

Chemical Properties of the Leinamycin–Guanine Adduct in DNA

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The reaction of the antitumor agent leinamycin with thiols converts this natural product into an episulfonium ion that alkylates the N7-position of guanine residues in double-stranded DNA. It is reported here that depurination of this adduct is unusually facile, occurring with a half-life of about 3.5 h at pH 7 and 37 °C in duplex DNA. This is one of the most rapid depurination reactions ever observed for an N7-alkylguanine residue. The rate constant for the depurination reaction was measured at several temperatures, and the activation parameters were calculated from the data. The energy of activation (E_a) for this reaction is 24.6 kcal/mol, and the Arrhenius A value is $1.2 \times 10^{13} \text{ s}^{-1}$. These values correspond to a $\Delta H^\ddagger = 24.0 \text{ kcal/mol}$ and $\Delta S^\ddagger = -0.78 \text{ eu}$ and are consistent with the expected unimolecular ($D_N + A_N$) mechanism for the depurination reaction. Changes in ionic strength (0–500 mM NaCl) or pH (3–8) do not significantly alter the rate of depurination, and the base excision repair protein Aag, which removes a variety of N7-alkylguanine residues from duplex DNA, does not excise the leinamycin–guanine adduct. Possible biological implications of this rapid depurination process are considered. Finally, during the course of these studies, the release of hydrolyzed leinamycin (**4**; Scheme 1) from leinamycin-modified DNA was observed. This result suggests that leinamycin may be a reversible DNA alkylating agent.

Introduction

Leinamycin (**1**) is a structurally novel *Streptomyces*-derived natural product that possesses potent cytotoxic and antitumor properties (for example, an IC_{50} of 27 nM against HeLa S3 cells) (1–3). The reaction of leinamycin with a thiol converts the natural product to an episulfonium ion (**2**; Scheme 1) that selectively alkylates the N7-position of guanine residues in double-stranded DNA to yield the covalent adduct **3** (3–5). N7-Alkylguanine lesions such as that formed by leinamycin are chemically unstable, with the most common mode of decomposition involving hydrolysis of the glycosidic bond between the alkylated base and the 2'-deoxyribose residue (depurination; Scheme 2) (6–9). The rate at which different N7-alkylguanine residues undergo depurination from duplex DNA varies widely depending upon the nature of the N7-substituent (6).

As part of an effort to better understand the molecular basis of leinamycin's potent cytotoxicity, we set out to characterize the chemical stability of the guanine adduct formed by this compound in double-stranded DNA and the monomeric nucleoside. We report here that depurination of this N7-alkylguanine lesion is unusually facile. These studies may ultimately help explain the potent biological activity of leinamycin, as they provide information regarding the nature of the DNA lesion(s) that confront the cell's repair, transcription, replication, and signal transduction machinery. In addition, during the course of these studies, we observed the release of hydrolyzed leinamycin (**4**; Scheme 1) from leinamycin-

modified DNA. This result suggests that leinamycin may be a reversible DNA alkylating agent.

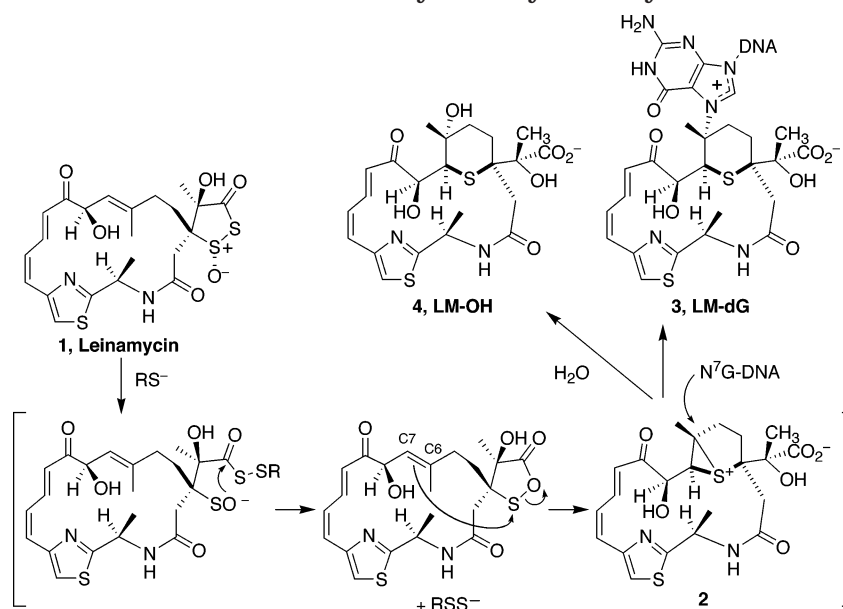
Materials and Methods

Materials. All buffers, ethanol, *n*-butanol, 2'-deoxyguanosine, trifluoroethanol, and 2-mercaptoethanol were purchased from Aldrich Chemical (St. Louis, MO) and were used as purchased unless otherwise stated. Herring sperm DNA was purchased from Boehringer Mannheim or Roche. UV-vis melting studies confirmed that the DNA was in the double-helical form. Analysis of the DNA by 1% agarose gel electrophoresis, alongside a series of size markers, shows that the average size of the duplex fragments is between 50 and 200 bp. The 2-mercaptoethanol was used fresh to minimize oxidative dimerization. HPLC grade trifluoroethanol was purified by fractional distillation stored dry. Leinamycin was generously provided by researchers at Kyowa Hakko Kogyo, Ltd. All solvents used were HPLC grade or higher and were purchased through Fischer Scientific, Inc.

DNA Alkylation by Activated Leinamycin. To a solution of herring sperm DNA (3.83 μmol bp) and leinamycin (0.2 μmol ; 40 μL of 5 mM stock solution in MeCN) in 0.46 mL of sodium phosphate buffer, pH 7.0, at 4 °C was added cold 2-mercaptoethanol (1.2 μL of a 250 mM stock solution), followed by vortex mixing (final concentrations: 7.6 mM bp DNA, 400 μM leinamycin, and 600 μM 2-mercaptoethanol). These conditions for preparing leinamycin-modified DNA were analogous to the procedure outlined previously by Asai and co-workers (4). The reaction mixture was incubated at 4 °C for 4 h, and the resulting alkylated DNA was extracted with *n*-butanol (2 \times 500 μL) to remove unreacted leinamycin and leinamycin byproducts. The aqueous layer was then ethanol precipitated with 0.3 M sodium acetate and 70% ethanol, followed by three washings with cold 70% ethanol (10). The resulting greenish-yellow DNA pellet was dried briefly under a stream of nitrogen and stored at –20 °C until further use. Different alkylation conditions (e.g., using 80 μM leinamycin), which were expected to afford different yields

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Scheme 1. DNA Alkylation by Leinamycin



of leinamycin adduct per DNA base pair, did not result in significantly different depurination kinetics.

Release of Leinamycin-Derived Products from DNA.

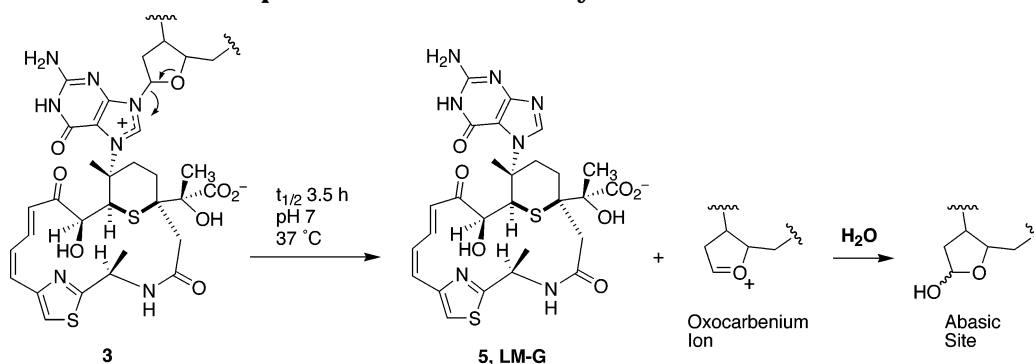
The leinamycin-treated DNA described above was dissolved in sodium phosphate buffer (50 mM, pH 7) at the desired temperature for the particular kinetic run. In some experiments, the buffer pH was varied or NaCl was added. At selected time points, a 5 μ L aliquot was removed from the assay mixture and diluted with 45 μ L of water. This mixture was then extracted with butanol (2 \times 50 μ L), and the organic extracts were combined, dried under vacuum, redissolved in 20% methanol/water, and analyzed by HPLC using a reverse phase Supelcosil LC-18 column (5 μ m, 250 mm \times 2.1 mm). For the kinetic runs, the products were eluted at a flow rate of 0.2 mL/min using a continuous gradient over 23 min running from 10% solvent B to 30% solvent B, where solvent A is 25 mM potassium phosphate buffer, pH 2.3, and solvent B is acetonitrile. Thereafter, a 2 min ramp to 40% solvent B was employed and maintained for 12 min, followed by a 2 min ramp to the initial solvent composition for a 25 min reequilibration. The analytes were detected by monitoring the UV absorbance at 324 nm. The LM-G adduct (5) elutes at 22–23 min under these conditions. The leinamycin–guanine adduct (5) was isolated using a different HPLC method. This method was identical to that describe above, except that pump A solvent was 0.5% formic acid in water, pH 2.3. The leinamycin–guanine adduct eluting at 26 min was collected and dried under vacuum. The resulting residue was dissolved in 20% methanol:water and then mixed with an equal volume of 0.5 M aqueous NH₄OH and then subjected to negative ion ESI-MS and ESI-MS/MS using a TSQ-7000 mass spectrometer with an API 2 source equipped with

the performance pack. All data were interpreted with Finnigan's Xcalibur software version 1.2. Typical mass spectra parameters were as follows: capillary temperature, 250 $^{\circ}$ C; spray voltage, 3.33 kV; sheath gas, 40 psi; and flow rate, 10 μ L/min. The major ion was observed at m/z of 628, consistent with the expected leinamycin–guanine adduct. MS/MS collision-induced dissociation experiments employing a collision energy of 50 eV at an argon pressure of 1.5 mTorr revealed that the ion at m/z 628 fragmented to yield a new ion at m/z 150, corresponding to the mass of the guanine residue, consistent with loss of the leinamycin fragment from the original leinamycin–guanine adduct. The structure of the leinamycin–guanine adduct was confirmed using high-resolution electrospray mass spectrometry: calcd for C₂₇H₃₀N₇O₇S₂, 628.1648; found, 628.1617.

The leinamycin hydrolysis product (4), released from leinamycin-alkylated DNA, was identified in these experiments based upon its HPLC retention time as compared with authentic material prepared by the method of Asai et al. (4). The identity of 4 released from leinamycin-modified DNA was further confirmed by observing that the material decomposed to yield a characteristic set of products identical to that produced by decomposition of authentic 4 (11).

Rate constants for the depurination of the leinamycin–guanine adduct from duplex DNA under various conditions were obtained by fitting the HPLC data for the appearance of the product (5) to the integrated rate expression for a first-order process: $P_t = c + A_0(1 - e^{-kt})$ where P_t is the HPLC peak area of depurination product at time = t , A_0 is the concentration of starting material at time zero (which is equal to the concentration of P at infinite time), and c is the peak area of the product at the beginning of the experiment. Fitting was performed using

Scheme 2. Depurination of the Leinamycin–Guanine Residue in DNA



Datafit version 7.1 (Oakdale Engineering). It was observed that the concentration of leinamycin-modified DNA does not significantly alter depurination kinetics. Average values and standard errors were calculated using results obtained from at least three separate experiments. Reaction half-lives were calculated using the equation $t_{1/2} = (\ln 2)/k$. The activation parameters were obtained from the depurination rate constants measured at different temperatures using the Arrhenius equation: $\ln k = -E_a/RT + \ln A$. Thus, a plot of $\ln k$ vs $1/T$ has a slope equal to $-E_a/R$ and an intercept equal to $\ln A$. The activation enthalpy (ΔH^\ddagger) was calculated using the equation $E_a = \Delta H^\ddagger + RT$, and the activation entropy (ΔS^\ddagger) was calculated using the relationship $\Delta S^\ddagger = 4.575 \log A - 60.53$ (12). The entropy of activation (ΔS^\ddagger) was expressed in terms of entropy units (eu), which correspond to $\text{cal degree}^{-1} \text{mol}^{-1}$. Alternatively, ΔH^\ddagger and ΔS^\ddagger can be calculated from an Eyring plot of $\log(k_r/T)$ vs $1/T$ where ΔH^\ddagger (in cal/mol) = slope(-4.576) and ΔS^\ddagger can be calculated using the equation $\Delta S^\ddagger = 2.303R \log(k_r/T) + \Delta H^\ddagger/T - 2.303R \log \kappa/h$, where k_r is the reaction rate at a given temperature T , κ is the transmission coefficient, which is usually taken to be one, k is Boltzmann's constant, and h is Planck's constant (12).

UV-Vis Assay for the Depurination of the Leinamycin-Guanine Residue from Duplex DNA. DNA modified with activated leinamycin as described above was dissolved in 650 μL of sodium phosphate buffer (50 mM, pH 7) and placed in a 1 cm path length quartz cell to achieve a final DNA concentration of 5.8 mM bp. The cuvette was placed in a Hewlett-Packard diode array spectrophotometer 8452A equipped with a temperature-controlled Peltier cell holder, absorption spectra were collected over time at 60 $^\circ\text{C}$, and the change in absorbance at 380 nm was used to monitor depurination of the leinamycin-guanine adduct from the DNA. Rate constants were obtained from the data using the equation: $\ln([A_t]/[A_0]) = \ln[(Y_t - Y_{\text{inf}})/(Y_0 - Y_{\text{inf}})] = -kt$, where A_t is the concentration of starting material at time t , A_0 is the concentration of starting material at time zero, Y_t is the absorbance₃₈₀ at time t , Y_{inf} is the absorbance₃₈₀ at infinite time (when absorbance values are no longer changing in the reaction), and Y_0 is the absorbance at time zero (13). Thus, a plot of $\ln[(Y_t - Y_{\text{inf}})/(Y_0 - Y_{\text{inf}})]$ vs t gave a line whose slope equals $-k$. The reaction follows first-order kinetics, generating linear plots over the course of 3–5 half-lives. Alternatively, the rate constant for depurination of the leinamycin-guanine adduct was obtained by fitting the UV-vis absorbance data for the decrease in absorbance at 380 nm to the equation $Y_t = Y_0 + (Y_{\text{inf}} - Y_0)(1 - e^{-kt})$ using Datafit version 7.1.

Experiments to Determine Whether the Base Excision Repair Enzyme 3-Methyladenine DNA Glycosylase (Aag) Catalyzes Release of the Leinamycin-Guanine Residue from Duplex DNA. Leinamycin-alkylated DNA prepared as described above was dissolved in 110 μL of "Aag buffer" (Trevigen) containing either 3 or 7.5 units of Aag endonuclease (Trevigen). The solutions were incubated at 37 $^\circ\text{C}$. At various time points, 5 μL of solution was withdrawn and diluted to 50 μL with the same buffer and then extracted with butanol ($2 \times 50 \mu\text{L}$). The butanol extracts were dried under vacuum, and the residue was dissolved in 100 μL of 20% methanol in water and analyzed by HPLC as described above.

Synthesis and Properties of the Monomeric Leinamycin-2'-Deoxyguanosine Nucleoside Adduct. 2'-Deoxyguanosine (7.4 mg, 26 μmol) was dissolved in 2,2,2-trifluoroethanol (1.3 mL) with sonication (1 h) to yield a 20 mM solution of the nucleoside. A portion of this 2'-deoxyguanosine solution (75 μL) was added to a dry leinamycin residue (77 μg , 0.15 μmol) in a 1.5 mL eppendorf tube, and the mixture was cooled in an ice bath. The alkylation reaction was initiated by the addition of cold, neat 2-mercaptoethanol (2 μL , 28 μmol), and the mixture was incubated at 4 $^\circ\text{C}$ for 3 days. At that time, HPLC analysis of the reaction mixture revealed the formation of a single major product. A portion of the reaction mixture was characterized by mass spectroscopy (using a Finnigan TSQ-7000 Electrospray Mass Spectrometer). The mixture was introduced by direct

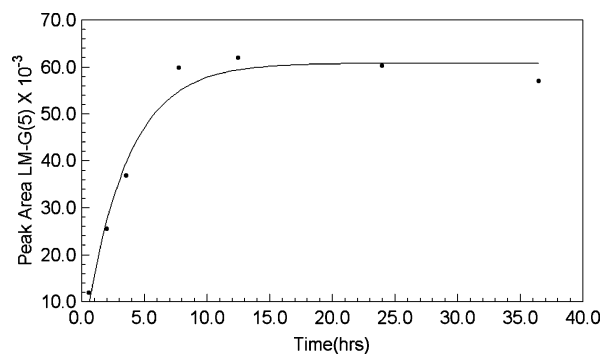


Figure 1. Time course for the release of LM-G (5) from double-stranded DNA at 37 $^\circ\text{C}$ (50 mM sodium phosphate buffer, pH 7). The yield of the depurination product at each time point was measured based on its HPLC peak area as described in the Materials and Methods.

infusion at a flow rate of 10 $\mu\text{L}/\text{min}$. The mass range of 17.5 to 1200 Th was observed with a scan duration of 1.5 s for a total of 100 scans, and positive ions were detected using the following conditions: capillary temperature, 250 $^\circ\text{C}$; source temperature, 70 $^\circ\text{C}$; capillary voltage, 0.9 kV; sheath gas, 40 psi; and flow rate, 10 $\mu\text{L}/\text{min}$. An ion with m/z 746, consistent with the anticipated product 6, was observed. The rate of depurination reaction of leinamycin-2'-deoxyguanosine adduct (6) was measured by HPLC as described above.

Results

Alkylation of Double-Stranded DNA by Leinamycin. Mixed sequence, double-stranded DNA (7.6 mM bp, average fragment size 50–200 bp in length) was incubated in aqueous buffer (10 mM potassium phosphate, pH 7, containing a final concentration of 5% acetonitrile) containing a mixture of leinamycin (400 μM) and 2-mercaptoethanol (600 μM) for 4 h at 4 $^\circ\text{C}$ (14). Following the alkylation reaction, excess leinamycin and leinamycin byproducts were removed from the alkylated DNA by two butanol extractions, ethanol precipitation, and washing with a cold solution of 70% ethanol/water (10).

Release of Leinamycin-Derived Products from Modified DNA. Previous work has shown that heating DNA (65 $^\circ\text{C}$, 30 min) that has been treated with activated leinamycin leads to the release of the leinamycin-guanine adduct (LM-G, 5; Scheme 2) as the sole thermally labile lesion (4); however, the properties of this adduct in duplex DNA under physiologically relevant conditions have not previously been examined. In our experiments, we monitored the release of leinamycin-DNA adducts using HPLC. During incubation of the alkylated DNA at 37 $^\circ\text{C}$ in sodium phosphate buffer (50 mM, pH 7), we observed the release of a single major product containing the leinamycin chromophore (with a prominent absorbance maxima near 350 nm). On the basis of previous work (4), we suspected that this was the depurination product LM-G (5). Indeed, negative ion ESI(-)-MS, high-resolution ESI(-)-MS, and authentic synthesis confirmed that the major product released from leinamycin-modified DNA is the N7-guanine adduct 5. We monitored the appearance of the LM-G product released from duplex DNA over more than 10 half-lives (Figure 1). Fitting the resulting data to the equation for a first-order reaction [$P_t = c + A_0(1 - e^{-kt})$] as described in the Materials and Methods yields a rate constant of $0.20 \pm 0.03 \text{ h}^{-1}$ for the release of LM-G from duplex DNA at 37 $^\circ\text{C}$ (50 mM sodium phosphate, pH 7). This

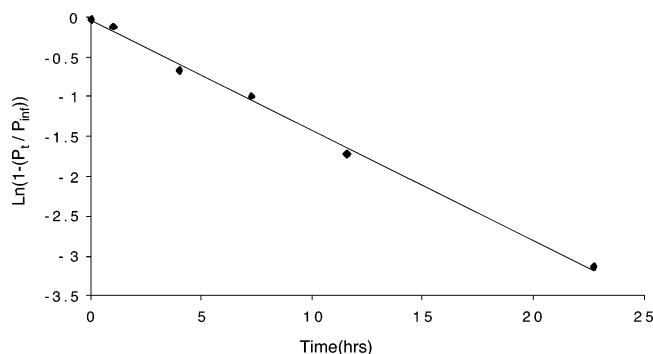


Figure 2. Plot of $\ln[1 - (P_t/P_{inf})]$ vs time, where P_t is the product (**5**, LM-G) released from double-stranded DNA at time t and P_{inf} is the yield of product at “infinite” time, when there is no further increase in product yield. The reactions were conducted in 50 mM sodium phosphate buffer, pH 7, at 37 °C. For clarity, data from a single run are shown. The average slope and standard error for the line obtained from three separate runs was 0.2 ± 0.03 .

corresponds to a half-life of 3.5 ± 0.5 h. An identical value is obtained by plotting $\ln[1 - (P_t/P_{inf})]$ as shown in Figure 2.

Interestingly, we observed the time-dependent release of a second, minor product from leinamycin-modified DNA in this experiment. From its HPLC retention time, we recognized it as the product resulting from the hydrolysis of activated leinamycin (LM-OH, **4**; Scheme 1). The identity of this product was confirmed by comparison to material prepared by authentic synthesis (*4*). Although this compound (**4**) is formed by hydrolysis of activated leinamycin (**2**) in the initial DNA alkylation reaction (Scheme 1), it is clear that the product observed in this experiment is not an impurity carried over from the initial alkylation reaction because it is not present at early times in the analysis and its concentration increases over time.

The release of hydrolyzed leinamycin (**4**) from leinamycin-modified DNA was an unexpected and intriguing finding, because hydrolytic attack on the alkyl substituents of N7-alkylguanine residues is not a common reaction (*6*). In the Discussion, we consider possible chemical mechanisms for the formation of this product. Overall, LM-OH (**4**) and LM-G (**5**) were the only leinamycin-derived products detected in these experiments.

We also developed a UV–vis spectrophotometric assay to monitor loss of the leinamycin–guanine adduct from duplex DNA. This convenient assay capitalizes on the fact that the leinamycin–N7-guanine adduct in duplex DNA displays a characteristic absorbance maximum near 370 nm (*15*), which is replaced by an absorbance at 350 nm for the LM-G free base when the lesion is lost from the duplex due to depurination. In practice, we followed the release of LM-G from leinamycin-modified DNA by monitoring the loss of the adduct’s absorbance maximum at 380 nm (Figure 3). The reaction at 60 °C shows a clean isosbestic point indicating that only two chemical species are present during the reaction. Analysis of the time course for this reaction at 60 °C in 50 mM sodium phosphate at pH 7 reveals a rate constant of 3.0 ± 0.02 h⁻¹ for depurination of the leinamycin–guanine adduct ($t_{1/2} = 0.23 \pm 0.001$ h), in good agreement with the results obtained from HPLC analysis at this temperature (Table 1).

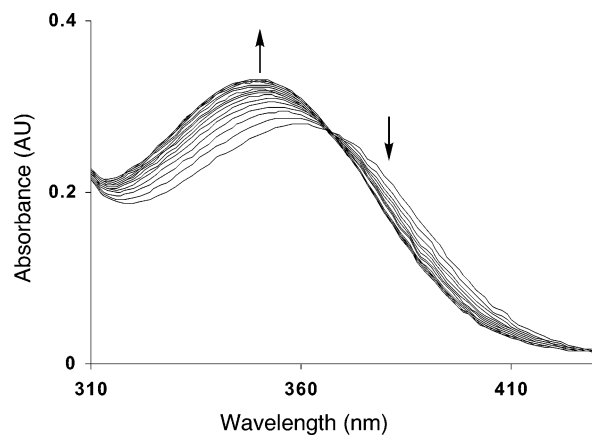


Figure 3. Time-dependent change in the UV–vis spectrum of the leinamycin–guanine residue as it depurinates from double-stranded DNA at 60 °C (50 mM sodium phosphate buffer, pH 7).

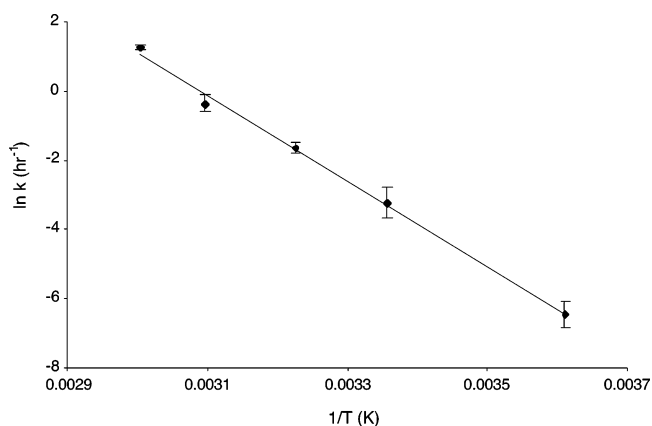


Figure 4. Arrhenius plot for depurination of the leinamycin–guanine residue from double-stranded DNA (50 mM sodium phosphate buffer at pH 7).

Table 1. Half-Lives for Depurination of the Leinamycin–Guanine Residue from Double-Stranded DNA At Different Temperatures and Activation Parameters for the Reaction (50 mM Sodium Phosphate, pH 7)

temp (°C)	half-life (h)
4	440
25	18
37	3.5
50	1.0
65	0.2
Activation Parameters	
E_a	24.6 kcal/mol
A	1.2×10^{13} s ⁻¹
ΔH^\ddagger	24.0 kcal/mol
ΔS^\ddagger	-0.78 eu

Temperature Dependence for Depurination of the Leinamycin–Deoxyguanosine Adduct: Determination of Activation Parameters. Determination of the activation parameters (E_a and the Arrhenius preexponential A value, or ΔH^\ddagger and ΔS^\ddagger) for a given reaction affords the ability to predict the reaction rate at various temperatures and may provide insight regarding reaction mechanisms (*16*). To obtain activation parameters for depurination of the leinamycin–deoxyguanosine adduct, we determined the rate constant of the reaction at five different temperatures using the HPLC assay described above (Table 1). A plot of $\ln k$ vs $1/T$ (Arrhenius plot; Figure 4) provided the activation

energy, $E_a = 24.6$ kcal/mol, and the preexponential factor, $A = 1.2 \times 10^{13} \text{ s}^{-1}$. From the data, it is also possible to calculate the enthalpy of activation, $\Delta H^\ddagger = 24.0$ kcal/mol, and the activation entropy, $\Delta S^\ddagger = -0.78$ eu (16).

For the purposes of comparison, we note that Osbourne and Phillips recently reported an E_a of 24.9 kcal/mol for the depurination of N⁷-methylguanine from double-stranded DNA (17). An Arrhenius A value of $5.32 \times 10^{11} \text{ s}^{-1}$, a ΔH^\ddagger of 25.5 kcal/mol, and a ΔS^\ddagger of -8.9 eu can be calculated from their data. For further points of reference, the activation energy for acid-catalyzed depurination of guanine residues in double-stranded DNA is 31 (18) and 28 kcal/mol in single-stranded oligonucleotides (19). Various groups have reported ΔH^\ddagger values of 22–26 kcal/mol and ΔS^\ddagger values of -11 to $+12$ eu for acid-catalyzed depurination of nucleoside derivatives (8, 20–22).

Effects of Ionic Strength and pH on the Fate of the Leinamycin–Guanine Adduct. Ionic strength and pH can affect depurination rates of N7-alkylguanine residues (6). Therefore, we examined the effects of these variables on the depurination of the leinamycin–deoxyguanosine adduct. We measured rate constants for the depurination reaction in 50 mM sodium phosphate, pH 7, in the presence of 100, 300, and 500 mM NaCl. Our results indicate that changes in ionic strength do not markedly affect the reaction rate. Similarly, the rate of depurination at pH 6 or 8 is not significantly different than that observed at pH 7. In addition, we observe that the final yield of LM-G (5) does not change substantially across the pH range 6–8. Interestingly, we find that the depurination rate of the leinamycin–guanine adduct does not change substantially at pH 3. This result is somewhat surprising because the rate at which N7-methyl-2'-deoxyguanosine undergoes depurination is reported to increase by about a factor of 4 as one moves from pH 7 to pH 3 (see Figure 2 in ref 8).

Does the Base Excision Repair Enzyme 3-Methyladenine DNA Glycosylase (Aag) Catalyze Depurination of the Leinamycin–Guanine Residue in Duplex DNA? The mammalian base excision repair enzyme 3-methyladenine DNA glycosylase (Aag) catalyzes the removal of a variety of N7-alkylguanine lesions from duplex DNA, including 7-methylguanine, 7-hydroxyethylguanine, 7-(2-ethoxyethyl)guanine, and 7-(2-chloroethyl)guanine (23–27). While it is not clear that a large lesion such as **3** can be accommodated by the active site of Aag (28), we nonetheless examined whether this enzyme can catalyze the removal of the leinamycin–guanine adduct from duplex DNA. Using HPLC to monitor the reaction, we find that Aag does not significantly accelerate the loss of LM-G (5) from DNA. Control experiments on methylated plasmid DNA substrates indicated that the Aag used in these experiments was, in fact, active. Thus, our results indicate that the LM-G adduct in duplex DNA is not a substrate for Aag.

Synthesis and Stability of the Leinamycin–dG Nucleoside Adduct. The structural environment of N7-alkylguanine lesions drastically influences the rate at which depurination occurs (6). For a given N7 adduct, the rate of depurination becomes progressively slower as one moves from nucleosides, to single-stranded DNA, to double-stranded DNA (6). To examine the inherent chemical stability of the glycosidic bond that joins the modified base and the sugar residue in the leinamycin-2'-deoxyguanosine lesion, we set out to prepare and characterize the nucleoside adduct of leinamycin (6). It

has previously been reported that activated leinamycin (2) cannot alkylate either single-stranded DNA or the monomeric 2'-deoxyguanosine nucleoside (4, 29). Indeed, we confirm that under conditions typically used for the alkylation of double-stranded DNA (e.g., 100 μM **1**, 1.5 equiv of thiol, 1–5 mM DNA base pairs, and 50 mM sodium phosphate, pH 7.2, 37 °C), leinamycin does not alkylate the monomeric nucleoside, dG (1 mM). Under these conditions, activated leinamycin primarily undergoes hydrolysis to LM-OH (4; Scheme 1). However, we were able to identify a set of "forcing conditions" that allows alkylation of monomeric 2'-deoxyguanosine by activated leinamycin. These conditions employ high concentrations of both the nucleophile (dG) and the electrophile (activated leinamycin, 2) in the nonnucleophilic solvent 2,2,2-trifluoroethanol. This reaction yields LM-dG (6) as the only significant product. The structure of the product was confirmed by ESI(–)-MS and by the subsequent observation that the compound decomposes to yield the characteristic depurination product LM-G (5).

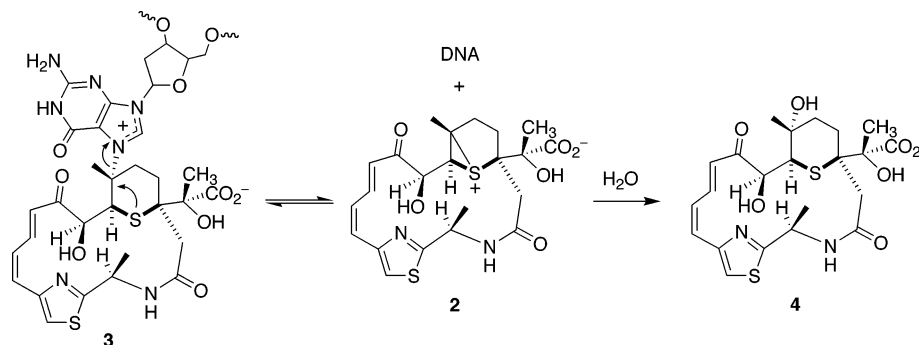
As anticipated, the nucleoside adduct **6** decomposes via depurination in buffered aqueous solution at pH 7 and 37 °C. Also, as expected, the depurination reaction is faster than that seen for the same adduct in double-stranded DNA. Monitoring the reaction by HPLC as described above, we determined the rate constant of this reaction to be $1.4 \pm 0.2 \times 10^{-2} \text{ min}^{-1}$, which corresponds to a half-life of 61 ± 10 min. We measured the rate constant for depurination at several temperatures and calculated the activation parameters for the reaction from these data: $E_a = 24.1$ kcal/mol, A value = $2.3 \times 10^{13} \text{ s}^{-1}$, $\Delta H^\ddagger = 24.0$ kcal/mol, and $\Delta S^\ddagger = 5.8$ eu.

Discussion

Leinamycin forms a structurally unique N7-alkylguanine adduct in double-stranded DNA (4). It is well-known that alkylation of the N7-position of guanine residues markedly accelerates depurination over the background rate seen for the native base (with a rate constant of $3 \times 10^{-11} \text{ s}^{-1}$, the $t_{1/2}$ for spontaneous depurination of purines in duplex DNA is approximately 730 years at pH 7.4 and 37 °C) (6, 18). Accordingly, we find that under physiologically relevant conditions, depurination is the primary decomposition reaction observed for the leinamycin–guanine adduct in double-stranded DNA. The activation parameters determined for this reaction are similar to those reported for other depurination reactions (8, 17–22) and are consistent with the unimolecular mechanism shown in Scheme 2, formally classified as a $D_N + A_N$ reaction (30, 31).

The rate at which N7-alkylguanine residues undergo depurination in double-stranded DNA varies widely, with half-lives ranging from 3 to 150 h under physiological conditions (6). We find that depurination of the leinamycin–guanine adduct from double-stranded DNA occurs with a half-life of 3.5 h at pH 7, 37 °C, making this one of the fastest depurination reactions ever reported. The reason(s) for the exceptionally facile depurination of the leinamycin–guanine adduct is not obvious. In general, the factors that affect the rate at which N7-alkylguanine residues undergo depurination are known; however, it is not possible at this time to completely rationalize the observed depurination rates for various N7-alkylguanine lesions in duplex DNA (6). It seems likely that further studies of the unusual adduct formed

Scheme 3. Reversible DNA Alkylation by Leinamycin



by leinamycin stand to reveal useful insights regarding the factors that determine the rates at which N7-alkylguanine residues undergo depurination in double-stranded DNA.

While the chemical basis for the rapid depurination of the leinamycin–guanine residue remains uncertain, this study nonetheless provides a foundation for considering the origin of leinamycin's potent cytotoxicity. Our results indicate that leinamycin is an efficient reagent for the rapid generation of abasic sites in DNA under physiological conditions. Abasic sites, and the strand breaks that they spontaneously generate (32), are cytotoxic (33–36) and may be of special biological relevance (37) if produced in a rapid burst that overwhelms the capacity of the cellular repair enzyme apurinic endonuclease (APE) to remove this lesion from DNA (34, 35). Furthermore, it is possible that leinamycin's ability to generate oxidative stress (38–42) via release of a persulfide residue (RSS⁻; Scheme 1) may potentiate the toxicity of the abasic sites generated by depurination of the leinamycin–guanine adduct. This suggestion is spurred by recent work indicating that conditions of oxidative stress may inhibit the repair function of APE (43), thus rendering cells hypersensitive to the effects of abasic sites (34, 35).

Despite the strikingly fast rate for depurination of the leinamycin–guanine residue, it is important to realize that with a depurination half-life of 3.5 h, substantial amounts of the adduct remain attached to DNA for more than 10 h (more precisely, 12.5% of the adduct remains bound to the DNA after 10.5 h). Therefore, the parent adduct (3) may have ample opportunity to contribute to the cytotoxicity of leinamycin—especially in rapidly dividing cancer cells whose faulty DNA damage checkpoints allow them to attempt cell division even after exposure to DNA damaging agents (44). It is clear from literature precedents that bulky N7-alkylguanine lesions can have profound biological consequences including genotoxicity and cytotoxicity (37, 45–51). Of course, when considering the possible biological effects of the leinamycin–guanine adduct, it is important to remember that the *in vivo* lifetime of any DNA lesion depends not only on its chemical properties but also on its rate of removal by cellular repair systems such as nucleotide excision repair (52). Finally, we note that some N7-alkylguanine residues can undergo ring opening to yield 5-*N*-alkyl-2,6-diamino-4-hydroxyformamidopyrimidine (alkyl-FAPy-G) lesions that are potentially mutagenic or cytotoxic (6, 46, 53, 54). Further studies are required to determine whether this type of DNA lesion is formed by leinamycin under physiological conditions.

Interestingly, during these studies, we observed the unexpected release of hydrolyzed leinamycin (4) from duplex DNA that bears the leinamycin adduct. In principle, this product could be formed by either direct S_N2 attack of water (or hydroxide) on a leinamycin–DNA adduct or via an S_N1 reaction. It is well-known that S_N2 reactions generally do not occur at tertiary centers (55). Therefore, it is likely that the release of 4 from leinamycin-treated DNA occurs by an S_N1 mechanism involving neighboring group participation of the sulfide moiety to regenerate activated leinamycin (2) as proposed in Scheme 3.

The release of 4 from leinamycin-modified DNA suggests that leinamycin is a reversible DNA alkylating agent. Reversible DNA alkylation has been reported for only a handful of other agents. The spirocyclopropylhexadienone-containing antibiotics duocarmycin and CC-1065 reversibly alkylate the N3-position of adenine residues in duplex DNA (14, 56, 57) and the imine-forming antibiotic ecteinascidin 743 reversibly alkylates the exocyclic N²-nitrogen atom of guanine residues in DNA (58). Rokita and co-workers have characterized reversible alkylation of the N1-position of 2'-deoxyadenosine by a quinone methide (59). In addition, recent evidence indicates that malondialdehyde adducts can be reversibly transferred between different DNA nucleophiles (60, 61). Reversible DNA alkylation allows equilibration (or “shuffling”) of adducts to the most thermodynamically favored locations in DNA (58) and may allow the transfer of adducts from DNA to other biological nucleophiles such as chromatin proteins or DNA binding transcription factors. These aspects of the leinamycin–DNA adduct will be examined in future studies.

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