

(+)-CC-1065/(+)-CPI-CDPI$_2$ Binding Directionality: 3′ → 5′
Selectivity: three base-pair selectivity = 5′-(AA<u>A</u>)-3′ = 5′-(TT<u>A</u>)-3′ ≥ 5′-(TA<u>A</u>)-3′ > 5′-(AT<u>A</u>)-3′
: fourth base-pair sensitivity = 5′-(A/TXX<u>A</u>)-3′ >> 5′-(G/CXX<u>A</u>)-3′
: fifth base-pair sensitivity = 5′-(A/TXXX<u>A</u>)-3′ > 5′-(G/CXXX<u>A</u>)-3′
· preceding base-pair sensitivity = 5′-(NNXX<u>A</u>Pu)-3′ ≥ 5′-(NNXX<u>A</u>Py)-3′
High Affinity Sequences: 5′-(T/AT/ATT<u>A</u>)-3′, 5′-(A/TA/TTA<u>A</u>)-3′, and 5′-(T/AAAA<u>A</u>)-3′ ≥ 5′-(T/ATAA<u>A</u>)-3′

This interpretation of the alkylation selectivity remains close to and further refines the initial sequence-selectivity attributed to the (+)-CC-1065 DNA alkylation[4] but differs somewhat from the more recent interpretation

Table 1. Summary of Covalent Alkylation Sites for (+)-CC-1065/(+)-CPI-CDPI$_2$.

| Sequence | no. AS/no. TS[a] | High Affinity[b] | Low Affinity[b] |
|---|---|---|---|
| 5′-(NNAAAN)-3′ | 25/39 (64%) | 10 (26%) | 15 |
| 5′-(NNTTAN)-3′ | 12/19 (63%) | 7 (37%) | 5 |
| 5′-(NNTAAN)-3′ | 9/19 (47%) | 4 (22%) | 5 |
| 5′-(NNATAN)-3′ | 4/18 (22%) | 0 (00%) | 4 |
| 5′-(NNTTAN)-3′ | | | |
| 5′-(NATTAN)-3′ | 4/4 (100%) | 3 (75%) | 1 |
| 5′-(A/TATTAN)-3′ | 3/3 (100%) | 3 (100%) | 0 |
| 5′-(G/CATTAN)-3′ | 1/1 (100%) | 0 (00%) | 1 |
| 5′-(NTTTAN)-3′ | 5/6 (83%) | 4 (67%) | 1 |
| 5′-(A/TTTTAN)-3′ | 4/4 (100%) | 4 (100%) | 0 |
| 5′-(G/CTTTAN)-3′ | 1/2 (50%) | 0 (00%) | 1 |
| 5′-(NG/CTTAN)-3′ | 3/9 (33%) | 0 (00%) | 3 |
| 5′-(NGTTAN)-3′ | 3/5 (60%) | 0 (00%) | 3 |
| 5′-(NCTTAN)-3′ | 0/4 (00%) | 0 (00%) | 0 |
| 5′-(NNTTAPu)-3′ | 4/6 (67%) | 3 (50%) | 1 |
| 5′-(NNTTAPy)-3′ | 8/13 (62%) | 4 (31%) | 4 |
| 5′-(NNAAAN)-3′ | | | |
| 5′-(NAAAAN)-3′ | 17/21 (81%) | 8 (38%) | 9 |
| 5′-(A/TAAAAN)-3′ | 13/14 (93%) | 6 (43%) | 7 |
| 5′-(G/CAAAAN)-3′ | 4/7 (57%) | 2 (29%) | 2 |
| 5′-(NTAAAN)-3′ | 4/6 (67%) | 1 (17%) | 3 |
| 5′-(A/TTAAAN)-3′ | 3/4 (75%) | 1 (25%) | 2 |
| 5′-(G/CTAAAN)-3′ | 1/2 (50%) | 0 (00%) | 1 |
| 5′-(NG/CAAAN)-3′ | 4/12 (33%) | 1 (08%) | 3 |
| 5′-(NGAAAN)-3′ | 1/5 (20%) | 0 (00%) | 1 |
| 5′-(NCAAAN)-3′ | 3/7 (43%) | 1 (14%) | 2 |
| 5′-(NNAAAPu)-3′ | 19/30 (63%) | 8 (27%) | 11 |
| 5′-(NNAAAPy)-3′ | 6/9 (67%) | 2 (22%) | 4 |
| 5′-(NNTAAN)-3′ | | | |
| 5′-(NTTAAN)-3′ | 2/3 (67%) | 1 (33%) | 1 |
| 5′-(A/TTTAAN)-3′ | 1/1 (100%) | 1 (100%) | 0 |
| 5′-(G/CTTAAN)-3′ | 1/2 (50%) | 0 (00%) | 1 |
| 5′-(NATAAN)-3′ | 5/9 (56%) | 3 (33%) | 2 |
| 5′-(A/TATAAN)-3′ | 3/5 (60%) | 1 (20%) | 2 |
| 5′-(G/CATAAN)-3′ | 2/4 (50%) | 2 (50%) | 0 |
| 5′-(NG/CTAAN)-3′ | 2/6 (33%) | 0 (00%) | 2 |
| 5′-(NGTAAN)-3′ | 1/4 (25%) | 0 (00%) | 1 |
| 5′-(NCTAAN)-3′ | 1/2 (50%) | 0 (00%) | 1 |
| 5′-(NNTAAPu)-3′ | 5/9 (56%) | 2 (22%) | 3 |
| 5′-(NNTAAPy)-3′ | 4/10 (40%) | 2 (20%) | 2 |
| 5′-(NNATAN)-3′ | | | |
| 5′-(NAATAN)-3′ | 2/5 (40%) | 0 (00%) | 2 |
| 5′-(A/TAATAN)-3′ | 1/3 (33%) | 0 (00%) | 1 |
| 5′-(G/CAATAN)-3′ | 1/2 (50%) | 0 (00%) | 1 |
| 5′-(NTATAN)-3′ | 0/6 (00%) | 0 (00%) | 0 |
| 5′-(A/TTATAN)-3′ | 0/4 (00%) | 0 (00%) | 0 |
| 5′-(G/CTATAN)-3′ | 0/2 (00%) | 0 (00%) | 0 |
| 5′-(NG/CATAN)-3′ | 2/7 (29%) | 0 (00%) | 2 |
| 5′-(NGATAN)-3′ | 0/0 - | - | - |
| 5′-(NCATAN)-3′ | 2/7 (29%) | 0 (00%) | 2 |
| 5′-(NNATAPu)-3′ | 3/14 (21%) | 0 (00%) | 3 |
| 5′-(NNATAPy)-3′ | 1/4 (25%) | 0 (00%) | 1 |

[a]Number of alkylated sites (no. AS)/number of total sites (no. TS) available. [b]Intensity of alkylation at the sites, high = high affinity site observed at lowest agent concentrations, low = low affinity site observed at higher agent concentrations. The expressed % is that of the number of high affinity alkylation sites (no. HAAS)/number of total sites (no. TS) available.

provided by the Hurley and UpJohn groups.[33] As detailed elsewhere,[7,12] we attribute the strict A-T preference for the first three-base pairs to represent a combination of the initial 3′-adenine alkylation site and the required noncovalent stabilization provided by the agents central subunit and its two-base pair A-T binding selectivity.[7] Similarly, the strong preference for the A or T fourth base may be attributed to the 3.5 base-pair binding site size required to accommodate the minor groove binding of the agents first two subunits and the A-T binding preference that extends less strongly to the fourth base-pair.[12,34-35] As discussed elsewhere,[7] the right-hand subunit of the agents may not be required to be bound in the minor groove and consequently the A-T selectivity of the fifth base-pair proved to be weaker (5′-A/T > 5′-G/C) and may reflect, as in the high affinity sequences, the preferential but not required binding of the third subunit within the minor groove.[7,12] No sensitivity to the sixth 5′-base was detected in our examination of the data.

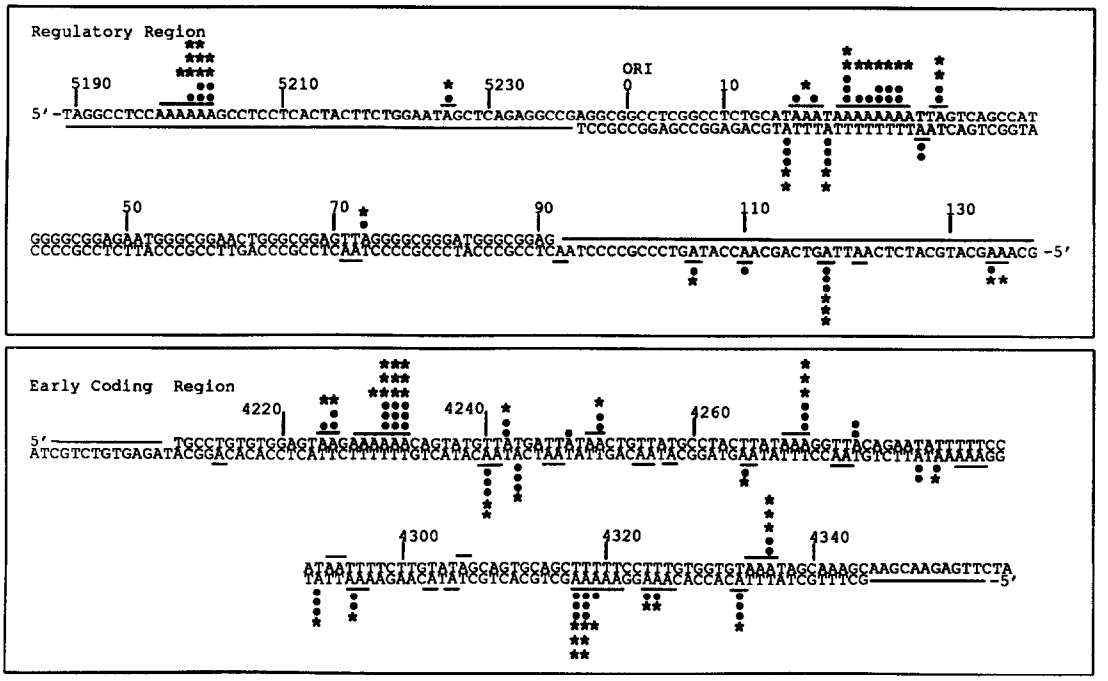



Figure 1. Summary of cleavage sites for (+)-1, (+)-2, (+)-3, and (±)-11b. • Represents cleavage by (+)-CC-1065 and (+)-CPI-CDPI$_2$ [(+)-1 and (+)-2], * represents cleavage by duocarmycin A, $C_1$-$C_2$ (3,5, and 7), and the underlined adenines represent alkylation sites for (±)-11b. The relative intensity of cleavage is shown by the number of symbols above a given site. The SV40 nucleotide numbering system is according to Tooze,[32] using the replication origin (ORI) as reference. Two regions of the SV40 genome were used for analysis: one region spans nucleotide no. 5189 to 138 and includes the replication origin and part of the SV40 regulatory sequences, the other includes a segment of the SV40 early genes and sequences representing strong agent cleavage sites. The data derived from c1346 is not shown.

**(+)-*N*-BOC-CPI, (+)-*N*-Acetyl-CPI, and (+)-CC-1065.** In initial studies, the DNA alkylation properties of (+)-**9** and (+)-**10**, simple derivatives of the authentic left-hand subunit of (+)-CC-1065, were compared to those of (+)-CC-1065, Figures 2-3. The CPI derivatives were shown to alkylate DNA when incubated at 4°C (24 h) but required concentrations of $10^5$ - $10^7$x that of (+)-CC-1065 for observation and the alkylation profile of (+)-**10**

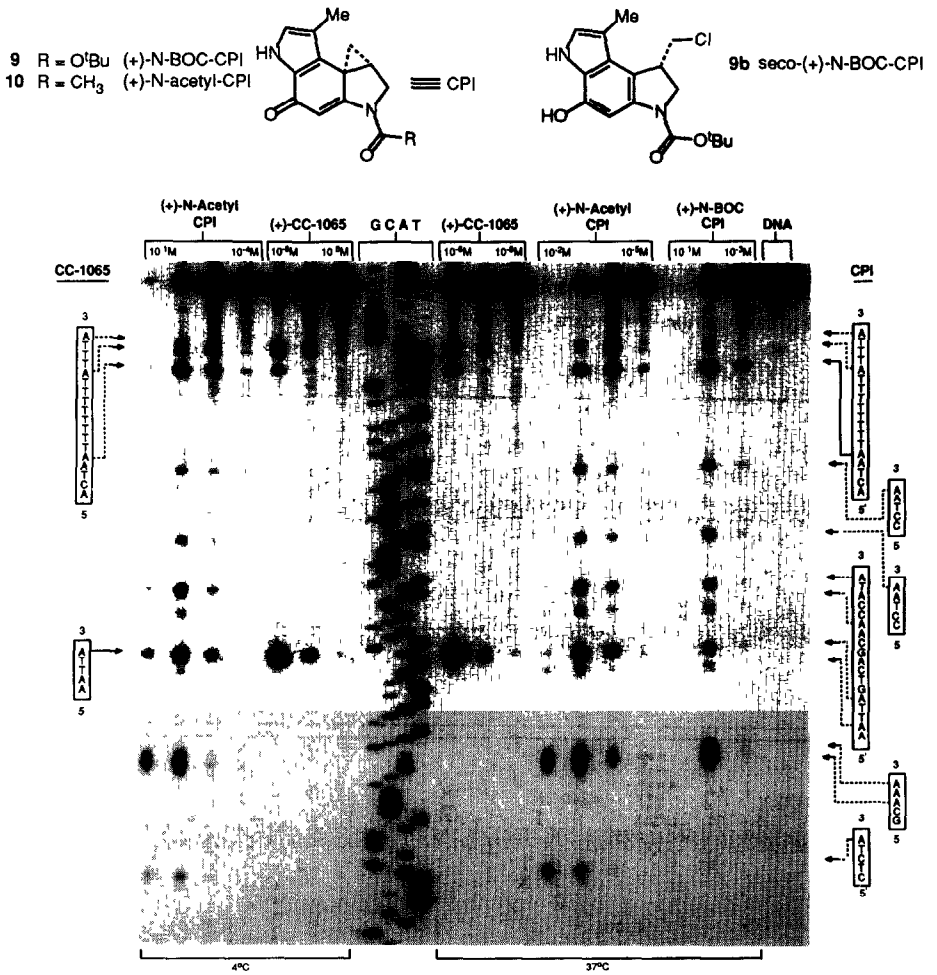



Figure 2. Thermally-induced strand cleavage of double-stranded DNA (SV40 fragment, 144 b.p.; nucleotide no. 138-5238, clone w794) after 24 h incubation of agent:DNA at 4°C or 37°C followed by removal of unbound agent and 30 min incubation at 100°C; 8% denaturing poly(acrylamide) gel and autoradiography. Lanes 1-4, (+)-*N*-acetyl-CPI (**10**, 4°C, 2.5 x $10^{-1}$ - 2.5 x $10^{-4}$ M); lanes 5-7, (+)-CC-1065 (**1**, 4°C, 1 x $10^{-6}$ - 1 x $10^{-8}$ M); lanes 8-11, Sanger G, C, A, and T reactions; lanes 12-14, (+)-CC-1065 (**1**, 37°C, 1 x $10^{-6}$ - 1 x $10^{-8}$ M); lanes 15-18, (+)-*N*-acetyl-CPI (**10**, 37°C, 2.5 x $10^{-2}$ - 2.5 x $10^{-5}$ M); lanes 19-21, (+)-*N*-BOC-CPI footprinted as *seco*-(+)-*N*-BOC-CPI (**9b**, 37°C, 2.5 x $10^{-1}$ - 2.5 x $10^{-3}$ M); lane 22, control DNA.

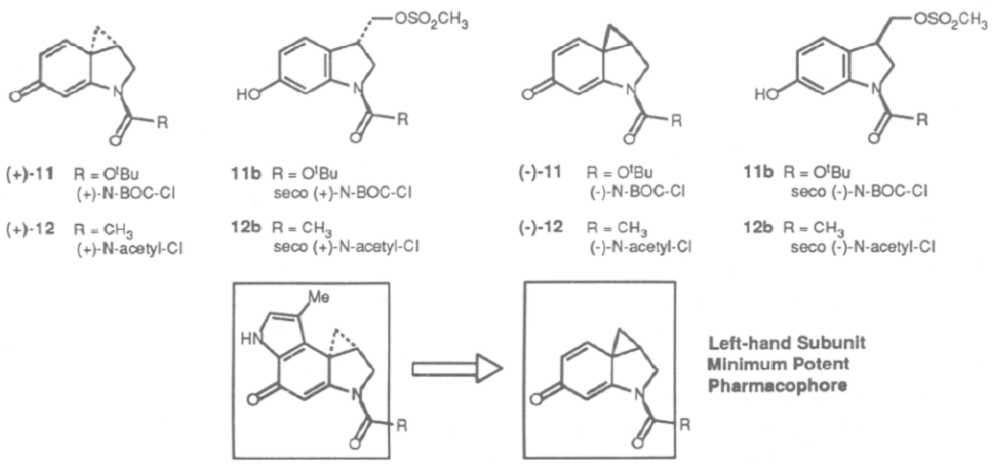
proved to be approximately 100x more intense than that of (+)-**9**. When (+)-**9** and (+)-**10** were incubated with DNA under more vigorous conditions (37°C, 24 h), the intensity of the DNA alkylation increased without significantly altering the selectivity. Even at 37°C, the thermally-induced DNA strand scission by (+)-**9** and (+)-**10** was weak requiring $10^4$ - $10^7$x the concentration of (+)-CC-1065. More significant was the recognition that the DNA alkylation selectivity for (+)-**9** and (+)-**10** proved distinct from that of (+)-CC-1065 (4°C or 37°C). While significant distinctions may be made between the two simple derivatives of CPI, the two agents alkylate the minor (+)-CC-1065 site of 5′-d(ACTAA)-3′ as their major alkylation site, a number of additional sites not alkylated by (+)-CC-1065 are alkylated, and the three (+)-CC-1065 high affinity sites [5′-d(AATTA)-3′ > 5′-d(ATTTA)-3′, 5′-d(TTTTA)-3′] constitute less prominent or minor alkylation sites for the simple CPI subunits. Thus, although the

comparable behavior of (+)-*N*-acetyl-CPI at low agent concentration (lane 18, Figure 2 or lane 16, Figure 3) and (+)-CC-1065 at high agent concentration (lane 12, Figure 2 or lane 6, Figure 3) have been interpreted to represent a demonstration of the comparable alkylation properties of the agents,[5-6,33] the examination of the full profile of DNA alkylation by the agents within w794 DNA has been interpreted by us to illustrate that the alkylation selectivity of the simple derivatives of (+)-CPI is less selective than and readily distinguished from that of (+)-CC-1065.[12]

**(±)-*N*-Acetyl-CI, (±)-*N*-BOC-CI, (+)-*N*-BOC-CI, and (-)-*N*-BOC-CI.** The examination of the DNA alkylation properties of simple derivatives of CI constituting the parent alkylation subunit of the (+)-CC-1065 left-hand segment proved similarly revealing. The DNA alkylation profile of racemic (±)-*N*-acetyl-CI (**12**) and (±)-*N*-BOC-CI (**11**) proved remarkably similar to that of (+)-*N*-acetyl-CPI (**10**, Figure 3) and (+)-*N*-BOC-CPI (**9**, Figures 2 and 5), respectively. Like *N*-acetyl-CPI versus *N*-BOC-CPI, *N*-acetyl-CI may be considered more selective than *N*-BOC-CI for the alkylation sites common with (+)-CC-1065.[33] Consistent with the relative reactivity of the CI versus CPI agents, the relative selectivity among the alkylation sites available to (±)-*N*-acetyl-CI or (±)-*N*-BOC-CI is lower than that observed with (+)-*N*-acetyl-CPI or (+)-*N*-BOC-CPI although the same alkylation sites are common to both classes of agents.[37] Consequently, the DNA alkylation selectivity for the simple alkylating subunit of the two classes of agents (CI versus CPI), while not being identical, has proven remarkably comparable. This demonstrated comparable behavior of simple derivatives of CI and CPI, the additional comparable behavior of more advanced analogs of CC-1065 containing the CI and CPI alkylation subunits, and the observation of cytotoxic activity with the CI-based agents served to demonstrate that CI constitutes the minimum pharmacophore[36] of the CC-1065 alkylation subunit (CPI).[9]

A comparison of the DNA covalent alkylation properties of (±)-*N*-BOC-CI (**11**) with racemic (±)-*seco*-*N*-BOC-CI (**11b**) revealed a comparable if not indistinguishable profile of covalent alkylation for the two agents, Figure 4 As in prior studies, this suggests that the agents such as **11b** and **12b** productively close to the cyclopropane agents prior to alkylation[4-6]or may serve as effective alkylating agents in their own right but display DNA alkylation properties comparable to the parent agents.[9,35] Importantly, this demonstration of the indistinguishable DNA alkylation properties of **11** and **11b** suggests that the stable agents **11b** may function in a manner comparable to the reactive and unstable CI agents. In addition, a comparison of the optically-active and racemic *seco*-*N*-BOC-CI agents revealed a nearly identical profile of alkylation for the three agents, Figure 5. As such, the sites of alkylation available to *N*-BOC-CI proved independent of the absolute configuration of the agent and suggest a relatively nondiscriminate alkylation event. Consistent with the relative reactivity of the electrophile, **11** and **11b** alkylate DNA under milder conditions (4°C versus 37°C) than (+)-*N*-BOC-CPI (**9**) but the observed alkylations proceed at comparable agent concentrations. A summary of results derived from the full examination of the alkylation selectivity of (±)-**11b** within 750 base-pairs of double-stranded DNA is provided in Table 2. In total, 40-45% of the adenines in the DNA examined are alkylated by (±)-**11b** within a single 10-fold concentration range. In each instance of the alkylation sites detected through the thermally-induced strand cleavage, the alkylation proved to be adenine flanked by a single 5'-A or T base with no apparent sensitivity to either sequence: 5'-(A<u>A</u>)-3' ≅ 5'-(T<u>A</u>)-3'.[38] In addition, (±)-**11b** exhibited a weak [5'-(A/TA<u>A</u>)-3' > 5'-(G/CA<u>A</u>)-3'] or nonapparent [5'-(A/TT<u>A</u>) ≅ 5'-(G/CT<u>A</u>)-3'] sensitivity to the third 5'-base, no sensitivity to the fourth - sixth 5'-bases, and a weak or nonapparent sensitivity to the 3'-base preceding the alkylation site. As such, the alkylation selectivity of (±)-*N*-BOC-CI proved distinct from that of (+)-CC-1065.

**(+)- and (-)-CI-CDPI$_1$, (+)- and (-)-CI-CDPI$_2$, and (+)-CC-1065.** The examination of the DNA alkylation profile of (+)- and (-)-CI-CDPI$_1$ (**13**) and (+)- and (-)-CI-CDPI$_2$ (**14**) versus that of (+)-CC-1065 proved



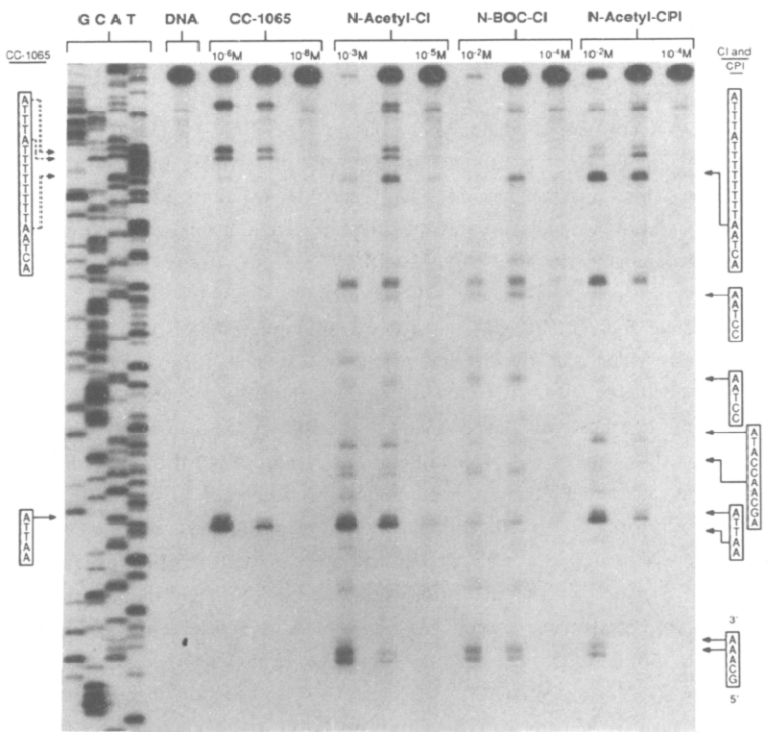




Figure 3. Thermally-induced strand cleavage of double-stranded DNA (SV40 DNA fragment, 144 b.p.; nucleotide no. 138-5238, clone w794) after 24 h incubation of agent:DNA at 4°C followed by removal of unbound agent and 30 min incubation at 100°C; 8% denaturing poly(acrylamide) gel and autoradiography. Lanes 1-4, Sanger G, C, A, and T reactions; lane 5, control DNA; lanes 6-8, (+)-CC-1065 ((+)-1, 1 x $10^{-6}$ - 1 x $10^{-8}$ M); lanes 9-11, (±)-*N*-acetyl-CI ((±)-**12b**, 2.5 x $10^{-3}$ - 2.5 x $10^{-5}$ M); lanes 12-14, (±)-*N*-BOC-CI ((±)-**11b**, 2.5 x $10^{-2}$ - 2.5 x $10^{-4}$ M); lanes 15-17, (+)-*N*-acetyl-CPI ((+)-**10**, 2.5 x $10^{-2}$ - 2.5 x $10^{-4}$ M).

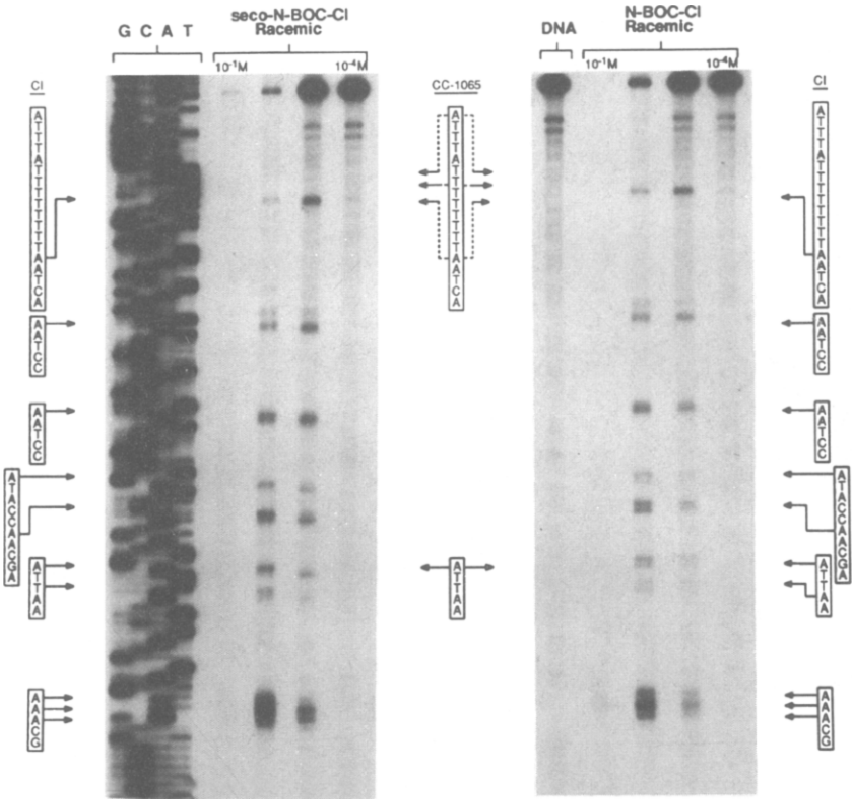



**Figure 4.** Thermally-induced strand cleavage of double-stranded DNA (SV40 DNA fragment, 144 b.p.; nucleotide no. 138-5238, clone w794) after 24 h incubation of agent:DNA at 4°C followed by removal of unbound agent and 30 min incubation at 100°C; 8 % denaturing poly(acrylamide) gel and autoradiography. Lanes 1-4, Sanger G, C, A, and T reactions; lanes 5-8, (+)-*seco*-*N*-BOC-CI (2.5 x $10^{-1}$ - 2.5 x $10^{-4}$ M); lane 9, control DNA; lanes 10-13, (+)-*N*-BOC-CI (2.5 x $10^{-1}$ - 2.5 x $10^{-4}$ M).

Table 2. Summary of Covalent Alkylation Sites for (+)-*N*-BOC-CI.

| Sequence | Total Sites | Alkylated Sites |
|---|---|---|
| 5′-(NNA<u>A</u>N)-3′ | 83 | 48 (58%) |
| 5′-(NAA<u>A</u>N)-3′ | 39 | 24 (61%) |
| 5′-(NTA<u>A</u>N)-3′ | 19 | 16 (84%) |
| 5′-(NGA<u>A</u>N)-3′ | 11 | 3 (27%) |
| 5′-(NCA<u>A</u>N)-3′ | 14 | 5 (36%) |
| 5′-(NNA<u>A</u>Pu)-3′ | 54 | 33 (61%) |
| 5′-(NNA<u>A</u>Py)-3′ | 29 | 15 (52%) |
| 5′-(NNT<u>A</u>N)-3′ | 53 | 22 (41%) |
| 5′-(NAT<u>A</u>N)-3′ | 18 | 13 (72%) |
| 5′-(NTT<u>A</u>N)-3′ | 19 | 3 (16%) |
| 5′-(NGT<u>A</u>N)-3′ | 7 | 4 (57%) |
| 5′-(NCT<u>A</u>N)-3′ | 9 | 2 (22%) |
| 5′-(NNT<u>A</u>Pu)-3′ | 29 | 19 (65%) |
| 5′-(NNT<u>A</u>Py)-3′ | 24 | 3 (12%) |

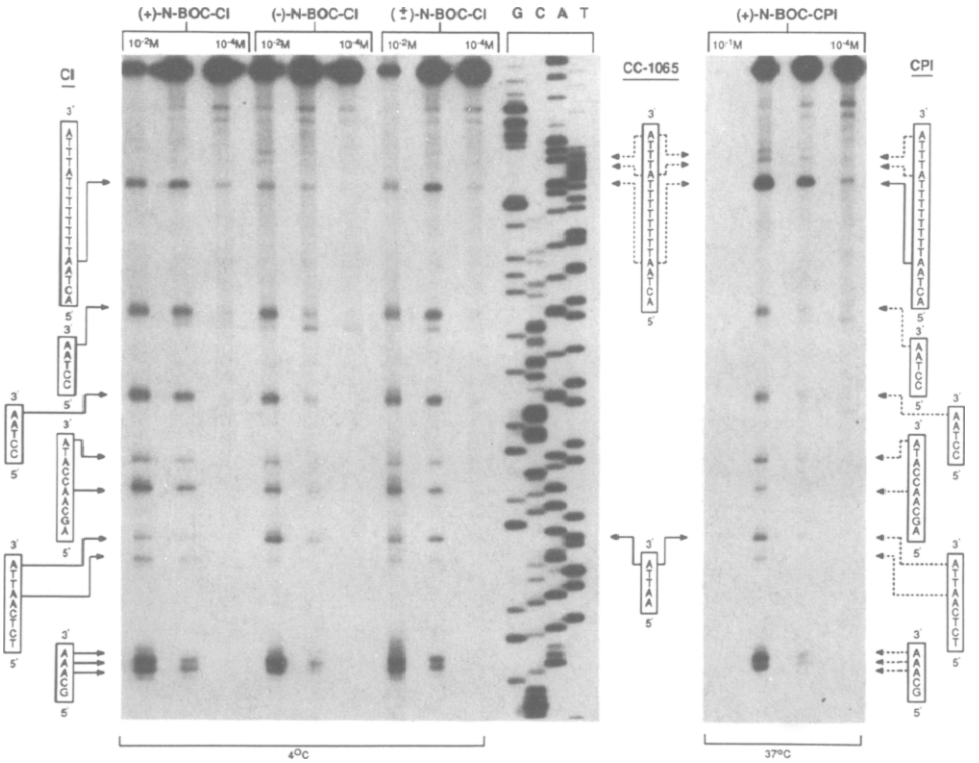


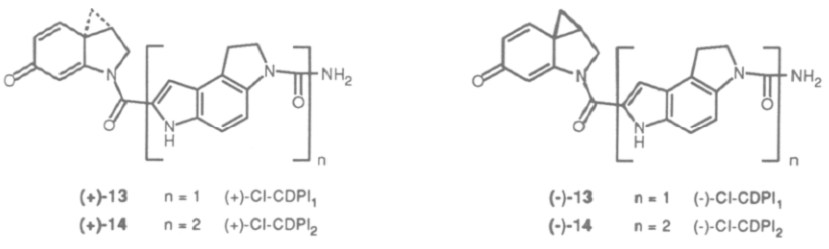

Figure 5. Thermally-induced strand cleavage of double-stranded DNA (SV40 DNA fragment, 144 b.p.; nucleotide no. 138-5238, clone w794) after 24 h incubation of agent:DNA at 4°C or 37°C followed by removal of unbound agent and 30 min incubation at 100°C; 8% denaturing poly(acrylamide) gel and autoradiography. Lanes 1-3, *seco*-(+)-*N*-BOC-CI ((+)-**11b**, 4°C, 1 x $10^{-2}$ - 1 x $10^{-4}$ M); lanes 4-6, *seco*-(-)-*N*-BOC-CI ((-)-**11b**, 4°C, 1 x $10^{-2}$ - 1 x $10^{-4}$ M); lanes 7-9, *seco*-(±)-*N*-BOC-CI ((±)-**11b**, 4°C, 1 x $10^{-2}$ - 1 x $10^{-4}$ M); lane 10-13, Sanger G, C, A, and T reactions; lanes 14-17, *seco*-(+)-*N*-BOC-CPI ((+)-**9b**, 37°C, 2.5 x $10^{-1}$ - 2.5 x $10^{-4}$ M).

to illustrate a prominent role for the noncovalent binding selectivity of the agents, Figure 6.[9] Like (+)-CC-1065, each of the agents exhibit a more selective alkylation of DNA than that of the parent, optically-active CI agents (Figure 5) that we have attributed to the restriction in the number of available alkylation sites by the noncovalent binding selectivity of the agents. The prominent sites of alkylation for (+)-CI-CDPI$_1$ and (+)- and (-)-CI-CDPI$_2$ proved to be the identical and prominent sites of alkylation for (+)-CC-1065. With the natural enantiomers, 100-1000x the concentration of (+)-CC-1065 was required for (+)-CI-CDPI$_2$ and (+)-CI-CDPI$_1$ to exhibit the comparable extent of DNA alkylation at 4°C presumably due to their relative stability to the assay conditions but their alkylation profiles proved to be remarkably similar to that of (+)-CC-1065. The single prominent alkylation site of (+)-CC-1065 within the 144 base-pairs of w794 DNA proved to be the high affinity site for (+)-CI-CDPI$_1$ and (+)-CI-CDPI$_2$ and the relative intensity and selectivity of the (+)-CI-CDPI$_2$ alkylation proved slightly greater than that of (+)-CI-CDPI$_1$ ((+)-CI-CDPI$_2$ > (+)-CI-CDPI$_1$, intensity and selectivity).

The alkylation profile for the unnatural enantiomer series of agents proved more distinct. (-)-CI-CDPI$_2$ exhibits a DNA alkylation profile that is decidedly more selective than that of (-)-*N*-BOC-CI (Figure 5), that proved essentially identical in selectivity and intensity to that of the natural enantiomer ((+)-CI-CDPI$_2$), and that

was remarkably comparable to that of (+)-CC-1065. In contrast, (-)-CI-CDPI$_1$ exhibits a DNA alkylation profile that is far more selective than that of (-)-*N*-BOC-CI (Figure 5) and that is unique from that of the natural enantiomers of (+)-CI-CDPI$_n$ or (+)-CC-1065.

Consequently, the incorporation of the reactive CI electrophile (Figure 5) into the optically-active agents CI-CDPI$_2$ provided agents with decidedly more intense and sequence-selective double-stranded DNA alkylation properties. We attribute the increased alkylation selectivity that is characteristic of (+)-CC-1065 and which is conveyed to even the exceptionally reactive CI electrophile to the prominent and preferential A-T rich minor groove noncovalent binding selectivity of the agents[7,9,12-14] which effectively restricts the number of available covalent alkylation sites.



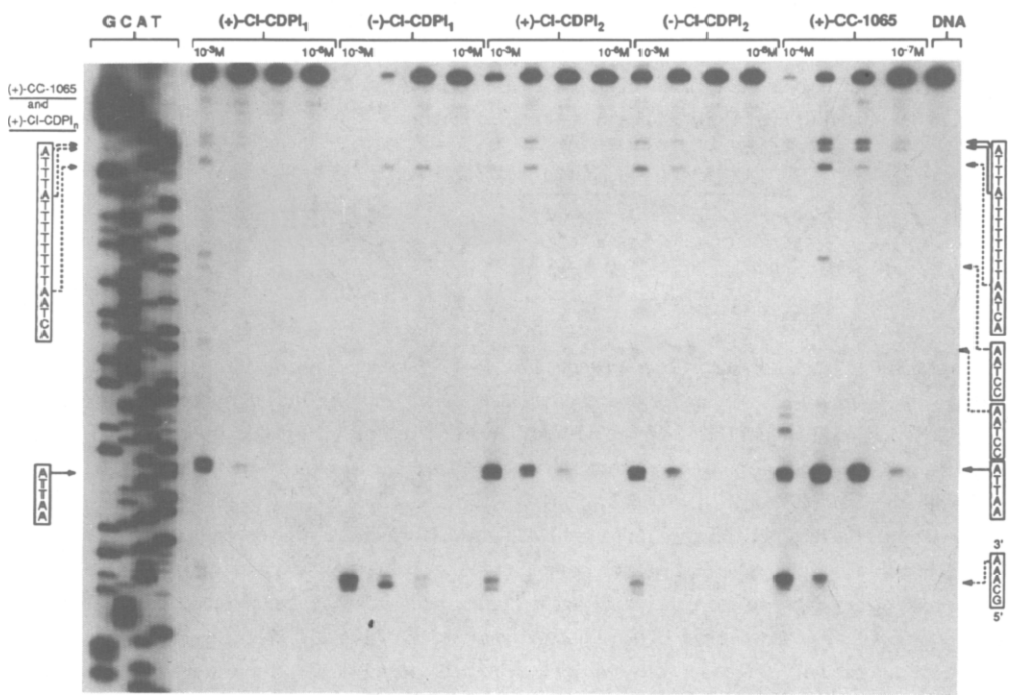
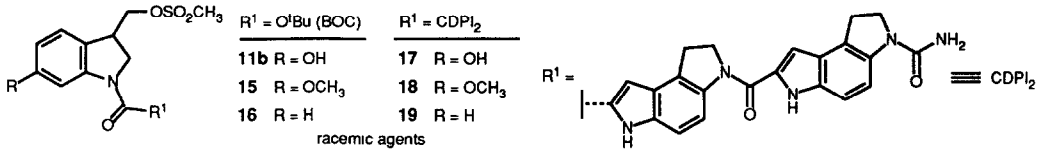




Figure 6. Thermally-induced strand cleavage of double-stranded DNA (SV40 fragment, 144 b.p.; nucleotide no. 138-5238, clone w794) after 24 h incubation of agent:DNA at 4°C followed by removal of unbound agent and 30 min incubation at 100°C; 8% denaturing poly(acrylamide) gel and autoradiography. Lanes 1-4, Sanger G, C, A, and T reactions; lanes 5-8, (+)-CI-CDPI$_1$ ((+)-**13**, 5.6 x $10^{-4}$ - 5.6 x $10^{-7}$ M); lanes 9-12, (-)-CI-CDPI$_1$ ((-)-**13**, 5.6 x $10^{-4}$ - 5.6 x $10^{-7}$ M); lanes 13-16, (+)-CI-CDPI$_2$ ((+)-**14**, 5.6 x $10^{-4}$ - 5.6 x $10^{-7}$ M); lanes 17-20, (-)-CI-CDPI$_2$ ((-)-**14**, 5.6 x $10^{-4}$ - 5.6 x $10^{-7}$ M); lanes 21-24, (+)-CC-1065 (**1**, 5.6 x $10^{-5}$ - 5.6 x $10^{-8}$ M); lane 25, control DNA.

***Seco*-CI Agents: Nature of the Alkylation Agent.** Past efforts have emphasized the unique involvement of the activated cyclopropane as the electrophilic functionality responsible for the selective DNA alkylation. In the instances of the observation of DNA alkylation properties with precursor (seco) agents lacking the preformed cyclopropane ring but which possess capabilities for cyclopropane closure, the implications have been that closure to the cyclopropane precedes observation of the sequence-selective DNA alkylation and is the putative agent responsible for the biological activity. The facile closure of the agents examined to date (CPI precursors) supported this expectation that the Ar-3′ alkylation with closure to the cyclopropane agents could be projected to occur under the conditions of assay.

In addition, in the course of the continued evolution of the studies of the Hurley and UpJohn groups and our own more recent complementary studies, distinctions in the extent of the (+)-CC-1065 alkylation selectivity and its potential structural origin have been detailed.[12,33] Most prominent among these distinctions has been the extent of the role that the (+)-CC-1065 noncovalent binding selectivity may play in determining the DNA alkylation selectivity. For the natural enantiomer, we have suggested a prominent role for the binding selectivity that may be expected to occur preferentially within the narrower, sterically more accessible A-T rich minor groove versus the wider, sterically less accessible G-C rich minor groove.[9] In contrast, the UpJohn and Hurley groups have suggested that the (+)-CC-1065 alkylation constitutes a sequence dependent covalent alkylation essentially independent of binding selectivity.[33] This has been suggested to be the result of a (Lewis) acid-catalyzed DNA alkylation requiring autocatalytic activation of the alkylation reaction (carbonyl complexation or protonation) by a strategically located phosphate in the DNA backbone two base-pairs removed from the alkylation site in the 5′ direction. In efforts to resolve these distinctions, we have examined the DNA alkylation properties of a range of seco agents **15-19** related to the CI agents **11** and **14** detailed herein.




The profiles of DNA alkylation for the agents **11b, 15-19**[39] were examined with w794 DNA and the results are illustrated in Figures 7-8. A number of important conclusions may be drawn from the comparative study. The DNA alkylation profiles of **11b, 15-16** are identical (Figure 7), indistinguishable from that of (±)-*N*-BOC-CI (**11**, Figure 4), and independent of the absolute configuration of the agent ((+)-**11b** = (-)**11b** = (±)-**11b**, Figure 5). Consistent with the reactivity of **11** ($t_{1/2}$ = 5.2 h, pH = 7; 35 sec, pH = 3), we have interpreted this profile of covalent alkylation as a relatively nondiscriminant alkylation event which has proven comparable to that of (+)-*N*-BOC-CPI (common alkylation sites, less selectivity among the available sites, Figures 4-5). The methyl ether **15** and the agent **16** cannot close to the putative CI agent **11** illustrating that the cyclopropane is not obligatory[35] for observation of the CI/CPI characteristic DNA alkylation and that the DNA alkylation by **15-16** cannot be subject to the proposed autocatalytic phosphate activation of a sequence dependent alkylation event. Similarly, the DNA alkylation profiles of **17-19** are identical (Figure 8), indistinguishable from that of (+)-CI-$CDPI_2$ (**14**, Figure 6), independent of the absolute configuration of the agent ((+)**14** = (-)-**14** = (±)-**14**, Figure 6), strikingly similar to that of (+)-CC-1065/(+)-CPI-$CDPI_2$, confined to A-T rich regions of DNA, and substantially more selective than that of **11, 15-16**. The methyl ether **18** and the agent **19** cannot close to the putative agent **14** illustrating that the cyclopropane is not obligatory[35] for observation of the selective DNA alkylation reaction characteristic of

(+)-CC-1065 and that the selective DNA alkylation reaction of **18-19** cannot be the result of a sequence dependent autocatalytic phosphate activation (complexation/protonation) of the alkylation event. The results are, however, consistent with a prominent role for the (+)-CC-1065 noncovalent binding selectivity within the narrower, sterically more accessible A-T rich minor groove effectively restricting the number of available alkylation sites (accessible hydrophobic binding,[7,9,12-14] selectivity: (+)-**14** = (+)-**17-19** >> (+)-**11** = (+)-**15-16**). Thus, in addition to illustrating that the cyclopropane is not obligatory for observation of the CI characteristic covalent alkylation, the studies highlight the nonselectivity of the simple alkylation event in the absence of noncovalent binding selectivity, illustrate a prominent role for agent binding selectivity for agents that possess such capabilities, and demonstrate that an autocatalytic phosphate activation may not be uniquely responsible for the nonselective or selective covalent alkylations.

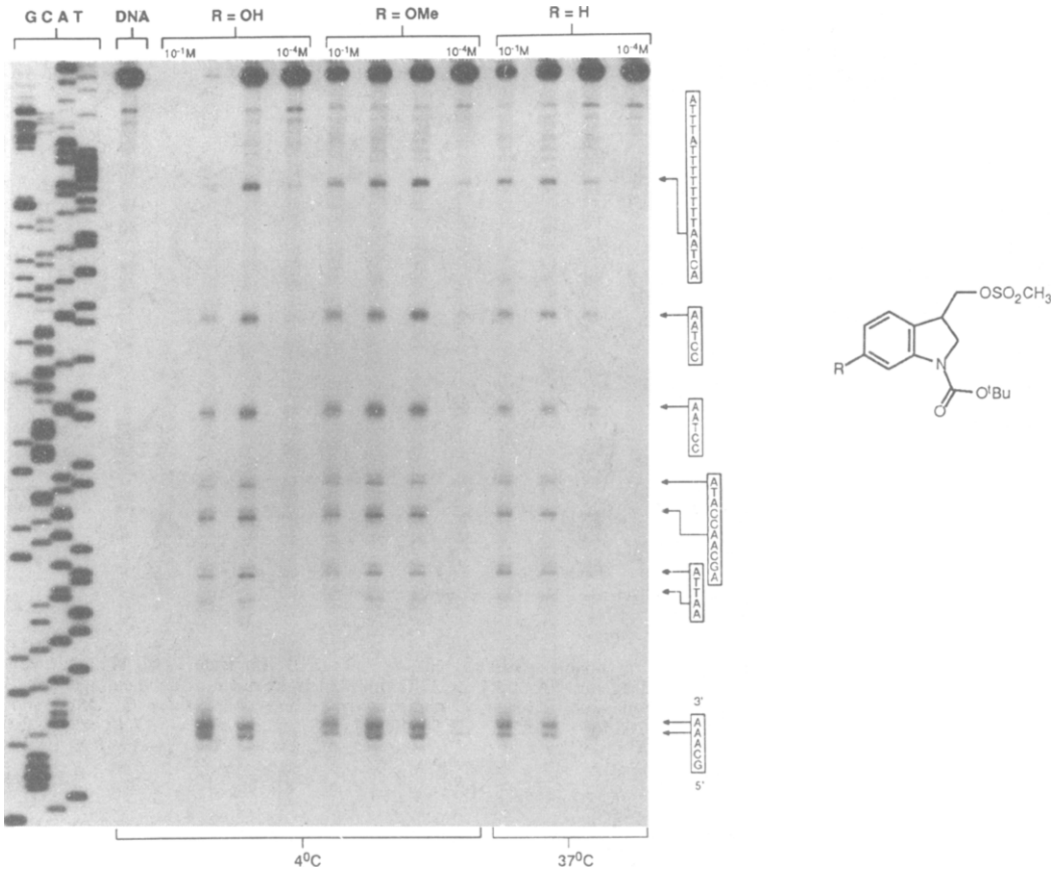



Figure 7. Thermally-induced strand cleavage of double-stranded DNA (SV40 DNA fragment, 144 b.p.; nucleotide no. 138-5238, clone w794) after 24 h incubation of agent:DNA at 4°C or 37°C followed by removal of unbound agent and 30 min incubation at 100°C; 8% denaturing poly(acrylamide) gel and autoradiography. Lanes 1-4, Sanger G, C, A, and T reactions; lane 5, control DNA; lanes 6-9, (+)-**11b** (R = OH, 2.5 x $10^{1}$ - 2.5 x $10^{-4}$ M, 4°C), lanes 10-13, (+)-**15** (R = OMe, 2.5 x $10^{1}$ - 2.5 x $10^{-4}$ M, 4°C); lanes 14-17, (+)-**16** (R = H, 2.5 x $10^{1}$ - 2.5 x $10^{-4}$ M, 37°C).

The relative intensity of DNA alkylation by **11b**, **15-16** and **17-19** appears to be related qualitatively to the reactivity of the electrophile which presumably benefit from aryl participation in the solvolytic reactivity of the agent through intermediate phenonium ion generation.[40] However, this may also be interpreted as representing an inverse relationship between the actual electrophile reactivity (phenonium ion reactivity) and the DNA alkylation intensity/cytotoxic potency. Although less obvious, the potential significance of this alternative interpretation should not be dismissed in light of the recent demonstration of the inverse relationship for agents possessing the preformed electrophilic cyclopropane.[13-14]

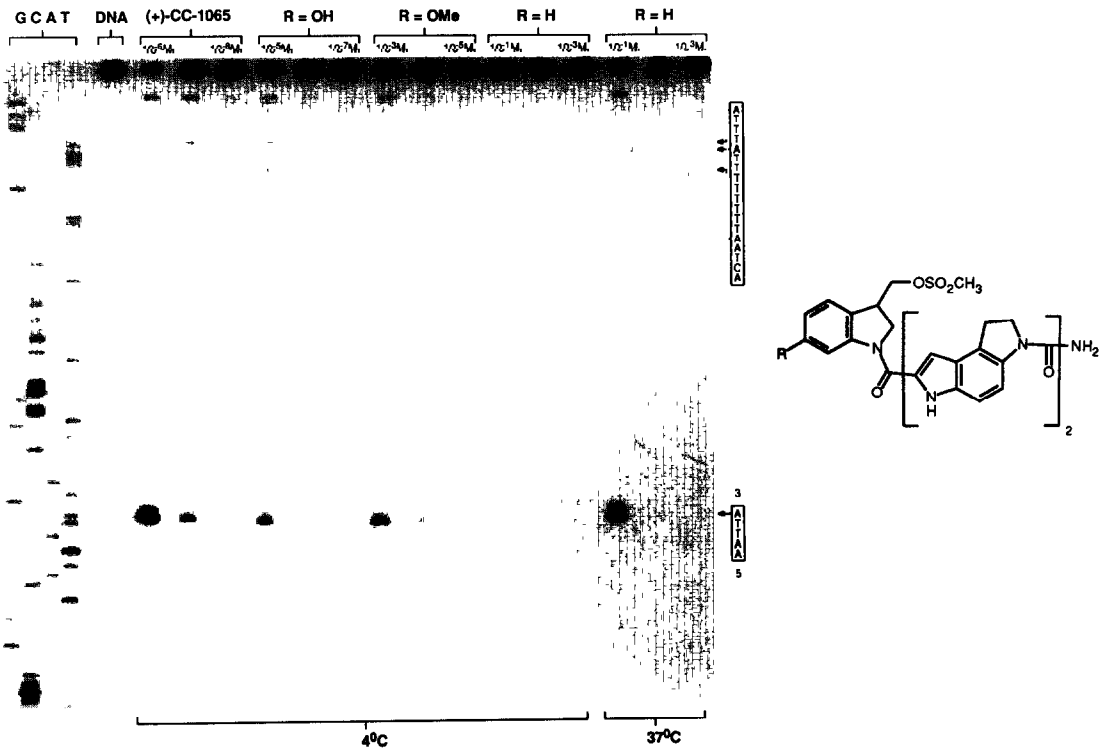
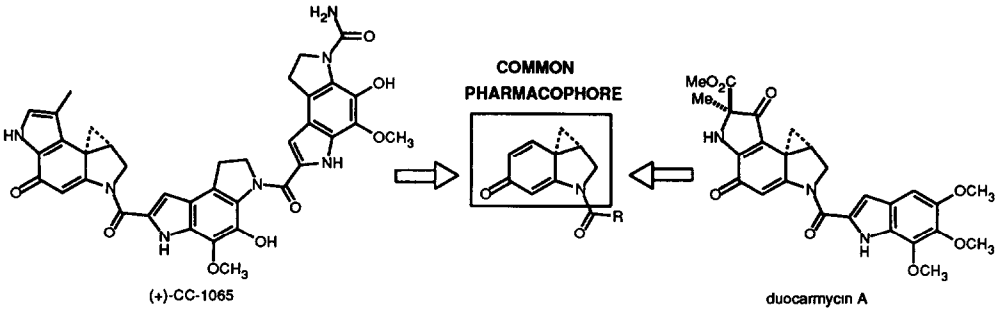



Figure 8. Thermally-induced strand cleavage of double-stranded DNA (SV40 DNA fragment, 144 b.p.; nucleotide no 138-5238, clone w794) after 24 h incubation of agent:DNA at 4°C or 37°C followed by removal of unbound agent and 30 min incubation at 100°C; 8% denaturing poly(acrylamide) gel and autoradiography. Lanes 1-4, Sanger G, C, A, and T reactions; lane 5, control DNA; lanes 6-8, (+)-CC-1065 (**1**, 2.5 x $10^{-6}$ - 2.5 x $10^{-8}$ M, 4°C); lanes 9-11, (±)-**17** (R = OH, 2.5 x $10^{-5}$ - 2.5 x $10^{-7}$ M, 4°C); lanes 12-14, (±)-**18** (R = OMe, 2.5 x $10^{3}$ - 2.5 x $10^{-5}$ M, 4°C); lanes 15-17, (±)-**19** (R = H, 2.5 x $10^{-1}$ - 2.5 x $10^{3}$ M, 4°C); lanes 18-20, (±)-**19** (R = H, 2.5 x $10^{1}$ - 2.5 x $10^{-3}$ M, 37°C).

**(+)-CC-1065 and the Duocarmycins.** The structural similarities between the duocarmycins (**3-8**)[15-16] and (+)-CC-1065 suggested that the agents may be acting by a common or related mechanism initiated with the irreversible alkylation of DNA. Although the full details of a demonstration and subsequent study of the DNA alkylation properties of the duocarmycins within w794, w836, c988, c820, and c1346 double-stranded DNA (750 base-pairs) have been presented elsewhere,[34-35] the results of this study illustrate that the duocarmycins display DNA alkylation properties (selectivity and intensity) strikingly similar to that of (+)-CC-1065, Figure 1 Further,

the results of the study demonstrated that CI constitutes the minimum potent pharmacophore[35-36] of the duocarmycin left-hand subunit and, thus, the common pharmacophore[35-36] relating the duocarmycin/CC-1065 alkylation subunits.[41]




Duocarmycin Binding Directionality: 3′ → 5′

Selectivity: Duocarmycin A = $C_2$ = $C_1$

: three base-pair selectivity = 5′-(AAA)-3 > 5′-(TTA)-3′ > 5′-(TAA)-3′ > 5′-(ATA)-3′

: fourth base-pair selectivity = 5′-(A/TXXA)-3′ >> 5′-(G/CXXA)-3′

: preceding base-pair sensitivity = 5′-(NXXAPu)-3′ ≥ 5′-(NXXAPy)-3′

High Affinity Sequences: 5′-(A/TAAA)-3′ and 5′-(A/TTTAPu)-3′

Intensity (4°C): (+)-CC-1065 ≥ Duocarmycin A (ca. 1-5x).

Table 3. In Vitro Cytotoxic Activity.

| Agent | Configuration | L1210, $IC_{50}$ (μg/ml) |
|---|---|---|
| (±)-1, CC-1065 | - | 0 00001 |
| (+)-1 | natural | 0.00001 |
| (-)-1 | enantiomer | 0.00001 |
| (+)-3, duocarmycin A | natural | 0.00002 |
| (+)-9, *N*-BOC-CPI | natural | 0.1 |
| (±)-11, *N*-BOC-CI | - | 10 |
| (±)-11b, *seco*-*N*-BOC-CI | - | 0.2 |
| (+)-11b | natural | 0.04 |
| (-)-11b | enantiomer | 0 2 |
| (±)-13, CI-CDPI$_1$ | - | 0.007 |
| (+)-13 | natural | 0.009 |
| (-)-13 | enantiomer | 0.02 |
| (±)-13b, *seco*-CI-CDPI$_1$ | - | 0.007 |
| (+)-13b | natural | 0.007 |
| (-)-13b | enantiomer | 0 007 |
| (±)-14, CI-CDPI$_2$ | - | 0.002 |
| (+)-14 | natural | 0.006 |
| (-)-14 | enantiomer | 0.02 |
| (±)-17, *seco*-CI-CDPI$_2$ | - | 0 001 |
| (+)-17 | natural | 0.001 |
| (-)-17 | enantiomer | 0 002 |
| (±)-15 | - | 1 |
| (±)-16 | - | > 10 |
| (±)-18 | - | 0 004 |
| (±)-19 | - | 0.5 |

$IC_{50}$, inhibitory concentration for 50% cell growth relative to untreated controls; L1210, mouse lymphocytic leukemia.

**In Vitro Cytotoxic Activity.** The results of the comparative in vitro cytotoxic evaluation of the CI-based agents are summarized in Table 3 and are striking in that the agents are comparable in cytotoxic potency to most existing or clinically useful agents even though their stability in aqueous solution is limited.[8] Consistent with trends depicted in the intensity of the DNA alkylation, substantial and incremental increases in the cytotoxic potency were observed within the CI-CDPI$_n$ series (n = 2 > n = 1 >> n = 0). In sharp contrast to observations made with the CPI-based agents,[12] both enantiomers of each of the CI-based agents exhibit cytotoxic activity that proved relatively independent of the absolute configuration of the agent although the agents that possess the natural absolute configuration of (+)-CC-1065 consistently displayed slightly more potent activity. Consistent with trends depicted in the DNA alkylation intensity of the agents, CI-CDPI$_1$ and CI-CDPI$_2$ proved to be approximately 1000-100x less potent than (+)-CC-1065 (CI-CDPI$_2$ > CI-CDPI$_1$ >> *N*-BOC-CI). Thus, (1) the natural enantiomers of the CI-based agents exhibit potent cytotoxic activity that has proven less potent than that of the corresponding CPI-based agent ((+)-*N*-BOC-CI < (+)-*N*-BOC-CPI, (+)-CI-CDPI$_1$ < (+)-CPI-CDPI$_1$, (+)-CI-CDPI$_2$ < (+)-CPI-CDPI$_2$) and presumably reflects their relative stability to the assay conditions, (2) unlike the behavior of the CPI-based agents, both enantiomers of the CI-based agents possess potent and comparable cytotoxic activity, (3) the stable *seco*-CI agents that possess alkylation capabilities possess cytotoxic activity and potency consistent with their relative intensity of DNA covalent alkylation, and (4) the cytotoxic potency of the CI agents exhibit substantial and incremental increases within the CI-CDPI$_n$ series (n = 2 > n = 1 >> n = 0).

**Conclusions.** An alternative approach to the generation of singly $^{32}$P-end-labeled double-stranded DNA has been detailed and offers a number of technical and strategic advantages over the conventional use of restriction fragments. Most prominent among these are the replenishable source of single-stranded DNA templates and singly end-labeled duplex without the necessity of preparative gel electrophoresis for duplex isolation. As such, the approach permits the generation of substantial quantities of end-labeled double-stranded DNA suitable for its use in the evaluation of an extensive series of related agents. The use of this strategy in the study of the DNA alkylation properties of (+)-CC-1065, the duocarmycins, and a series of CI-based analogs have led to.

(1) a refined definition of the (+)-CC-1065 DNA alkylation selectivity,

(2) a definition of the (±)-*N*-BOC-CI DNA alkylation selectivity,

(3) a definition of the duocarmycin DNA alkylation selectivity,[35]

(4) the demonstration that CI constitutes the minimum potent pharmacophore of the (+)-CC-1065 and duocarmycin alkylation subunits and, thus, the common pharmacophore relating the two classes of antitumor agents,[9,35]

(5) the demonstration that the cyclopropane is not obligatory to observation of the characteristic CI DNA alkylation selectivity,

(6) the demonstration that a sequence dependent autocatalytic activation of the DNA alkylation event by a strategically located phosphate (carbonyl protonation/complexation) may not be uniquely responsible for the alkylation selectivity, and

(7) the observation of results consistent with the prominent role for a noncovalent binding selectivity contribution to the (+)-CC-1065 DNA alkylation selectivity.[35]

## Experimental[42]

**General Methods.** All glassware, plasticware, and solutions were autoclaved prior to use Adjustments to pH were made with hydrochloric acid or sodium hydroxide. Ethanol precipitations were carried out by adding *t*-RNA as a carrier (1 μL, 10 μg/μL), a buffer solution containing salt (0.1 volume, 2 M NaCl in TE) and ethanol (2 5 volumes). The solution was gently vortex mixed and chilled at -70°C in an ethanol dry-ice bath for 20 minutes. The DNA was reduced to a pellet at 4°C by centrifugation for 15 minutes, washed with -20°C ethanol

(70% in TE containing 0.2 M NaCl), and recentrifuged for 15 minutes. The pellets were dried in a Savant Speed Vac concentrator. TE buffer consists of 10 mM Tris, 1 mM EDTA, pH = 7.6. Kinase buffer (10x) consists of 500 mM glycine (pH = 9.5), 100 mM $MgCl_2$, 50 mM dithiothreitol in 50% aqueous glycerol. Agents **1-2**,[11] **9-10**,[11] **11-11b**,[8] **13-14**,[8] **15**,[8,35] and **17-18**[8] were prepared as detailed elsewhere.

**M13 Cloning Strategy.** Purified SV40 nucleosomal DNA was shotgun cloned into the *Hinc* II or *Sma* I sites of the M13 vector mp10 and transformed into competent JM103 cells following standard procedures.[27-29] Phage colonies containing SV40 DNA were identified and sequenced. From the library of available clones five were chosen for phage and single-stranded DNA preparations used for the experiments described herein. Such a shotgun approach is not necessary and was performed for experiments detailed elsewhere.[28,29]

**Preparation of Template DNA.** Phage pellets isolated from JM103-infected cells[27] were suspended in TE buffer (pH 7.6). Protein was removed from the DNA using buffered, water-saturated phenol. The DNA was concentrated by precipitation with ethanol.

**5'-End-Labeling of M13 Universal Primer.** M13 universal primer (5'-d(GTAAAACGACGGCCAGT)-3', 1 μL, 6 x $10^{-3}$ $A_{260}$/μL) was treated with γ-$^{32}$P-ATP (1 μL, 6000-7000 Ci/mmol), T-4 polynucleotide kinase (1 μL, 10 u/μL) in water (24 μL), and kinase buffer (3 μL, 10x). The phosphorylation reaction was conducted at 37°C (3 h) and stopped with the addition of EDTA (2.5 μL, 250 mM). To remove ADP and unreacted γ-$^{32}$P-ATP, the primer was precipitated with ethanol, chromatographed on DE-52 cellulose as previously described,[30] and resuspended in 100 μL TE.

**Double-Stranded DNA Synthesis.** The primer was annealed to the template in a small microfuge tube containing an aliquot of the single-stranded M13mp10 clone (30 μL), 5'-$^{32}$P-end-labeled universal primer from above (5 μL), DNA synthesis buffer (7.5 μL), and water (7 5 μL). [DNA synthesis buffer (10x) consisted of Tris hydrochloride (500 mM, pH = 7.2), $MgSO_4$ (100 mM), dithiothreitol (1 mM) and bovine serum albumin (500 μg/mL)]. The solution was warmed at 100°C in a water bath for 5 minutes. To allow the solution to cool slowly for 3 h, the heat source to the water bath was turned off. The condensate containing the annealed primer was collected by centrifugation.

Double-stranded DNA synthesis and the sequencing reactions were conducted concurrently. 5 Units of the Klenow fragment of DNA polymerase I were added to the annealed primer-template duplex at room temperature and 4 x 2.4 μL aliquots were removed for dideoxynucleotide sequencing reactions performed as previously described.[28,31] To the remainder, 14.8 μL of a solution containing dNTPs (2 mM in each dATP, dGTP, dCTP, and dTTP) was added in order to initiate the extension of the primer by the Klenow fragment. The reaction mixture was incubated for 15 minutes at 47°C and followed with an additional aliquot (14.8 μL) of the dNTP mix which was maintained for 16 minutes at 47°C. The DNA synthesis was stopped with the addition of EDTA (5 μL, 250 mM). The double-stranded DNA product was precipitated with ethanol and chilled at -70°C. Prior to reducing the precipitated DNA to a pellet, it was necessary to warm the mixture for 5 minutes at room temperature to solubilize residual monomeric nucleotides since they often inhibit DNA digestion by restriction enzymes. The collected DNA pellets were dissolved in water (25 μL).

To generate a homogeneous length of singly 5'-$^{32}$P-end-labeled DNA, the double-stranded DNA was cleaved with *Eco* RI (2 μL, 20,000 units/mL, Bethesda Research Laboratories) as described.[25] The cleavage reaction was terminated by EDTA (3 μL, 250 mM) addition. The DNA was precipitated with ethanol and subsequently dissolved in TE buffer (210 μL) for agent binding studies.

**Agent DNA Binding and Gel Electrophoresis.** Eppendorf tubes containing the double-stranded DNA (9 μL, from above) were treated with the agent in a solution of DMSO (1 μL, at the specified concentration). Agent concentrations were measured by UV (Varian Cary 210 UV-Visible spectrophotometer) using known extinction coefficients. The reaction was mixed by vortexing and brief centrifugation, and subsequently incubated at 4°C or 37°C for 24 hours. DNA was separated from unbound agent by ethanol precipitation and resuspended in TE buffer (20 μL). In the case of the use of high concentrations of insoluble agents ((±)-**19**, $10^{-1}$ - $10^{-2}$ M), unbound agent was removed by diluting the agent:DNA complex with TE (75 μL) and extracting the solution with buffer-saturated phenol:$CHCl_3$:*i*-amyl alcohol (25:24:1, 200 μL). After washing the organic layer with TE (25 μL), the agent:DNA complex was isolated from the combined aqueous fractions through ethanol precipitation. The tube was sealed with Teflon tape, warmed at 100°C for 30 minutes to induce cleavage at the alkylation sites, allowed to cool to room temperature, and centrifuged to remove debris. Formamide-dye was then added (10 μL) to the supernatant. Prior to electrophoresis, the samples were warmed to 100°C for 5 minutes, placed into an ice bath, centrifuged, and the supernatant was loaded onto the gel. Sanger dideoxynucleotide sequencing reactions were run as standards adjacent to the agent:DNA reactions. Gel electrophoresis was carried out using an 8% sequencing gel (19:1 acrylamide: *N,N'*-methylenebisacrylamide; 8 M urea). Formamide dye contained xylene cyanol FF (0.03%), bromophenol blue (0.03%), and aqueous $Na_2EDTA$ (8.7%, 250 mM). Electrophoresis running buffer (1 x TBE) contained Tris base (100 mM), boric acid (100 mM), and $Na_2EDTA\,2H_2O$ (0.2 mM) dissolved in water. Gels were pre-run for 30 minutes to 1 hour with formamide dye prior to loading the samples. Autoradiography of dried gels was carried out at -70°C using Kodak X-Omat AR film and a Picker Spectra™ intensifying screen.

**1-Acetyl-6-hydroxy-3-(methanesulfonyloxymethyl)indoline (12b).** Phenol **11b**[8] (7.6 mg, 22 μmol) was treated with anhydrous 3 N hydrochloric acid in ethyl acetate (0.5 mL) at 24°C (30 min). The solvent was removed in vacuo to afford the crude, unstable indoline hydrochloride (quantitative) as a white semisolid. A solution of the crude indoline hydrochloride salt in tetrahydrofuran (0.4 mL) was treated with acetic anhydride (8.4 μL, 88 μmoL, 4 equiv) at 24°C under argon and the mixture was stirred for 10 h (24°C). The solvent was

removed in vacuo. Chromatography (0.5 x 5 cm $SiO_2$, 10-66% THF-hexane gradient elution) afforded **12b** (5.3 mg, 6.3 mg theoretical, 84%) as a white solid. Mp 175-176°C; $^1H$ NMR (DMSO-$d_6$, 300 MHz, ppm) 9.40 (bs, 1H, OH), 7.61 (d, 1H, $J$ = 2.2 Hz, C7-H), 7.12 (d, 1H, $J$ = 8.1 Hz, C4-H), 6.41 (dd, 1H, $J$ = 2.2, 8.1 Hz, C5-H), 4.37 (dd, 1H, $J$ = 5.1, 9.7 Hz, C2-H), 4.24 (dd, 1H, $J$ = 7.6, 9.6 Hz, C2-H), 4.23 (dd, 1H, $J$ = 9.8, 11.0 Hz, C*H*HOSO$_2$CH$_3$), 3.89 (dd, 1H, $J$ = 5.1, 10.9 Hz, CH*H*OSO$_2$CH$_3$), 3.69 (m, 1H, C3-H), 2.15 (s, 3H, COCH$_3$); IR (KBr) $\nu_{max}$ 3146 (br), 1638, 1606, 1542, 1508, 1498, 1448, 1348, 1282, 1168, 986, 960, 878 cm$^{-1}$; UV (MeOH) $\lambda_{max}$ 254 (ε 10000), 294 nm (ε 5000); EIMS, *m/e* (relative intensity) 285 (M$^+$, 2), 189 (3), 176 (6), 146 (35), 134 (57), 96 (63), 81 (61), 79 (base); CIMS, *m/e* 286 (M$^+$ + H, base); CIHRMS, *m/e* 286.0734 ($C_{12}H_{15}NO_5S$ + H$^+$ requires 286.0749).

**1-(*tert*-Butyloxycarbonyl)-3-(methanesulfonyloxymethyl)indoline (16).** A vigorously stirred solution of 1-(benzenesulfonyl)-3-(hydroxymethyl)indoline[11] (24 mg, 83 μmoL) in toluene (700 μL) at 23°C under argon was treated with a solution of sodium bis(2-methoxyethoxy)aluminum hydride (Redal-H, 150 μL, 3.4 M in toluene, 510 μmoL, 6 equiv). The solution was warmed at 100°C (bath temperature) under argon for 3 h. The reaction mixture was cooled to 0°C and quenched by careful addition of ice. The reaction solution was diluted with saturated aqueous $NaHCO_3$ (10 mL, nitrogen saturated) and extracted with ethyl acetate (3 x 10 mL, nitrogen saturated). The combined organic extracts were dried ($Na_2SO_4$) under nitrogen. The solvent was removed in vacuo to afford crude indoline which was immediately dissolved in 1.0 mL of tetrahydrofuran at 23°C under argon and treated with di-*tert*-butyl dicarbonate (57 μL, 250 μmoL, 3 equiv). After 20 h, the solvent was removed in vacuo and the residue was purified by flash chromatography (0.8 x 9 cm $SiO_2$, 0-25% ether-hexane gradient elution) to afford 1-(*tert*-butyloxycarbonyl)-3-hydroxymethylindoline (11 mg, 20 mg theoretical, 55%). The alcohol (10 mg, 40 μmol) dissolved in methylene chloride (500 μL) was treated sequentially with triethylamine (11.2 μL, 80 μmol, 2 equiv) and methanesulfonyl chloride (5 μL, 64 μmol, 1.6 equiv) at 0°C under argon. After 8 h at 0°C, the reaction mixture was dissolved in methylene chloride (10 mL) and washed with cold 1 N $H_3PO_4$. After extraction of the aqueous layer with methylene chloride (2 x 10 mL), the combined organic phases were dried ($Na_2SO_4$) and concentrated in vacuo. Flash chromatography ($SiO_2$, 0-25% ether-hexane, gradient elution) afforded **16** (12.7 mg, 13.1 mg theoretical, 97%) as a colorless oil. $^1H$ NMR ($CDCl_3$, 300 MHz, ppm) 7.86 (br s, 1H, C7-H), 7.26 (m, 2H, C4-H and C6-H), 6.97 (apparent t, 1H, $J$ = 7.3 Hz, C5-H), 4.37 (dd, 1H, $J$ = 9.8, 5.5 Hz, C*H*HOSO$_2$CH$_3$), 4.22 (dd, 1H, $J$ = 9.8, 8.1 Hz, CH*H*OSO$_2$CH$_3$), 4.09 (dd, 1H, $J$ = 11.7, 9.8 Hz, C2-H), 3.89 (dd, 1H, $J$ = 11.7, 4.6 Hz, C2-H), 3.73 (m, 1H, C3-H), 2.95 (s, 3H, OSO$_2$CH$_3$), 1.55 (s, 9H, OC(CH$_3$)$_3$); IR (neat) $\nu_{max}$ 2977, 2361, 2345, 1701, 1636, 1488, 1395, 1174, 956, 753 cm$^{-1}$; EIMS, *m/e* (relative intensity) 327 (5), 271 (11), 227 (13), 131 (31), 130 (52), 118 (88), 57 (base); CIMS (isobutane), *m/e* (relative intensity) 328 (M + H$^+$, 1), 327 (M$^+$, 1), 273 (12), 272 (base); EIHRMS, *m/e* 327.1140 ($C_{15}H_{21}NO_5S$ requires 327.1140).

**7-[[7-[[2,3-dihydro-3-[[(methylsulfonyl)oxy]methyl]-1*H*-indol-1-yl]carbonyl]-1,6-dihydrobenzo[1,2-*b*:4,3-*b*']dipyrrol-3(2*H*)-yl]carbonyl]-1,6-dihydrobenzo[1,2-*b*:4,3-*b*']dipyrrole-3(2*H*)-carboxamide (19).** A solution of **16** (7 mg, 21 μmol) in 3 N hydrochloric acid in ethyl acetate (1 mL) was stirred under argon for 45 min at 23°C. The solvent was removed in vacuo to afford the crude indoline hydrochloride as a colorless semisolid. A mixture of the crude indoline hydrochloride, 1-[(3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI, 12.3 mg, 64 μmol, 3 equiv), and $CDPI_2$[10] (10.4 mg, 21 μmol, 1 equiv) was stirred in anhydrous *N,N*-dimethylformamide under nitrogen for 30 h. The solvent was removed in vacuo and the residue was washed with water (5 x 5 mL) to afford **19** (6 mg, 13.7 mg theoretical, 44%) as a light tan powder. Flash chromatography (0.6 x 6 cm $SiO_2$, 1-10% methanol-chloroform) afforded pure **19**. Mp > 240°C; $^1H$ NMR (DMSO-$d_6$, 300 MHz, ppm) 11.83 (s, 1H, pyrrolo-NH), 11.55 (s, 1H, pyrrolo-NH), 8.28 (m, 1H, C4'-H), 8.23 (m, 1H, C7-H), 7.96 (d, 1H, $J$ = 8.8 Hz, C4''-H), 7.47 (d, 1H, $J$ = 7.4 Hz, C5'-H), 7.35 (m, 1H, C6-H), 7.22 (d, 2H, C5''-H, C4-H), 7.13 (m, 2H, C5-H and C1'-H), 6.96 (s, 1H, C1''-H), 6.10 (br s, 2H, NH$_2$), 4.75 (apparent t, 1H, $J$ = 10.2 Hz, C*H*HOSO$_2$), 4.64 (t, 2H, $J$ = 7.8 Hz, C7'-H$_2$), 4.48 (br d, 1H, $J$ = 5.8 Hz, C2-H), 4.38 (m, 1H, CH*H*OSO$_2$), 4.25 (apparent t, 1H, $J$ = 7 Hz, C2-H), 3.95 (m, 2H, C7''-H), 3.76 (m, 1H, C3-H), 3.40 (m, 2H, C8'-H), 3.25 (m, 2H, C8''-H), 3.17 (s, 3H, OSO$_2$CH$_3$); IR (KBr) $\nu_{max}$ 3399, 2933, 1701, 1617, 1507, 1481, 1459, 1411, 1343, 1283, 1037, 957, 805, 760 cm$^{-1}$; FABMS (3-nitrobenzyl alcohol), *m/e* 639 (M$^+$ + H); FABHRMS (3-nitrobenzyl alcohol), *m/e* 639.2052 ($C_{33}H_{30}N_6O_6S$ + H$^+$ requires 639.2026).

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36. The definition of pharmacophore reads "the group of atoms in the molecule of a drug responsible for the drug's action".
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38. The observed alkylations at adenine (> 70 alkylation sites) were found to be flanked by a 5′-A or -T base with one exception, cf. Figure 1.
39 The samples of **15** and **18** were purified by HPLC (Alltech 10 x 250 mm, 10 μ econosil column, flow rate = 3.0 mL/min; For **15**, 30% EtOAc-hexane, $R_T$ = 17 min (**15**), $R_T$ = 25 min (**11b**); for **18**, 10% DMF in $CH_2Cl_2$, $R_T$ 22 min (**18**), $R_T$ = 25 min (**17**)) and determined to be free of contaminant phenol **11b** or **17** (**15** ≥ 99.94% pure, ≤ 0.05% **11b**; **18**, ≥ 99.9% pure, ≤ 0.1% **17**). The relative intensity of the footprint of **15** and **18** cannot be attributed to a phenol contamination of the samples (requires ca. 10-100% **11b** in **15** and ca. 1% **17** in **18**).
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42 Sufficient quantities of the M13mp10 derived single-stranded DNA templates containing the DNA fragments detailed herein (clones w794, w836, c988, c820, c1346) may be obtained upon request.