Altromycin B Threads the DNA Helix Interacting with Both the Major and the Minor Grooves To Position Itself for Site-Directed Alkylation of Guanine N7†

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Abstract: The pluramycins are a class of antitumor antibiotics for which a detailed structural investigation of their interaction with DNA is lacking. Using altromycin B as a prototypical pluramycin, we have characterized the drug's interaction with the self-complementary DNA duplex [d(GAAG*TACTTC)]$_2$ diadduct (* denotes the site of covalent modification) by two-dimensional NMR and have gained considerable insight into the role played by the drug's glycosidic substituents in sequence selectivity. The drug intercalates into the DNA molecule and stacks to the 5′ side of the modified guanine, thereby placing a disaccharide into the minor groove and a monosaccharide into the major groove. As a result of these interactions, the epoxide is positioned in the major groove of the DNA to perform electrophilic attack on N7 of guanine.

Introduction

The members of the pluramycin family of antitumor antibiotics (Figure 1) are *Streptomyces*-derived natural products that show *in vitro* activity against a wide variety of tumor cell lines, including P388 leukemia, adenocarcinoma, M5076 ovarian sarcoma, Lewis lung carcinoma, and human LS174T colon cancer.[1] As a group, they can be considered as being structurally elaborated from the anthracycline class of antibiotics, i.e., by adding a pyran ring to the anthraquinone chromophore and various possible saccharide moieties and epoxide functionalities to positions on the conjugated ring system.[2] Those members bearing an epoxide substituent attached to the C2 position on the pyran ring have the ability to covalently modify DNA.[1,3] On the basis of mass spectroscopy, NMR, and gel-electrophoretic studies performed on the heat-induced depurination reaction of these agents adducted to DNA, the mechanism of covalent modification has been determined to be the electrophilic attack of the epoxide on N7 of guanine. This N7 alkylation results in opening of the epoxide functionality to form cationic lesions on the DNA molecule (Figure 2).[4]

The compounds of this family of antibiotics range in structural complexity from sapurimycin, the most simple representative, to hedamycin and altromycin B, the two most complex members. Altromycin B and hedamycin each represent a structurally distinct subgroup within the pluramycin family of compounds. The classical pluramycins, represented by hedamycin, have amino sugars attached to the C8 *and* C10 positions. Attached to the C2 position can be a vinyl substituent (kidamycin),[5] a vinyl epoxide (pluramycin A),[6] a single epoxide (epoxykidamycin),[7] or a double epoxide (hedamycin) (see Figure 1).[8] The altromycins, the newest members in the pluramycin family, lack the C8 amino sugar distinctive to their classical counterparts and are characterized by an amino disaccharide attached through the C10 position on the conjugated chromophore. Additionally, the altromycins can have a neutral methyl ester-linked 6-deoxy-3-*O*-methylaltrose attached to the chromophore C5 position.[1,9] Unlike the classical pluramycins, the altromycins do not vary the C2 single epoxide substituent.

Despite the many similarities among this family of DNA-reactive ligands, the two families of pluramycins exhibit different DNA sequence selectivities in sites of covalent modification. The varying reactivity of different members to the same sequences appears to be modulated by glycoside substitution on the main chromophore at positions C5, C8, and C10. While hedamycin and pluramycin exhibit a high reactivity

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† Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear overhauser effect; NOESY, nuclear overhauser effect spectroscopy; ROESY, rotating frame nuclear overhauser effect spectroscopy; DQF-COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; tppi, time proportional phase increment; EDTA, ethylenediaminetetraacetic acid.

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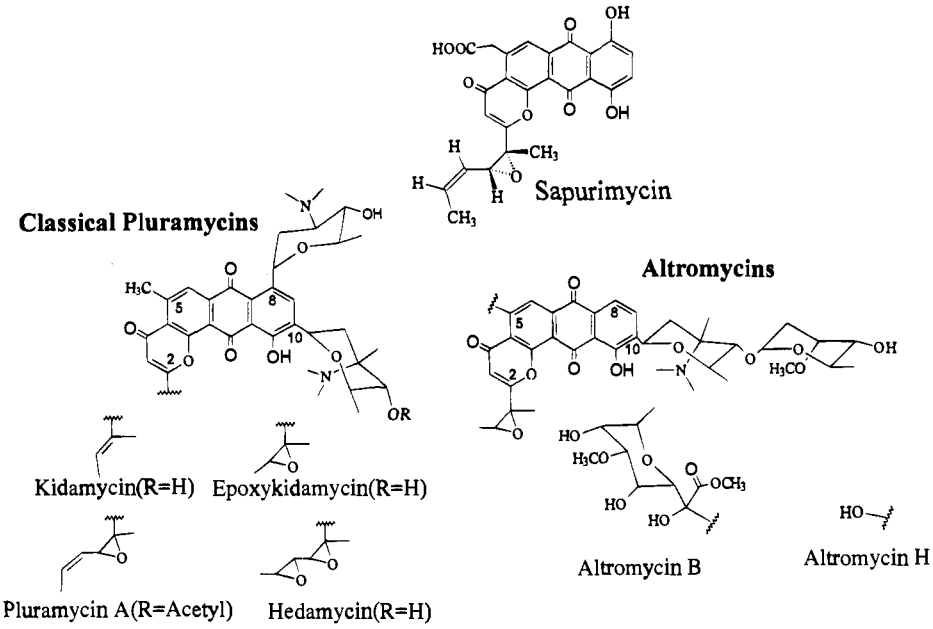

Figure 1. Structures of representative pluramycin antibiotics. The family subdivides by structure into two distinct groups: the classical pluramycins (depicted by kidamycin, epoxykidamycin, pluramycin A, and hedamycin) and the newly discovered altromycins (depicted by altromycins B and H). Sapurimycin represents the simplest analogs.

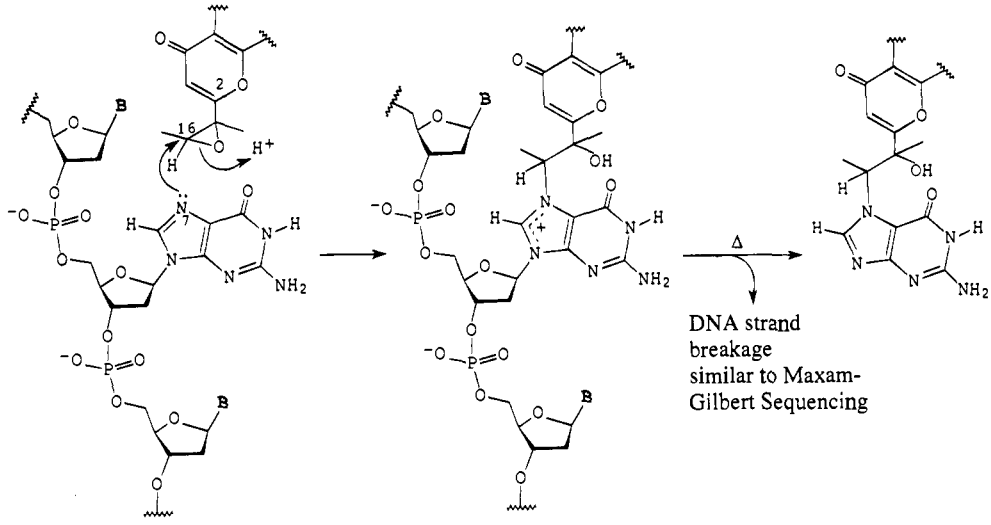

Figure 2. Proposed mechanism of covalent modification of DNA by pluramycin-type compounds. The N7 of guanine performs nucleophilic attack on the epoxide, forming a cationic lesion on the DNA. Subsequent thermal depurination results in DNA strand breakage.[4]

toward 5′(C/T)G sequences, the altromycins demonstrate selectivity for 5′AG sequences.[3,10]

In this structural study, the self-complementary [d-(GAAG*TACTTC)]$_2$ decamer, which contains two highly reactive altromycin B intercalation/alkylation sites (underlined and starred), has been reacted with 2 equiv of altromycin B to form a C2-symmetric bisadduct (Figure 3). Two-dimensional NMR is used to confirm the proposed mechanism of covalent modification of guanine, to structurally determine the three-dimensional interaction of altromycin B with the DNA molecule, and to identify key interactions between chromophore substituents and DNA, directing the DNA sequence recognition by the drug molecule. The altromycin B chromophore intercalates through the DNA helix to the 5′ side of the covalently modified guanine, interacting with both major and minor grooves through its sugar moieties. It appears that the interactions of the C5 neutral altrose moiety with the major groove and the amino disaccharide with the minor groove orient the epoxide to react with N7 of guanine in a sequence-dependent fashion.

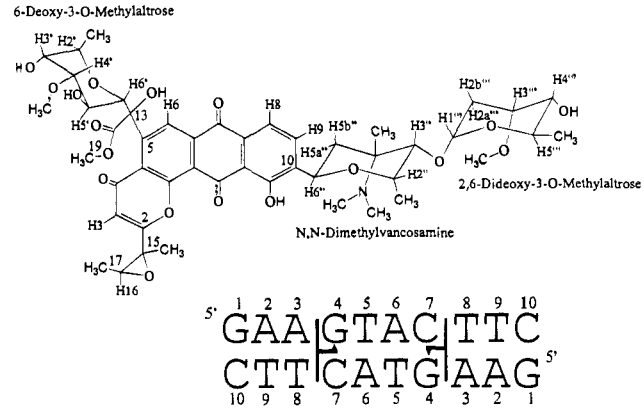

Figure 3. Structures and numbering schemes of altromycin B and the DNA decamer used in this study. Shown are sites of intercalation (3A, 4G) and covalent modification (4G).

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Table 1. Comparison of the Altromycin B Chemical Shifts in the Bis(altromycin B)−[d(GAAGTACTTC)]$_2$ Diadduct and the Free Drug

	Chemical Shifts (ppm) of Altromycin B	
^{1}H assignment[a]	bis-adduct[b]	free drug[c]
H3	6.85	6.52
H6	8.04	8.69
H8	6.80	7.85
H9	7.70	7.98
15CH_3	1.58	1.93
H16	4.83	3.35
17CH_3	1.70	1.25
19OCH_3	3.47	3.69
H2′	4.21	4.31
2′CH_3	1.62	1.73
H3′	*d*	3.96
H4′	3.68	3.59
4′OCH_3	3.47	3.60
H5′	4.51	4.66
H6′	4.40	4.56
H2″	4.31	4.32
2″CH_3	1.37	1.65
H3″	4.20	3.77
4″CH_3	1.54	1.04
4″$N(CH_3)_2$	2.95	2.47
H5″a	2.32	2.48
H5″b	2.11	1.25
H6″	5.28	5.53
H1‴	5.07	4.79
H2‴a	2.23[e]	2.31
H2‴b	2.03[e]	1.78
H3‴	3.88	3.62
3‴OCH_3	3.47	3.40
H4‴	3.86	3.27
H5‴	4.27	4.09
5‴CH_3	1.30	1.26

[a] For numbering, see Figure 3. [b] Chemical shifts referenced to H_2O in 99.9% D_2O. [c] Chemical shifts in chloroform.[12] [d] Not assigned in this study. [e] Distinguished from each other by chemical shift arguments and may be interchanged.

Results

Three oligonucleotides, [d(GAAGCTTC)]$_2$, [d(GAAGTACTTC)]$_2$, and [d(GATAGTACTATC)]$_2$, were initially prepared and reacted with altromycin B. The octamer, which lacked the two-base-pair spacer between modified guanines, was too unstable to adequately study. In the one-dimensional exchangeable and nonexchangeable spectra of the 2:1 bis(altromycin B)−[d(GAAGTACTTC)]$_2$ diadduct, there initially exists only one set of proton resonances representing one adduct species. The existence of only four nonterminal imino resonances, coupled with two-dimensional analysis of the nonexchangeable spectra, reveals a retention of self-complementarity of the duplex palindrome upon adduct formation. Both the dodecamer and the decamer yielded similar C2-symmetric bisadducts with moderate chemical stability. However, each degraded over the span of several days from a singular set of resonances to a complex mixture of signals resulting from depurination of the cationic guanine adduct. Additionally, samples were diluted in an attempt to diminish signal broadening due to aggregation at the expense of signal intensity. Both the decamer and dodecamer were subjected to initial study by NMR experiments, and the decamer, being less complex spectrally, was characterized further.

Assignment of the Altromycin B Proton Resonances. The altromycin B proton resonances (Table 1) are divided into three regions: the C10 sugar moiety, the C13 sugar moiety, and the chromophore region bearing the site of covalent attachment to the DNA molecule. Protons of each region were assigned using TOCSY, ROESY, and NOESY experiments.[11] The aromatic H8 and H9 resonances, on the conjugated chromophore, adjacent to the C10 disaccharide, were identified by mutual, through-bond coupling and by NOE connectivities to the H6″ sugar proton. In the C10-linked, *N,N*-dimethylvancosamine (designated with double prime numbering), the H6″ proton coupled to both H5a″ and H5b″ resonances and showed ROESY connectivities to the 2″ methyl (doublet), identified through coupling to the H2″ proton. The H5a″ and H5b″ were differentiated on the basis of a ROESY cross peak (strong NOESY connectivity) between H6″ and H5b″ and an absent ROESY connectivity (weak NOESY connectivity) between H6″ and H5a″. The 4″ dimethylamino, easily identified by chemical shift and one-dimensional resonance intensity, was used to identify the 4″ methyl (singlet) and H3″ resonances through ROESY connectivities. The C3″-linked 2,6-dideoxy-3-*O*-methylaltrose proton resonances (designated in triple prime numbers) were easily assigned through mutual coupling between adjacent protons around the sugar ring. The H2a‴ and H2b‴ could only be tentatively distinguished from each other on the basis of correlation to the chemical shifts of the free drug.[12]

In the C13-linked 6-deoxy-3-*O*-methylaltrose, the 2′ methyl (doublet) coupled to the H2′ proton. The H4′, H5′, and H6′ were all identified through mutual coupling to each other and by the through-space ROESY connectivities between H4′ and the 2′ methyl. The H3′ proton assignment remains problematic and has not been assigned in this experiment. The H6 aromatic proton, located near this region on the chromophore, was identified through NOE connectivities between itself and the 2′ methyl, H2′, H4′, H5′, and H6′ proton resonances.

At the site of covalent attachment, the 17 methyl (doublet) was assigned through coupling to the H16 proton and NOE connectivities to the 4GH8 proton resonance. The 15 methyl (singlet) was then assigned on the basis of long-range coupling to both the 17 methyl and H16. The H3 aromatic proton resonance could be identified by NOE connectivities to both former epoxide methyls. NMR results of this study strongly argue that the stereochemistry at the site of covalent attachment is *R* at the C14 chiral center and *S* at the C16 chiral center. It is known from published ROESY data on the free drug[12] that the two epoxide methyls are trans to each other. The existence of only a single adduct species in this study reveals that the nucleophilic addition goes by an S_N2 mechanism, which would result in the inversion of the stereochemistry at the C16 center. This information limits the stereochemistry possibilities to the diastereomeric pair, C14*R*,C16*S* or C14*S*,C16R (a and b in Figure 4). Pathway a, associated with the C14*R*,C16*S* stereochemistry, was chosen on the basis of a medium ROESY cross peak between the H3 aromatic proton and the 17 methyl resonance (strong NOESY connectivity) and an absent ROESY H3−15 methyl cross peak (weak NOESY connectivity). Also, the 17 methyl−15 methyl cross peak is quite weak in the ROESY spectrum and of medium intensity in the NOESY spectrum. In the case of pathway b, associated with the C14*S*,-C16*R* stereochemistry, one would expect the H3−15 methyl connectivity to be greater than the H3−17 methyl connectivity and the 17 methyl−15 methyl to be quite intense. On the basis of these arguments, the as yet undetermined absolute stereochemistries for the free drug would be *R* for both C14 and C16 chiral centers.

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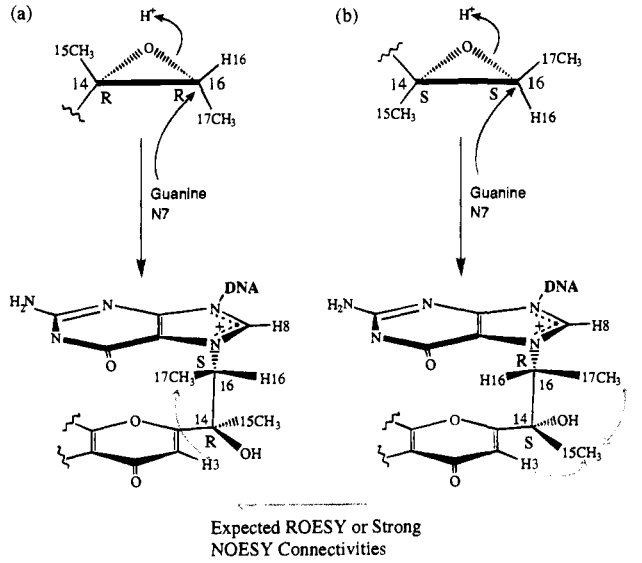

Figure 4. Two possible pathways (a and b) for epoxide alkylation of DNA by altromycin B. Shown are the expected through-space connectivities for each respective stereoisomer and final stereochemical consequences.

DNA Proton Assignments. DNA assignments of the 10-mer duplex and its bis(altromycin) diadduct were achieved through established methods[13] by identifying sequential NOESY connectivities between bases and creating walks leading from the 5′ end to the 3′ end of the DNA molecule; these are reported in Table 2. The aromatic to aromatic, aromatic to H1′ (left walk in Figure 5), aromatic to H2′/H2″, and aromatic to H3′ (right walk in Figure 5) walks all demonstrate breaks at the 3A−4G and the 7C−8T steps, presumably due to the separation of the base pairs at this position to accommodate intercalation by the drug chromophore. The DNA walks also show some irregularities caused by unusual line broadening to the 5′ side of the alkylation site, specifically residues 2A and 3A. Bridging the break associated with the 7C−8T junction in the DNA's aromatic−H1′ walk is the altromycin H9 aromatic proton. The 7CH1′ connects to the drug's H9 proton, which in turn connects to the 8TH1′ traversing the intercalation site (seen as a dotted line on the left walk in Figure 5). These results unequivocally position the drug chromophore directly to the 5′ side of the covalently modified guanine between the 3A•8T and 4G•7C base pairs.

Altromycin B Is Covalently Attached at N7 of 4G. The chemical character of the DNA's 4GH8 proton resonance yields evidence confirming the site of covalent modification. The H8 aromatic proton of the drug-modified guanine (4G) is shifted to 9.7 ppm, which is 2.2 ppm downfield from its chemical shift in the unmodified duplex. Due to rapid exchange of this proton resonance with deuterium in D_2O, the aromatic proton was identified and characterized in NMR experiments performed in 95% H_2O. The acidic nature of this proton is the direct result of alkylation of guanine through N7 to form a cationic species in which the H8 proton has become labile (see Figure 2). This result agrees with previously reported N7-alkyl−guanine adducts, most notably the aflatoxin−DNA adduct,[14] in which the H8 proton of the modified guanine also resonates at 9.7 ppm.

Inter-Drug−DNA Connectivities in the Major Groove. In the major groove of the DNA molecule, the drug shows two regions of DNA NOESY contacts (summarized in Figure 6). Surrounding the site of alkylation at 4G, the proton resonances of the former epoxide show contacts to both sides of the intercalation site. The 15 methyl shows NOE connectivities to the 4GH8 and 3AH8 protons, reaffirming positioning of the intercalating chromophore between the 3A and 4G bases. The 17 methyl resonance shows NOE connectivity to the H8 proton of the modified guanine, placing the opened epoxide in proximity of N7 of 4G in the major groove.

The C13-linked 6-deoxy-3-*O*-methylaltrose associates on the other side of the major groove with the noncovalently modified DNA strand and spans the opened base pair between 7C and 8T. The hydrophobic side of the sugar appears to make van der Waal contacts with the hydrophobic pocket formed by 7C, the drug chromophore, and 8T. The 2′ methyl of this sugar shows NOE contacts with 7CH5, 7CH6, and the 8T methyl, and the H4′ and H6′ positioned on the same face of the sugar show contacts to the 8T methyl. Positioned on the opposite side of the sugar, the H2′ shows a connectivity to the 8T methyl, while the H5′ does not show any DNA contacts, presumably because it faces out of the major groove. In this orientation, the more hydrophilic side of the sugar orients away from the floor of the major groove to interact with the solvent.

Inter-Drug−DNA Connectivities in the Minor Groove. Positioning of the C10-linked *N,N*-dimethylvancosamine can be unequivocally made in the minor groove side of the DNA molecule, based on NOE contacts with 3AH2, projecting from the floor of the minor groove, and H1′ and H4′ resonances of 3A, 4G, 8T, and 9T, which are located on the DNA backbone facing into the minor groove (Figure 7). Based upon these NOE contacts, this positions this substituent deep in the minor groove making extensive contact with the DNA. The dimethylamino substituent of this sugar shows medium to strong connectivities to 3AH2, 8TH1′, 8TH4′, 9TH1′, and 9TH4′, which positions it in close proximity to interact with the 8T residue on the noncovalent strand of the DNA duplex.

Also located in the minor groove is the neutral 2,6-dideoxy-3-*O*-methylaltrose, which is attached to the C3″ position of the *N,N*-dimethylvancosamine. Unlike its neighboring amino sugar, this terminal sugar moiety makes only minimal specific contact with the DNA, showing only two weak NOE connectivities to the minor groove between the H2″a/b methylene of the drug sugar and 3AH4′ of the DNA molecule. Molecular modeling based on NOE constraints places this sugar toward the outside edge of the minor groove nearest to the strand containing the modified guanine and is in the proximity of 3A and 4G.

The proton resonances associated with 2A and 3A, which are both located to the 5′ side of the modified guanine, are particularly broad in comparison to the other resonances in the DNA duplex and altromycin B (see Figure 5). This phenomenon is duplicated in the bis(altromycin B)−[d-(GATAG*TACTATC)]$_2$ diadduct, affecting the equivalent residues 3T and 4A. Due to the minimal NOE contacts of the terminal neutral sugar with the minor groove of DNA, it is probable that this sugar is conformationally flexible, merely filling space in the minor groove but not making any specific interactions. The broadness of the 2A and 3A DNA resonances may be a result of a heterogeneous chemical environment caused by the 3″ sugar sampling multiple conformations in this region of the minor groove. This suggests that the fluctual motion of the 3″ neutral sugar sweeps out a greater area of the DNA minor groove, including the region near 2A, than revealed by restrained molecular modeling.

NMR-Derived Molecular Modeling of the Bis(altromycin B)−(10-mer) Diadduct. Through a 100 ps solvated molecular dynamics calculation using 48 inter-drug−DNA, 64 intra-drug,

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Table 2. Comparison of the DNA Proton Chemical Shifts[a] of the Bis(altromycin B)−[d(GAAGTACTTC)]$_2$ Duplex Diadduct with the Unmodified Duplex

base	H8/H6	H5/5CH$_3$	H2	H1′	H2′	H2″	H3′	H4′
1G	7.78 (−0.02)[b]			**5.44 (+0.22)**	2.26 (−0.18)	**2.40 (−0.25)**	4.70 (−0.12)	4.09 (−0.07)
2A	**7.94 (−0.26)**		absent[c]	**5.62 (−0.32)**	**2.37 (−0.40)**	**2.55 (−0.37)**	4.95 (−0.11)	4.27 (−0.14)
3A	8.10 (+0.04)		7.33 (−0.20)	6.20 (+0.18)	2.66 (−0.02)	**2.47 (−0.36)**	5.05 (−0.00)	4.37 (−0.09)
4G*	**9.70 (−2.2)**[d]			6.00 (+0.17)	2.42 (−0.00)	2.77 (+0.08)	4.82 (−0.06)	4.36 (−0.03)
5T	**7.37 (+0.22)**	1.30 (+0.02)		5.73 (−0.02)	2.00 (−0.05)	2.45 (−0.03)	4.80 (−0.06)	4.11 (−0.10)
6A	**8.04 (−0.21)**		7.20 (−0.20)	6.09 (−0.11)	**2.40 (−0.31)**	2.75 (−0.10)	5.00 (−0.02)	4.28 (−0.17)
7C	7.48 (+0.17)	5.09 (−0.11)		**5.62 (−0.21)**	**2.40 (+0.35)**	**2.27 (−0.23)**	**4.95 (+0.27)**	4.23 (−0.02)
8T	**7.80 (+0.35)**	**1.92 (+0.35)**		6.15 (+0.12)	**2.45 (−0.31)**	2.45 (−0.12)	5.04 (+0.17)	**4.40 (+0.23)**
9T	7.32 (−0.15)	1.65 (−0.07)		6.10 (−0.08)	2.10 (−0.08)	2.53 (−0.02)	4.78 (−0.14)	4.10 (−0.08)
10C	7.62 (−0.03)	5.77 (−0.03)		6.27 (−0.00)	2.27 (−0.02)	2.27 (−0.02)	4.60 (−0.00)	4.01 (−0.04)

[a] Referenced to H_2O in 99.9% D_2O unless otherwise noted. [b] Chemical shift differences: + and − represent upfield and downfield shifts, respectively. Shown in bold are those greater than 0.2 ppm. [c] Too broad to be observed. [d] Exchangeable proton observed in 95% H_2O.

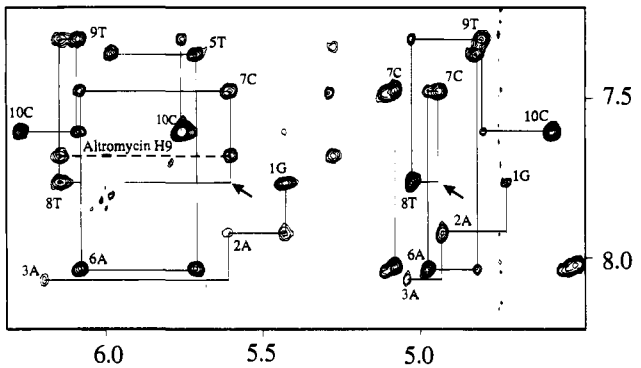

Figure 5. NOESY data (150 ms) showing the aromatic to H1′ (left) and aromatic to H3′ walks (right). Identified are the intra-residue connectivities with a base and number designation, breaks in the walks due to ligand intercalation with arrows, and the altromycin H9 resonance with a dotted line. Also shown are cytosine H5 connectivities to their own H6-proton and the 5′ neighbor's aromatic proton. For 7C and 10C, these are shown by a single vertical line.

and 118 intra-DNA constraints, a molecular model was achieved, summarizing the NMR results (Figure 8). In this model, the tetracyclic chromophore positions its axis in a perpendicular orientation to the two adjacent base pairs, easily spanning the helix to place glycosidic substituents into each groove. The DNA molecule undergoes extensive structural changes to accommodate this interaction by opening the two neighboring base pairs to twice their normal distance to stack on either side of the conjugated ring system. In addition, the minor groove has opened slightly, due to the insertion of the intercalating chromophore and interaction with the C10-linked disaccharide. In the major groove, the influence of the C5-linked altrose moiety remains mostly nondistortive, binding snugly against 7C and 8T. The site of covalent modification, 4G, distorts to accommodate N7 attachment of the drug chromophore through a two-carbon linker. To maintain an aromatic, planar bonding geometry of N7 and a tetrahedral, sp^3 bonding configuration of the attached C16, the covalently modified guanine is forced to tilt out of the base-pair plane toward both the major groove and the 5′ phosphate.

Due to the proximity of the C10 *N,N*-dimethylvancosamine to 8T, hydrogen bond formation is proposed between the protonated dimethylamino and 8TO2 functionalities. Direct hydrogen bonding to the thymine O2 carbonyl and hydrogen bonding mediated through a water molecule with the carbonyl are both consistent with NMR-derived constraints. However, hydrogen bonding to a water molecule, which bridges 8TO2 to 9TO1′, more favorably satisfies the modeling force field and is drawn from direct analogy to the interaction of the amino sugar with the DNA minor groove in doxorubicin−DNA crystal structures (see Figure 9).[15]

Although either stereoisomer at C13 is feasible in the molecular model, the currently undetermined stereochemistry of the C13 chiral center that joins the chromophore to the 6-deoxy-3-*O*-methylaltrose was arbitrarily modeled in the *R* configuration. In this position, the carbonyl of the methyl ester orients favorably to hydrogen bond to the external 7CH6 amino proton. Additionally, this stereochemistry orients the methoxy group out of the major groove to interact with the solvent. Positioning of a methoxy substituent facing out of a DNA groove to interact with the solvent is consistent with other DNA-reactive ligands, specifically CC-1065,[16] calicheamycin,[17] the altromycin C5-linked altrose, and nogalamycin.[18]

Discussion

Comparison to Other DNA Adducts. The results published in this study confirm earlier proposals on the mode of DNA interaction of altromycin B[4] and parallel results from concurrent NMR studies with the sapurimycin−DNA adduct[19] and the hedamycin−DNA adduct.[20] In all studies of the examples, the tetracyclic ring system is reported to orient perpendicular to the axes of the DNA base pairs in which it intercalates, positioning the epoxide functionality into the major groove to alkylate a guanine residue located on the 3′ face of the molecule. Covalent attachment has been pinpointed by long-range ^{1}H−^{15}N heterocorrelations between the drug molecule and N7 of guanine in the depurinated altromycin B−guanine adduct,[4] as well as by identification and characterization of an acidic H8 proton associated with the modified guanine in all three DNA adduct studies. Although the interaction of the chromophore and site of alkylation on the DNA in all three adducts are the same, the three compounds differ in that sapurimycin lacks glycoside interaction in either groove, altromycin B demonstrates sugar-mediated binding in both the major and the minor grooves, and hedamycin binds both the C8- and the C10-linked amino sugars in the minor groove.

The pluramycins demonstrate characteristics of two other classes of DNA-reactive ligands, combining them into a novel mode of interaction with the DNA molecule. Like the mutagens aflatoxin[21] and benzopyrene,[22] the pluramycins have a conju-

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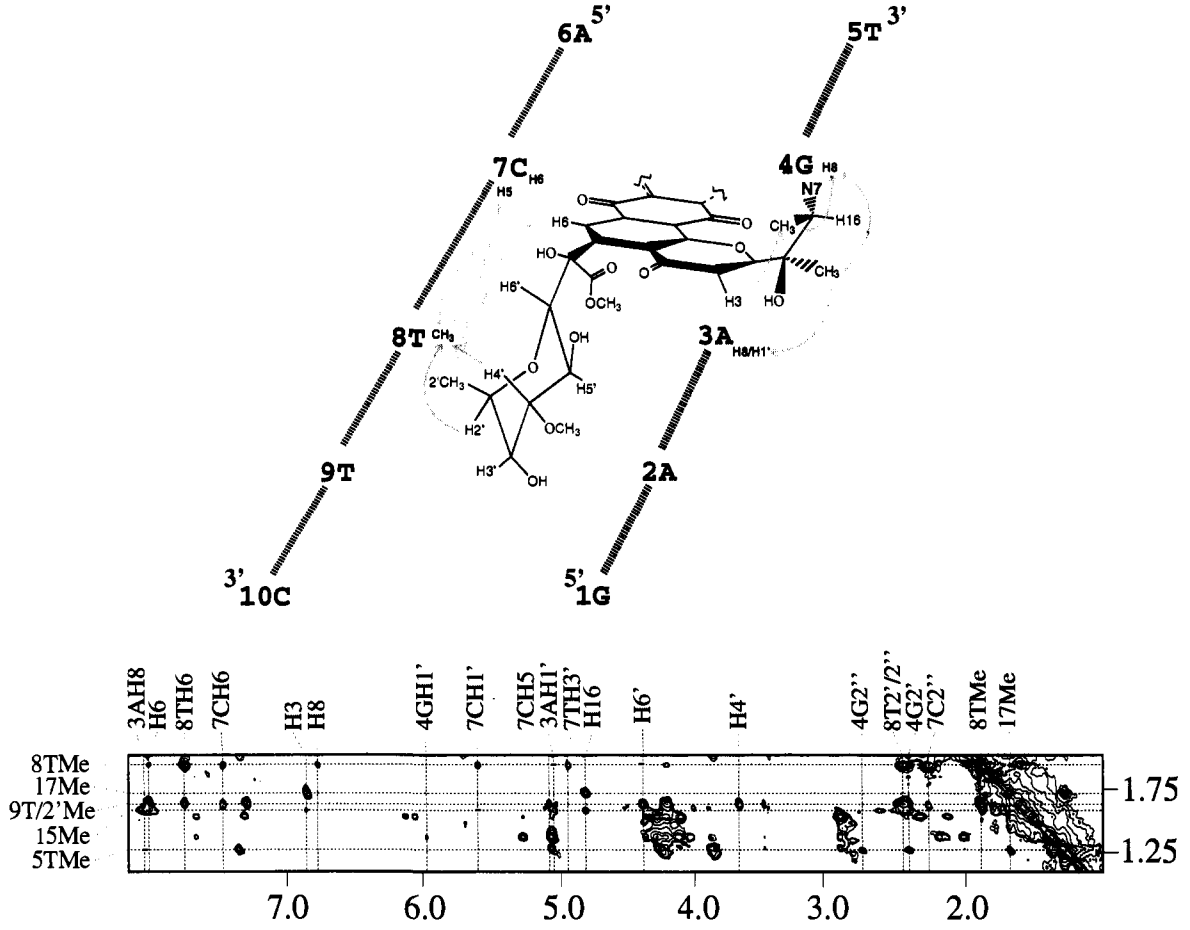

Figure 6. Summary of key NOESY connectivities (150 and 300 ms) (top) and NOESY data (150 ms) (bottom), which position the C5 sugar and the former C2 epoxide moiety of altromycin B in the major groove. The NOESY data (bottom) show connectivities of some of the proton and methyl resonances located in the major groove.

gated ring system that is optimal for stacking between DNA bases and epoxide moieties that can covalently attach the planar ligand to N7 of guanine. By placing its sugar substituents into both major and minor grooves of the DNA helix, altromycin B has developed an additional mode of DNA interaction, similar to the anthracycline nogalamycin. Nogalamycin, a noncovalent intercalating agent, exhibits an intercalating chromophore similar to the pluramycins with the addition of attached sugar moieties. These glycosidic substitutions give nogalamycin definite sequence binding selectivity and increased binding affinity by forming a fairly rigid "dumbbell" structure that interacts with both grooves of the DNA molecule.[18,23]

In contrast, the pluramycins demonstrate none of the binding selectivity exhibited by nogalamycin. In NMR studies in solution, while nogalamycin demonstrates specific binding to sites in oligomeric DNA,[23] the noncovalent pluramycin analog kidamycin binds with a high affinity but no observable selectivity to the oligomer $[d(GATGTACATC)]_2$.[24] Similar nonselective binding results are observed with the non-alkylating analogs neopluramycin and kidamycin in DNase I footprinting studies.[10] It is therefore proposed that, upon intercalation of the DNA molecule by the drug chromophore, the C5, C8, and C10 glycoside binding interactions primarily function in select sequences to orient and steer the normally unreactive epoxide into the proximity of the nucleophilic N7 of guanine in the major groove, thereby achieving increased DNA reactivity and sequence selectivity.

Rationale for Sequence Selectivity. By high-resolution gel electrophoresis studies using radiolabeled restriction enzyme fragments, the DNA alkylation selectivity has been determined for the pluramycin class of antibiotics and a full account is given in the accompanying paper.[10]

In NMR studies with hedamycin and altromycin B adducted to their respective highly reactive sequences, the amino sugars show a high degree of interaction with pyrimidines in the minor groove. Driving this interaction is the proposed hydrogen bond formation between the pyrimidine O2 carbonyl, which is highly negative and protrudes slightly from the floor of the minor groove, and the protonated dimethylamino substituent (see Figure 9). Considering the extensive contacts made by the C10 *N,N*-dimethylvancosamine with a pyrimidine to the 5′ side of the intercalation side in both modeled adducts, this is probably a fundamental interaction, giving rise to the two-base-pair sequence selectivity demonstrated by pluramycin analogs. The most reactive altromycin B sequence, 5′AG, places a thymine on the nonmodified strand in the 5′ base pair position. Sequence selective effects arise from the association of the *N,N*-dimethylvancosamine with this nonmodified strand to create a minor groove pocket for the terminal 6-deoxy-*O*-3-methylaltrose to fit. In contrast, in the low reactive 5′TG bonding site, the amino sugar would be limited to either hydrogen bonding to the thymine carbonyl, which would sterically crowd the terminal neutral sugar out of the minor groove, or forming a less optimal hydrogen bond with N3 of the base paired adenine on the floor of the minor groove.

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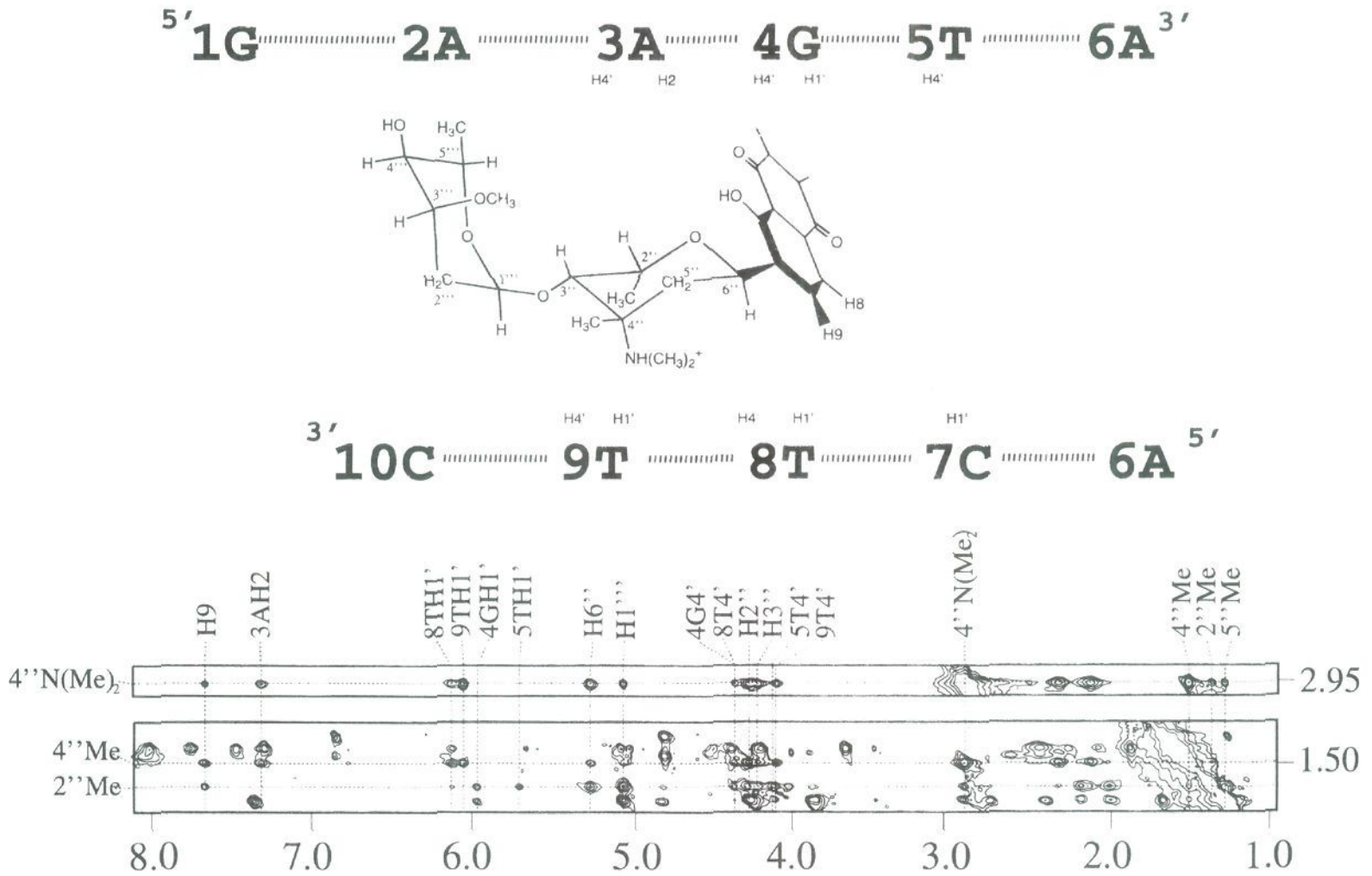

Figure 7. Summary of key NOESY connectivities (150 and 300 ms) (top) and NOESY data (150 ms) (bottom) between altromycin B's C10 disaccharide and the minor groove. In the top, medium and strong connectivities are shown in solid arrows and weak connectivities are shown in hatched arrows. The NOESY data show connectivities of some of the proton and methyl resonances located in the minor groove.

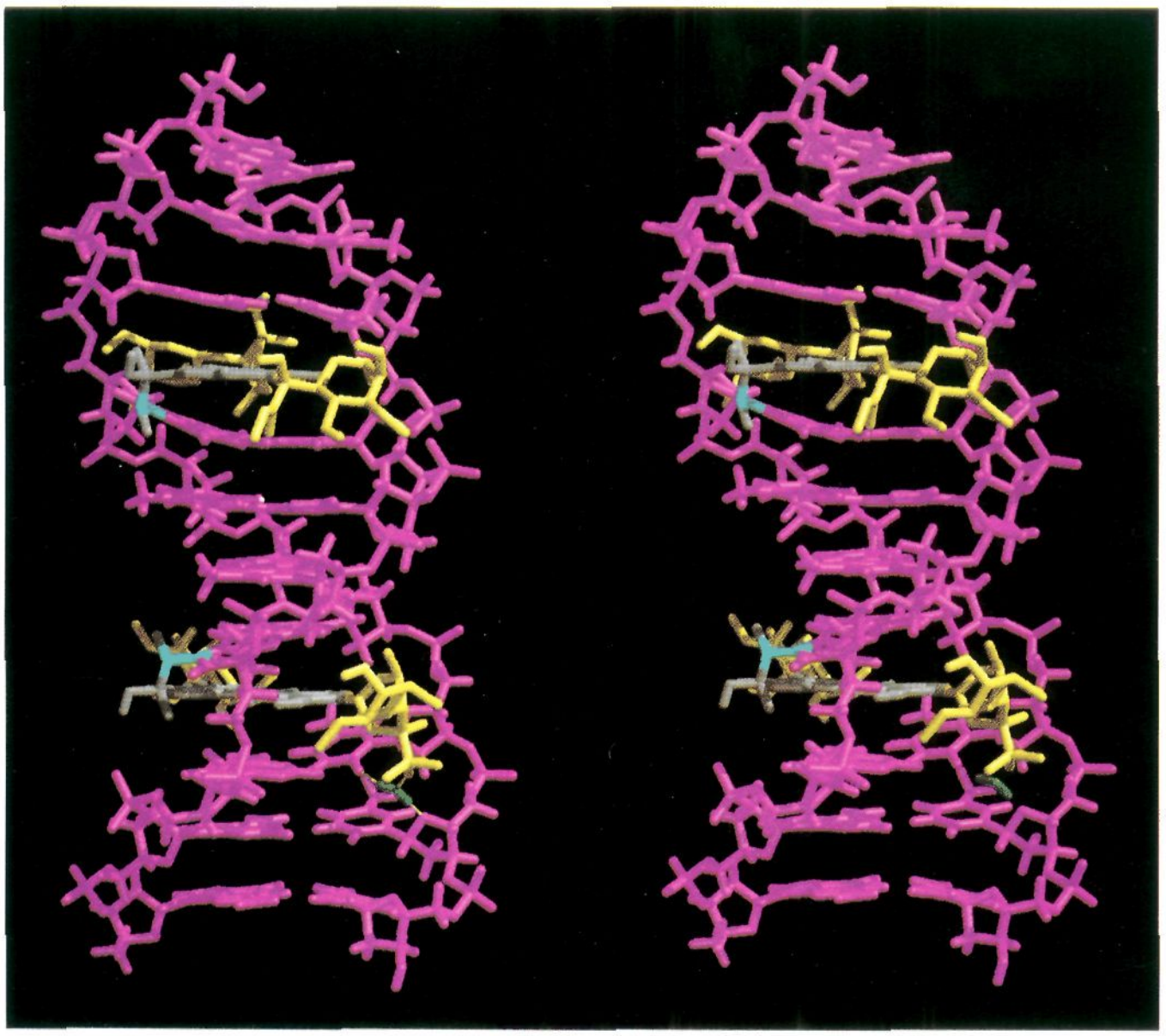

Figure 8. NMR-driven molecular model of the bis(altromycin B)−10-mer duplex adduct depicting intercalation of the drug chromophore into the DNA helix (gray), saccharide binding in both DNA grooves (yellow), and alkylation at N7 (cyan). The 10-mer DNA molecular is rotated so that the major groove faces out at the top of the helix and the minor groove faces out at the bottom of the helix.

The altromycin B C5 neutral sugar substituent also plays an important role in sequence selectivity, but through interactions in the DNA major groove (Figure 10). Altromycins lacking the C5 neutral sugar substituent, such as altromycin H,

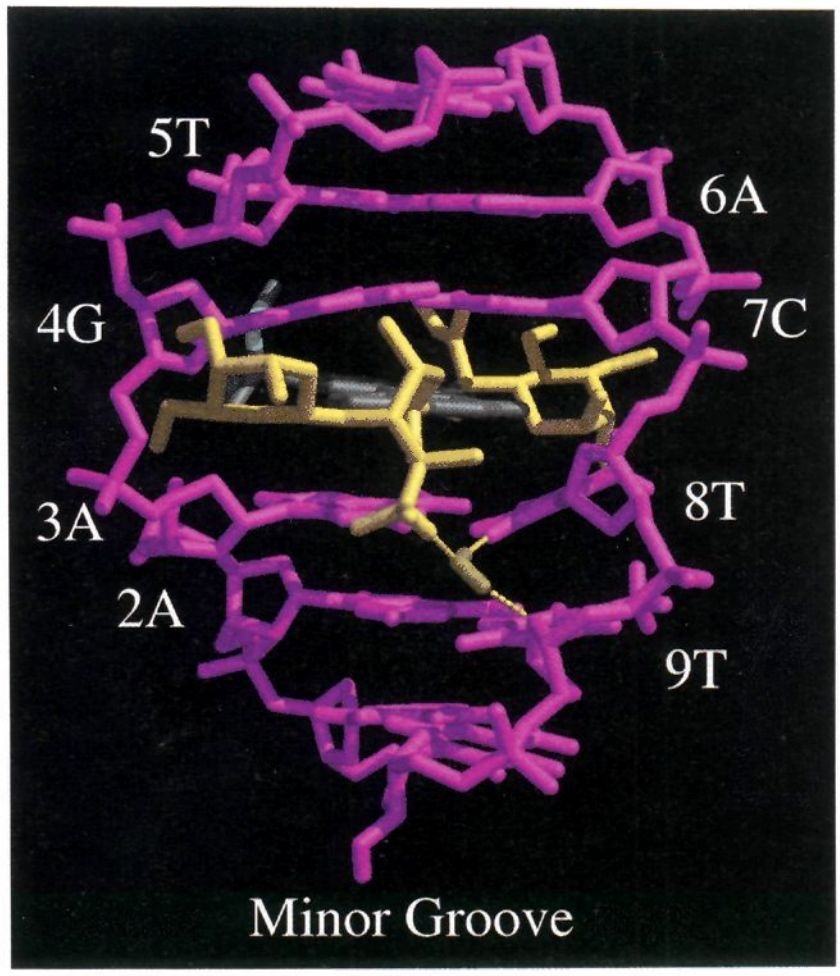

Figure 9. Molecular representation of the interaction of the altromycin B amino disaccharide in the minor groove. In green is a water molecule mediating the proposed hydrogen bond between the dimethylamino substituent and the 8TO2 carbonyl.

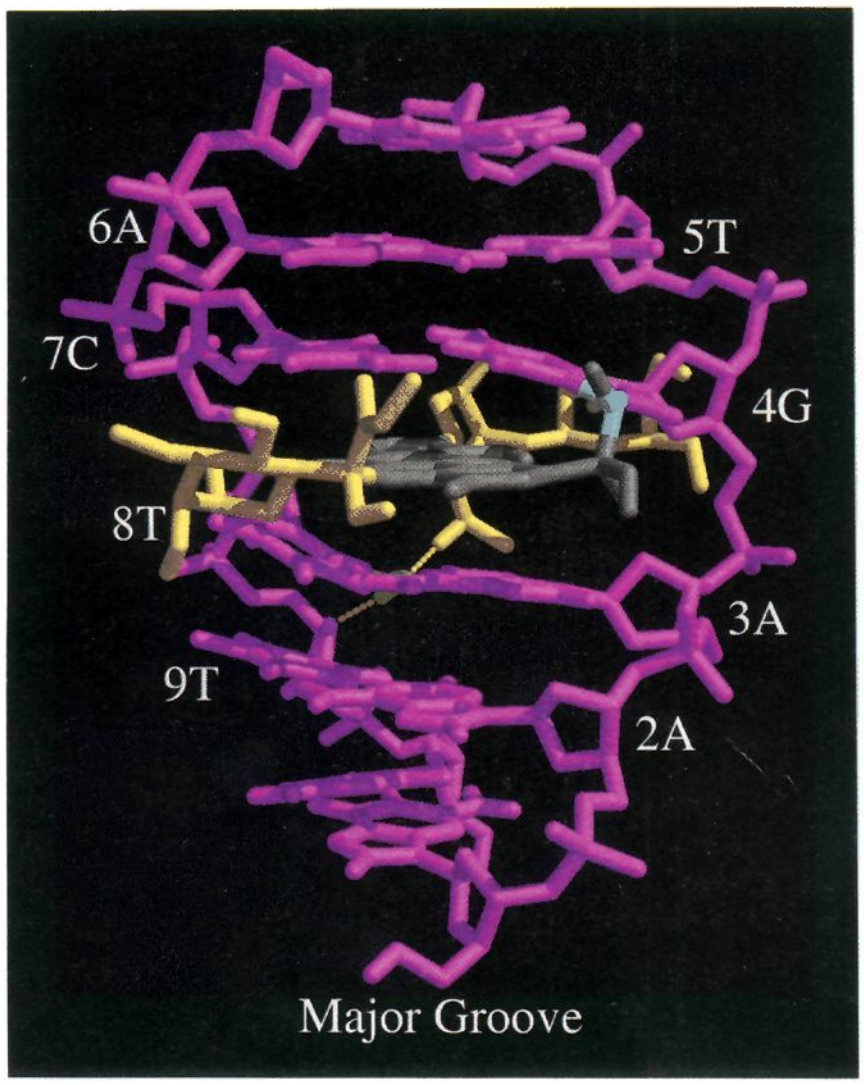

Figure 10. Molecular representation of the interaction of altromycin B in the major groove. Shown are the interaction of the C5 altrose with the noncovalently modified strand, site of covalent attachment to 4G, and tilting of 4G toward the major groove.

demonstrate decreased reactivity with DNA and a modified sequence selectivity compared to that of altromycin B.[10] The key interaction of the C5 substituent is between the hydrophobic face of the C5 sugar and the hydrophobic pocket formed by the drug, 7C, and 8T, which gives rise to a preference for a pyrimidine located on each side of the intercalation site on the noncovalent DNA strand. The lack of the C5 altrose moiety probably allows the altromycin H greater freedom for hydrogen bond formation in the minor groove to either nucleotide of the 5′ base pair and, consequently, less sequence selectivity than those altromycins subjected to steering by both the C3″ *and* C5 altrose substituents.

Drug Design. Although the DNA lesion produced by the pluramycins is remarkably similar to that of the aflatoxins, it should be noted that this class of agents also bears much resemblance to the clinically efficacious anthracycline adriamycin in both structure and mode of interaction with DNA. Due to the large structural variance exhibited by this family of natural products, it is hoped that future generations of synthetic analogs can maximize therapeutic properties while minimizing deleterious ones. As learned in the case of the cyclopropapyrroloindole family of DNA-reactive agents, a seemingly simple structural change, the removal of the ethano bridges of the nonalkylating subunits, yielded agents with potential therapeutic value and without the associated delayed lethality syndrome.[25] This structural elucidation of the interaction of altromycin B with DNA renders a foundation upon which rational design of pluramycin analogs can be undertaken. The tetracyclic chromophore has proven to be a versatile template upon which multiple substituents can be attached to vary biochemical activity. At the C2 position are attached electrophiles that can covalently modify DNA through nucleophiles positioned in the major groove. It has also been shown that glycosidic substitutions at the C5, C8, and C10 chromophore positions can modulate the C2 electrophile's reactivity and sequence selectivity through interactions in both major and minor grooves. By understanding the role in molecular recognition played by substituents attached at these three positions, it is reasonable to pursue the synthesis of compounds with modified sequence affinities or altered reactivities. On a more ambitious note, an understanding of the functional groups that interact in both DNA grooves offers inspiration for the design of agents that can selectively alkylate biologically relevant DNA−RNA hybrids or form novel groove to groove cross-links on the DNA molecule.

Conclusions

Through the study of altromycin B, we have gained insight into the interaction of this family of compounds with DNA for which prior structural information could only be speculated upon. The altromycins have proven to be a novel group of compounds, one that includes characteristics of other classes of DNA-reactive agents. Like the anthracycline nogalamycin, the altromycins intercalate through the DNA molecule via a threading mechanism, which positions saccharide structural groups into the minor groove as well as the major groove. In addition, these agents can alkylate N7 of guanine via an epoxide mechanism reminiscent of aflatoxin B in a sequence-selective fashion. This work has shown potential sugar-mediated recognition of the DNA molecule, which could affect sequence discrimination exhibited by these agents. To further understand the sequence recognition of DNA and the role played by different glycosidic substitutions of this family of compounds, a two-dimensional NMR characterization of a hedamycin−DNA adduct is in progress.

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Experimental Procedures

The self-complementary d(GAAGTACTTC) DNA strand was synthesized on an automated DNA synthesizer (Applied Biosystems 381A) using the solid-phase phosphoramidite method. The DNA synthesis was performed on a 10 μmol scale, leaving the final dimethoxytrityl group on the 5′ end of the DNA molecules. The crude sample was deprotected in concentrated NH_4OH at 55 °C overnight and purified by reverse-phase chromatography on a C18 column (Dynamax-300A). Purified DNA was then detritylated by dissolving in 80% acetic acid for 30 min, followed by an ether extraction to remove the acetic acid. The sample was then extensively dialyzed, and the buffer was adjusted to 10 mM NaH_2PO_4, 100 mM NaCl, and 0.5 mM EDTA (pH 6.8).

The altromycin B samples (Abbott Laboratories) were used without further purification. Being experimental DNA-reactive agents, drug samples were handled with extreme caution to minimize exposure. Drug−DNA adduct formation was achieved by titrating altromycin B, dissolved in deuterated methanol, into the purified DNA until a 2:1 ratio of drug to DNA had been achieved. The sample was mixed at 5 °C for several hours and then checked by one-dimensional NMR to confirm complete alkylation of the DNA. Because of rapid sample degradation due to depurination, multiple samples were prepared in this manner. Due to aggregation problems, NMR experiments were performed on 1.0−2.0 mM adduct concentrations at 30 °C. In between experiments, samples were stored inside NMR tubes at −70 °C to minimize decomposition.

All NMR experiments were performed on a Bruker AMX 500 spectrometer in 99.96% D_2O or 95% H_2O:5% D_2O. Phase-sensitive, tppi two-dimensional NOESY spectra in D_2O were obtained for two mixing times at 150 and 300 ms, and ROESY, TOCSY, and DQF-COSY experiments[11] were performed on samples dissolved in D_2O. For these experiments, 1 K data points were acquired in t_1 with a spectral width of 5000 Hz. To observe exchangeable protons, a two-dimensional NOESY spectrum was obtained with a 150 ms mixing delay in H_2O using a 1−1 echo pulse sequence[26] to suppress water. In this experiment, 1 K of data points were collected with a sweep width of 12 000 Hz.

Due to sample limitations, inter-proton distance constraints were calculated from a single 150 ms NOESY spectrum in D_2O, obtained with 32 scans per increment, a 6000 Hz sweep width, and a 5 s relaxation delay between scans. Data was multiplied by a $\pi/2$ shifted sine bell squared window function, zero filled to 4 K, and Fourier transformed using Felix 2.0 NMR data-processing software. Inter-proton cross peaks were integrated and sorted by volume into 16 distinct bins: four each for proton−proton cross peaks, methyl−proton cross peaks, dimethylamino−proton cross peaks, and methyl−dimethylamino/methyl−methyl cross peaks, corresponding to very strong, strong, medium, and weak connectivities. The upper and lower constraint boundaries for each respective group are 2.2−3.5, 2.6−4.0, 3.0−4.5, 3.5−5.5 Å; 2.2−3.5, 2.6−4.0, 3.0−5.0, 3.5−6.0 Å; 2.2−4.0, 2.6−4.5, 3.0−5.5, 3.5−6.5 Å; and 2.2−4.0, 2.6−4.5, 3.0−5.5, 3.5−6.5 Å. In this way, 118 DNA−DNA constraints, 48 DNA−altromycin B constraints, and 64 intra-drug constraints were generated for the complete bis-adduct system.

The SANDER (simulated annealing with NMR driven energy restraints) module of AMBER 4.0[27] was used to perform energy refinement with these NMR-derived distance constraints to derive a model of the altromycin B−[d(GAAG*TACTTC)]$_2$ diadduct. The AMBER force field was used with the addition of pseudo energy terms consisting of a flat well potential with parabolic boundaries for each interproton connectivity measured. Altromycin B partial atomic charges, calculated by MOPAC 5.0, were incorporated into the force field parameters for electrostatic energy calculations. Additionally, bond, torsion, and angle parameters calculated for carbohydrates[28] were included for energy calculations concerning the saccharide moieties in the drug molecule. The N7 of the modified guanine was parameterized assuming planar, sp^2 hybridization.

Initial DNA structures were created with the NUCGEN module of AMBER and adjusted in MIDAS[29] to form intercalation sites and to dock the altromycin B molecule. The altromycin B structure was generated and minimized using stereochemistries from previously reported NMR structural determinations.[12] Sodium counterions were added to the structure, positioned approximately 4.5 Å from each phosphate. The starting structure was then solvated with approximately 1000 water molecules, subjected to 30 ps of belly dynamics at 300 K (holding the drug−DNA adduct rigid), minimized, and then slowly heated to 300 K over 10 ps. In the initial period of molecular dynamics, inter-proton distance constraints were slowly incorporated to 25 kcal/(mol·Å^2). The system was maintained at 300 K for 90 ps and then minimized without constraints to obtain the reported structure.

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