Mechanism of Bleomycin: Evidence for 4′-Ketone Formation in Poly(dA-dU) Associated Exclusively with Free Base Release†

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ABSTRACT: Incubation of poly(dA-[3′-^{3}H]dU), poly(dA-[5′-^{3}H]dU), or poly(dA-[5′-^{3}H]dT) under a variety of conditions with activated bleomycin resulted in the production of free nucleic acid base, base propenal, and a small amount of ^3H_2O. Adjustment of the terminated reaction mixture to pH 10 and incubation at 95 °C resulted in a time-dependent increase in ^3H_2O to an amount equal to the amount of free base. If the terminated reaction mixture was incubated with $NaBH_4$ prior to the heat and alkaline treatment, the release of ^3H_2O was significantly inhibited. These results are consistent with the generation by activated bleomycin of a 4′-ketone yielding free base, with the exchange of the 3′- and 5′-hydrogens by enolization and with the alkaline-induced strand scission occurring from this intermediate.

In the preceding paper (Wu et al., 1985), we established that the reaction of bleomycin activated with either Fe(II) and O_2 or Fe(III) and H_2O_2 (Burger et al., 1982) and with poly(dA-[4′-^{3}H]dU) is subject to a large tritium selection effect ($k_H/k_T \sim 10$–12) during the formation of uracil and uracil propenal.[1] The isotope effect is, within experimental error, insensitive to the relative yields of uracil and uracil propenal generated under conditions of varying O_2 concentration. This strongly suggests that 4′-hydrogen abstraction is a rate-determining step in the formation of both products, thereby implicating a common precursor.

In the case of the formation of uracil propenal and the correlative immediate DNA strand scission (Burger et al., 1982), the scheme proposed by Giloni et al. (1981) involving a quench of the 4′-radical by a diatomic oxygen species and subsequent fragmentation is corroborated by our findings. Mechanistic and stereochemical aspects of the formation of base propenal have been investigated and are the subject of another paper (J. C. Wu et al., submitted for publication).

The mechanism of the formation of free nucleic acid base and concomitant generation of alkaline-labile sites (Burger et al., 1982) had been more problematic. In our initial study (Wu et al., 1983), we observed in the reaction of bleomycin–Fe(II)–O_2 and poly(dA-[3′-^{3}H]dU) the formation of ^3H_2O, which was associated exclusively with the release of uracil. On the basis of the reasonable and recently substantiated proposal of 4′-hydrogen abstraction also being responsible for the formation of uracil (Wu et al., 1985), we proposed a hydroxylation of the 4′-radical intermediate by a monooxygen species (Figure 1). The resulting 4′-hemiketal would then open with concomitant elimination of uracil. The residual carbohydrate moiety would be susceptible to alkaline-catalyzed β-elimination of the 3′-phosphate ester effecting strand scission.

A salient feature of this scheme is the formation of a 4′-ketone, which would account for the slow washout of tritium from the 3′-position. In light of the results of our preceding paper (Wu et al., 1985), we report here a detailed analysis of the bleomycin-induced tritium labilization from both the 3′- and 5′-positions of poly(dA-dU) and the 5′-position of poly(dA-dT), the suppression of the tritium washout by $NaBH_4$ treatment of the bleomycin-damaged DNA, and the stoichiometry of the release of tritium vis-ā-vis the formation of uracil. These results support the existence of a 4′-ketone accompanying the release of free base and are consistent with the initial hydroxylation of a bleomycin-derived 4′-radical in competition with the production of a 4′-intermediate leading to the formation of base propenal.

Materials and Methods

UTP, dATP, TTP, phosphodiesterase I, and phosphodiesterase II were obtained from P-L Biochemicals. Malondialdehyde bis(dimethyl acetal) was from Aldrich. Alkaline phosphatase (calf intestine) and 2-thiobarbituric acid were purchased from Sigma. DNA polymerase I large fragment (Klenow) was isolated by the procedure of Joyce & Grindley (1983). Exonuclease III was purchased from New England Biolabs. [5′-^{3}H]Thymidine was purchased from Moravek Biochemicals and converted to the 5′-triphosphate by standard procedures (Yoshikawa et al., 1967; Hoard & Ott, 1965). [3′-^{3}H]UTP was prepared by the procedure of Stubbe et al. (1981) and converted to [3′-^{3}H]dUTP as described in Wu et al. (1985). [5′-^{3}H]UTP was prepared by the procedure of Harris et al. (1984) and converted to [5′-^{3}H]dUTP (Wu et al., 1985). Poly(dA-[3′-^{3}H]dU), poly(dA-[5′-^{3}H]dU), and poly(dA-[5′-^{3}H]dT) were prepared as previously described and had specific activities of 3.6×10^6, 7.7×10^6, and 5.7×10^5 cpm/μmol, respectively, as determined by degradation to deoxynucleosides (Wu et al., 1985).

Bleomycin–Poly(dA-dU) Reaction Conditions. To effect variations in product partitioning, five different reaction

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[1] Trivial names and abbreviations: base propenal, the poly(dA–dU) products 3-(uracil-1′-yl)-2-propenal and 3-(adenin-9′-yl)-2-propenal; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography.

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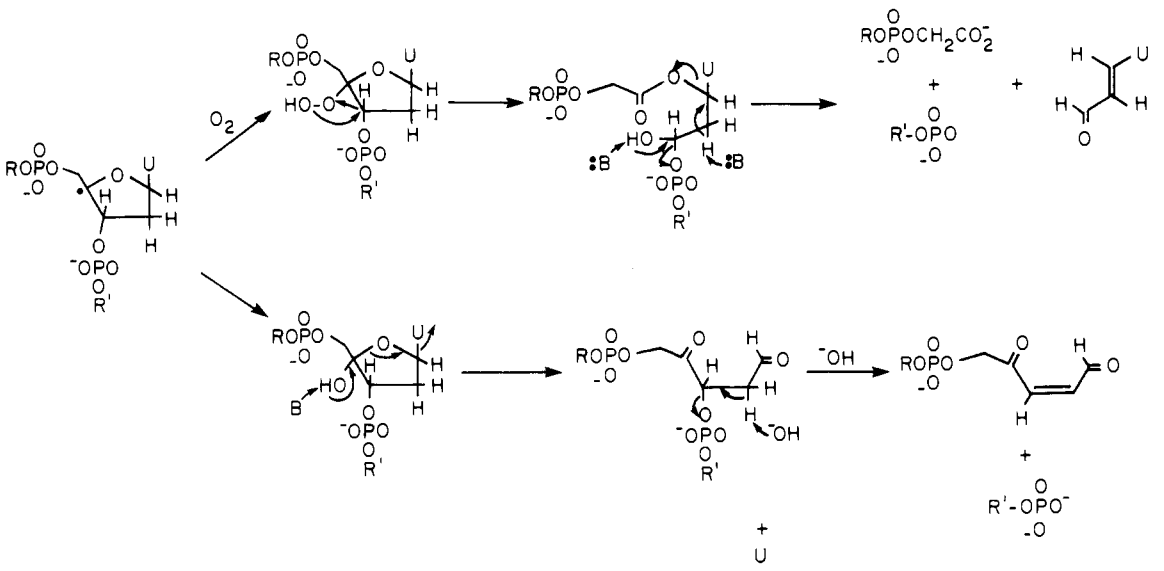

FIGURE 1: Proposed mechanism of degradation of poly(dA-dU) by activated bleomycin to form free base and base propenal. The intermediacy of a 4′-ketone is shown for the production on free base.

conditions were used in these experiments. The experimental protocol is described in detail in the preceding paper (Wu et al., 1985).

Bleomycin–Poly(dA-dT) Reaction Conditions. Conditions were identical with those described for poly(dA-dU) (Wu et al., 1985). Thymine and thymine propenal were isolated by HPLC chromatography on a Rainin Microsorb Short-One (3-μm) reverse-phase column using a linear gradient of methanol (0–100%) over 20 min (compound, retention time: thymine, 8.1 min; thymine propenal, 13.4 min). The yield of thymine was calculated from the UV absorbance of the collected peak (ϵ_{260} = 7.4 × 10^3 M^{-1} cm^{-1}) and of thymine propenal from the thiobarbituric acid assay (Burger et al., 1980).

Heat, Alkaline, and $NaBH_4$ Treatment of Complete Reactions. A typical reaction mixture contained in a final volume of 0.20 mL 0.5 mM bleomycin, 0.5 mM ferrous sulfate, 1.1 mM copolymer, and 0.01 M sodium phosphate (pH 7.5). The reaction was maintained at 0 °C for 30 min at which time it was quenched by addition of EDTA (final concentration of 10 mM), which lowered the pH to 5.5.

Fifty microliters of this sample was added to 450 μL of H_2O, and the solution was shell frozen and subjected to bulb to bulb distillation. The amount of radioactivity recovered in the distillate was determined by scintillation counting.

Fifty microliters of the reaction mixture was adjusted to pH 10 by addition of 2.5 μL of 1 M NaOH, and the sample was heated in a sealed polypropylene tube for 1 h at 95 °C. The sample was then cooled, shell frozen, and subjected to bulb to bulb distillation. The amount of radioactivity in the distillate was determined by scintillation counting.

A third 50-μL sample was made 0.1 M in $NaBH_4$ and was incubated at 0 °C for 30 min. The solution was then adjusted to pH 10 with 1 M NaOH, heated, and subjected to bulb to bulb distillation. The distillate was analyzed for ^3H_2O as described above.

The fourth 50-μL sample was analyzed directly by HPLC chromatography as described above. Uracil was quantitated by A_{260} (ϵ = 8.2 × 10^3 M^{-1} cm^{-1}) and uracil propenal by both A_{294} (ϵ = 3.1 × 10^4 M^{-1} cm^{-1}) and the thiobarbituric acid method (ϵ_{532} = 1.6 × 10^5 M^{-1} cm^{-1}) (Burger et al., 1980). Control experiments indicated that neither particulate flyover nor malondialdehyde were responsible for the tritium measured in the distillates.

Reaction of Bleomycin and [3′-^{3}H]Uracil Propenal. Uracil propenal (15 nmol) was incubated with ferrous sulfate and bleomycin at 3 atm of O_2 under reaction conditions D (Wu et al., 1985). The reaction was stopped with EDTA, and the base and heat treatment was identical with that described above.

Results and Discussion

Initial Studies of Tritium Release from Poly(dA-[3′-^{3}H]-dU) by Bleomycin. Our preliminary findings on the tritium release from poly(dA-[3′-^{3}H]dU) will be summarized here (Wu et al., 1983). In a typical experiment, approximately 10% of the total tritium was isolated as ^3H_2O under conditions that resulted in 55% of the total deoxyuridine converted to uracil and uracil propenal. The specific activity of the uracil propenal formed in the reaction was identical with the specific activity of the starting copolymer, which was consistent with the occurrence of the 3′-tritium at the nonexchangeable aldehydic position of the uracil propenal. These results strongly implied that the ^3H_2O could only be derived from a process associated with the release of uracil.

In light of the singularity of 4′-hydrogen abstraction by activated bleomycin, we reasoned that formation of a 4′-hydroxy species could account for uracil release and alkaline lability of the lesion (Figure 2). Moreover, the labilization of the 3′-tritium would not be due to a direct hydrogen abstraction by bleomycin as this would result in an immediate strand scission, inconsistent with experimental observations (Burger et al., 1982). However, production of ^3H_2O can be accounted for by solvent exchange of the now acidic proton (Figure 2). This hypothesis implies that (1) the 3′-tritium release should be time-dependent and possibly stoichiometric with the amount of uracil released, (2) the release of tritium should be abolished by chemical reduction of the 4′-ketone, and (3) similar behavior should be observed for the protons at the 5′-position, which also flanks the activating carbonyl.

Kinetics of Tritium Release from Poly(dA-[3′-^{3}H]dU). In our initial experiments, the release of ^3H_2O from the 3′-position constituted ~25% of the uracil observed. The significant amount of exchange probably reflected the conditions and procedures of our analysis. To explore this, we determined the time course of 3′-tritium release under a variety of conditions which were anticipated to accelerate a proton exchange (Figure 3). Adjustment of the terminated reaction mixture

Table I: Tritium Release from Poly(dA-[3′-^{3}H]dU) under Various Conditions[a]

reaction condition	uracil (nmol)	uracil propenal (nmol)	$^3H_0$ (nmol)[b]	^3H_f (nmol)[a]	$^3H_f - {}^3H_0$ (nmol)	$(^3H_f - {}^3H_0)$/ uracil ratio
A	10.0	1.4	0.26	13.0	12.7	1.27
B	7.6	11.2	1.6	9.7	8.1	1.06
C (O_2)	4.0	16.4	3.3	6.3	3.0	0.73
D	2.8	16.3	5.2	7.6	2.4	0.87
E	23.7	<0.8	ND	21.1	ND	0.89[d]

[a] See Wu et al. (1985) for reaction conditons A–E. [b] $^3H_0$ is the amount of ^3H_2O measured from unheated reactions. [c] ^3H_f is the amount of ^3H_2O measured from heat and alkali-treated reactions. [d] $^3H_0$ was not determined for this experiment, and ^3H_f was used directly to calculate the molar ratio of tritium to uracil. This does not introduce significant error because in anaerobic condition E the initial ^3H_2O release ($^3H_0$) is ≤2% of the heat-induced tritium.

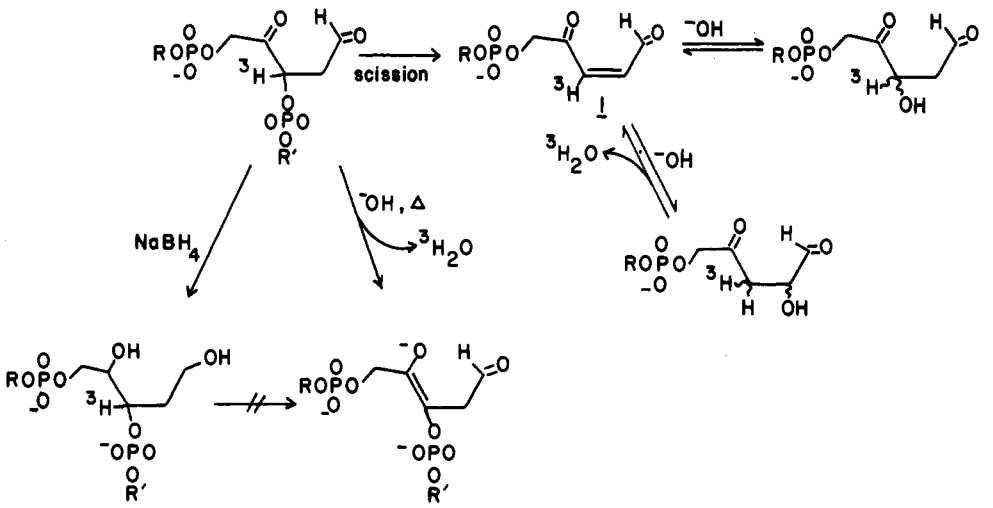

FIGURE 2: Proposed mechanism for free nucleic acid base production and the consequences of free base release: alkaline-labile strand scission, alkaline-catalyzed exchange of 3′- and 5′-^{3}H from appropriately tritiated copolymers, and prevention of alkaline-catalyzed exchange by $NaBH_4$.

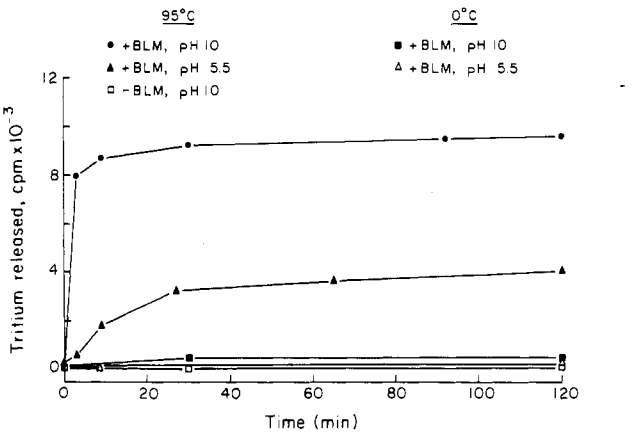

FIGURE 3: Effects of temperature and pH on the time course of ^3H_2O release from poly(dA-[3′-^{3}H]dU)–bleomycin reactions under conditions described in method A.

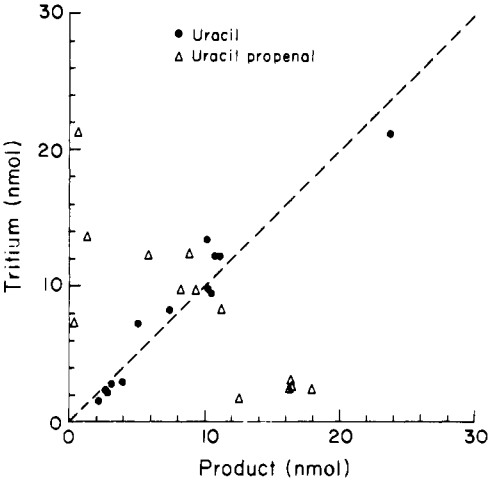

FIGURE 4: Correspondence of tritium release with uracil formation. Data are plotted for 13 experiments in which the yield of heat-inducible tritium, uracil, and uracil propenal were determined. The dashed line (slope = 1) predicts a theoretical 1:1 stoichiometric relationship.

to pH 10 and incubation at 95 °C resulted in a rapid release of tritium to solvent, which was complete in approximately 10 min. At pH 5.5 and 95 °C, the rate of ^3H_2O production was slower, and at 0 °C little release was observed. Tritium in the uracil propenal or in the undamaged copolymer was not labilized to solvent by these procedures. These findings confirm the exchangeability predicted by the hypothesis.

Stoichiometry of Tritium and Uracil Release from Poly(dA-[3′-^{3}H]dU). The stoichiometry of tritium and uracil release from poly(dA-[3′-^{3}H]dU) by activated bleomycin has been determined under a range of conditions detailed in the preceding paper (Wu et al., 1985). Conditions of O_2 concentration and bleomycin activation that resulted in uracil propenal to uracil ratios of 0.03–7 yielded tritium to uracil ratios of approximately 1 (Table I). The correlation of tritium release to the formation of uracil and not of uracil propenal is clearly demonstrated for 13 independent experiments in Figure 4.

An interesting phenomenon apparent in Table I is an elevation in the background of tritium release ($^3H_0$) with increasingly oxygen-rich reaction conditions (A–E). A possible explanation is the susceptibility of uracil propenal or a 3′-aldehydic precursor to oxidation to a carboxylic acid. This would result in the release of tritium in the absence of heat and alkaline treatment. Moreover, this would occur at the expense of uracil propenal. It is suggestive to note that if the amount of background tritium ($^3H_0$) is added to the total

Table II: Tritium Release Catalyzed by Base and Heat Treatment of Bleomycin–Poly(dA-[3′-^{3}H]dU) Reactions Is Abolished by $NaBH_4$

	reaction condition[a]	
	A	E
total deoxyuridine [nmol (cpm)]	47.4 (176389)	55.5 (193229)
uracil (nmol)	11.1	5.2
uracil propenal (nmol)	8.8	≤0.4
^3H_2O [nmol (cpm)]	0.89 (3325)	0.09 (322)
^3H_2O (OH^-, 95 °C) [nmol (cpm)][b]	13.0 (48558)	7.2 (25000)
^3H_2O (OH^-, 95 °C) after $NaBH_4$ [nmol (cpm)][b]	1.9 (7130)	0.71 (2471)
^3H_2O uracil	1.09	1.36

[a] See Wu et al. (1985) for reaction conditions A and E. [b] See Materials and Methods.

monomeric product formed (i.e., a correction for uracil propenal oxidized), then the total extent of reaction remains nearly constant for the most reproducible reaction conditions (B–E) with the increase in uracil propenal occurring at the expense of uracil. This supports the existence of a common intermediate whose partitioning is dependent upon the availability of O_2.

A control experiment was run to test the hypothesis that the production of ^3H_2O during the reaction was the result of oxidation of the uracil propenal to the corresponding acid. Incubation of purified 3′-[^{3}H]uracil propenal with bleomycin, Fe(II), and 3 atm of O_2 (conditions D), however, does not appear to result in significant tritium release over the time course of the reaction. This would imply that if 3′-aldehyde oxidation is responsible for the background tritium, then this process must occur via a DNA-bound intermediate and may be bleomycin-catalyzed. Regardless of the precise origin of this background, its subtraction from the total tritium released affords good 1:1 stoichiometry between the amount of uracil and exchangeable tritium.

Suppression of Tritium Release from Poly(dA-[3′-^{3}H]dU) by $NaBH_4$. If the lability of the 3′-tritium is due to a flanking carbonyl, then chemical reduction of the carbonyl in the damaged copolymer prior to heat and alkaline treatment should result in an inhibition of the exchange. The effect of $NaBH_4$ is shown in Table II. Under normal aerobic (A) and anaerobic conditions (E), heat and alkaline treatment results in a ^3H_2O to uracil ratio of approximately 1. Incubation of the reaction mixtures with $NaBH_4$ prior to heat and alkaline treatment suppresses the formation of ^3H_2O by nearly 90%. These observations give further support to the hypothesis shown in Figure 2 that this exchange is due to an enolization process.

Release of Tritium from Poly(dA-[5′-^{3}H]dT) and Poly(dA-[5′-^{3}H]dU). Figure 2 depicts that the 5′-hydrogens of the damaged copolymer will be susceptible to enolization. The data in Table III demonstrate similar properties to heat and alkaline exchange and $NaBH_4$ inhibition for the 5′-hydrogens of bleomycin-treated poly(dA-[5′-^{3}H]dT) as were observed for the 3′-tritiated copolymer. Under these normal aerobic conditions (A), the ratio of ^3H_2O released to thymine was found to be 1.3. On the basis of the errors involved in the spectrophotometric quantitation of thymine, this value does not differ significantly from the predicted value of 1.

The stoichiometry is further corroborated under anaerobic conditions (E) as shown in Table IV. In this case, the results clearly demonstrate that the release of ^3H_2O correlates only with the formation of thymine and not thymine propenal.

The same trends are observed with poly(dA-[5′-^{3}H]dU); however, the stoichiometry of ^3H_2O and uracil exhibited more variability. This is due in part to the greater difficulty associated with the spectrophotometric quantitation of uracil, which was also a factor in the errors in the calculation of the extents of reaction discussed in the preceding paper (Wu et al., 1985).

Table III: Tritium Release from Poly(dA-[5′-^{3}H]dT) Is Inducible by Heat and Base and Abolished by $NaBH_4$[a]

	+BLM	−BLM
total thymidine [nmol (cpm)]	45.2 (26001)	37.4 (21465)
thymine (nmol)	7.0	ND
thymine propenal (nmol)	8.0	ND
^3H_2O (unheated) [nmol (cpm)]	0.34 (193)	ND
^3H_2O (OH^-, 95 °C) [nmol (cpm)]	9.5 (5451)	0.09 (52)
^3H_2O (OH^-, 95 °C) after $NaBH_4$ [nmol (cpm)]	0.38 (219)	ND
^3H_2O/thymine	1.3	

[a] Reaction condition A (Wu et al., 1985).

Table IV: Tritium Production from Poly(dA-[5′-^{3}H]dT) Is Stoichiometric with Thymine Formation[a]

	+BLM	−BLM
total thymidine [nmol (cpm)]	46.6 (26775)	35.8 (20554)
thymine (nmol)	15.4	ND
thymine propenal (nmol)	<0.62	ND
^3H_2O (unheated) [nmol (cpm)]	0.38 (218)	ND
^3H_2O (OH^-, 95 °C) [nmol (cpm)]	15.81 (9087)	0.06 (31.7)
^3H_2O/thymine (molar ratio)	1.0	ND

[a] Reaction condition E (Wu et al., 1985).

Conclusions

The exchange of the 3′- and 5′-hydrogens of the bleomycin-damaged copolymer is best accounted for by a 4′-ketone intermediate. The results obtained upon $NaBH_4$ treatment substantiate this argument. In light of the findings reported in the preceding paper (Wu et al., 1985), this intermediate must be derived from the abstraction of the 4′-hydrogen and be the byproduct of the release of free nucleic acid base. A likely precursor then would be a 4′-hemiketal (Figure 1), which would ring open to eliminate free base and form the 4′-ketone. Precedent for this scheme may be found in the study of DNA damage by ionizing radiation (von Sonntag & Schulte-Frolinde, 1978).

The 1:1 stoichiometry of the release of ^3H_2O and uracil from poly(dA-[3′-^{3}H]dU) might be viewed as unexpected given the alkali-catalyzed β-elimination leading to strand scission that is in competition with the enolization (Figure 2). Alkali-catalyzed strand scission would yield intermediate **1**. While **1** might not be expected to exchange the 3′-hydrogen directly, addition of hydroxide to the α,β-unsaturation would reestablish the exchangeability of this proton. This, of course, would not be required for 5′-hydrogen exchange.

In conclusion, the major features of the reaction of bleomycin and a DNA model have been illuminated. These are (1) the rate-determining 4′-hydrogen abstraction responsible for the production of base and base propenal, (2) the oxygen-dependent partitioning of the common intermediate, and (3) the generation of a 4′-ketone associated with the release of free base. Experiments directed toward the elucidation of the carbohydrate fragment formed by the release of free base are in progress.

Registry No. Poly(dA-dU), 26780-70-1; poly(dA-dT), 25464-54-4; bleomycin, 11056-06-7; uracil propenal, 86798-57-4; thymine propenal, 85394-19-0; thymine, 65-71-4; uracil, 66-22-8.

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Structure of the Anthramycin–d(ATGCAT)$_2$ Adduct from One- and Two-Dimensional Proton NMR Experiments in Solution†

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ABSTRACT: One- and two-dimensional 400-MHz proton NMR experiments are used to examine the solution structure of the covalent adduct formed by the interaction of anthramycin methyl ether with the self-complementary deoxyoligonucleotide d(ATGCAT)$_2$. The concentration dependence of chemical shifts and nuclear Overhauser enhancement (NOE) experiments are utilized to assign the adenine H_2 protons within the minor groove for both free d(ATGCAT)$_2$ and the adduct. These studies demonstrate that one of the four adenine H_2 protons is in close proximity to the bound anthramycin and this results in its upfield shift of 0.3 ppm compared to the adenine H_2 protons of the free duplex. Effects of the covalent attachment of anthramycin to the d(ATGCAT)$_2$ duplex result in an increased shielding of selected deoxyribose protons located within the minor groove of the adduct, as demonstrated by two-dimensional autocorrelated (COSY) NMR techniques. Interactions between the protons of the covalently attached anthramycin and the d(ATGCAT)$_2$ duplex are determined by utilizing two-dimensional NOE (NOESY) techniques. Analysis of these data reveals NOE cross-peaks between the anthramycin methyl, H_6, and H_7 protons with specific deoxyoligonucleotide protons within the minor groove, thus allowing the orientation of the drug within the minor groove to be determined. Nonselective inversion recovery (T_1) relaxation experiments are used to probe the structural and dynamic properties of the anthramycin–d(ATGCAT)$_2$ adduct. These data suggest that the binding of anthramycin alters the correlation time of the d(ATGCAT)$_2$ duplex and stabilizes both of the internal A·T base pairs with respect to solvent exchange. The solution conformation of the anthramycin–d(ATGCAT)$_2$ adduct, as deduced from the NMR data, is in agreement with model-building studies [Hurley, L. H., & Petrusek, R. L. (1979) *Nature (London) 282*, 529–531; Petrusek, R. L., Anderson, G. L., Garner, T. F., Fannin, Q. L., Kaplan, D. J., Zimmer, S. G., & Hurley, L. H. (1981) *Biochemistry 20*, 1111–1119].

The antitumor activity of anthramycin (Figure 1A) has been attributed to its ability to interact with DNA resulting in the inhibition of the biosynthesis of nucleic acids (Kohn et al., 1968; Stefanovic, 1968; Horwitz et al., 1971; Glaubiger et al., 1974). The exact nature of this interaction with DNA has been the subject of numerous studies over the past several years. From these studies, anthramycin has been shown to form a labile covalent attachment to DNA spanning approximately three base pairs (Glaubiger et al., 1974; Kohn & Spears, 1970; Kohn et al., 1974). The stability of this bond is dependent upon the maintenance of the secondary structure of the DNA and is lost upon denaturation of the DNA by heating, by enzymatic digestion, or by lowering the pH to <7.0. Anthramycin is highly selective in binding to DNA, requiring the presence of both a double-strand DNA template and guanine (Hurley et al., 1979). This nonintercalative interaction results in the thermal stabilization of the duplex and is presumed to occur in the minor groove (Hurley & Petrusek, 1979; Petrusek et al., 1981).

Recently, nuclear magnetic resonance techniques were used to confirm the points of attachment of anthramycin to DNA (Graves et al., 1984). Analysis of ^{13}C NMR spectra demonstrated that the anthramycin forms a covalent linkage to the DNA at the C_{11} position. The site of attachment on the DNA was determined by using the self-complementary deoxyoligonucleotide d(ATGCAT)$_2$ as a DNA model. ^1H NMR studies confirmed the guanine NH_2 as the site of attachment to the DNA, consistent with the model proposed (Hurley & Petrusek, 1979; Petrusek et al., 1981). Upon formation of the adduct, a loss in the helical symmetry of the self-complementary deoxyoligoribonucleotide resulted in the doubling of the nucleotide resonances. The doubling, combined with superposition of anthramycin resonances, resulted in a complex spectrum that could not be unequivocally assigned, thus pre-

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