Sequence-Specific Cleavage of DNA by *N*-Bromoacetyldistamycin. Product and Kinetic Analyses

Brenda F. Baker and Peter B. Dervan*

Contribution No. 7802 from the Arnold and Mabel Beckman Laboratories of Chemical Synthesis, California Institute of Technology, Pasadena, California 91125. Received July 18, 1988

Abstract: *N*-Bromoacetyldistamycin (BD) is a designed molecule with two structural domains with distinct functions: sequence specific binding to double helical DNA and cleavage of the DNA backbone. An electrophilic bromoacetyl group is appended to the amino end of the tripeptide from the natural product distamycin A. Footprinting studies reveal that *N*-bromoacetyldistamycin binds within minutes to a 167 base pair (bp) restriction fragment at four A,T rich sites five base pairs in size, 5'-TTTAA, GTTTA, AAATT, and GAAAT-3'. After a reaction time of 5 h at 37 °C, cleavage occurs at a single adenine in the GTTTA site in this 167 bp restriction fragment following a piperidine workup procedure. Covalent attachment of BD occurs at N3 of adenine. 3-(Acetyldistamycin)adenine is the released product from the reaction of BD with a 15 base pair oligonucleotide duplex 5'-CGGTAGTTTATCACA-3' at 37 °C. The 3' and 5' DNA termini at the site of cleavage after piperidine treatment are phosphate groups. Alkylation of adenine in duplex DNA by BD is described by a first-order rate process. The rate constant for alkylation of N3 of adenine at the sequence 5'-GTTTA-3' is $k = 5.39 \times 10^{-6}$ s^{-1} ($t_{1/2}$ = 35.7 h) at 37 °C. The cleavage specificities of the synthetic molecule, *N*-bromoacetyldistamycin, and the natural product, CC-1065, were compared on the 167 bp restriction fragment. After a reaction time of 1 h at 37 °C, CC-1065 cleaves at 13 adenines whereas BD shows cleavage at one adenine. The differences in covalent binding specificity between CC-1065 and BD may be related to sequence dependent reactivity in both cases but sequence dependent binding being additionally important in the latter.

The design of sequence-specific cleaving molecules for double-helical DNA requires the attachment of DNA cleaving moieties to sequence-specific DNA binding molecules.[1] The cleavage specificity achieved by the hybrid molecule is determined by both the specificity of the cleavage moiety and the sequence specificity of binding. From a mechanistic point of view, one can consider three classes of DNA cleaving moieties: oxidative degradation of the sugar backbone, electrophilic modification of a base, or hydrolysis of the phosphodiester bond. The rates of all these reactions should be sequence dependent due to the sequence-dependent microheterogeneity of DNA. Using a design-synthesis approach, we would like to elucidate those chemical factors that control these rate differences and whether they will be large or small.

Design of sequence-specific DNA cleavage chemistry will benefit from a prior understanding of the chemical principles for the sequence-specific binding of small molecules to right-handed double-helical DNA.[1] Productive model building is possible because of recent progress in the development of analytical methods such as footprinting and affinity cleaving to determine the preferred binding locations, binding site sizes, and orientations of natural or synthetic sequence specific DNA binding molecules on DNA restriction fragments.[1] We have shown that for those cases where the sequence specificity of the binding moiety is not well understood, a *diffusible DNA* reactive species allows flexibility in the placement of the cleaving function next to the site of DNA binding. In contrast, a precise understanding of the DNA recognition moiety allows a *nondiffusible* cleaving species to be positioned proximal to the site of reaction on DNA.[2]

Electrophilic Modification of DNA. Cleavage of the DNA backbone may be achieved by electrophilic attack of purines followed by depurination under appropriate workup conditions.[3,4] One might expect differential reactivity of purines[5] (guanine and adenine) due to different intrinsic nucleophilicity of several heteroatom positions (e.g., N7 or N3) and different accessibility within each site due to the sequence-dependent structure of DNA. For the design of synthetic sequence-specific DNA cleaving molecules based on the attachment of electrophiles to sequence-specific DNA binding molecules,[2] proper positioning of an electrophile near nucleophilic centers on DNA, such as the N3 of adenine in the minor groove or the N7 of guanine in the major groove of DNA, requires some understanding of the molecular structure of the DNA binding unit complexed to DNA. There are examples of natural products, such as mitomycin C,[6] anthramycin,[7] and CC-1065,[8] which place an electrophile proximal to specific regions of double-helical DNA. Most relevant to this report, CC-1065 binds covalently to N3 of adenine in the minor groove of DNA, generally at sequences 5'AAAAA and 5'PuNTTA on the 3' side.[8] The crescent-shaped CC-1065 consists of three repeating pyrroloindole subunits, one of which contains a reactive cyclopropyl function.

We have shown previously by footprinting[9] and affinity cleaving methods[10] that the tripeptide from the natural product distamycin binds to five base pair sites with a preference for A,T rich regions.[11] In most cases the tripeptide can adopt two orientations at each binding site.[10] The molecular basis for recognition of DNA by netropsin (and by extension distamycin) is understood from X-ray[12] and NMR[13] analysis of the oligonucleotide d-

(1) (a) Dervan, P. B. *Science* **1986**, *232*, 464-471. (b) Dervan P. B. In *Nucleic Acids and Molecular Biology*; Eckstein, F., Lilley, D. M. J., Eds.; Springer-Verlag: Heidelberg, 1988; Vol. 2.

(2) (a) Baker, B. F.; Dervan, P. B. *J. Am. Chem. Soc.* **1985**, *107*, 8266-8268. (b) Dervan, P. B.; Baker, B. F. *Ann. N.Y. Acad. Sci.* **1986**, *471*, 51-59.

(3) Lawley, P. D.; Brookes, P. *Biochem. J.* **1963**, *89*, 127-138.

(4) (a) Maxam, A. M.; Gilbert, W. *Methods in Enzymology* **1980**, *65*, 499-560. (b) Maxam, A. M.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 560-564.

(5) Beranek, D. T.; Weis, C. C.; Swenson, D. H. *Carcinogenesis* **1980**, *1*, 595-606.

(6) Tomasz, M.; Lipman, R.; Verdine, G. L.; Nakanishi, K. *Biochemistry* **1986**, *25*, 4337-4344.

(7) Hurley, L. H.; Needham-VanDevanter, D. R. *Acc. Chem. Res.* **1986**, *19*, 230-237 and references cited there.

(8) (a) Reynolds, V. L.; McGovren, J. P.; Hurley, L. H. *J. Antibiot.* **1986**, *39*, 319-334. (b) Hurley, L. H.; Reynolds, V. L.; Swenson, D. H.; Petzold, G. L.; Scahill T. A. *Science* **1984**, *226*, 843-844. (c) Reynolds, V. L.; Molineux, I. J.; Kaplan, D. J.; Swenson, D. H.; Hurley, L. H. *Biochemistry* **1985**, *24*, 6228-6237. (d) Hurley, L. H.; Lee, C. S.; McGovern, J. P.; Warpehoski, M. A.; Mitchell, M. A.; Kelley, R. C.; Aristoff, P. A. *Biochemistry* **1988**, *27*, 3886.

(9) (a) Van Dyke, M. W.; Dervan, P. B. *Cold Spring Harbor Symposia on Quantitative Biology* **1983**, *47*, 347-353. (b) Van Dyke, M. W.; Dervan, P. B. *Nucl. Acids. Res.* **1983**, *11*, 5555-5567.

(10) (a) Schultz, P. G.; Taylor, J. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1982**, *104*, 6861-6863. (b) Taylor, J. S.; Schultz, P. G.; Dervan, P. B. *Tetrahedron* **1984**, *40*, 457-465.

(11) For a comprehensive review see: Zimmer, C. *Prog. Nuc. Acid Res. Mol. Biol.* **1975**, *15*, 285-318.

(12) Kopka, M. L.; Yoon, C.; Goodsell, D.; Pjura, P.; Dickerson, R. E. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1376-1380.

(13) Patel, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6424-28.

0002-7863/89/1511-2700$01.50/0

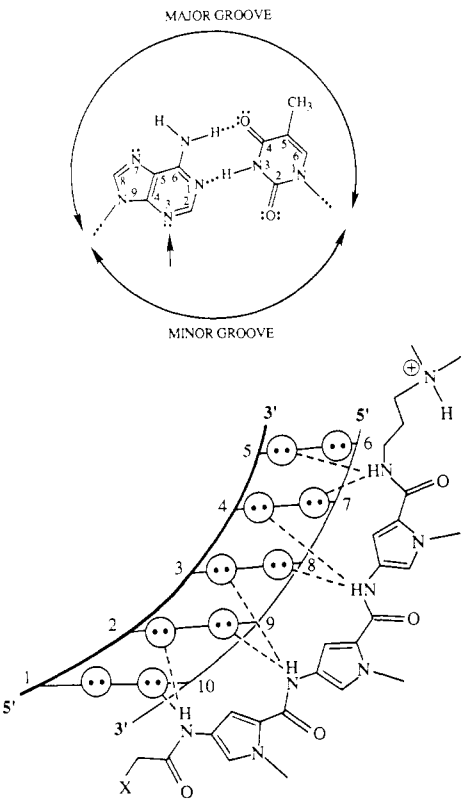


Figure 1. (Top) Adenine·thymine Watson–Crick base pairs. The arrow indicates the site of potential electrophilic attack at N3 of adenine from which cleavage of the sugar phosphate backbone will occur. (Bottom) Model of the tripeptide tris(*N*-methylpyrrole)carboxamide with an electrophile, α-bromoacetyl (X = Br), attached at the amino terminus, complexed to five-base pairs of A + T in the minor groove of right-handed DNA. Circles with two dots represent lone pairs of electrons on N3 of adenine and O2 of thymine at the edges of the bases.

(CGCGAATTCGCG):netropsin complex. In the crystal structure netropsin sits symmetrically in the center of the minor groove of A,T rich right-handed DNA.[12] Each of its three amide NH groups forms a bridge between adjacent adenine N3 or thymine O2 atoms on opposite strands. The basis of specificity of netropsin for contiguous sequences of A,T base pairs in B-DNA is provided by hydrogen bonding and close van der Waals contacts between adenine C2 hydrogens and CH groups on the pyrrole rings of the oligopeptide molecules. More recently, the structure of the oligonucleotide d(CGCAAATTTGCG) and distamycin cocrystal was solved to 2.2-Å resolution.[14]

On the basis of our understanding of distamycin and its interaction with double helical DNA, attachment of an electrophile on the amino end of the tripeptide, tris(*N*-methylpyrrole)-carboxamide, should place the electrophile at A,T rich sequences in the minor groove of DNA proximal to the N3 of adenine (Figure 1). We chose to attach α-bromoacetyl to the binding unit to create *N*-bromoacetyldistamycin (BD) (Figure 1). In preliminary work, we reported that BD at 5 μM concentration cleaves at a single adenine within a restriction fragment containing 167 base pairs of DNA (100 μM concentration in base pairs) after 5 h of reaction at 37 °C (pH 7.0), followed by a modified Maxam-Gilbert base-labilization DNA cleavage workup procedure.[2,4] The location of this adenine on the pBR322 plasmid is base pair 48. Footprinting under conditions of little observable cleavage by BD (5 μM concentration, 30 min, 37 °C) revealed that BD is binding four five-base pair sites on the 167 bp fragment, 5′-TTTAA, GTTTA, AAATT, and GAAAT-3′. These are the same sites bound by distamycin. The major cleavage site is contained within one of these four equilibrium binding sites on the complementary strand of the 5′-GTTTA-3′ site.

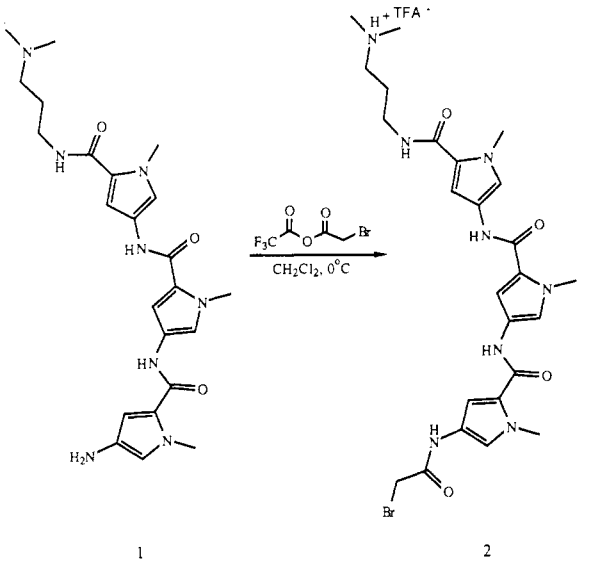


Figure 2. Synthetic scheme of *N*-bromoacetyldistamycin.

In order to gain some insight on the mechanism of cleavage of DNA by BD, we have now analyzed the products and the kinetics of this reaction. We report here the synthesis of BD, characterization of the DNA termini at the cleavage site, isolation and characterization of the released tripeptide–adenine adduct, and the rate constants for the reaction of BD at a single site on duplex DNA. Finally, the cleavage specificities of the synthetic BD and the natural product CC-1065 are compared.

Results and Discussion

Synthesis of *N*-Bromoacetyldistamycin. The synthetic scheme for *N*-bromoacetyldistamycin (**2**, BD) is shown in Figure 2. The tripeptide **1** is prepared in nine steps from *N*-methylpyrrole-2-carboxylic acid with a modified literature procedure.[10a,15] Reaction of the mixed anhydride,[16] trifluoroacetyl bromoacetic anhydride, with tripeptide diamine **1** in dichloromethane at 0 °C affords *N*-bromoacetyldistamycin (**2**) in 60% yield.

Dependence of DNA Cleavage on Time and Temperature. Footprinting studies reveal that *N*-bromoacetyldistamycin binds four A,T rich sites five base pairs in size on a 167 base pair restriction fragment from pBR322 plasmid.[2] A single cleavage site at adenine was observed at 37 °C within 5 h on this 167 bp DNA restriction fragment.[2] This result indicates unequal relative rates of reaction at the seven adenines proximal to the bound bromoacetyl moiety within the four equilibrium binding sites for the tripeptide. To assess these differences, the relative rates of cleavage have been investigated at longer reaction times and higher temperatures. Cleavage of the 167 bp restriction fragment by *N*-bromoacetyldistamycin was monitored by gel electrophoresis over a 40 h time period at 35 °C (Figure 3A). Cleavage is detected after 5 h at base pair 48, an adenine on the complementary strand of the binding site sequence 5′-GTTTA-3′. Secondary cleavage sites, base pairs 58 and 47, appear at adenine within the 5′-AAATT-3′ site (10 h) and 5′-GTTTA-3′ site (20 h) (Figure 3C).

The temperature dependence of the *N*-bromoacetyldistamycin reaction on the 167 bp DNA fragment was investigated up to 65 °C (Figure 3B) over a 5-h period. There is one cleavage site detected at 35 °C (bp 48), two at 45 °C (bp 48, 58), eight at 55 °C (bp 35, 47, 48, 58, 59, 89, 90, 91), and nine at 65 °C (bp 35, 47, 48, 58, 59, 60, 89, 90, 91) (Figure 3D). The reactions all occur at adenine and within the binding sites of the tripeptide. Curiously, the adenines deemed proximal to the electrophilic moiety from the model building (Figure 1) are not always the most reactive.

(14) Coll, M.; Frederick, C. A.; Wang, A. H.-J.; Rich, A. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8385–8389.

(15) Bialer, M.; Yagen, G.; Mechoulam, R. *Tetrahedron* **1979**, *34*, 2389–2391.

(16) Emmons, W. D.; McCallum, K. S.; Ferris, A. F. *J. Am. Chem. Soc.* **1953**, *75*, 6047–6048.

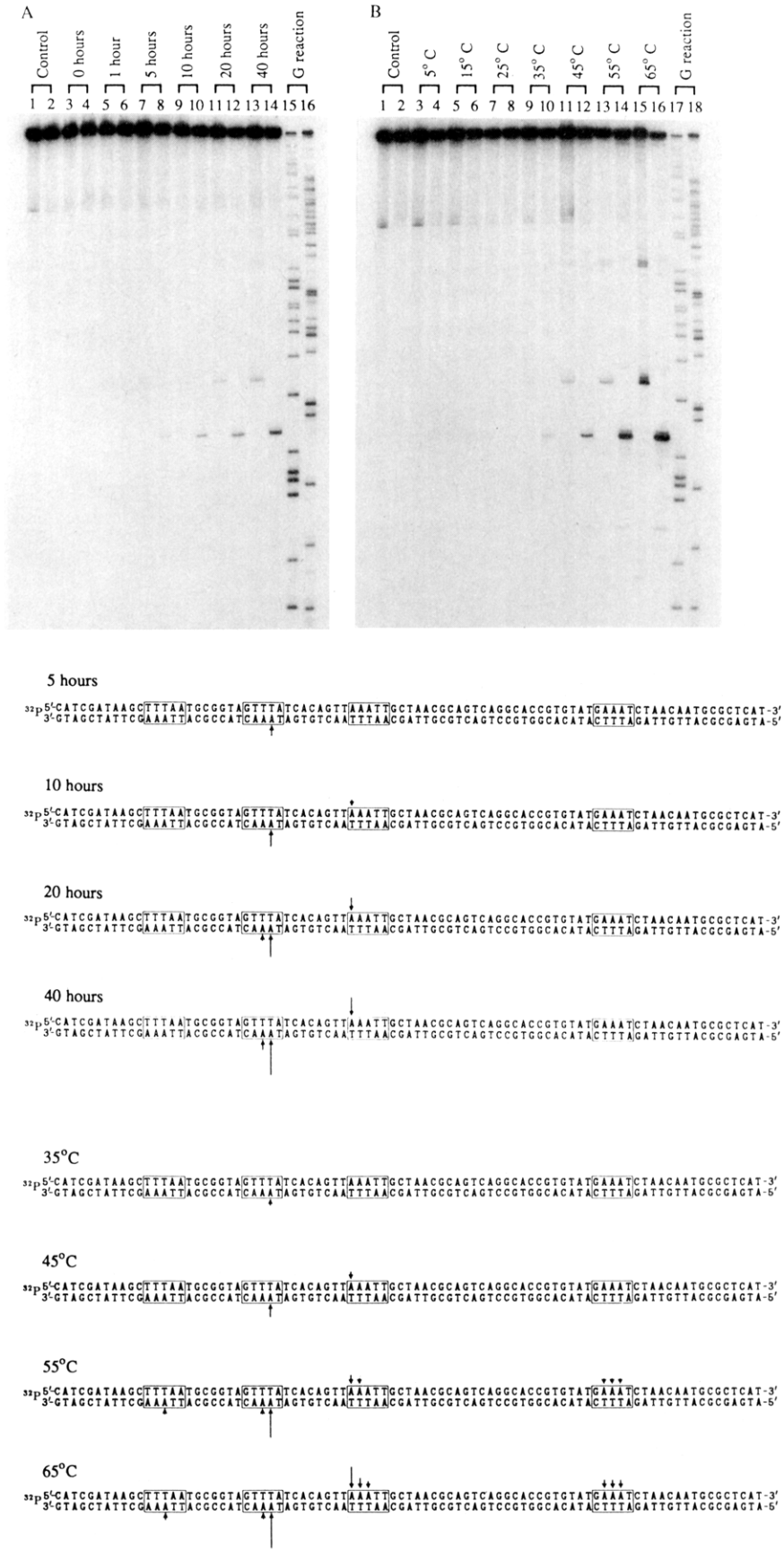


Figure 3. Autoradiograms of high-resolution polyacrylamide denaturing gels. Odd and even numbered lanes are DNA end-labeled with ^{32}P at the 5′ and 3′ position, respectively. (A) BD cleavage on the 167 bp restriction fragment through a 40 h time period at 35 °C. Lanes 1 and 2, DNA only; lanes 3 and 4, 0 h; lanes 5 and 6, 5 h; lanes 7 and 8, 10 h; lanes 9 and 10, 20 h; lanes 11 and 12, 40 h; lanes 13 and 14, G reaction. (B) BD cleavage on the 167 bp restriction fragment in 10 °C increments from 5 to 65 °C after 5 h. Lanes 1 and 2, 65 °C, DNA only; lanes 3 and 4, 5 °C; lanes 5 and 6, 10 °C; lanes 7 and 8, 15 °C; lanes 9 and 10, 25 °C; lanes 11 and 12, 35 °C; lanes 13 and 14, 45 °C; lanes 15 and 16, 55 °C; lanes 17 and 18, 65 °C; and lanes 19 and 20, G reaction. (C and D) Base pairs 22–108 from pBR322 plasmid. Arrows indicate sites and extent of DNA cleavage resulting from the reaction of BD on the 167 bp restriction fragment.

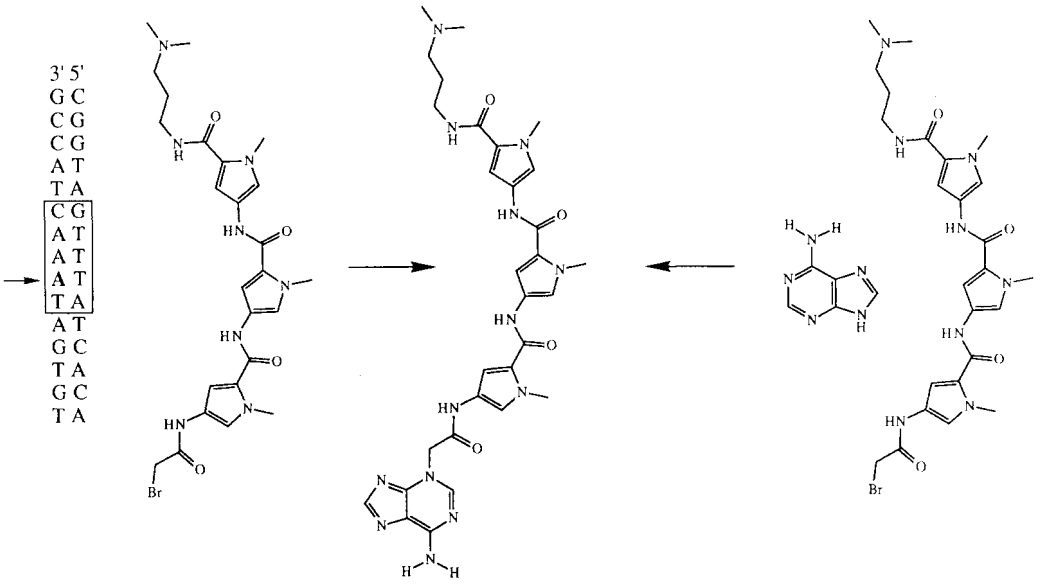


Figure 4. 3-(Acetyldistamycin)adenine is a common product from the reaction of adenine with BD and the oligonucleotide 15mer duplex with BD.

The site-dependent alterations in the DNA helical twist, base propeller twist, roll angle, and torsion angle undoubtedly allow differential steric and stereoelectronic effects to moderate the relative rates of intramolecular S_N2 displacement reaction within each discrete tripeptide binding site. The multiple contiguous cleavage sites (AAA) observed at 55 and 65 °C suggest that at these higher temperatures the tripeptide can *slide* as much as two base pairs within the binding site, or there is a conformational change at these sites which alters their reactivity.

***N*-Bromoacetyldistamycin Product Analysis.** If cleavage of the DNA backbone by BD results from alkylation of adenine at N3 followed by depurination, it should be possible to detect and isolate the tripeptide–adenine adduct. BD labeled with ^{14}C was used for microscale reactions to identify the released adduct. 3-(Acetyldistamycin)adenine, obtained from the reaction of adenine with BD and characterized by ^1H, ^{13}C, and ^{15}N NMR, was compared with the released products from the reaction of duplex DNA (15 base pair oligonucleotide, 5′-CGGTAGTTTATCACA-3′) and BD. The DNA substrate (15mer) contains the binding site 5′-GTTTA-3′ and flanking sequences in the 167 bp restriction fragment in which the major cleavage at adenine 48 is observed. After 96 h at 37 °C, a single compound is released from the reaction of the 15mer and ^{14}C labeled BD. This product comigrates by TLC with a synthetic product isolated from the reaction of adenine with BD. Verification of the common identity of the BD-oligonucleotide adduct and the BD–adenine adduct was obtained by reverse phase HPLC by coinjection of the synthetic tripeptide–adenine adduct with the BD-oligonucleotide reaction mixture. The ultraviolet–visible and the proton NMR spectra of the adenine-BD product and the released DNA-BD product are identical. A combination of ^{15}N labeled adenine and ^{13}C labeled acetyldistamycin reveal that this common product is 3-(acetyldistamycin)adenine (**8**, DA) which results from the reaction between the N3 of adenine and the methylene carbon of the acetyl group on BD (Figure 4).

NMR Assignment of the Structure of the Released Acetyldistamycin–Adenine Adduct. The proton NMR spectrum of the tripeptide–adenine adduct is consistent with covalent attachment of BD at N3 of adenine based on the difference in chemical shifts for H2 (8.31 ppm) and H8 (7.69 ppm) of the adenine moiety (Figure 5).[17] In order to unequivocally prove that the nucleophile of this reaction is N3 of adenine, [3-^{15}N]adenine (99% enriched) and *N*-bromo[2-^{13}C]acetyldistamycin (99% enriched) were synthesized. If alkylation occurs at the N3 position, coupling through

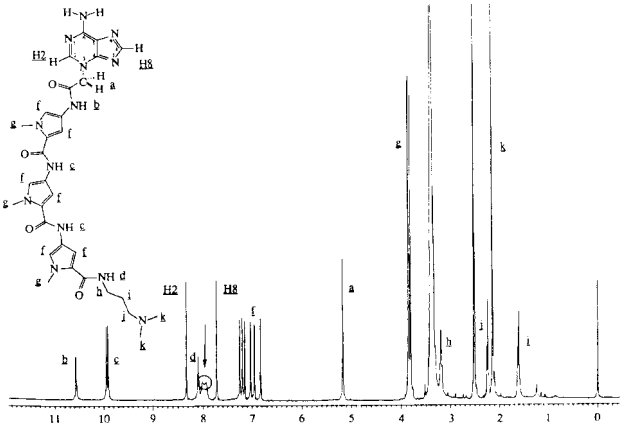


Figure 5. Proton NMR spectrum of the synthetic 3-(acetyldistamycin)adenine.

a $^{15}N3$-$^{13}C2$ bond should be observed. ^{15}N labeled adenine at N3 was synthesized in eight steps from imidazole by modification of established procedures.[18] ^{13}C labeled at the bromoacetyl methylene of BD was synthesized from the ^{13}C labeled TFA mixed anhydride.

Three isotopic combinations of 3-(acetyldistamycin)adenine (DA), 3-([2-^{13}C]acetyldistamycin)adenine, 3-(acetyldistamycin)[3-^{15}N]adenine, and 3-([2-^{13}C]acetyldistamycin)[3-^{15}N]adenine, were compared by proton, carbon, and nitrogen NMR (Figure 6). Assignments of H2 and H8 protons of the adenine are supported by the proton spectra of the ^{15}N labeled adducts. A doublet ($^2J_{H-N}$ = 6.2 Hz) is observed at 8.31 ppm and is interpreted as H2 coupled to $^{15}N3$. This value is in agreement with other coupling constants observed for pyrrolic nitrogens and protons.[19] The H8 proton of adenine of the ^{15}N labeled adduct, 3-(acetyldistamycin)[3-^{15}N]adenine, appears as a singlet at 7.69 ppm. The acetyl methylene protons of the distamycin moiety shift downfield from 3.98 (BD) to 5.18 ppm in 3-(acetyldistamycin)adenine. This assignment is supported by the proton spectra of 3-([2-^{13}C]acetyldistamycin)adenine, which show a splitting of the methylene protons to a doublet, $^1J_{H-C}$ =

(17) (a) Ishino, M.; Sakaguchi, T.; Morimoto, I.; Okitsu, T. *Chem. Pharm. Bull.* **1981**, *29*, 2403–2407. (b) Yang, N. C.; Chang, C. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 5250–5254.

(18) (a) Balaban, J. E.; Pyman, F. L. *J. Chem. Soc.* **1922**, *121*, 947–958. (b) Barrio, M. C. G.; Scopes, D. I. C.; Holtwick, H. B.; Leonard, N. J. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 3986–3988. (c) Leonard, N. J.; Carraway, K. L.; Helgeson, J. P. *J. Heterocycl. Chem.* **1965**, *2*, 261–297.

(19) (a) Witanowski, M.; Stefaniak, L.; Webb, G. A. *Annu. Rep. NMR Spectrosc.* **1981**, *11B*, 115. (b) Wiemer, D. F.; Scopes, D. I. C.; Leonard, N. J. *J. Org. Chem.* **1976**, *41*, 3051–3053.

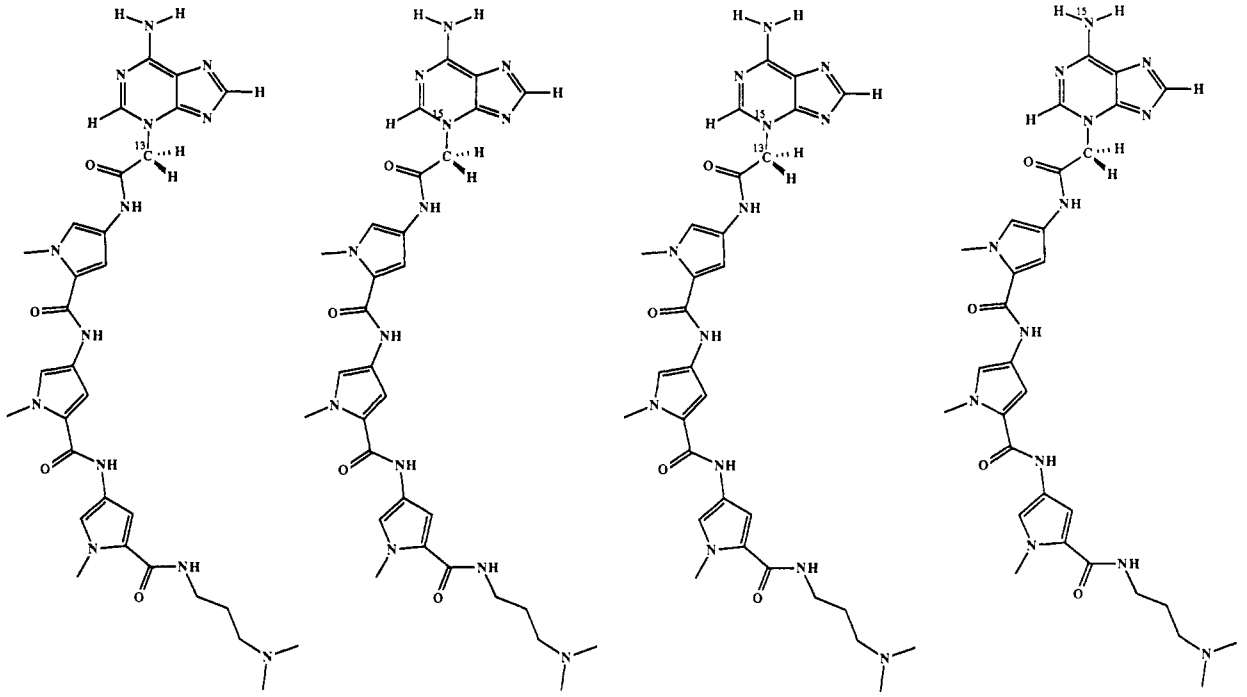


Figure 6. Synthetic isotopically labeled 3-(acetyldistamycin)adenine adducts: 3-([2-^{13}C]acetyldistamycin)adenine; 3-(acetyldistamycin)[3-^{15}N]adenine; 3-([2-^{13}C]acetyldistamycin)[3-^{15}N]adenine; and 3-(acetyldistamycin)[6-^{15}N]adenine.

142.8 Hz. The characteristic peak assignments for the unlabeled adduct are given in Figure 5.

The nitrogen spectra of 3-(acetyldistamycin)[3-^{15}N]adenine shows a singlet at −221.21 ppm (Figure 7A) and the double-labeled compound (2-^{13}C, 3-^{15}N) shows a doublet at −221.51 and an ^{15}N–^{13}C coupling constant of 11.0 Hz (Figure 7B). In the proton-decoupled carbon spectra of the [2-^{13}C]distamycin–adenine adduct, we observe a singlet at 50.53 ppm (Figure 7C). In the proton decoupled carbon spectra of the ^{13}C2 and ^{15}N3 adduct we observe a doublet at 50.55 ppm where $^{1}J_{C-N}$ = 11.0 Hz (Figure 7D). These coupling constants are within the range of values observed for one bond coupling between ^{13}C and ^{15}N.[20] These results establish the site of alkylation of adenine by BD as N3.

Tautomer of the Acetyldistamycin–Adenine Adduct. Two broad singlets, each equivalent to one proton at 8.02 and 7.95 ppm, appear in the proton spectrum of 3-(acetyldistamycin)adenine (arrow in Figure 5). Two imino tautomers are possible, one with proton at N6 and the other at the imidazole nitrogens N7 or N9.[21] Alternatively, the two singlets in the NMR could be assigned as two nonequivalent amine protons at N6. This latter situation would be indicative of a barrier to rotation around the nitrogen–carbon bond caused by resonance[22] or self base pairing[23] at the concentrations required for NMR studies.

[6-^{15}N]Adenine (99% enriched) and the corresponding 3-(acetyldistamycin)[6-^{15}N]adenine were synthesized[24] in order to unequivocally determine the number of protons attached to the exocyclic nitrogen (N6), i.e., determine the tautomeric species of the adduct. By ^{15}N NMR the imino form, which has one proton, will display a doublet (from ^{15}NH) and the amino form, which has two protons, will display a triplet (from $^{15}NH_2$). The nitrogen spectrum of 3-(acetyldistamycin)[6-^{15}N]adenine displays a triplet at −282 ppm with a coupling constant of 90.9 Hz (Figure 7E). This is evidence for two protons bonded to the exocyclic nitrogen. The coupling constant is of the same magnitude as those of exocyclic amines of analogous heterocycles. For adenine $^{1}J_{N-H}$ = 90 Hz, for 1-methylcytosine $^{1}J_{N-H}$ = 90 Hz,[22a] and for 5-azacytidine $^{1}J_{N-H}$ = 90 Hz.[22b] Therefore, 3-(acetyldistamycin)adenine exists in the amino form.

To determine whether the nonequivalence observed in the proton spectra was due to a barrier to bond rotation or self base pairing, the dependence of concentration on chemical shifts was examined. The proton spectrum of the ^{15}N labeled adenine adduct at 130 mM shows a pair of doublets or quartet. As the concentration is decreased the peaks coalesce. At 2.5 mM a single doublet with an N–H coupling constant of 90.2 Hz is observed indicating that the protons are equivalent. The results at lower concentrations demonstrate that alkylation at the N3 site of adenine by BD does not create a barrier of rotation around the C–N exocyclic bond. The nonequivalent protons at high concentrations are likely not due to base stacking (this would alter the chemical shifts of H2 and H8) but due to selfbase-pairing as observed in crystal structures of similar compounds like adenine, 9-methyladenine, and deoxyadenosine.[23]

DNA Product Analysis. By analogy to the known alkylation–depurination studies on DNA,[3,4] the 3′ and 5′ termini of the DNA substrate at the site of alkylation–depurination and cleavage after piperidine workup should be phosphate groups. Accordingly, we find that the DNA fragments resulting from cleavage of DNA by BD comigrate by gel electrophoresis with cleavage products from the reaction with dimethyl sulfate known to terminate in phosphoryl groups (Figure 8). To confirm the presence of a terminal phosphate at the site of cleavage, the 5′ (and 3′) ^{32}P labeled oligonucleotide fragment was allowed to react with the appropriate phosphatase. T4 polynucleotide kinase hydrolyzes a 3′ phosphate to a hydroxyl group and calf intestinal phosphatase hydrolyzes a 5′ phosphate. Conversion of the phosphate group to a hydroxyl decreases the electrophoretic mobility of the DNA

(20) For example, the coupling constant of neat pyrrole is $^{2}J_{N-H}$ = 4.5 Hz and that of N2 and H3 for 2-methylindazole measures as $^{2}J_{N-H}$ = 4.3 Hz in dimethyl sulfoxide. These values are in contrast to two bond coupling constants of pyridinic nitrogens and protons. For example, the coupling constant for pyridine measures as $^{2}J_{N-H}$ = 11 Hz in acetone,[19a] that of N3 and H2 of adenine as $^{2}J_{N-H}$ = 13 Hz in D_2O,[19a] and that of N2 and H3 of indazole as $^{2}J_{N-H}$ = 12.8 Hz in dimethyl sulfoxide.[19b]

(21) (a) Gonnella, N. C.; Roberts, J. D. *J. Am. Chem. Soc.* **1982**, *104*, 3162–3164. (b) Chenon, M. T.; Pugmire, R. J.; Grant, D. M.; Panzica, R. P.; Townsend, L. B. *J. Am. Chem. Soc.* **1975**, *97*, 4627–4636. (c) Gonnella, N. C.; Nakanishi, H.; Holtwick, J. B.; Horowitz, D. S.; Kanamori, K.; Leonard, N. J.; Roberts, J. D. *J. Am. Chem. Soc.* **1983**, *105*, 2050–2055.

(22) (a) Becker, E. D.; Miles, H. T.; Bradley, R. B. *J. Am. Chem. Soc.* **1965**, *87*, 5575–5582. (b) Roberts, J. D.; Sullivan, G. R.; Pang, P. P.; Leonard, N. *J. Org. Chem.* **1981**, *46*, 1014–1016.

(23) Voet, D.; Rich, A. *Prog. Nuc. Acid Res. Mol. Biol.* **1970**, *10*, 183–265.

(24) (a) Gao, X.; Jones, R. A. *J. Am. Chem. Soc.* **1987**, *109*, 1275–1278. (b) Gao, X.; Jones, R. A. *J. Am. Chem. Soc.* **1987**, *109*, 3169–3171.

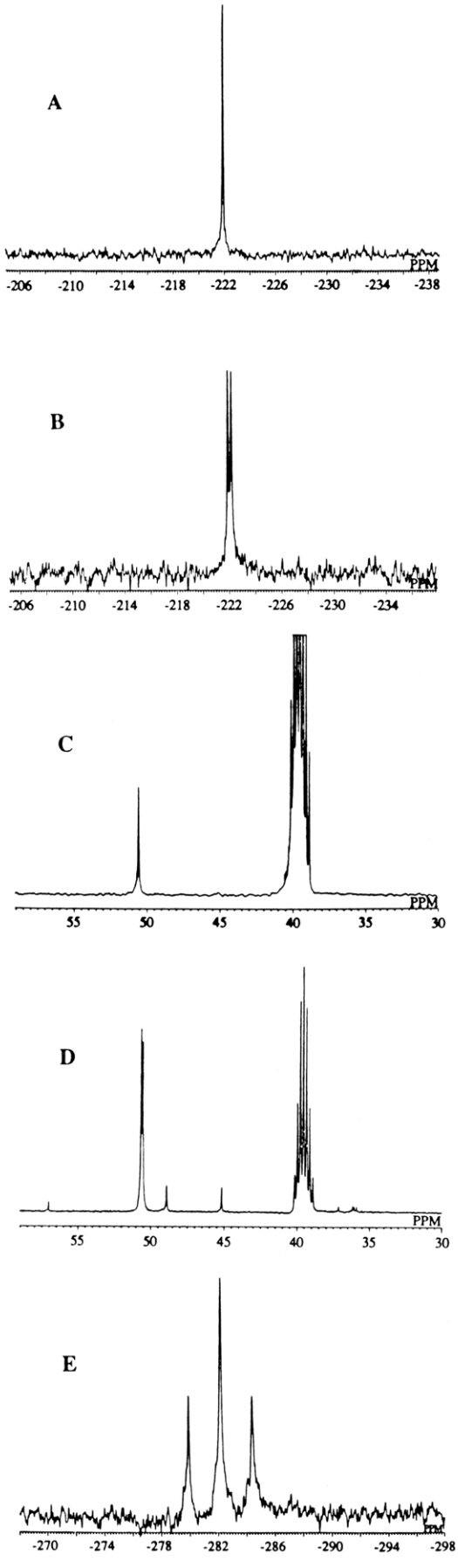


Figure 7. Nitrogen-15 spectra of (A) 3-(acetyldistamycin)[3-^{15}N]adenine and (B) 3-([2-^{13}C]acetyldistamycin)[3-^{15}N]adenine; carbon-13 spectra of (C) 3-([2-^{13}C]acetyldistamycin)adenine and (D) 3-([2-^{13}C]acetyldistamycin)[3-^{15}N]adenine; nitrogen-15 spectrum of (E) 3-(acetyldistamycin)[6-^{15}N]adenine.

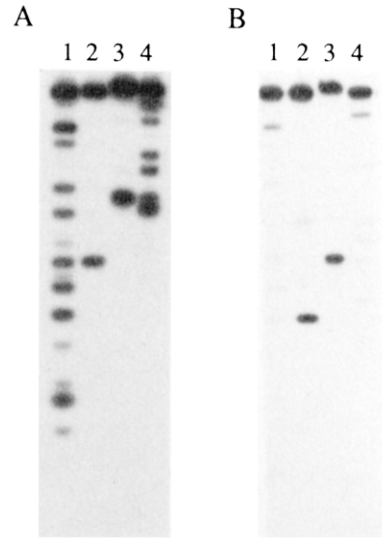


Figure 8. Characterization of the DNA termini at the cleavage site by analysis of altered electrophoretic mobilities after enzymatic dephosphorylation: (A) 3′ end analysis using T4 polynucleotide kinase; (B) 5′ end analysis using calf alkaline phosphatase. Lane 1, G + A reaction products; lane 2, BD + 15mer reaction products; lane 3, BD + 15mer reaction products treated with phosphatase; and lane 4, G + A reaction products treated with phosphatase.

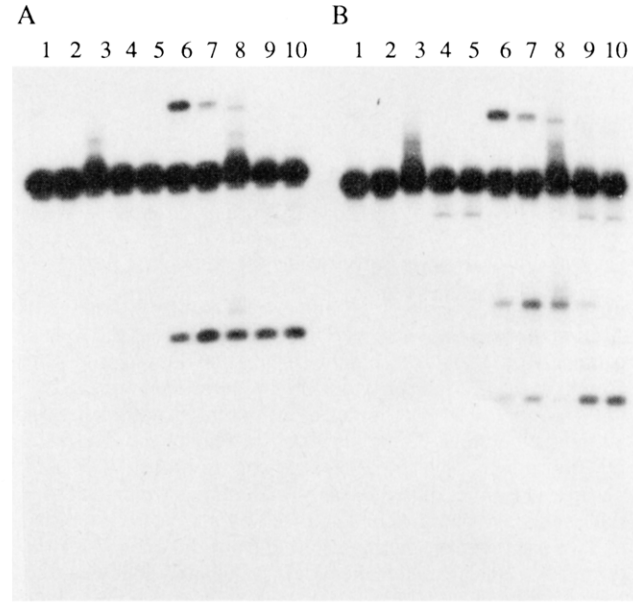


Figure 9. Autoradiogram of the sequential analysis of the 15 base pair oligonucleotide-BD reaction (A) 3′-^{32}P end-labeled 15mer and BD, (B) 5′^{32}P end-labeled 15mer and BD. Incubation times: 0 h (lanes 1–5) and 24 h (lanes 6–10). Sequential analysis: incubation (lanes 1 and 6), heat, 15 min, 90 °C (lanes 2 and 7), 1 M piperidine, 5 min, 25 °C (lanes 3 and 8), 1 M piperidine, 15 min, 90 °C (lanes 4 and 9), and 1 M piperidine, 30 min, 90 °C (lanes 5 and 10).

fragment. The two types of termini, hydroxyl and phosphoryl, on DNA fragments of similar length are resolved by gel electrophoresis using a 20% polyacrylamide gel. Comparison of DNA cleavage products (lanes 2) and DNA cleavage products treated with phosphatases (lanes 3) is shown in Figure 8 confirming that the 5′ and 3′ termini at the cleavage site are phosphate groups.

To further characterize the intermediates in the reaction by which BD cleaves the phosphodiester backbone, the DNA products at each stage of the reaction and workup procedure were monitored by their gel electrophoretic mobilities. Analysis was carried out on the 15 base pair duplex 5′-CGGTAGTTTATCACA-3′. This strand, labeled either at the 3′ or 5′ end with ^{32}P, was analyzed by 20% polyacrylamide gel electrophoresis (Figure 9).

A one-to-one mixture of the 15mer and BD (10 μM each) in 100 mM NaPhosphate buffer (pH 7.0) was incubated at 37 °C

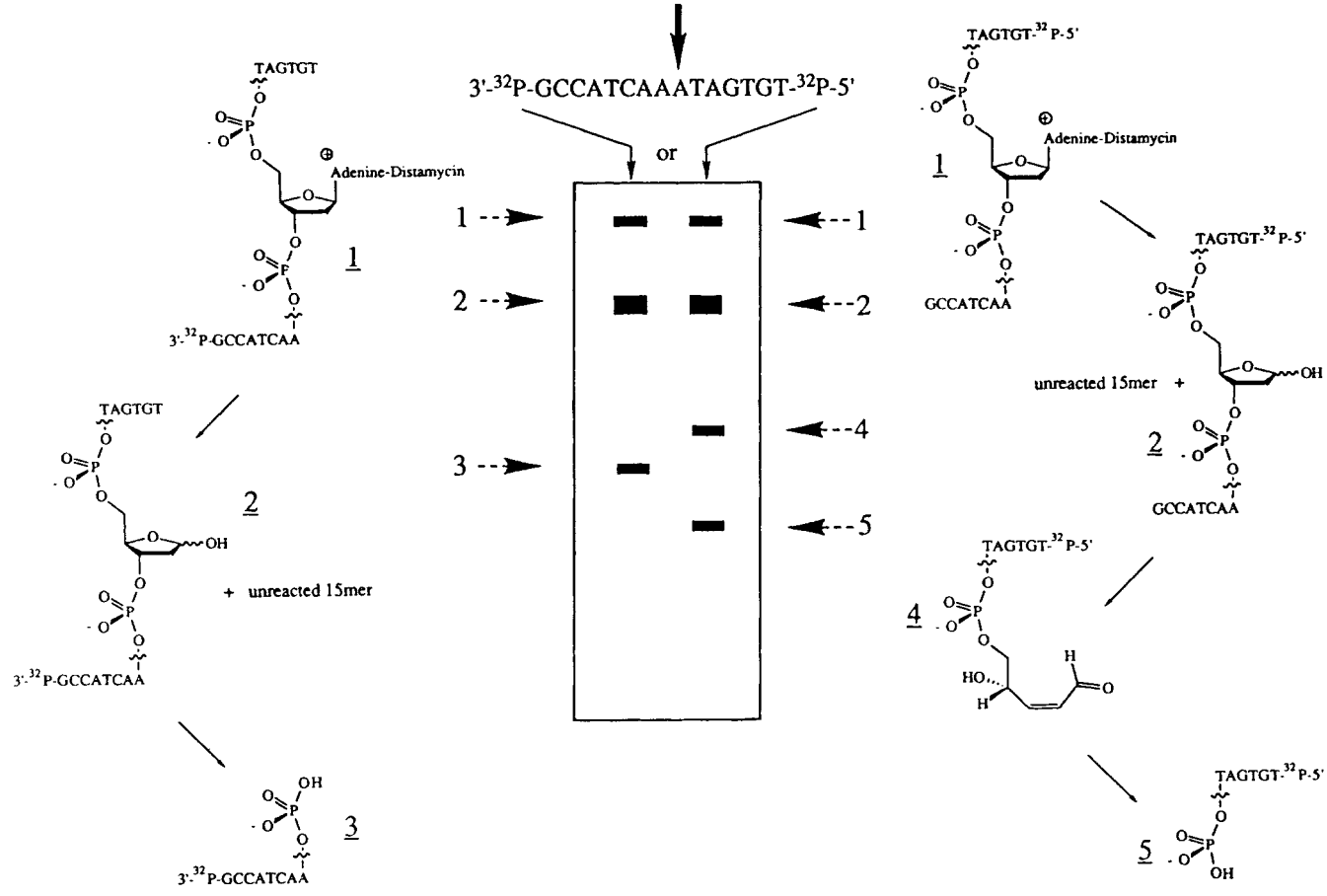


Figure 10. Diagram of the gel band assignments based on the sequential analysis of the 15 base pair oligonucleotide BD reaction: (1) acetyldistamycin covalently attached to the 15mer, (2) unreacted 15mer and potentially the depurinated 15mer, (3) 6mer with a 5′ phosphate at the site of cleavage, (4) 8mer with a 3′ sugar residue at the site of cleavage, and (5) 8mer with a 3′ phosphate at the site of cleavage.

for 0 (Figure 9, lanes 1–5) and 24 h (Figure 9, lanes 6–10). Aliquots for gel analysis were removed at each of the following consecutive steps: lanes 1 and 6, incubation; lanes 2 and 7, heat, 15 min at 90 °C; lanes 3 and 8, 1 M piperidine, 5 min at 25 °C; lanes 4 and 9, 1 M piperidine, 15 min at 90 °C; and lanes 5 and 10, 1 M piperidine, 30 min at 90 °C. Many of the intermediate DNA products that precede and lead to the final 3′ and 5′ phosphate termini of the two DNA fragments where cleavage of the backbone occurs were visualized by gel electrophoresis.

Two products are distinguishable from the analysis of the 5′ termini (3′ labeled oligonucleotide) immediately following the 24 h time period at 37 °C Figure 9A, lane 6). Reading top to bottom on the autoradiogram, the slow migrating band corresponds to the DNA strand with acetyldistamycin covalently attached to it assigned by detection of ^{14}C in the band after reacting the 15mer with labeled BD ([2-^{14}C]2). The middle intense band migrates at a rate corresponding to the intact oligonucleotide 15mer. This band could also include depurinated oligonucleotide. The fastest migrating band is a 6mer, the cleaved DNA product. Heating the reaction at 90 °C for 15 min almost completes depurination and cleavage of the strand (lane 7). Complete cleavage requires piperidine and heat (Figure 9A, lanes 9 and 10).

Analysis of the 3′ termini (5′ labeled strand) shows three distinct products following a 24 h reaction time (Figure 9B, lane 6). Reading top to bottom on the autoradiogram the slowest moving band is assigned to the 15mer–acetyldistamycin adduct. The next strong band is the intact 15mer. The band that follows is believed to be an 8mer with the partial sugar residue remaining at the 3′ end after the β-elimination step. The final band is the 8mer following loss of the sugar residue. Piperidine and heat complete depurination and hydrolysis of the sugar yielding the 8mer as the only product. The control lanes demonstrate that the cleaved DNA and the preceding products are not a result of the presence of BD prior to the heat and base workup procedure. Product assignments for each band are summarized in Figure 10.

Kinetic Analysis. The reaction of *N*-bromoacetyldistamycin (BD) with double helical DNA has four experimentally distinguishable steps: binding to the double helical DNA, covalent attachment to adenine, depurination of the distamycin–adenine adduct, and strand cleavage. In order to investigate the rates of these different steps at a single reaction site, the 15 base pair oligonucleotide duplex, 5′-CGGTAGTTTATCACA-3′, was utilized as the DNA substrate.

Binding/Complexation. The percentage of *N*-[1-^{14}C]acetyldistamycin (AcD) and *N*-bromo[2-^{14}C]acetyldistamycin bound to the 15mer was quantitated by equilibrium dialysis. The *N*-[1-^{14}C]acetyldistamycin was utilized to determine the time required for diffusion and equilibration across the dialysis membrane and to test whether the α-bromo substitutent alters the binding affinity of the ligand. The binding constants for *N*-bromoacetyldistamycin and *N*-acetyldistamycin with the 15 base pair duplex 5′-CGGTAGTTTATCACA-3′ (100 mM sodium phosphate, pH 7.0) are $K_{BD} = 5.5 \times 10^5$ (±0.4) M^{-1}, and $K_{AcD} = 8.8 \times 10^5$ (±0.4) M^{-1}, respectively.[25]

Alkylation/Covalent Attachment. The alkylation rates were determined by monitoring simultaneously the disappearance of the reactant, BD, and the appearance of the product, 3-(acetyldistamycin)adenine (DA), by reverse phase HPLC in the presence of the 15 base pair oligonucleotide duplex, 5′-CGGTAGTTTATCACA-3′. Peak identity was assigned by ultraviolet spectra of each compound as it eluted from the column and by co-injection comparisons with the authentic compounds bromoacetyl-, *N*-

(25) The equilibration time for *N*-acetyldistamycin (10 μM in 100 mM NaPhosphate, pH 7.0 at 37 °C) between chambers is approximately 6 h, which is much less than the time required for significant covalent reaction of BD with DNA ($t_{1/2}$ = 36 h at 37 °C).[26]

(26) Baker, B. F. Ph.D. Dissertation, California Institute of Technology, 1988.

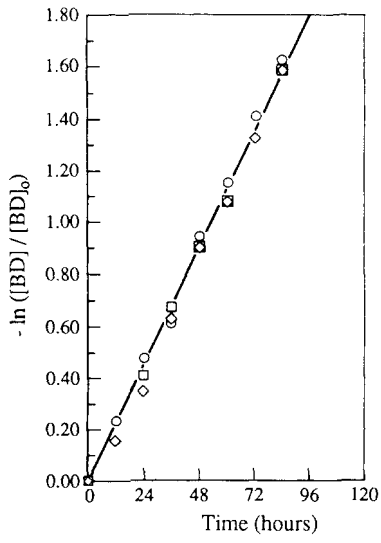


Figure 11. Rate of alkylation of the 15 base pair oligonucleotide (100 μM) by BD (10 μM) at 37 °C, 100 mM sodium phosphate (pH 7.0). Plot of time vs $-\ln([BD]/[BD]_0)$. Symbols represent three separate runs.

hydroxyacetyldistamycin, and 3-(acetyldistamycin)adenine (BD, HOD, DA). Changes in concentrations through time were quantitatively determined by peak area and with reference to an internal standard. In the presence of excess DNA (100 μM 15mer) the trace amount of *N*-hydroxyacetyldistamycin (HOD) remained constant throughout the reaction. Therefore, hydrolysis of *N*-bromoacetyldistamycin (BD) (10 μM) to the corresponding alcohol does not occur and we presume this is due to sequestering of BD by the duplex DNA.

The rate of disappearance of BD is described by the following first-order rate expression: $-\ln [BD]/[BD]_0 = kt$. On the basis of this, one may consider a minimal mechanistic scheme of prior complexation of BD with duplex DNA followed by a first-order reaction within the complex to afford the covalently bound distamycin to N3 of adenine.

$$\text{DNA} + \text{BD} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{DNA·BD} \xrightarrow{k_2} \text{DNA–D}$$

As the ratio of DNA:BD is increased, the rate constant (k) for disappearance of BD increases, suggesting that more BD is bound productively for reaction.[27] Above a 10:1 ratio of 15mer:BD (100 μM 15mer:10 μM BD), k is constant. Equilibrium dialysis measurements indicated that >98.0% of BD is bound to the 15mer duplex under identical conditions. Three samples (100 μM 15mer, 10 μM BD) were analyzed for the rate of alkylation at adenine 48 on the 15mer duplex by BD at 37 °C (Figure 11). From $-\ln [BD]/[BD]_0 = kt$, we find that $k_{BD} = 5.39 \times 10^{-6}$ s^{-1} (±0.03, σ = 0.998) ($t_{1/2}$ = 35.7 h).

Cleavage of the 167 bp fragment with a series of halogen substituted acetyl groups (Cl, Br, I) on distamycin reveals that the rate of cleavage is affected by the leaving group but the site of cleavage is not. Curiously, the rate of disappearance of *N*-iodoacetyldistamycin (ID) appeared slower than BD on the 167 bp restriction fragment. The rate of alkylation of DNA by ID is too slow at 37 °C to be conveniently measured and rate comparisons were limited to 45 °C. HPLC analysis of the reaction with oligonucleotide indicates that the only reaction product is 3-(acetyldistamycin)adenine. Thus the decrease in reactivity is not caused by an increase in the rate of hydrolysis (competitive binding by *N*-hydroxyacetyldistamycin) or degradation. The observed first-order rate constants for disappearance of ID and BD at 45 °C (100 μM 15mer, 10 μM BD or ID) were $k_{ID} = 8.89 \times 10^{-6}$ s^{-1} (±0.10, σ = 0.995) ($t_{1/2}$ = 21.6 h) and $k_{BD} = 1.46 \times 10^{-5}$ s^{-1} (±0.02, σ = 0.998) ($t_{1/2}$ = 13.2 h). The relative rate of alkylation, k_{ID}/k_{BD}, is 0.6.[28]

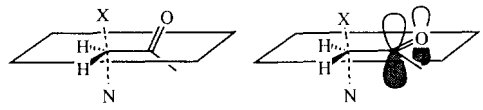


Figure 12. Transition-state models that display the different geometries for displacement of the halogen (X) in the minor groove due to steric constraints (left) in comparison to displacement free in solution due to stereoelectronic factors (right).

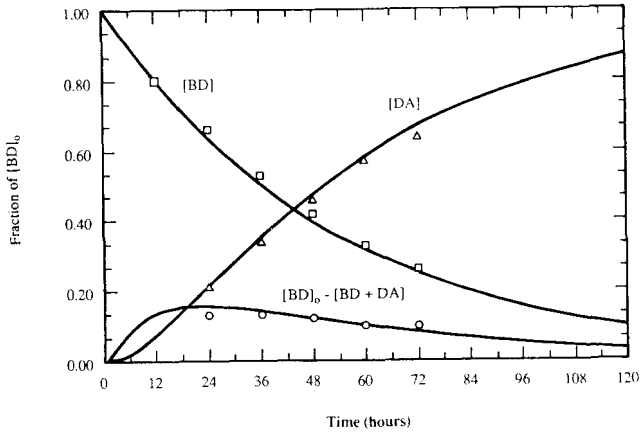


Figure 13. Amounts of the three distamycin reaction species through time, *N*-bromoacetyldistamycin (BD), distamycin covalently bound to the DNA duplex ($[BD]_0$–[BD + DA]), and 3-(acetyldistamycin)adenine (DA). $-d[BD]/dt = k_2[DNA·BD]$, $d[DNA–D]/dt = k_2[DNA·BD] - k_3[DNA–D]$, and $d[DA]/dt = k_3[DNA–D]$. Calculated points (lines) are derived from the integrated forms of the rate equations which yields the concentration of each species at any given time: $[DNA·BD] = [DNA·BD]_0 e^{-k_2 t}$; $[DNA–D] = [DNA·BD]_0\{k_2/(k_3 - k_2)\}(e^{-k_2 t} + e^{-k_3 t})$; $[DA] = [DNA·BD]_0\{1 + (1/(k_2 - k_3))(k_3 e^{-k_2 t} + k_2 e^{-k_3 t})\}$, where $k_2 = 3.95 \times 10^{-6}$ s^{-1} and $k_3 = 2.16 \times 10^{-5}$ s^{-1}. Data points (symbols) are obtained from the HPLC chromatograms.

The relative rate of reactivity of BD and ID is the inverse of what one would expect from relative leaving group abilities in SN2 reactions. One might imagine that in the absence of DNA, the optimal orientation for displacement of the halogen bond is orthogonal to the plane of the carbonyl group due to stereoelectronic arguments. However, when BD and ID are bound to DNA, the haloacetyl group is constrained by the walls of the minor groove. One could imagine that the halogen leaving group is oriented more toward the plane of the carbonyl in this case. The larger iodo atom might have a higher steric barrier to approach the optimal orthogonal geometry in the transition state for displacement (Figure 12). Alternatively, the rate differences for BD and ID could be differential solvation of the anionic leaving group as it emerges from the minor groove of DNA.

The relative rate of disappearance of *N*-chloroacetyldistamycin (CD) was measured on the 15mer at 45 °C (100 μM 15mer, 10 μM CD). The rate of alkylation is very slow for this compound and the derived rate constant is only an estimate based on the initial rate of disappearance of *N*-chloroacetyldistamycin. Four separate samples were examined at 48 h from which initial rates were determined. Assuming that the chloro reaction adheres to first-order kinetics, $k_{CD} \sim 8.06 \times 10^{-7}$ s^{-1}, $t_{1/2}$ = 239 h, and $k_{CD}/k_{BD} \simeq 0.06$ at 45 °C.

Depurination/Base Release. Alkylation at N3 of adenine leads to purine labilization and hydrolysis of the *N*-glycosidic bond.[29]

(27) Baker, B. R. *Design of Active Site Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active Site*; John Wiley and Sons, Inc.: New York, 1967; pp 122–126.

(28) Relative rate studies were conducted on the binding site 5′-AAATT-3′ (positions 58 and 59) in a 21mer oligonucleotide duplex ^{32}P5′-TCA-CAGTTAAATTGCTAACGC-3′ and analyzed by gel electrophoresis. Cleavage analysis by this method gives $k_{ID}/k_{BD} = 0.6$.

(29) (a) Lindahl, T.; Nyberg, B. *Biochemistry* **1972**, *11*, 3610–3618. (b) Grossman, L.; Grafstrom, R. *Biochimie* **1982**, *64*, 577–580. (c) Loeb, L. A.; Preston, B. D. *Annu. Rev. Genet.* **1986**, *20*, 201–230. (d) Manoharan, M.; Ransom, S. C.; Mazumder, A.; Gerlt, J. A.; Wilde, J. A.; Withka, J. A.; Bolton, P. H. *J. Am. Chem. Soc.* **1988**, *110*, 1620–1622.

The first-order rate constant for release of the 3-(acetyldistamycin)adenine adduct (DA) is dependent on the concentration of the covalent DNA–distamycin complex. Assuming alkylation of adenine followed by depurination involves two consecutive first-order rate processes,[30] the rate equations can be analyzed for the appearance and disappearance of the three distamycin

$$\text{DNA·BD} \xrightarrow{k_2} \text{DNA–D} \xrightarrow{k_3} \text{DNA + 3-(acetyldistamycin)adenine}$$

reaction species—BD, distamycin covalently bound to the DNA duplex (DNA-D), and the released 3-(acetyldistamycin)adenine product. $-d[BD]/dt = k_2[DNA\cdot BD]$, $-d[DNA–D]dt = k_2[DNA\cdot BD] - k_3[DNA–D]$, $d[DA]/dt = k_3[DNA–D]$. Integration of the rate equations yields the concentrations of each species at any given time (Figure 13). Iterative fitting of the integrated formulas to the experimental HPLC data was done to determine if the reaction was of this type and, if so, to determine k_3, the rate constant for depurination. The concentrations of BD and DA were determined at each time point. The difference in concentration of [BD] and [DA] affords a measure of buildup and decay of a DNA–distamycin covalent intermediate.

Four separate reactions conducted at initial concentrations of 100 μM 15mer and 10 μM BD in 100 mM NaPhosphate (pH 7.0) at 37 °C were analyzed. The final product DA was not detected until after 24 h. The existence of such an induction period is an indication that the released product DA is not formed directly in the reaction but via some intermediate. This intermediate is assigned as the covalent acetyldistamycin–DNA adduct based on the results acquired in the sequential analysis of the DNA product by gel electrophoresis (Figure 9 and 10).

The rate constants derived after iterative fitting are $k_2 = 5.39 \times 10^{-6}$ s^{-1} and $k_3 = 2.16 \times 10^{-5}$ s^{-1} (Figure 13). The data are consistent with two consecutive first-order processes—alkylation at N3 and then adduct release. The derived rate constants indicate that alkylation is the rate-determining step of the cleavage reaction at 37 °C. Since the rate constants for alkylation and depurination are relatively close only a small buildup of the covalent distamycin–DNA adduct is observed. The amount of the distamycin–DNA adduct passes through a maximum at 24 h and then diminishes. This observation indicates that the sole BD product in this system under these reaction conditions (37 °C) is the released product 3-(acetyldistamycin)adenine. Alkylation of other sites, such as the O2 of thymine, would likely result in an increase in the amount of a distamycin–DNA adduct with time.

A Comparison to CC-1065. The synthetic *N*-bromoacetyldistamycin and the natural product antibiotic CC-1065 have many features that are common. Both are crescent-shaped molecules with an electrophile at one end that binds covalently at N3 of adenine in the minor groove of DNA at 37 °C. However, the rates and covalent binding specificities of these two molecules are quite different. The cleavage specificities of *N*-bromoacetyldistamycin and CC-1065, were compared on the 167 bp restriction fragment. Each compound was allowed to react with the ^{32}P-labeled DNA (3′ and 5′) at 37 °C for 1 h (10 mM NaPhosphate pH 7.0, 100 μM bp calf thymus DNA). Then each reaction mixture was heated for 15 min at 90 °C in the reaction buffer and again for 15 min at 90 °C in 1 M piperidine. Sites of cleavage were determined by high-resolution denaturing gel electrophoresis (Figure 14A). After 1 h, CC-1065 cleaves the DNA at 13 adenines (lanes 9 and 10). On the other hand, after 1 h under the same reaction conditions and workup procedure, we observe a minute amount of cleavage at adenine, base pair 48, by BD (lanes 5 and 6). After 10 h (lanes 7 and 8) BD approaches the same degree of cleavage at one adenine as observed with CC-1065 at several sites after a 1 h reaction time. Clearly, the absolute rates of covalent binding are significantly different for BD vs CC-1065 (Figure 14B). The difference in covalent binding specificity reflects differences in both the binding specificity and sequence dependent reactivity of BD vs CC-1065. In the case of BD, sequence dependent binding is evident from footprinting studies which reveal that equilibrium binding to four A,T rich sites five base pairs in size occurs within minutes on the 167 bp DNA fragment.[2] Seven adenines within these four sites are proximal to the bromoacetylmethylene and are suitable candidates for covalent reaction. On a slower time scale (hours), sequence dependent covalent reaction within these four bound sites is evident by preferred alkylation at one adenine. The combination of sequence dependent binding and sequence dependent reactivity by BD affords a highly discriminating sequence specific DNA cleaving molecule. Researchers at Upjohn and Austin have recently shown that the cyclopropyl subunit of CC-1065 *alone* has sufficient structural information to mediate the sequence specificity of the entire CC-1065 molecule.[8d] In the case of CC-1065, covalent binding specificity appears to be determined mostly by the sequence dependent reactivity of the alkylating subunit.[8d] One could argue then that the differences in covalent binding specificity between CC-1065 and BD are related to sequence dependent reactivity *in both cases* but sequence dependent binding being additionally important in the latter.

Conclusion

In summary, we have described the synthesis of *N*-bromoacetyldistamycin and the time/temperature dependence of the cleavage specificity on DNA restriction fragments. The structure of the released adenine adduct, 3-(acetyldistamycin)adenine, has been characterized and together with the 3′ and 5′ phosphate termini at the cleavage site are consistent with the mechanism being alkylation at N3 of adenine in the minor groove of DNA at distamycin binding sites which are five base pairs in size. The rate of disappearance of BD reveals that the covalent attachment step is slow ($t_{1/2}$ = 36 h at 37 °) with respect to noncovalent complexation to the thermodynamically favored binding sites (minutes). The preferred alkylation at one adenine within four binding sites which totals 20 base pairs and contains seven adenines proximal to the bromoacetyl moiety reveals sequence dependent site reactivity in combination with sequence dependent binding in mediating the overall alkylation sequence specificity. This combination of sequence specific binding coupled to sequence dependent reactivity affords a designed molecule with covalent specificity that appears to surpass that of the natural product CC-1065. Finally, the unexpected reversal of relative rates of reactivity for bromo- vs iodoacetyldistamycin lead us to speculate that, for reactive molecules bound in the groove of duplex DNA, stereoelectronic and/or differential solvation factors might alter, in some instances, the energetically favorable pathways usually seen for substrates free in solution.

Experimental Section

Reagents and Materials. HPLC grade DMF was dried over 3 Å molecular sieves. Silica gel 60 (230–400 mesh) was from EM Science, adenine and calf thymus DNA was from Sigma, and [2-^{13}C]bromoacetic acid (99.8% enriched) was from MSD Isotopes. [2-^{14}C]Bromoacetic acid (52 mCi/mmol), [1-^{14}C]glycolic acid sodium salt (8.8 mCi/mmol), and [1-^{14}C]acetic anhydride (30 μCi/umol) were from Amersham. H^{15}NO$_3$ (99.9% enriched) was from Merck Sharpe and Dohme. Enzymes were from Boehringer Mannheim, and CC1065 was a gift from Dr. Wendell Wierenga of the UpJohn Company.

Instrumentation. Infrared spectra were recorded on a Shimadzu Model IR-435 infrared spectrometer. High resolution positive ion fast atom bombardment mass spectra were performed by the Midwest Center for Mass Spectrometry at the University of Nebraska. Ultraviolet–visible spectra were obtained on a Beckman Model 25 UV–vis spectrophotometer and a Perkin-Elmer Lambda 4C UV–vis spectrophotometer. Proton, carbon, and nitrogen NMR spectra were acquired from a JEOL GX400 and are recorded in parts per million internally from tetramethylsilane (proton), dimethyl sulfoxide (carbon), and externally from 1 M Na^{15}NO$_3$ (nitrogen). The [3-^{15}N]adenine adduct experiments utilized the G40T10 probe, a pulse delay of 5 s, and a pulse width of 45 μs and were proton decoupled w/o NOE. The [6-^{15}N]adenine adduct experiments utilized the G40MU probe, a pulse delay of 1.0 s, and pulse width of 135 μs and

(30) Levine, I. N. *Physical Chemistry*, 2nd ed.; McGraw-Hill Book Co.: New York, 1983; pp 506–507.

(31) (a) Storm, D. R.; Koshland, D. E., Jr. *J. Am. Chem. Soc.* **1972**, *94*, 5815–5825. (b) Menger, F. M. *Tetrahderon* **1983**, *39*, 1013–1040. (c) Kirby, A. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 183–279. (d) Harris, J. M.; McManus, S. P., Eds. *Nucleophilicity*; American Chemical Society: Washington, D.C., 1987; Adv. Chem. Ser. Vol. 215.

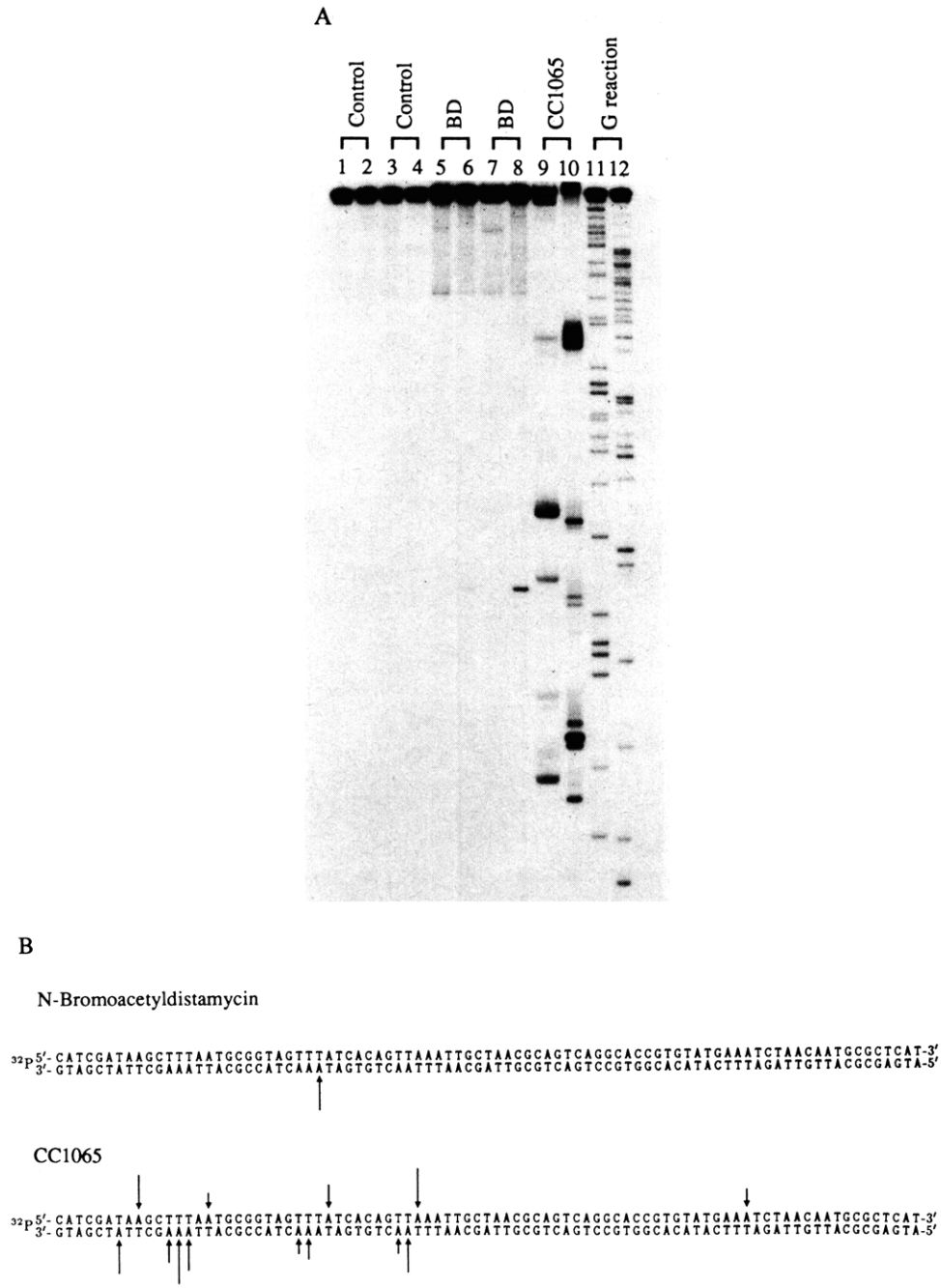


Figure 14. (A) Autoradiogram of a high-resolution polyacrylamide gel which compares the cleavage sites of CC1065 to BD on the 167 bp restriction fragment. Odd and even numbered lanes are DNA end-labeled with ^{32}P at the 5′ and 3′ position, respectively. Final concentrations are 100 μM bp calf thymus DNA and 5 μM cleaving reagent in 10 mM sodium phosphate, pH 7.0, at 37 °C. (B) Histogram of sites of cleavage of CC1065 and BD between base pairs 22 and 108 of the pBR 322 plasmid. Arrows indicate sites and extent of DNA cleavage resulting from the reaction of BD (10 h at 37 °C) and CC1065 (1 h at 37 °C) on the 167 bp restriction fragment.

were proton coupled w/o NOE. A Beckman System 1 Plus DNA Synthesizer was utilized for synthesis of oligonucleotides. Semipreparative high-pressure liquid chromatography was performed on a Beckman/Altex system with use of a C18 reverse phase column.

Kinetics and Thermodynamics. Temperature was controlled with a Forma Scientific Model 2006 temperature bath. Equilibrium dialysis experiments were carried out in a 5-cell equilibrium dialyzer (Spectrum). Scintillation counting was conducted on the Beckman 3801 system. The Hewlett Packard HP1090M work station was utilized for high-pressure liquid chromatography. Densitometry was performed by an LKB Ultroscan XL laser densitometer.

Tris(*N*-methylpyrrole)diamine 1. A solution of 0.5 g of the tripeptide nitro compound[10b,15] in 250 mL of dimethyl formamide was hydrogenated over 100 mg of 5% Pd/C at atmospheric pressure for 12 h. The mixture was filtered through Celite, concentrated under reduced pressure, and chromatographed (35% $MeOH/CH_2Cl_2$ saturated with ammonia) to afford amine **1**: ^1H NMR (Me_2SO-d_6) δ 1.64 (m, 2), 2.20 (s, 6), 2.32 (t, 2), 3.19 (m, 2) 3.74 (s, 3), 3.81 (s, 3), 3.84 (s, 3), 6.28 (d, 1), 6.39 (d, 1), 6.84 (d, 1), 7.02 (d, 1), 7.20 (d, 1), 7.22 (d, 1), 8.10 (t, 1), 9.64 (s, 1), 9.90 (s, 1); IR (KBr) 3300, 3100, 1640, 1580, 1520, 1462, 1430, 1400, 1345, 1258, 1200, 1100, 1050; *m/z* for $C_{23}H_{32}O_3N_8$, calcd 469.266, obsd 469.267.

***N*-Bromoacetyldistamycin (2).** To a solution of 108 mg (0.46 mmol) of bromoacetyltrifluoroacetic anhydride in 90 mL of CH_2Cl_2 (0 °C under argon) was added 178 mg (0.35 mmol) of amine **2** in 4 mL of CH_2Cl_2. The reaction was allowed to stir for 5 min and 35 μL (0.46 mmol) of trifluoroacetic acid was added. After 5 min the reaction was complete by TLC (1.5% NH_4OH/MeOH). The product was precipitated from solution with diethyl ether and then washed several times with ether. Ether was removed under reduced pressure and the white solid (98% *N*-bromoacetyldistamycin and 2% *N*-hydroxyacetyldistamycin) was stored at −20 °C. Yield 60%. ^1H NMR ($MeSO_2$-d_6) δ 1.84 (m, 2, aliphatic), 2.80 (s, 6, aliphatic *N*,*N*-dimethyls), 3.08 (m, 2, aliphatic), 3.25 (m, 2, aliphatic), 3.82 (s, 3, pyrrole *N*-methyl), 3.85 (s, 6, pyrrole *N*-methyls), 3.98 (s, 2, acetyl), 6.94 (s, 2, pyrrole), 7.06 (d, 1, pyrrole), 7.18 (d, 1, pyrrole), 7.20 (d, 1, pyrrole), 7.24 (d, 1, pyrrole), 8.17 (t, 1,

aliphatic chain carboxamide), 9.40 (s, 1, pyrrole carboxamide), 9.95 (s, 1, pyrrole carboxamide), 10.36 (s, 1, acetamide); UV max (H_2O) 242, 303 (ϵ 35 000 cm^{-1} M^{-1}); IR (KBr) 3300, 1640, 1580, 1530, 1460, 1430, 1400, 1260, 1200, 1170, 1130, 1105; m/z for $C_{25}H_{35}O_4N_8{}^{79}Br$, calcd 590.196, obsd 590.191.

***N*-Bromo[2-^{13}C]acetyldistamycin ([2-^{13}C]2).** The same procedure as for **2** was utilized, except the mixed anhydride was 99.8% ^{13}C enriched at position 2 of the bromoacetyl fragment: ^{1}H NMR ($MeSO_2$-d_6) δ 1.82 (m, 2), 2.78 (d, 6), 3.06 (br s, 2), 3.24 (m, 2), 3.77, 4.16 (d, 2, $^1J_{H-C}$ = 153.9 Hz), 3.80 (s, 3), 3.84 (s, 6), 6.93 (s, 2), 7.04 (s, 1), 7.16 (s, 1), 7.18 (s, 1), 7.22 (s, 1), 8.16 (t, 1), 9.25 (br s, 1), 9.90 (s, 1), 9.93 (s, 1), 10.34 (s, 1); ^{13}C NMR ($MeSO_2$-d_6) δ 29.78; m/z for $C_{24}{}^{13}CH_{35}O_4N_8{}^{79}Br$, calcd 591.200, obsd 591.188.

***N*-Bromo[2-^{14}C]acetyldistamycin ([2-^{14}C]2).** Bromo[2-^{14}C]acetyltrifluoroacetic anhydride (14 mg (60 μmol), 0.5 μCi/μmol synthesized from bromo[2-^{14}C]acetic acid) in 0.5 mL in CH_2Cl_2 was added to 23 mg (50 μmol) of amine **1** in 5 mL of CH_2Cl_2 at 0 °C under argon. After 2 min 100 μL of trifluoroacetic acid was added, solvent was removed, and the residue was taken up into 0.5 mL of methanol and 0.5 mL of dichloromethane. Diethyl ether (10 mL) was added and 28 mg (40 μmol) of the white precipitate was collected and washed with ether. Yield 80%.

***N*-Chloroacetyldistamycin (3).** The same procedure for the synthesis of **2** was followed by using chloroacetyltrifluoroacetic anhydride. ^{1}H NMR (Me_2SO-d_6) δ 1.84 (m, 2), 2.79 (s, 6), 3.07 (m, 2), 3.25 (m, 2), 3.82 (s, 3), 3.85 (s, 6), 4.19 (s, 2), 6.94 (d, 1), 6.95 (d, 1), 7.06 (d, 1), 7.18 (s, 1), 7.20 (s, 1), 7.24 (s, 1), 8.17 (t, 1), 9.45 (br s, 1), 9.92 (s, 1), 9.95 (s, 1), 10.29 (s, 1); UV max (H_2O) 240, 302 (ϵ 35 000 cm^{-1} M^{-1}); IR 3300, 1680, 1640, 1580, 1530, 1460, 1430, 1400, 1260, 1200, 1170, 1130, 1105; m/z for $C_{25}H_{34}O_4N_8Cl$, calcd 545.239, obsd 545.239.

***N*-Iodoacetyldistamycin (4).** The same procedure for the synthesis of **2** was followed with iodoacetyltrifluoroacetic anhydride. ^{1}H NMR (Me_2SO-d_6) δ 1.84 (m, 2), 2.79 (s, 6), 3.08 (m, 2), 3.25 (m, 2), 3.79 (s, 2), 3.82 (s, 3), 3.85 (s, 6), 6.92 (d, 1), 6.95 (d, 1), 7.06 (d, 1), 7.17 (s, 1), 7.18 (s, 1), 7.23 (s, 1), 8.16 (t, 1), 9.34 (br s, 1), 9.91 (s, 1), 9.94 (s, 1), 10.29 (s, 1); UV_{max} (H_2O) 240, 304 (ϵ 35 000 cm^{-1} M^{-1}); IR (KBr) 3300, 1640, 1580, 1540, 1465, 1435, 1405, 1260, 1200, 1175, 1130, 1105; m/z for $C_{25}H_{34}O_4N_8I$, calcd 637.175, obsd 637.174.

***N*-Hydroxydistamycin (5).** Glycolic acid (7.5 mg, 0.10 mmol) and 13 mg (0.10 mmol) of *N*-hydroxybenzotriazole were mixed in 1 mL of DMF for 5 min. Next 45 mg (0.10 mmol) in 0.75 mL of DMF of amine **1** was added, followed by 24 mg (0.12 mmol) of dicyclohexylcarbodiimide. The reaction was allowed to stir at room temperature for 6 h and then concentrated under reduced pressure. The product was purified by flash chromatography with 15% $MeOH/CH_2Cl_2$ saturated with ammonia to yield 43 mg (0.082 mmol) of **5** (82%): ^{1}H NMR (Me_2SO-d_6) δ 1.65 (q, 2), 2.24 (s, 6), 2.38 (t, 2), 3.19 (q, 2), 3.80 (s, 3), 3.83 (s, 3), 3.84 (s, 3), 3.95 (d, 2), 5.59 (t, 1), 6.84 (d, 1), 7.04 (s, 2), 7.18 (d, 1), 7.20 (d, 1), 7.24 (d, 1), 8.09 (t, 1), 9.66 (s, 1), 9.89 (s, 1), 9.92 (s, 1); UV_{max} (H_2O) 235, 303 nm; m/z for $C_{25}H_{34}O_5N_8$, calcd 527.273, obsd 527.274.

***N*-Hydroxy[1-^{14}C]acetyldistamycin ([1-^{14}C]5).** [1-^{14}C]Glycolic acid sodium salt (0.44 μg, 5.68 μmol) was transferred in water to a 3-mL reaction vial. The water was removed by lyopholization, and 2.7 mg (5.68 μmol) of amine **1**, 0.77 mg (5.68 μmol) of *N*-hydroxybenzotriazole (HBT), and 1.2 mg (5.68 μmol) of dicyclohexylcarbodiimide (DCC) in dichloromethane were added to the vial giving a total volume of 400 μL. Approximately 1 mg of 18-crown-6 ether was added. After 5 h, 1.2 mg (6 μmol) of DCC and 0.81 mg (0.6 μmol) of HBT was added. After 20 h the solvent was removed and the reaction mixture was flash chromatographed through a pipet with 15% $MeOH/CH_2Cl_2$ saturated with ammonia to yield 2.5 mg (84%) of [1-^{14}C]**5** (8.8 μCi/μmol).

[1-^{14}C]Acetyldistamycin ([1-^{14}C]6). Amine **1** (4.2 mg, 9 μmol) and 11 μL (80 μmol) of triethylamine in approximately 2 mL of dichloromethane was introduced into an enclosed ampule containing 0.86 mg (8.3 μmol) of [1-^{14}C]acetic anhydride. An additional 3.7 mg (8 μmol) of the tripeptide amine was added. The reaction mixture was removed by pipet, the solvent was carefully removed, and the product was isolated by preparative thin-layer chromatography, 1% NH_4OH/MeOH. Verification of the product was determined by cospotting on TLC with the characterized unlabeled *N*-acetyldistamycin.

[6-^{15}N]Adenine (7). [^{15}N]Benzamide was reduced to [^{15}N]benzylamine with $LiAlH_4$ in dry diethyl ether. 6-Chloropurine (1.23 g, 8 mmol), 0.98 g (9 mmol) of [^{15}N]benzylamine, and 3.34 mL (24 mmol) of triethylamine were refluxed for 36 h in 100 mL of absolute ethanol. The product was preabsorbed onto silica and flash chromatographed with 1.5% $MeOH/CHCl_3$. [6-^{15}N]Benzyladenine (1.8 g, 8 mmol) was added to a 2:2:3 mixture of $CH_2Cl_2/CH_3CN/H_2O$ (180:180:280 mL) in a 1-L flask, followed by 6.84 g (32 mmol) of $NaIO_4$ and then 55 mg of ruthenium oxide (RuO_2/H_2O). The heterogeneous mixture was allowed to reflux for 3 h. Flash silica was added and the solvent was removed under reduced pressure. The absorbed material was flashed over 2 in. of silica with 1.5% NH_4OH/MeOH to remove the ruthenium. The remaining salts were removed by filtration and the filtrate was concentrated. Benzyl derivatives were extracted with diethyl ether. The product **7** was flash chromatographed with 1.5:1.5:7.0 EtOH/MeOH/$CHCl_3$ (or alternatively, 10 to 30% $MeOH/CH_2Cl_2$ saturated with ammonia) affording 0.62 g (58%) of **7**. ^{1}H NMR (Me_2SO-d_6) δ 7.12 (d, 2, $^1J_{H-N}$ = 89.8 Hz) 8.09 (s, 1), 8.12 (s, 1); ^{15}N NMR (Me_2SO-d_6) δ −293 (t); m/z for $C_5H_6N_4{}^{15}N$, calcd 137.059, obsd 137.059.

Synthesis of 3-(Acetyldistamycin)adenine (8): (a) 3-(Acetyldistamycin)adenine, (b) 3-([2-^{13}C]Acetyldistamycin)adenine, (c) 3-(Acetyldistamycin)[3-^{15}N]adenine, (d) 3-([2-^{13}C]Acetyldistamycin)[3-^{15}N]adenine, (e) 3-(Acetyldistamycin)[6-^{15}N]adenine. Adenine (130 mg, 0.96 mmol) and 84 mg (0.12 mmol) of **2** in 40 mL in dimethylformamide were allowed to reflux for 1 h. Solvent was removed under reduced pressure and the product was purified by preparative TLC with 1.5% NH_4OH/MeOH (yield 28%). ^{1}H NMR (Me_2SO-d_6) **8a** δ 1.59 (m, 2), 2.12 (s, 6), 2.21 (t, 2), 3.18 (m, 2), 3.78 (s, 3), 3.81 (s, 3), 3.83 (s, 3), 5.18 (s, 2), 6.81 (d, 1, J = 1.7 Hz), 6.93 (d, 1, J = 1.3 Hz), 7.01 (d, 1, J = 1.3 Hz), 7.13 (d, 1, J = 1.7 Hz), 7.17 (d, 1, J = 1.7 Hz), 7.23 (d, 1, J = 1.3 Hz), 7.69 (s, 1), 7.91 (br s, 1) 7.98 (br s, 1), 8.06 (t, 1), 8.31 (s, 1), 9.89 (s, 1), 9.92 (s, 1), 10.51 (s, 1). $\Delta\delta_{2,8}$ = 0.62. Deviations from **8a** due to isotopic labels: **8b** and **8d** δ 5.32, 4.96 (d, 2, $^1J_{H-C}$ = 142.76 Hz) instead of 5.18 (s, 2); **8c** and **8d** δ 8.30 (d, 1, **8c** $^2J_{H-N}$ = 6.4 Hz, **8d** $^2J_{H-N}$ = 6.0 Hz) instead of 8.31 (s, 1); **8e** δ 7.97 (br d, 2, $^1J_{N-H}$ = 90.4 Hz) instead of 7.97 (br s, 2); ^{13}C NMR (Me_2SO-d_6) **8b** δ 50.53, **8d** δ 50.62, 50.51 ($^1J_{C-N}$ = 11.0 Hz); ^{15}N NMR (Me_2SO-d_6) **8c** δ −221.91 (s). **8d** δ −221.88, −221 (d, $^1J_{N-C}$ = 11.0 Hz), **8e** δ −282 (t, $^1J_{N-H}$ = 90.9 Hz); UV_{max} (H_2O) 290; IR (KBr) 3400, 2900, 2500, 1920, 1660, 1620, 1450, 1350, 990, 830, 695; m/z for (**8a**) $C_{30}H_{38}N_{13}O_4$, calcd 644.317, obsd 644.318, (**8b**) $C_{29}{}^{13}C_1H_{38}N_{13}O_4$, calcd 645.320, obsd 645.319, (**8c**) $C_{30}H_{38}N_{12}{}^{15}N_1O_4$, calcd 645.314, obsd 645.314, (**8d**) $C_{29}{}^{13}C_1H_{38}N_{12}{}^{15}N_1O_4$, calcd 646.317, obsd 646.317, (**8e**) $C_{30}H_{38}N_{12}{}^{15}N_1O_4$, calcd 645.314, obsd 645.313.

3-([2-^{14}C]Acetyldistamycin)adenine (9). [2-^{14}C]**2** (4.4 mg, 3.1 μmol) and 18.5 mg (25 μmol) of adenine in 0.78 mL of dimethylformamide were heated at 75 °C for 20 h. The DMF was removed under vacuum and the product was purified by flash chromatography twice, 10–20% $MeOH/CH_2Cl_2$ saturated with ammonia (step gradient) and then with 1% NH_4OH/MeOH. Finally 1.6 mg (32%) of product was recovered (1 μmol, 0.5 μCi/μmol).

Preparation of the 3′ and 5′ Labeled 167 Base Pair Restriction Fragment. Supercoiled pBR322 plasmid was digested with *Eco* RI restriction endonuclease and was labeled at the 3′ end with [α-^{32}P]dATP using the Klenow fragment of DNA Polymerase I. A separate batch of *Eco* RI digested plasmid was labeled at the 5′ end by removing the 5′ phosphate with calf intestine alkaline phosphatase (CAP) and replacing it with a radioactive phosphate, [γ-^{32}P]dATP, using T4 polynucleotide kinase. After labeling, the DNA was digested with *Rsa* I to yield four restriction fragments. The 516 and 167 bp fragments were separated by gel electrophoresis and isolated by elution from the gel.

Time and Temperature Dependence of BD Cleavage Specificity. Time course of DNA cleavage was examined on the 167 bp restriction fragment (*Eco* RI/*Rsa* I) of plasmid pBR322. BD at 5 μM concentration was allowed to react at 35 °C with both the 3′ and 5′ end-labeled restriction fragment in 10 mM sodium phosphate buffer to pH 7.0 containing 100 μM in bp sonicated calf thymus DNA for 0, 1, 5, 10, 20, and 40 h. The control reaction in the absence of BD was for 40 h. The reactions were ethanol precipitated and the pellets were dissolved in 10 μL of 10 mM sodium phosphate buffer and heated for 15 min at 90 °C. Piperidine (40 μL, 1.4 M) was added and the solution was heated again for 15 min at 90 °C. Piperidine was removed by several lyophilizations. For temperature studies, BD at 5 μM concentration was allowed to react for 5 h with both the 3′ and 5′ labeled restriction fragments as above, except that the temperature was varied in 10-deg increments from 5 to 65 °C. The control reaction was performed at 65 °C and the reactions were worked up under the same procedure as the time course reactions.

Cleavage of the 167 Base Pair Restriction Fragment by BD and CC1065. The cleavage specificities of BD and CC1065 were compared on the 167 bp restriction fragment (*Eco RI*/*Rsa* I) of plasmid pBR322. BD and CC1065 at 5 μM concentrations were allowed to react at 37 °C with both the 3′ and 5′ labeled restriction fragment in 10 mM sodium phosphate buffer at pH 7.0 containing μM in bp sonicated calf thymus DNA. CC1065 was allowed to react with DNA for 1 h and BD for 1 and 10 h. The control DNA in the absence of CC1065 or BD was incubated at 37 °C for 10 h. Reaction workup was as above for the time course studies.

High-Resolution Gel Electrophoresis of the 167 Base Pair Restriction Fragment. Samples were dissolved in 3 μL of 75% formamide, heat denatured at 90 °C for 1 min, chilled immediately to 0 °C, and elec-

trophoresed through an 8% 1:20 cross-link polyacrylamide gel (50% urea). Gels were transferred to Whatman 3 mm cellulose paper and dried prior to autoradiography on Kodak x-Omat AR film.

Oligonucleotides. The 15 base pair oligonucleotide 5′-CGGTAGTTTATCACA-3′ and its complement were synthesized with phosphoramidite chemistry and purified by polyacrylamide gel electrophoresis followed by electroelution. DNA was then ethanol precipitated and dialyzed against doubly distilled water. Annealing of the oligonucleotides was achieved by mixing 1 equiv each of the two complementary strands in 100 mM sodium phosphate buffer at pH 7.0. Mixed strands were heated at 65 °C for 15 min and cooled to room temperature over 15 min and then to 4 °C over 15 min. Before addition of other reactants the annealed oligonucleotides were allowed to warm to room temperature.

5′ Labeling of Oligonucleotides. Ten picomoles of each single stranded oligonucleotide was reacted with 2 units polynucleotide kinase (PNK) and 20 pmol of [γ-^{32}P]dATP for 30 min at 37 °C. Excess ATP was removed by centrifuging the reaction mixture twice through Sephadex G-10-120. Solvent was removed by lyophilization. Labeled oligonucleotide was again purified by polyacrylamide gel electrophoresis followed by electroelution.

3′ Labeling of Oligonucleotides. Three and one-half nanomoles each of the single stranded 15mer 5′-CGGTAGTTTATCACA-3′ and the complementary 12mer 5′-TGTGATAAACA-3′ were mixed and reannealed at a concentration of 60 μM in 100 mM sodium phosphate buffer at pH 7.0. The following compounds were then added to a final volume of 100 μL and the specified final concentrations $MgCl_2$ (6.6 mM), mercaptoethanol (1 mM), 2′-deoxycytidine-5′-triphosphate (1 mM), [α-^{32}P]-2′-deoxyguanosine-5′-triphosphate (100 μCi), and DNA polymerase I-Klenow (15 units). This reaction was incubated at 37 °C for 20 min and then mixed with 100 μL formamide and loaded onto at 20% polyacrylamide preparative gel for purification as above.

Sequential Analysis of Oligonucleotide Products. 15mer (10 μM): BD (1:1) in 100 mM sodium phosphate buffer (pH 7.0) was incubated at 37 °C for 0 and 24 h. Aliquots for gel analysis were removed at each stage of the workup procedure as described in the legend of Figure 9 and analyzed by gel electrophoreses.

Enzymatic Characterization of DNA Termini. 15mer (10 μM): BD (1:1) in 100 mM sodium phosphate buffer (pH 7.0) was incubated at 37 °C for 48 h. The resulting reaction mixtures were worked up by cleavage procedures cited above. Salts that inhibit phosphatases were removed by chromatography through G10-120 Sephadex. For 5′ end analysis, the cleaved 3′ labeled oligonucleotide was dissolved in calf alkaline phosphatase (CAP) buffer (50 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) and then heated at 65 °C for 5 min and chilled immediately to 0 °C. Ten units of CAP were added and the reaction mixture was incubated at 37 °C for 30 min. Water was removed by lyophilization and the sample was prepared for gel electrophoresis. CAP activity was monitored by comparison with DNA reactions containing 5′ phosphate termini generated from Maxam-Gilbert G+A sequencing reaction under the same conditions. For 3′ end analysis the cleaved 5′ labeled oligonucleotide was dissolved in 50 mM Tris–HCl, 10 mM $MgCl_2$, heated for 5 min at 65 °C, and then chilled immediately to 0 °C. DTT (5 mM) and 10 units of polynucleotide kinase were added and the reaction was incubated at 37 °C for 1 h. Water was removed by lyophilization and the sample was prepared for gel electrophoresis. The 3′ phosphatase activity of polynucleotide kinase was verified as above by comparison with 3′ phosphate termini from chemical sequencing G+A reaction. Gel electrophoresis was conducted on 1:20 cross-link 20% polyacrylamide gels (50% urea).

BD Product Analysis [^{14}C]-BD/15mer. Complementary single strands of the 15mer (133 μM) were mixed in 133 mM sodium phosphate buffer at pH 7.0 (0.6 mL). The double-stranded oligonucleotide was formed according to the above procedure. After warming the solution to room temperature 1 equiv of [2-^{14}C]**2** (0.07 mg/0.4 mL of H_2O) was added, giving a final concentration of 100 μM BD and 100 μM oligomer in 1 mL of 100 mM sodium phosphate. The complex was allowed to equilibrate at room temperature for 10 min, heated, and maintained at 37 °C for 96 h, after which a 200-μL aliquot was removed and lyophilized for analysis. BD and products were extracted two times from the dried DNA reaction with 100 μL of 1% NH_4OH/MeOH. The extract was condensed to approximately 20 μL and spotted on 0.25 mm Silica Gel 60 plate (Merck). This corresponds to 2–4 nCi of labeled material per lane. The control in the absence of oligonucleotide was subjected to the same reaction conditions and workup procedure. The TLC runs using 1% NH_4OH/MeOH were allowed to develop until the solvent front moved 4 cm from the origin. Autoradiography of the TLC plates was performed at room temperature on Kodak X-Omat AR film.

Calf Thymus DNA/BD Reaction. Isolation and Purification of the Adenine Adduct. In 1380 mL of 10 mM neutral sodium phosphate solution, 911 mg of sonicated calf thymus DNA and 48 mg of *N*-bromoacetyldistamycin were stirred for 56 h at 48 °C. Water was removed by lyophilization. BD products were extracted from the DNA with 1.5% NH_4OH/MeOH to isolate the product, which by TLC corresponded to the 3-(acetyldistamycin)adenine adduct obtained from the reaction of adenine and BD. Further purification of the released adduct was achieved by semipreparative HPLC using an Altex Ultrasphere ODS column (10 mm × 25 cm). A two-step gradient was employed, step 1 consisting of 5–30% acetonitrile over 15 min, followed by 30–100% acetonitrile in 10 min vs 100 mM triethylammonium formate pH 3.1. The flow rate was 14 mL/min: Partial ^{1}H NMR (Me_2SO-d_6, δ 5.15 (s), 6.81 (d), 6.93 (d), 7.01 (d), 7.13 (d), 7.17 (d), 7.23 (d), 7.71 (s), 8.06 (s), 8.31 (s), 9.88 (s), 9.91 (s), 10.50 (s).

Equilibrium Dialysis. The apparatus utilized has a five-cell carrier which rotates at 5, 10, or 20 revolutions and can be placed inside of a temperature-controlled water bath. The membrane between each cell compartment was Spectra/Por 3, which has a molecular weight cutoff of 3500 (the 15mer duplex has a weight of 9900 and the ligands are approximately 600). For the final step of conditioning, the membranes were soaked in the dialysis buffer, 100 mM NaPhosphate at pH 7.0, for at least 15 min. Each compartment has an effective volume of 1 mL. All experiments were conducted at 37 °C at a rotation rate of 10 rpm. Two compounds were examined, *N*-[1-^{14}C]acetyldistamycin and *N*-[2-^{14}C]bromoacetyldistamycin, for their affinity toward the 15 base pair oligonucleotide duplex 5′-CGGTAGTTTATCACA-3′. Determination of the equilibration time was done with the nonreactive *N*-[1-^{14}C]-acetyldistamycin, which is approximately the same size as *N*-bromoacetyldistamycin. One compartment of each cell was filled with 1 mL of the dialysis buffer. The other compartment was filled with 1 mL of 10 μM *N*-acetyldistamycin in 100 mM NaPhosphate at pH 7.0. After times of 1, 2, 4, 6, and 8 h the solutions in each compartment of one of the remaining five cells were removed and transferred to scintillation vials. The empty compartments were then washed two times with 1 mL of dialysis buffer and the washes were transferred to the appropriate and complimentary vial. To each vial containing 3 mL of the aqueous carbon-14 solution was added 7 mL of the scintillation fluid. The quenching affects in counting were monitored by the H number method and the confidence level for channel two on the instrument was set to 2%. The initial cpm value, time zero, was 50 930 (approximately 75 nCi).

The reannealed 15mer was mixed with the ligand to a total volume of 1.2 mL by the following addition procedure with vortexing between each step 600 μL of 15mer (200 μM in 100 mM NaPhosphate), 420 μL of H_2O, 60 μL of 1 M NaPhosphate at pH 7.0, and 120 μL of the labeled ligand (100 μM). As before, 1 mL of the dialysis buffer was put in one compartment of the cell and 1 mL of the oligo/ligand mixture was placed and sealed in the other compartment. On the basis of the equilibration time experiment, samples were dialyzed for 8 h at 37 °C. Samples were removed and counted by the same procedure as given above.

Rate of Alkylation/Disappearance of the Reactant. Each reaction mixture was made up separately at room temperature in a 0.6-mL polypropylene microcentrifuge tube. The following protocol was used for determination of the rate of alkylation of adenine-48 by BD (100 μM 15mer:10 μM BD). The total volume was 100 μL for each sample. H_2O (52 μL) was added to a 20-μL solution of 15mer duplex (500 μM) in neutral 100 mM sodium phosphate, and then 8 μL of 1 M sodium phosphate, 10 μL of 100 μM BD, and 10 μL of the 140 μM 3-nitrobenzenesulfonic acid were added to the reaction tube. The complete mixture was vortexed. Aliquots (10 μL) were removed by pipet and transferred to a polypropylene autosampler tube for injection on the HPLC column. The remaining solution was immediately placed into the 37 °C temperature bath.

Method of Rate Measurements. The disappearance of the reactant, *N*-haloacetyldistamycin, was monitored by HPLC/UV spectroscopy. At each time interval 10 μL of the reaction mixture was injected onto a Hewlett Packard Hypersil ODS 5 μm column (100 × 2.1 mm) and separated with use of the following gradient program. Solvent A was 100 mM triethylamine formate at pH 3.1. Solvent B was acetonitrile. Gradient program: 5 to 30% B in 15 min, 30 to 100% B in 10 min, 100 to 5% B in 5 min, followed by 5% B for 10 min to re-equilibrate the column. The amounts of compounds in the injected samples were determined by peak area at 260 nm, band width of 4 nm, with respect to the internal standard peak area. The relative retention times of bromodistamycin and 3-(acetyldistamycin)adenine with respect to the internal standard 3-nitrobenzenesulfonic acid (1.0) was 3.0 and 2.0, respectively. Under the chromatographic conditions, it was assumed that all of the ligand noncovalently associated with the DNA is separated and accounted for.

Measurement of the Extinction Coefficients. Because the spectrum of 3-(acetyldistamycin)adenine (DA) is solvent and pH dependent, the extinction coefficients for both the reactant, *N*-bromoacetyldistamycin, and released product, 3-(acetyldistamycin)adenine, were determined under the HPLC buffer conditions at which they each eluted. The

extinction coefficients for BD and DA were determined in their respective HPLC elution solvents as determined by the elution time and the corresponding gradient program. For BD this occurs at 17% acetonitrile in 100 mM triethylammonium formate at pH 3.1. In this system BD was found to have the following molar absorptivities: ϵ (cm^{-1} M^{-1}) for BD 36 500 (λ_{max}, 304 nm), 22 300 (270 nm), 21 700 (260 nm). For DA, the solvent composition at elution is 11% acetonitrile in 100 mM triethylammonium formate at pH 3.1 DA (0.600 mg) was dissolved in 1.0 mL of absolute methanol to give a 932 μM solution. Four aliquots of 21 μL were diluted to 1000 μL with the elution buffer to yield a concentration of 20 μM. The blank sample was 21 μL of methanol diluted to 1000 μL with the elution buffer. ϵ for DA: 26 800 (304 nm), 28 400 (270 nm), 23 800 (260 nm), 31 800 (λ_{max}, 280 nm).

Acknowledgment. We are grateful to the National Institutes of Health (GM-27681) for support of this research.

Communications to the Editor

Chromodorolide A, a Rearranged Diterpene with a New Carbon Skeleton from the Indian Ocean Nudibranch *Chromodoris cavae*

Eric J. Dumdei, E. Dilip de Silva, and Raymond J. Andersen*

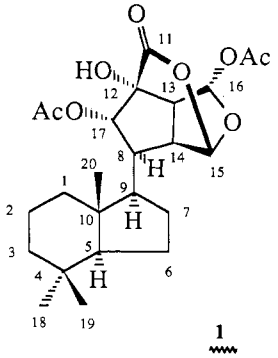
Departments of Chemistry and Oceanography
University of British Columbia
Vancouver, British Columbia, Canada V6T 1W5

M. Iqbal Choudhary and Jon Clardy*

Department of Chemistry—Baker Laboratory
Cornell University, Ithaca, New York 14853-1301

Received November 21, 1988

Nudibranchs are shell-less molluscs that rely on an arsenal of exotic secondary metabolites to thwart predation.[1] Most of the defensive substances are sequestered by the nudibranchs from the sponges, soft corals, hydroids, and other sessile marine invertebrates that make up their diets. A family of rearranged spongian diterpenes, acquired from dietary sponges, are deployed for defensive purposes by tropical nudibranchs in the genus *Chromodoris*.[2] We have examined the skin chemistry of *Chromodoris cavae*[3] collected in the Indian Ocean, and we now wish to report the structure of chromodorolide A (**1**), a putative repellent.



1

The nudibranchs (90 individuals) were collected from the waters near Jaffna on the northern coast of Sri Lanka. Freshly collected whole animals were immersed in methanol/dichloromethane (1:1) and stored in a freezer. The organic solvents were decanted from thawed samples and evaporated in vacuo to give an oily residue that was partitioned between water and dichloromethane. Fractionation of the dichloromethane soluble materials by silica gel flash (step gradient CH_2Cl_2 to Et_2O) and preparative silica gel thin layer (CH_2Cl_2/Et_2O 88:12, R_f 0.42) chromatographies gave pure chromodorolide A (**1**)[4] (colorless needles from hot MeOH, mp 133–134 °C, 61 mg).

Table I. Partial ^1H and ^{13}C NMR Assignments for Chromodorolide A (**1**)[a]

carbon no.	^1H	^{13}C
5	0.72, dd, J = 7.5, 12.6 Hz	57.3 (CH)
8	2.17, ddd, J = 2.9, 6.9, 12.1 Hz	45.1 (CH)
9	1.26, m	51.7 (CH)
11		172.6 (C)
12	3.61 (OH)	80.4 (C)
13	2.96, dd, J = 1.1, 5.4 Hz	52.7 (CH)
14	2.76, ddd, J = 3.5, 5.4, 6.9 Hz	46.5 (CH)
15	5.59, dd, J = 1.1, 3.5 Hz	104.2 (CH)
16	6.67, s	95.9 (CH)
17	5.04, d, J = 2.9 Hz	79.2 (CH)
18	0.80, s	33.4 (CH_3)
19	0.77, s	21.0 (CH_3)
20	0.45, s	13.4 (CH_3)
$MeCO_2$		168.0 (C)
$MeCO_2$	1.60, s	20.4 (CH_3)
$MeCO_2$		169.5 (C)
$MeCO_2$	1.80, s	20.4 (CH_3)

[a] Spectra were recorded in C_6D_6 at 400(^1H) and 75(^{13}C) MHz. Chemical shifts are in ppm from internal TMS. ^{13}C assignments are based on HETCOR data.

The molecular formula of chromodorolide A (**1**), $C_{24}H_{34}O_8$, was determined by analysis of its mass spectral and NMR data. A ^{13}C APT experiment[5] revealed 24 carbons attached to a total of 33 hydrogen atoms, and the ^1H NMR spectrum ($CDCl_3$) contained resonances at δ 2.09 (s, 3 H) and 2.13 (s, 3 H) that could be assigned to acetate methyl protons. The highest mass peak at m/z 390.2043 daltons ($C_{22}H_{30}O_6$ ΔM + 0.1 mmu) in the EIHRMS of **1** could, therefore, be attributed to a M^+ − HOAc fragment ion.

Deshielded ^{13}C NMR resonances at δ 95.9 (CH) and 104.2 (CH) indicated the presence of two ketal functionalities in **1**, typical of rearranged spongian diterpenes.[2] An IR band at 3461 cm^{-1} and a ^{13}C resonance at δ 80.4 (C) revealed a tertiary alcohol. Three ester carbonyl resonances (Table I), two of them assigned to the acetate functionalities, were also apparent in the ^{13}C NMR spectrum. The absence of additional carbonyl or olefinic ^{13}C resonances implied that chromodorolide A (**1**) had to be penta-

(1) (a) Williams, D. E.; Andersen, R. J. *Can. J. Chem.* **1987**, *65*, 2244. (b) Gustafson, K.; Andersen, R. J. *Tetrahedron* **1985**, *41*, 1101. (c) Faulkner, D. J. In *Biomedical Importance of Marine Organisms*; Fautin, D. G., Ed.; California Academy of Sciences: San Francisco, CA, 1988; pp 29–36. (d) Karuso, P. In *Bioorganic Marine Chemistry*; Scheuer, P. J., Ed.; Springer Verlag: Berlin, 1987; Vol. 1, pp 31–60.

(2) (a) Hochlowski, J. E.; Faulkner, D. J.; Matsumoto, G. K.; Clardy, J. *J. Org. Chem.* **1983**, *48*, 1141. (b) Molinski, T. F.; Faulkner, D. J. *J. Org. Chem.* **1986**, *51*, 2601. (c) Molinsky, T. F.; Faulkner, D. J.; Cun-Leng, H.; Van Duyne, G. D.; Clardy, J. *J. Org. Chem.* **1986**, *51*, 4564. (d) Carmely, S.; Cojocaru, M.; Loya, Y.; Kashman, Y. *J. Org. Chem.* **1988**, *53*, 4801.

(3) Identified by Sandra Millen, Zoology Department, UBC. See: Eliot, C. *Proc. Zool. Soc. London* **1904**, 380. A voucher sample is deposited at UBC.

(4) **1**: IR 3461, 1770, 1748 (sh), 1220 cm^{-1}; ^{13}C (C_6D_6) δ 13.4 (CH_3), 20.1 (CH_2), 20.4 (CH_3), 20.4 (CH_3), 21.0 (CH_3), 21.0 (CH_2), 26.7 (CH_2), 33.0 (C), 33.4 (CH_3), 40.0 (CH_2), 41.2 (CH_2), 42.3 (C), 45.1 (CH), 46.5 (CH), 51.7 (CH), 52.7 (CH), 57.3 (CH), 79.2 (CH), 80.4 (C), 95.9 (CH), 104.2 (CH), 168.0 (C), 169.5 (C), 172.6 (C); CIMS m/z 468 ($M^+ + NH_4$).

(5) Patt, S. L.; Shoolery, J. N. *J. Magn. Reson.* **1982**, *46*, 535.

0002-7863/89/1511-2712$01.50/0