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Facile formation of a crosslinked adduct between DNA and the daunorubicin derivative MAR70 mediated by formaldehyde: Molecular structure of the MAR70-d(CGT^{n}ACG) covalent adduct

(antitumor drug/rational drug design/DNA crosslink/x-ray diffraction/DNA conformation)

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ABSTRACT **MAR70 is a synthetic derivative of the anticancer drug daunorubicin that contains an additional sugar, attached to the O$^{4'}$ of daunosamine. When MAR70 was crystallized with the DNA hexamer d(CGT^{n}ACG), where ^{n}A is 2-aminoadenine, a covalent methylene bridge was formed between the N$^{3'}$ of daunosamine and the N^{2} of 2-aminoadenine. This spontaneous reaction occurred through the crosslinking action of formaldehyde. The crosslink was demonstrated by the three-dimensional structure of the 2:1 adduct between MAR70 and d(CGT^{n}ACG) solved at 1.3-Å resolution by x-ray diffraction analysis. The perfect juxtaposition of the two amino groups in the complex provides a template for efficient addition of formaldehyde. This adduct structure is compared with the analogous structure at 1.5-Å resolution of the complex of MAR70-d(CGTACG), in which no formaldehyde addition was observed. In both complexes, two MAR70 molecules bind to the DNA hexamer double helix; the elongated aglycon chromophore is intercalated between the CpG steps and spans the G·C Watson–Crick base pairs. The disaccharides occupy nearly the entire minor groove of the distorted B-DNA hexamer double helix. The second sugar is in contact with the sugar-phosphate backbone and does not affect the binding interactions of the daunorubicin portion to DNA. The structure allows us to model the binding to DNA of drugs having more extensive oligosaccharides. In addition, it suggests that placing a reactive (e.g., alkylating) functional group at the N$^{3'}$ amino position of daunorubicin might be a fruitful route for designing anticancer drugs.**

Daunorubicin (Dau) and doxorubicin (Dox) (Fig. 1) are important anticancer drugs currently in widespread clinical use (1, 2). Despite the success of these drugs, there have been problems associated with the undesirable side effects of cardiotoxicity and drug resistance. For these reasons, intense efforts have been made to improve the pharmacological properties of anthracycline compounds by modifying either the aglycone or the amino sugar. This approach has resulted in the preparation of literally hundreds of synthetic or semisynthetic compounds, some of which seem to have better anticancer activities (3). Another approach is to search for new antibiotics, including anthracycline antibiotics, from different microbial sources; many of those drugs, while maintaining the similar aglycone chromophore, have more complicated chemical structures. For example, nogalamycin contains two sugar moieties attached to rings A and D, respectively. The interactions of nogalamycin with DNA have recently been elucidated by x-ray crystallography (4, 5) and NMR studies (6–8). Another related class of anthracycline antibiotics contains longer sugar moieties, exemplified by aclacinomycin A (9), viriplanin (10), or chromomycin A_3 (11). Some drugs, such as esperamicin (12) and calicheamicin (13), cut DNA very efficiently. How these sugar moieties interact with the DNA double helix remains mostly unanswered.

A very different class of antitumor antibiotics acts by forming covalent adducts between the drug and DNA. For example, mitomycin C crosslinks two adjacent guanines of the sequence CpG of B DNA via the N^{2} amino groups (14, 15). Similarly, CC-1065 binds to the narrow minor groove of A+T-rich sequences in a way not unlike distamycin and forms a covalent adduct at N^{3} of adenine in a sequence-specific manner (ref. 16; for review, see ref. 17). These crosslinking reactions require critical spatial positioning between the nucleophile and the electrophile of the two molecules.

Because the biological activities of these drugs are probably closely related to their DNA-binding affinity and sequence specificity, knowing how these drug molecules interact with their target DNA would help to better understand the structure–function relationships and to improve the design of agents based on these correlations. Toward this goal, we have determined the three-dimensional structure of molecular complexes between several antitumor drugs and DNA oligomers by high-resolution x-ray diffraction analysis (4, 18–20). These studies have provided valuable information regarding the role of various functional groups of the drug molecules.

In this paper we present the high-resolution (1.3 Å) molecular structure of a covalent adduct formed between the Dau derivative MAR70 (Fig. 1) and the DNA hexamer d(CGT^{n}ACG) (MAR70-T^{n}A) (where ^{n}A is 2-aminoadenine), mediated through a formaldehyde (HCHO) molecule. This adduct forms readily with high yield during the crystallization steps, likely due to the trace amounts of HCHO in the 2-methyl-2,4-pentandiol (2-MPD) solvent. We compare this unusual adduct structure to the analogous structure of complex MAR70-d(CGTACG) (MAR70-TA), in which no HCHO addition was observed.§

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Abbreviations: ^{n}A, 2-aminoadenine; Dau, daunorubicin (daunomycin); Dox, doxorubicin (adriamycin); MAR70, synthetic compound with 4′-epi-2′-deoxyfucose attached to the O$^{4'}$ of daunosamine; MAR70–T^{n}A, MAR70–d(CGT^{n}ACG) complex; MAR70–TA, MAR70-d(CGTACG) complex; 2-MPD, 2-methyl-2,4-pentandiol; A, T, G, C, ^{n}A indicate respective bases or corresponding nucleotides.

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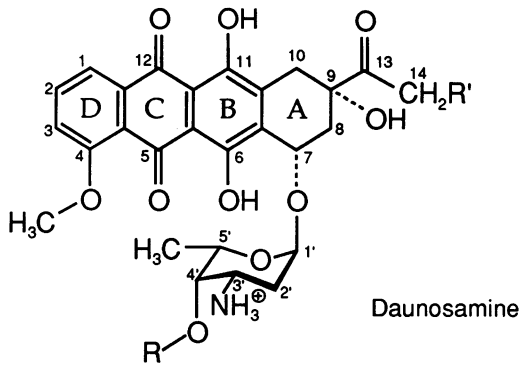
§The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 [reference: (CGTDCG)/MAR70, 1D35; and (CGTACG)/MAR70, 1D36].



Daunorubicin R = R' = H

Doxorubicin R = H; R' = OH

MAR70 R = (4'-Epi-2'-deoxyfucose); R' = H

FIG. 1. Molecular formulas of anthracycline antibiotics, Dau, Dox, and MAR70. These compounds contain an aglycon chromophore with four fused rings (A–D); rings B–D are unsaturated, whereas ring A is semisaturated. MAR70 is a synthetic compound with an additional sugar, 4'-epi-2'-deoxyfucose, attached to the $O^{4'}$ of the daunosamine; it has similar activity as Dau.

EXPERIMENTAL

Oligonucleotides d(CGTACG) and d(CGT^{n}ACG) were synthesized according to a published procedure (21). MAR70 hydrochloride was dissolved in water as stock solutions for crystallization. The MAR70–T^{n}A complex was crystallized from a mixture containing 1.2 mM d(CGT^{n}ACG) (single strand), 4 mM $BaCl_2$, 30 mM sodium cacodylate (pH 6.0), 2.5 mM spermine, 1.2 mM MAR70 plus 5% (vol/vol) 2-MPD. The solution was equilibrated with 40% (vol/vol) 2-MPD at room temperature (≈25°C) by vapor diffusion. The MAR70-TA complex was crystallized under similar conditions, except that $BaCl_2$ was replaced with 20 mM $MgCl_2$. The crystals were in space group $P4_12_12$ with respective unit cell dimensions $a = b = 28.12$ (1) and $c = 52.98$ (4) Å for T^{n}A complex and $a = b = 28.01$ (1) and $c = 53.11$ (3) Å for TA complex. The crystal was mounted in a sealed glass capillary with a droplet of mother liquor. Data were collected on a Rigaku (Japan) AFC-5R rotating anode x-ray diffractometer at 25°C using the ω-scan mode with CuK_α radiation to a resolution of 1.3 Å (MAR70-T^{n}A) and 1.5 Å (MAR70-TA). There were 2599 and 2168 independent reflections seen at the 2.0 $\sigma(F)$ level above background after Lorentz-polarization, empirical absorption, and decay corrections for the two data sets. Coordinates from the Dau-d(CGTACG) structure (19) were used as the starting model and refined by the Konnert–Hendrickson constrained-refinement procedure (22). A series of Fourier maps was calculated to locate the second sugar and solvent water molecules in the crystal lattice. During refinement of the MAR70-T^{n}A structure, we noticed a well-defined electron density bridged between N^2 of ^{n}A and $N^{3'}$ of MAR70. This electron density could not be interpreted as solvent or ion, as it was too close to both nitrogen atoms. We concluded that this density was best assigned as a methylene bridge resulting from the crosslinking reaction of the HCHO and refined as such. The MAR70-T^{n}A structure was refined to a final R factor of 17.4% at 1.3-Å resolution with the root-mean-square differences in bond distances of 0.013 Å from the ideal values. The final R factor is 16.4% at 1.5-Å resolution with a root-mean-square value of 0.025 Å for the MAR70-TA structure. One hydrated sodium ion coordinated to N^7 of the G^6 residue was located in both complexes, as in the Dau-d(CGTACG) complex (19). No other ions (Mg^{+2}, Ba^{+2}, or spermine) could be unambiguously identified.

We used the color-test method of chromotropic acid to determine HCHO in 2-MPD solvent (Aldrich, 11210-0, 99%) (23). Ten microliters of 99% 2-MPD was added to 2 ml of 12 M sulfuric acid containing some solid chromotropic acid. After 30 min of heating at 60°C, the solution became yellow. Adding 100 μl or more of 2-MPD resulted in a deep orange color. For comparison, a low concentration of HCHO gave a yellowish-brown color, and a high concentration gave a deep-purple color. This experiment suggests that certain impurities in 2-MPD can cause a crosslinking reaction to produce coloration of chromotropic acid. We note that even if HCHO constitutes only 0.001% of the 2-MPD stock used in the crystallization reservoir (30 ml of 40% 2-MPD), it represents a 1000-fold molar excess over MAR70 in the dip (2.5 μl at 7 mM concentration). We have also demonstrated the crosslinking between Dau and DNA oligonucleotides mediated by HCHO by HPLC.

RESULTS AND DISCUSSION

Molecular Structure. Both structures were determined and refined at high resolution, which allow us to have an unambiguous view of not only the 2:1 complexes but also the surrounding solvent molecules. Fig. 2 compares the difference Fourier ($F_o - F_c$) maps of the disaccharide moiety, which shows the additional electron density connected to the $N^{3'}$ atom in the MAR70–T^{n}A complex. Location and geometry of the extra density are consistent with a methylene group linking the $N^{3'}$ atom of daunosamine and the N^2 atom of ^{n}A. As control, the structure of MAR70-TA shows no such density, which rules out the possibility of problems with impure MAR70 or other artifacts.

The three-dimensional structure of the 2:1 complex of MAR70-T^{n}A is shown in Fig. 3. It is remarkably similar to MAR70-TA with a root-mean-square difference of 0.209 Å, excluding the N^2 and methylene groups. In fact, the Dau portion of both structures is almost identical to the original Dau-TA structure (20) with respective root-mean-square values of 0.170 Å (T^{n}A complex) and 0.189 Å (TA complex). This fact suggests that the second sugar does not affect the binding of the Dau portion to DNA, and the methylene crosslink does not perturb the structure to any significant extent.

In both complexes, two MAR70 molecules are intercalated between the CpG steps at both ends of a distorted B-DNA double helix. The elongated aglycon chromophore (rings A–D) penetrates the DNA double helix with ring D protruding into the major groove and the disaccharide lying in the minor groove. The pucker in ring A has the O^9 atom deviating from the mean plane the most (0.48 Å for T^{n}A complex and 0.44 Å for TA complex) and aligns the axial O^9 hydroxyl group to form two hydrogen bonds with the N^2 amino group and N^3 atom of the guanine G^2, as for the Dau and Dox complexes (19, 20). The C^{13} keto group attached to the C^9 position is bridged to the O^2 of C^1 by a first-shell water molecule. The O^7 of the glycosyl linkage receives a weak hydrogen bond from the NH_2 of G^2 (3.12 Å for T^{n}A complex and 3.13 Å for TA complex).

The intercalation causes the inner G·C base pairs (G^2·C^{11}) to buckle significantly (18.4° for the T^{n}A complex and 17.8° for the TA complex). The helical twist angle of the CpG step across the intercalator in the MAR70-T^{n}A and MAR70-TA complexes is 37.1° and 37.6°, indicating a slight over-winding

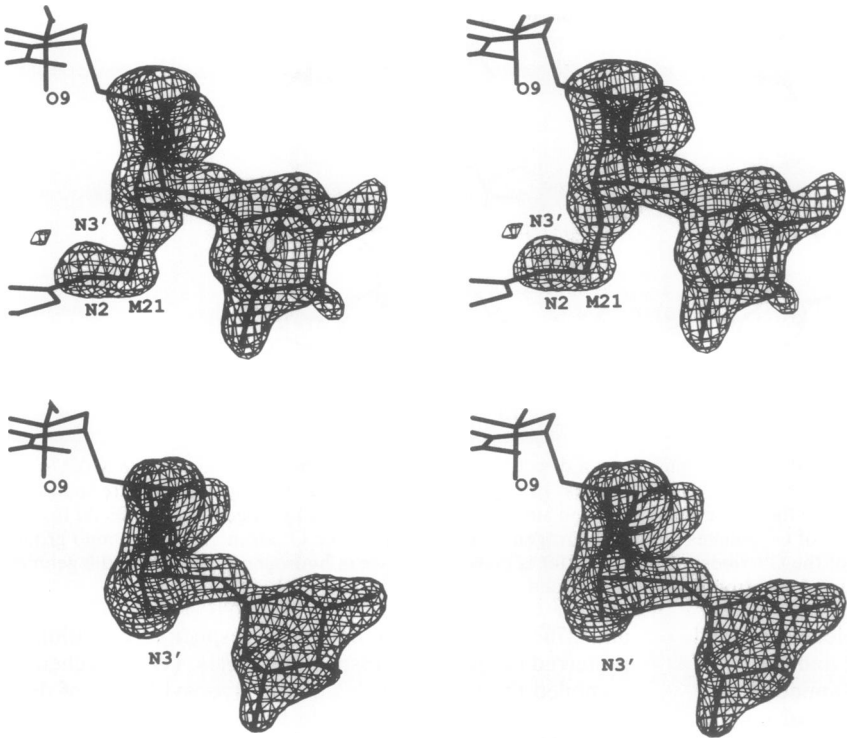


FIG. 2. Stereoscopic views of the difference Fourier ($F_o - F_c$) electron density maps of the MAR70-d(CGT^{n}ACG) (*Upper*) and MAR70–d(CGTACG) (*Lower*) complexes, displayed by FRODO/TOM (24). The disaccharides are omitted from the phase contributions. The high-resolution nature of the structure is visible by the donut shape of the sugar density. Additional atoms (N^2 of ^{n}A and the methylene group M21) were omitted in the former complex. The extra electron density bridged between N^2 of $^n A^{10}$ and $N^{3'}$ of daunosamine can be seen.

(1 to ≈2°) relative to B-DNA. The rest of the steps have a combined unwinding angle of −8.4° in MAR70-T^{n}A and −9.0° in MAR70-TA. Therefore, the overall unwinding angle of the DNA helix from MAR70 intercalation is estimated at only about −8°, significantly lower than the value (−26°) seen for the simple-intercalator ethidium (25). This low DNA unwinding angle associated with MAR70 is a common feature of anthracycline drugs, as discussed (4, 19). Despite the fact that the minor groove is occupied by the disaccharide, its groove width is only slightly affected.

Formaldehyde Crosslinks Drug and DNA. Although HCHO is well-known for its crosslinking capacity (23), it is still quite surprising to see MAR70 crosslinked to d(CGT^{n}ACG), especially because no explicit HCHO was added in the crystallization dips. This observation may be explained as follows. Fig. 4 shows a portion of the complex from a direction perpendicular to the plane of the aglycon ring. As seen in other Dau-DNA structures (19, 20), daunosamine is positioned with its $N^{3'}$ amino group approaching the edge of the base pairs, such that weak hydrogen bonds are formed between $N^{3'}$ and DNA. In the MAR70-TA structure, $N^{3'}$ is 3.31 Å from the O^2 of C^{11} cytosine base and 3.41 Å from $O^{4'}$ of C^{11} deoxyribose. An interesting consequence of the second sugar in the drug is that it creates a cavity between the fucoses

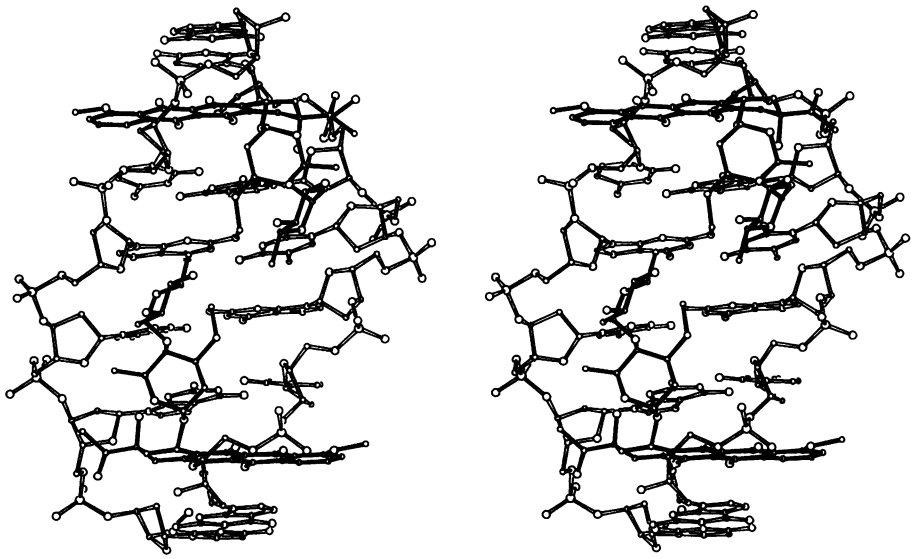


FIG. 3. A view of the MAR70-d(CGT$^n A^{10}$CG) complex looking into the minor groove. Two MAR70s (filled-in bonds) are intercalated between the CpG steps of the distorted right-handed B-DNA hexamer duplex (open bonds) with the aglycon chromophore penetrating through the helix. Their disaccharides lie in and nearly fill up the minor groove of the hexamer duplex, displacing many first-shell water molecules. The tight fit of drug to DNA results in a significant buckle in the C^{11}-G^2 base pair (18.4°). Each MAR70 covers a little over 3 base pairs. The fucose sugars point away from the base pairs, approaching the deoxyribose of the $^n A^4/^n A^{10}$ residues. The $O^{5'}$ of fucose is close to the $O^{4'}$ of $^n A^4$ (3.12 Å). Note that there is a cavity near the bottom of the two T^{n}A base pairs.

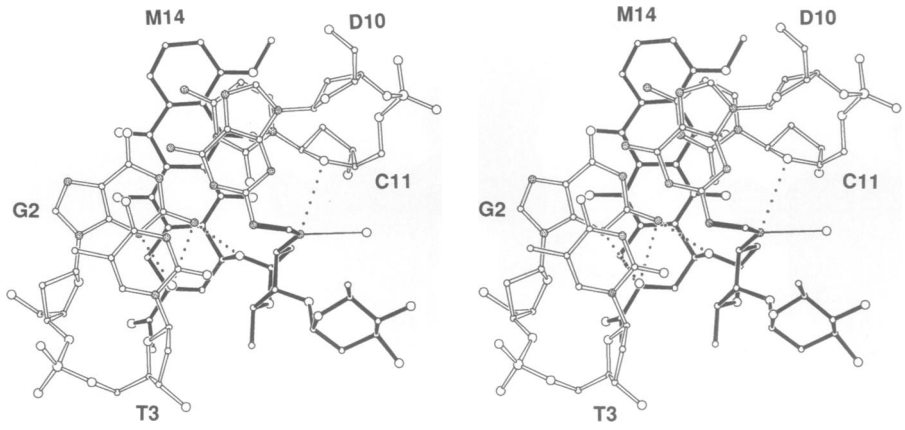


FIG. 4. A view of the intercalated MAR70 (M14) and two base pairs from a direction perpendicular to the base plane in the MAR70–T ^{n}A complex. Hydrogen bonds between MAR70 and DNA are shown as dotted lines. In the minor groove, three hydrogen bonds are found. The O^9 hydroxyl group donates a hydrogen bond (2.80 Å) to the N^3 of guanine G^2 and simultaneously receives a hydrogen bond (3.03 Å) from the N^2 amino group of the same guanine. In addition, N^2 of G^2 donates another hydrogen bond (3.13 Å) to the O^7 atom. The $N^{3'}$ amino group is 3.35 Å to O^2 of C11 cytosine and 3.44 Å to the $O^{4'}$ of the C^{11} ribose. Only the latter is considered a weak hydrogen bond due to the geometry of the N-H...O vector. A water molecule showing strong electron density is included. D10 is $^{n}A^{10}$.

and the two central A·T base pairs. A water molecule is found in this cavity bridging the $N^{3'}$ of daunosamine and the N^3 of adenine A^{10}. When adenine is replaced by 2-aminoadenine $^{n}A^{10}$, the two amino groups ($N^{3'}$ and N^2) are in close contact (3.09 Å), leaving no room for the water molecules. A pocket devoid of water is thus created and surrounded by base pairs on one side, the drug disaccharide on the second side, and deoxyriboses of A^{10}, C^{11} plus the symmetry-related fucose on the third side. This pocket affords a perfect template for an electrophilic attack on amino groups (which become highly reactive in this shielded environment) by an agent such as HCHO. We stress that the second sugar is important in its role as a picket fence for excluding water and rigidifying daunosamine. Under similar crystallization conditions, our preliminary refinement of the 4′-deoxyDau-d(CGT ^{n}ACG) complex indicates that the crosslinking reaction has occurred to a significantly lesser extent (unpublished data).

The crosslinking reaction by HCHO in the MAR70–T ^{n}A complex appears quite effective, as judged by the occupancy and the temperature factor of the methylene group from the crystallographic refinement. The result also suggests that the reaction is sequence specific. Only 5′-^{n}ACG and 5′-GCG have the proper drug-binding conformation to place the N^2 amino group of the first ^{n}A or guanine in the triplet sequence near the $N^{3'}$ of MAR70. To determine whether the N^2 of guanine is nearly as active as the N^2 of ^{n}A, we investigated the effect of HCHO on the 2:1 complexes of Dau-d(CGCGCG) and Dau-d(CGT ^{n}ACG) by adding four equivalents of HCHO and monitoring the reaction by HPLC. HPLC profiles clearly showed that in both complexes all Dau comigrated with DNA after a short incubation, thus indicating an efficient crosslinking reaction (31).

2-MPD is widely used as a precipitating agent in the crystallization of biological macromolecules. Our results point out the potential hazard of trace impurities in 2-MPD, including HCHO, for the biological function of proteins and nucleic acids. For example, in enzymes some amino acid side chains may be juxtaposed so that a crosslinking reaction may occur from the presence of the HCHO, thereby inhibiting enzyme activity.

Oligosaccharides as DNA Binders. The glycosyl linkages of the disaccharide in the drug complex adopt the preferred conformation frequently seen in oligosaccharides. Both sugars in MAR70 are in the chair conformation. The torsion angles around the glycosyl ether linkage [$C^{5'}$—$C^{4'}$—$O^{4'}$—$C^{1'F}$ (τ_1) and $C^{4'}$—$O^{4'}$—$C^{1'F}$—$C^{2'F}$ (τ_2); F is fucose] between the two sugars are 268°, 160° for TA complex and 276°, 151° for T ^{n}A complex, respectively, within the preferred range for the respective angles. (We searched and compiled the torsion angles of the glycosyl bonds of disaccharides using 79 disaccharide fragments from the current Cambridge Data Base. There are two preferred regions for the τ_1 and τ_2 torsion angles. The most frequently occurring region (52%) is 230° ± 50° for τ_1 and 160° ± 20° for τ_2. The next most frequently occurring region (24%) is 225° ± 20° for τ_1 and 220° ± 30° for τ_2.) This combination of τ_1 and τ_2 makes the two sugars nearly perpendicular to each other.

Recently, a number of newly discovered antitumor antibiotics have been found to contain extensive oligosaccharide moieties that may play an important role in DNA binding. NMR and x-ray crystallographic studies showed that chromomycin, containing a disaccharide and a trisaccharide unit, is preorganized into a dimer (mediated by a magnesium ion) and bound to the minor groove of DNA in a distorted A-DNA conformation (26, 27). The sugar moieties in chromomycins adopt a rigid conformation in the complex. Another intriguing class of antibiotics, esperamicin (12) and calicheamicin (13), possess a potent DNA-cleaving function. Their oligosaccharide moieties are believed to guide the DNA-cutting portion (the so-called "warhead") to the sugar-phosphate backbone in a specific manner. An NMR study has shown that they adopt a rigid, extended conformation (28).

The present structures reinforce the above observations and suggest that the glycosyl linkage in an oligosaccharide adopts a well-defined and preferred conformation. We can now use the knowledge accumulated from the structural studies of various anthracycline drugs bound to DNA to predict the modes of action of other more complicated drugs—e.g., aclacinomycin—with DNA. Further, they provide useful information on the interactions between anthracycline derivatives and DNA. They point out that the $N^{3'}$ amino group is a reactive functional group proximal to many nucleophilic positions (e.g., N^3 of adenine, N^2 of guanine) of the DNA double helix. When these amino groups are brought into a hydrophobic environment, not unlike the active site of an enzyme, their reactivities toward nucleophiles increase. In MAR70, this effect was achieved by properly juxtaposing the second fucose in the minor groove. We suggest that the extensive oligosaccharides in calicheamicin not only direct the "warhead" to the DNA backbone but also create a pocket for the cutting reaction.

In conclusion, we suggest that it may be useful to attach certain functional groups (e.g., alkylating) at the $N^{3'}$ amino position of Dau/Dox molecules. These new molecules may

be able to alkylate the N^3 position of adenine very effectively, thereby increasing the therapeutic index in ways similar to CC-1065, an extremely potent anticancer agent that alkylates at the N^3 of adenine (16, 17). It is also interesting to point out that the highly potent $N^{3'}$-modified derivative of Dox, 3'-(3-cyano-4-morpholinyl)-3'-deamino-Dox, forms a covalent adduct to DNA *in vivo* with the loss of the cyano group (29). Although the exact nature of this adduct is yet to be determined, the mechanism associated with the aldehyde-mediated adduct found in the present structure may be worth noting. For example, another highly potent anthracycline, barminomycin, has been shown to contain an aldehyde group attached to $O^{4'}$ of the daunosamine sugar (30). Finally, one may use the efficient HCHO crosslinking between Dau/Dox and DNA to locate the highly specific drug-binding sites in footprinting experiments.

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