Quantification of Formaldehyde-Mediated Covalent Adducts of Adriamycin with DNA†

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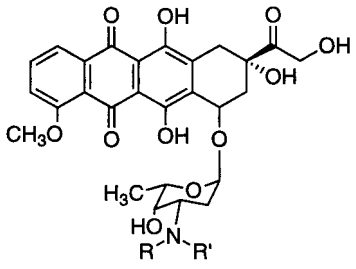
ABSTRACT: Duplex DNA incubated with adriamycin, dithiothreitol (DTT), and Fe^{3+} under aerobic, aqueous conditions yields double-stranded (DS) DNA bands by denaturing polyacrylamide gel electrophoresis (DPAGE) analysis, characteristic of DNAs which are interstrand cross-linked. Another laboratory has provided evidence that formaldehyde produced under these conditions promotes the covalent linkage of adriamycin to one strand of DNA and suggested that this complex results in the anomalous DPAGE behavior. We provide herein strong support for this interpretation. We show: (a) that mixtures of DNA and adriamycin incubated with DTT/Fe^{3+}, H_2O_2, or formaldehyde all show DS DNA bands on DPAGE, (b) that the DS DNA bands and the formaldehyde-mediated lesion (detected by an indirect, GC-MS analysis) form with similar time courses, and in similar amounts, and (c) that the DNA in the DS DNA bands contains approximately one such lesion per DNA, whereas the single-stranded DNA is devoid of it. These results further support the interpretation that adriamycin does not create interstrand cross-links in DNA, and that the DS DNA observed in DPAGE experiments derives from the formaldehyde-mediated monoadduct.

The anthracycline antibiotic adriamycin (**1a**)



1a: R, R' = H (Adriamycin)
1b: R = Me, R' = H
1c: R, R' = Me

has been a widely used anticancer drug for more than 20 years (*1*−*3*). It has been used in the treatment of many cancers, including leukemia, Hodgkin's and non-Hodgkin's lymphomas, and carcinomas of the breast, lung, and ovary (*4*). Despite its prevalence, the mode of action for this drug is not precisely understood (*5*). Numerous studies have indicated that the activity of adriamycin stems from its interactions with DNA (*6*−*10*). Also, adriamycin is known to inhibit topoisomerase II (*11*−*14*). Topoisomerase II and adriamycin form a ternary drug−DNA−enzyme complex which traps DNA strand passage intermediates, leading to single- and double-strand breaks in DNA (*4*). The structure of this ternary complex is unknown. Wang and co-workers, however, have reported the single-crystal X-ray structure of both the noncovalent, intercalation complex of adriamycin with DNA as well as a formaldehyde-mediated covalently linked complex (*15*, *16*).

DNA duplex I: 5' TTG AAG CAA CGA AGT T
3' AAC TTC GTT GCT TCA A

In a seemingly unrelated system, Phillips and co-workers have investigated the reactions of adriamycin with duplex DNA in the presence of reducing agents (*17*, *18*). Initially, transcriptional blockages were observed at guanine residues of 5′-GC sequences in DNA that had previously been incubated with adriamycin, dithiothreitol (DTT),[1] and Fe^{3+} (*19*). In subsequent experiments, they observed that DNA which was treated with reductively activated adriamycin displayed electrophoretic mobility similar to that of double-stranded (DS) DNA when subjected to denaturing polyacrylamide gel electrophoresis (DPAGE) (*17*). Deoxyinosine replacement experiments revealed that N2 of deoxyguanosine played a primary role in the formation of this denaturation-resistant DS DNA (*17*, *18*). For example, an 8-fold reduction in the amount of the DS DNA band was seen when one deoxyguanosine in the [5′-d(GC)]$_2$ duplex was replaced by deoxyinosine (*18*). They concluded that the major component of the DS DNA was the result of an interstrand cross-link between the N2 atoms of the two guanines. Because alkylation of DNA is a characteristic of several antitumor agents and toxins, we attempted to characterize these interstrand cross-links.

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[1] Abbreviations: DTT, dithiothreitol; DS DNA, double-stranded DNA; DPAGE, denaturing polyacrylamide gel electrophoresis; GC-MS, gas chromatography−mass spectrometry; TBE, tris−borate−EDTA.

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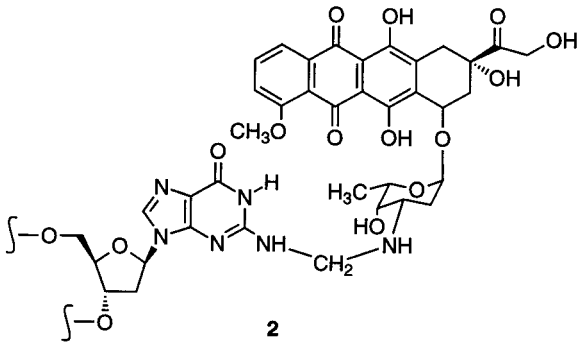
While this research was in progress, Koch and co-workers related in an insightful study (*20*, *21*) the observations of Phillips and co-workers to the formaldehyde-mediated covalent complex of adriamycin and DNA provided by Wang and co-workers (*15*, *16*). Koch's laboratory determined that reactions of DNA duplex [5′-d(GCGCGCGC)]$_2$ with adriamycin, activated by either DTT/Fe^{3+}, H_2O_2, or CH_2O, all gave the same adriamycin−DNA complex. Mass spectrometric analysis revealed the complex to have a molecular mass corresponding to the combined mass of single-stranded DNA, adriamycin, and a methylene unit, minus the mass of two hydrogen atoms. This mass is consistent with the formaldehyde-mediated lesion that Wang and co-workers had studied by X-ray crystallography. Koch and co-workers suggested that the formaldehyde results from H_2O_2-promoted oxidation of Tris buffer or Baeyer−Villiger oxidation of adriamycin itself. The H_2O_2 is generated by reduction of O_2 in the DTT/Fe^{3+} mixture (*20*, *21*). They have also investigated other redox reaction systems and demonstrated that they form the same adriamycin−DNA complex as the previously mentioned activating agents (*22*). The structure that Wang and co-workers determined for the formaldehyde-mediated coupling of adriamycin to DNA has adriamycin linked through its amino group to N2 of a deoxyguanosine residue through a methylene group (**2**)



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(*15*, *16*). The methylene group in this aminal presumably has its origin in formaldehyde. This is consistent with the studies by Chaires and co-workers, who determined that N2 of deoxyguanosine in DNA, the amino group of adriamycin, and formaldehyde are necessary and sufficient for lesion formation (*23*).

Koch and co-workers have proposed that the DS DNA bands reported by Phillips are not due to interstrand cross-links, but rather have their origin in resistance to denaturation caused by **2**. This is consistent with the increased melting point of DNA upon incorporation of **2** compared to adriamycin noncovalently bound to DNA (*23*). Koch and co-workers further suggest that important hydrogen bonding interactions between adriamycin's 9-OH and N2 of dG on the nonalkylated strand create a "virtual cross-link." This explains the 5′-GC sequence selectivity of the DS DNA formation as well as the lability of the lesion observed by Phillips and co-workers.

While this manuscript was in final preparation, Zeman, Phillips, and Crothers published a study that characterized by NMR the lesion resulting from the incubation of DNA with adriamycin activated by either a DTT/Fe^{3+} mixture or formaldehyde (*24*). It was concluded in both cases that the major product was **2**. They also determined that incorporation of lesion **2** greatly decreased the rate of strand exchange for duplex DNA in the presence of excess single-stranded DNA which has a complementary sequence (*24*). There are, however, no experimental data which conclusively show that lesion **2** is responsible for the DS DNA observed by DPAGE.

Despite strong circumstantial evidence supporting the explanation by Koch and co-workers concerning the structural origin of the DS bands, potential contradictions remain unresolved. Chaires demonstrated that the formaldehyde-mediated coupling of daunorubicin to DNA occurs at guanine in *any* sequence. Despite this, Phillips' experiments (which we have confirmed) have shown that only DNA containing the sequence [5′-d(GC)]$_2$ affords the DS bands by DPAGE. Given these circumstances, it seems reasonable to seek further evidence that the formaldehyde-mediated covalent linkage is the origin of the DS DNA bands. We provide this evidence herein. In the course of this study, we have developed a quantitative and sequence-independent assay for lesion **2** using gas chromatography−mass spectrometry (GC-MS). Using this assay, we provide further structural evidence for **2** as the causative lesion of DS bands in DPAGE. We conclusively show that lesion **2** is necessary for the formation of DS DNA seen by DPAGE analysis of DNA which has been incubated with adriamycin, activated by either DTT/Fe^{3+}, H_2O_2, or CH_2O. These results further support the interpretation of Koch et al. that adriamycin does not create interstrand cross-links in DNA, and that the DS DNA observed in DPAGE experiments derives from the covalent monoadduct **2**.

EXPERIMENTAL SECTION

Materials and Methods. Materials and their sources were as follows: DNA synthesis reagents, Applied Biosystems; [γ-^{32}P]ATP, DuPont; adriamycin, Sigma; T4 polynucleotide kinase, Amersham or Boehringer Mannheim; 40% 19:1 acrylamide:bisacrylamide solution, Bio-Rad; alkaline phosphatase (calf intestinal), Boehringer Mannheim; and phosphodiesterase I (*Crotalus adamanteus* venom), Pharmacia. All other reagents were commercial and used as received. Water was purified on a Millipore Milli-Q deionizer. Oligonucleotides were synthesized on an Applied Biosystems Model 392 synthesizer. Samples were concentrated in vacuo with a Savant speed vacuum concentrator. UV spectra were measured on a Hewlett-Packard 8452A or a Perkin-Elmer Lambda 4a spectrophotometer. All analytical gel electrophoresis was conducted on a Hoeffer thermojacketed Poker Face gel stand connected to a Fischer Scientific Model 90 refrigerated circulator. Gel loading solution consisted of 5 M urea and 0.5% xylene cyanol. 1 × TBE buffer used for gel electrophoresis was 90 mM Tris/90 mM boric acid (pH 7.5) and 1.8 mM Na_2EDTA. Gels were dried using a Bio-Rad Model 583 gel dryer onto Whatman 3M paper; autoradiograms were with Fuji RX film. For phosphorimagery, dried gels were scanned by a Molecular Dynamics 400A PhosphorImager, and data analysis was performed using Molecular Dynamics Image Quant software (on an Intel 80486 microprocessor). GC-MS analysis of derivatized samples was performed using a Hewlett-Packard 5890 gas chromatograph and a Micromass Trio2000 quadrupole mass spectrometer. GC-MS was conducted using a DB-5 column,

a source temperature of 200 °C, and an oven temperature gradient as follows: 140 °C for 1 min, increase at 25 °C/min to 215 °C, increase at 6 °C/min to 240 °C, increase at 70 °C/min to 280 °C, hold at 280 °C for 3 min. Autosampling of 2 μL injections was performed using a Hewlett-Packard 7674a autosampler, and data analysis was performed using Windows based Micromass MassLynx software. HPLC was performed on an SSI 200B/220B dual pump system with an SSI controller and a Rainin UV-D II detector (output to a Linear 255/M recorder and an HP 3390A electronic integrator). Solvent gradients were run at 10 mL/min for preparative separations (Rainin, 300 Å, C_{18}, 250 mm × 21.4 mm column) and 1 mL/min for analytical separations (Alltech, 5-μm, C_{18}, 250 mm × 4.6 mm Econosphere column). Gradient A: solvent A, 10 mM triethylammonium acetate (pH 7.0); solvent B, 50% 10 mM triethylammonium acetate and 50% acetonitrile; a 10 min linear gradient from 60% A to 40% A, isocratic 40% A for 15 min, and a 5 min linear gradient to initial conditions. Gradient B: solvent A, 50 mM ammonium acetate (pH 4.0); solvent B, acetonitrile; isocratic 50% A. Electrospray ionization mass spectra (ESMS) was performed using a Kratos Analytical Ltd. Profile Mass Spectrometer and Mach 3 software.

Synthesis and Purification of Oligodeoxynucleotides. Each of the oligodeoxynucleotides used to form DNA duplex I was synthesized using standard phosphoramidite methodology without removing the dimethoxytrityl group on the 5′ end. After 12−14 h of deprotection with concentrated ammonium hydroxide at 55 °C, the sample was concentrated and purified by preparative-scale HPLC using gradient A. After concentration of the purified DNA, the DNA was detritylated by treatment with 10% acetic acid for 1 h. To ethanol precipitate the DNA, 3 M sodium acetate (pH 5.2) was added to bring the aqueous solution to a concentration of 0.3 M, and cold ethanol was added (−20 °C) such that the final volumetric ratio of EtOH to H_2O was 85:15. This was kept at −20 °C for 1 h and centrifuged for 15 min at 4 °C. The supernatant was removed, and the resulting pellet was dried using a speed-vac.

Preparation of Radiolabeled DNA. 5′-d(TTG AAG CAA CGA AGT T) was 5′-radiolabeled using the following procedure: 0.1 ODU of DNA was placed in a microfuge tube and diluted to 14 μL with water. Added to this was 2 μL of 10× kinase buffer [0.5 M Tris (pH 7.5), 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA], 1 μL (10 μCi) of [γ-^{32}P] ATP, and 1 μL (3 units) of T4 polynucleotide kinase. This was incubated at 37 °C for 30 min. Radiolabeling was stopped by heating the reactions at 90 °C for 5 min followed by ethanol precipitation.

Reactions of DNA with Adriamycin in the Presence of either CH_2O, DTT/Fe^{3+}, or H_2O_2. 5′-Radiolabeled 5′-d(TTG AAG CAA CGA AGT T) was annealed to 5′-d(AAC TTC GTT GCT TCA A) by heating to 90 °C for 5 min and cooling to room temperature over approximately 1 h. Reaction mixtures were comprised of duplex DNA (100 μM) and adriamycin [200 μM, $\epsilon_{(480)} = 11\,500$] in buffered solution (40 mM Tris base, pH 8.0, 50 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA) and were incubated in the dark at 37 °C. In order that both DPAGE and GC-MS analysis could be performed, 2−20 μL aliquots were taken in each case and quenched by ethanol precipitation. CH_2O: reaction mixtures (40 μL volume, giving 2−20 μL aliquots) were incubated with varying concentrations of CH_2O and allowed to progress for 24 h. DTT/Fe^{3+}: reactions were identical except for the absence of CH_2O and the presence of 7 mM dithiothreitol and 40 μM $FeCl_3$. H_2O_2: reaction mixtures were identical except for the absence of CH_2O and the presence of 5 mM H_2O_2. Reactions with either DTT/Fe^{3+} or H_2O_2 were allowed to proceed for 96 h and were done in triplicate [200 μL initial volume, giving 2−20 μL aliquots at each of 5 time points (0, 24, 48, 72, and 96 h)].

Aliquots of 20 μL from reactions which had been ethanol precipitated were dissolved in gel loading buffer (5 M urea, 0.5% xylene cyanol) and analyzed by DPAGE. DPAGE was conducted on a 20% gel (19:1 acrylamide:bisacrylamide) with 8 M urea in 1 × TBE buffer (0.35 mm thick, 33 cm × 41 cm, using either 20- or 40-toothed comb). Gels were run at 2000 V with the refrigerated circulator kept at 15 °C. They were run until the xylene cyanol dye had run 10 cm (approximately 3 h). The gel was transferred to Saran Wrap and filter paper, dried for 40 min at 80 °C, and visualized by either autoradiography or phosphorimagery.

*Synthesis of N-Methyladriamycin (**1b**).* The reaction mixture was assembled in no particular order and was comprised of adriamycin (100 μM), $NaCNBH_3$ (67 μM), and CH_2O (2.5 mM) in 2:1 CH_3CN/H_2O. After 1.5 h at room temperature, the reaction mixture was analyzed by analytical HPLC using gradient B. This gave three broad peaks, the first of which was determined by comparison of retention times and co-injection to be adriamycin. The second and third peaks were determined by ESMS to be *N*-methyladriamycin (**1b**) ($MH^+ = 558.5$) and *N*,*N*-dimethyladriamycin (**1c**) ($MH^+ = 572.5$), respectively. Subsequent GC-MS analysis of adriamycin and **1b** after borohydride reduction and acetylation showed three major ions for each (**5a**: *m*/*e* 56, 98, 158; **5b**: *m*/*e* 70, 112, 172). This is consistent with methylation having occurred at the amino group.

GC-MS Analyses. Ten microliter solutions of either adriamycin, *N*-methyladriamycin (**1b**), or a 1:1 mixture of both were subjected to GC-MS analysis. The procedure that follows is similar to methodology by Blakeney et al. (*25*). First, 5 μL of 10 mM glucosamine was added as an internal standard. Then 50 μL of aqueous 5 M $NaBH_4$ was added, the microfuge used as the reaction vessel was pierced with a needle in order to prevent pressure from building up, and the reduction was allowed to progress at 65 °C for 1.5 h. Fifty microliters of glacial acetic acid was added to consume the excess $NaBH_4$; 100 μL of *N*-methylimidazole and 1 mL of acetic anhydride were added to acetylate the amino sugars. After 30 min at room temperature, the acetic anhydride was quenched by the addition of 2 mL of H_2O, and the acetylated amino sugars were extracted with 1 mL of CH_2Cl_2. The extracted CH_2Cl_2 was removed by speed-vac and the sample dissolved in 100 μL of CH_3CN and subjected to GC-MS analysis. After monitoring the total ion current (TIC) of the GC-MS, the retention times of **5a**, **5b**, and the internal standard were determined. After this, single-ion monitoring (SIM) was used to more precisely measure the most intense signals for **5a** (98.1), **5b** (112.1), and the internal glucosamine standard (84.1).

Known concentrations of **1b** (determined by UV/Vis assuming that **1b** and adriamycin have the same extinction coefficient) were mixed with the internal standard glucosamine and subjected to borohydride reduction, acetylation,

and GC-MS analysis. The ratios of the size of these peaks were then used to determine the concentration of **5b** detected in the subsequent experiments. Treatment of samples from the DTT/Fe^{3+}, H_2O_2, and CH_2O activated reactions was the same as above, except that an ethanol-precipitated DNA pellet was the original sample.

Preparative Scale Reactions of DNA with Adriamycin and either DTT/Fe^{3+} or H_2O_2. Nonradiolabeled DNA duplex I was reacted with either DTT/Fe^{3+} or H_2O_2 at the same concentrations as the analytical reactions (1 mL volume). After ethanol precipitation, the DNA was dissolved in gel loading buffer and purified on 20% DPAGE (1.5 mm thick, 14 × 16 cm, using a 5-toothed comb). The gel was run at 350 V at room temperature until the xylene cyanol dye had run 4 cm (approximately 2 h). The single-stranded (SS) and double-stranded (DS) DNA bands for each activating agent were located by UV shadowing and isolated from the gel by a crush and soak procedure: gel slices were crushed with a glass rod into fine particles and incubated at 4 °C for 30 min with elution buffer [500 mM ammonium acetate (pH 7.0), 10 mM $MgCl_2$, and 10 mM Na_2EDTA]. The cold temperature and short duration of the elution procedure were required to minimize decomposition of lesion **2**. The solution containing DNA was separated from gel particles by centrifugation, and the DNA was desalted using a Waters Sep-Pak C_{18} cartridge: a Sep-Pak was attached to a plastic syringe (10 mL size) and washed with 10 mL of CH_3CN followed by 10 mL of H_2O. The aqueous solution containing DNA sample was then loaded to the cartridge through the syringe. The Sep-Pak was sequentially washed with 10 mL of 10 mM ammonium acetate and 10 mL of H_2O; and the DNA eluted with 4 mL of 25% aqueous CH_3CN which was subsequently frozen and concentrated to dryness.

Pelleted DNAs isolated from SS and DS DNA were each brought up in 40 μL of H_2O and split into 2−20 μL aliquots. For each sample, the internal standards glucosamine and $NaBH_4$ were added to one aliquot, with subsequent derivatization and GC-MS analysis. The other 20 μL aliquot had 4 μL of alkaline phosphatase (4 units) and 1 μL of phosphodiesterase I (1 unit) added and incubated at 37 °C in the dark overnight in order to enzymatically digest the DNA. The concentrations of digested DNA duplex I [$\epsilon_{(260)} = 360\,000$] and adriamycin [$\epsilon_{(480)} = 11\,500$] in the original 20 μL aliquots were then determined by bringing the volume of the solutions to 1 mL and measuring the UV/Vis spectrum (Table 1).

RESULTS

DNA Admixed with Adriamycin and DTT/Fe^{3+}, H_2O_2, or CH_2O Yields DS DNA by DPAGE Analysis. If lesion **2** is the source of the DS DNA observed by Phillips and co-workers (*17*, *18*), and if activation by either DTT/Fe^{3+}, H_2O_2, or CH_2O leads to the production of lesion **2** as reported by Koch and co-workers (*20*, *21*), then activation by any one of the above three agents should result in this DS DNA. Similar to those experiments, 5′-radiolabeled DNA duplex I (100 μM) was incubated (40 mM Tris base, pH 8.0, 50 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA) in the dark at 37 °C with or without adriamycin (200 μM) and various other reagents (Figure 1). After ethanol precipitation and drying, samples were dissolved in 5 M aqueous urea loading buffer and analyzed by 20% DPAGE. The DS DNA (approximately half the mobility of single strands, Figure 1) was quantified by phosphorimagery. DS DNA was seen for each of the three activating agents in the indicated yield: DTT/Fe^{3+}, 19%; H_2O_2, 6%; CH_2O, 73%, but only when adriamycin was present. Incubation of the DNA with adriamycin alone yielded only single-stranded bands. This demonstrated that activation of adriamycin by either H_2O_2 or CH_2O afforded the DS DNA bands, similar to the observations of Phillips and co-workers with DTT/Fe^{3+} activation (*17*, *18*). This is consistent with the suggestion that lesion **2** is responsible for the DS DNA.

Methylated Adriamycin Derivatives Can Be Detected and Quantified by GC-MS Analysis. Attempts to isolate the conjugate of adriamycin with deoxyguanosine implicit in **2** following enzymatic digestion of the DNA phosphodiester backbone returned only unmodified nucleosides and adriamycin. We presume that free of duplex DNA, the aminal in **2** is quite vulnerable to aqueous hydrolysis. We elected, therefore, to detect **2** indirectly, by trapping the iminium ion intermediate **3** in this hydrolysis (Scheme 1). An indirect GC-MS assay was developed as follows. Adriamycin and *N*-methyladriamycin (**1b**, prepared by reductive methylation of adriamycin) were independently and in admixture treated with $NaBH_4$ (5 M, 60 °C, 1.5 h). Excess $NaBH_4$ was quenched by acetic acid, and the resulting mixture, presumed to contain the reduced sugars (**4a** and **4b**), was acetylated by a combination of acetic anhydride and 1-methylimidazole in preparation for GC-MS analysis (*26*). After quenching with water and extraction with dichloromethane, the acetylated compounds (**5a** and **5b**) were subjected to GC-MS analysis. The compounds were easily separated, and the mass spectra showed three major ions (**5a**: *m*/*e* 56, 98, 158; **5b**: *m*/*e* 70, 112, 172). The increase of 14 mass units for each of the peaks in **5b** versus **5a** is the result of the additional methyl group. Using **1b** and an internal standard of glucosamine, GC-MS analysis in the single-ion monitoring (SIM) mode showed a linear relationship between the amount of **5b** present and the ion current.[2]

*Gel and GC-MS Analysis of DNA and Adriamycin Activated by CH_2O, DTT/Fe(III), or H_2O_2 Shows a Correlation between the Amounts of DS DNA in DPAGE and **5b**.* We assayed mixtures of DNA duplex I, adriamycin, and varying concentrations of formaldehyde using both the DPAGE (Figure 2A) and GC-MS (Figure 2B) assays. The fraction of DNA which was shifted to the DS DNA band in DPAGE increased with increasing formaldehyde concentrations, consistent with the idea that formaldehyde is required of DS DNA bands. That the yield of this reaction saturated at less than 100% DS DNA could reflect dissociation during DPAGE of DNA containing adriamycin covalent adducts or a lower stability toward dissociation of DNA containing certain adriamycin adducts in a structurally heterogeneous mixture. The concentration of **5b** in these same reaction mixtures, determined by GC-MS quantitation, also rose with increasing formaldehyde concentration, saturating at roughly 1.2 adducts per DNA duplex. Control experiments did not

[2] Quantification of **5b** was determined in all GC-MS experiments by using the least-squares regression line for the *N*-methyladriamycin standards versus its GC-MS response. The error bars in GC-MS experiments represent the 95% confidence limits in the value of the slope of that line.

detect any **5b** when DNA was removed from the reaction mixture (data not shown). Given that the reaction mixtures contained no more than 2.0 adriamycin molecules per duplex DNA, the lower limit for the efficiency of this indirect detection method is thus 60%.

If every covalent linkage of adriamycin to DNA resulted in production of DS DNA in the DPAGE assay, one would predict that under single-hit conditions the concentrations of DS DNA and covalent lesions would be identical at each formaldehyde concentration. In other words, a plot of [**5b**] vs [DS DNA] would be linear with slope 1.0. In fact, [**5b**] is roughly twice the concentration of DS DNA at low concentrations of CH_2O (Figure 2C). One interpretation is that lesion **2** is formed at guanines at other sequences than 5′-GC, resulting in relatively lower stabilization of the duplex, without increasing the magnitude of the DS DNA band. Lesion **2** is thus necessary, but not sufficient, for the observation of DS DNA when analyzed by DPAGE.

A similar series of experiments was conducted using DTT/Fe^{3+} or H_2O_2 as the activating agents. DNA duplex I (100 μM) was incubated (same buffer conditions) in the dark at 37 °C with adriamycin (200 μM) activated by either DTT/Fe^{3+} or H_2O_2. The progress of each reaction was analyzed by both DPAGE and GC-MS. DPAGE showed a gradual increase with time in the amount of DS DNA for either DTT/Fe^{3+} or H_2O_2 (Figure 3A). Consistent with DS DNA detected by DPAGE having its origin in **2**, **5b** could be detected and quantified by GC-MS for each of the time points (Figure 3B,C). Formation of **5b** required both the activating agent and adriamycin (data not shown).

The correlation of the amounts of **5b** with DS DNA for both DTT/Fe^{3+} and H_2O_2 at various time points is shown in Figure 4A,B, respectively, overlaid with the data for formaldehyde at various concentrations. For the DTT/Fe^{3+} activation, there is a significantly larger amount of **5b** found per DS DNA than was the case for formaldehyde. It is important, however, that there is at least as much **5b** detected in each case to cause the amount of DS DNA seen. In other words, some lesions that yield **5b** in the GC-MS analysis in the DTT/Fe^{3+} activation appear not to stabilize DS DNA against denaturation in DPAGE. It may be that reductive methylation of adriamycin that does not involve intermediate **2** is enhanced under these reaction conditions. **5b** is formed in mixtures containing $NaBH_4$, DTT, adriamycin, and CH_2O (data not shown). When DTT was omitted, the level of **5b** fell below the detection limit of the assay. In these control experiments, approximately one-fourth of the quantity of **5b** needed to account for the deviation from the CH_2O data was seen. This control is quite imperfect, though, because DNA is known to bind adriamycin very tightly, potentially altering its ability to interact with DTT. There are other potential reasons for the detection of elevated **5b** levels in the DTT/Fe^{3+} reactions.[3] In contrast, the amount of **5b** detected per DS DNA at each time point was for H_2O_2 activation essentially identical to that seen with formaldehyde. For both DTT/Fe^{3+} and H_2O_2 activation, the correlation between the amounts of **5b** with DS DNA is consistent with **2** being the cause of the appearance of DS DNA bands.

*DS DNA Bands from DPAGE Possess the Adriamycin Chromophore and Yield **5b** on Reduction.* To provide direct evidence for the presence of lesion **2** in the DS DNA from DPAGE, both single-stranded (SS) and DS DNA were isolated and analyzed. DNA duplex I (100 μM) was incubated (40 mM Tris base, pH 8.0, 50 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA) in the dark at 37 °C for 96 h with adriamycin (200 μM) activated by either DTT/Fe^{3+} or H_2O_2. After ethanol precipitation, the samples were purified by DPAGE. The material eluted from the SS and DS bands was analyzed by UV/Vis spectroscopy (Table 1). DNA was enzymatically digested prior to this analysis in order to avoid the hypochromic effect associated with intercalation and allow accurate determination of the concentration of adriamycin (*27*). For the DS DNA from either DTT/Fe^{3+} or H_2O_2 activation, 1.4 and 1.1 mol equiv of adriamycin was found per duplex DNA, respectively. For the SS bands, the amount of adriamycin was below the level of detection.

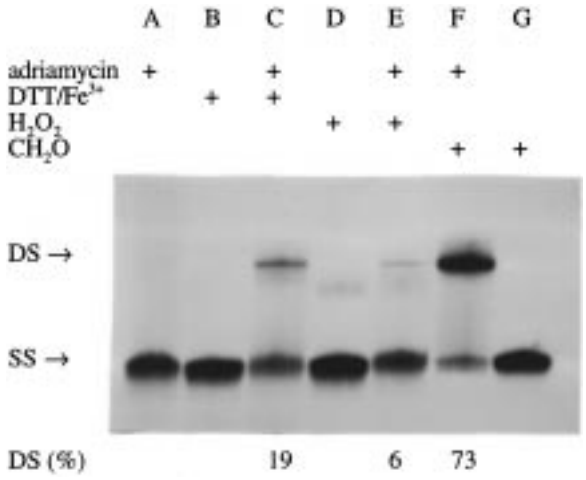


FIGURE 1: DPAGE analysis of DNA duplex I and adriamycin. All reactions were performed in the dark at 37 °C using 100 μM DNA duplex I in Tris buffer. (A) 200 μM adriamycin; (B) 7 mM DTT, 40 μM $FeCl_3$; (C) 7 mM DTT, 40 μM $FeCl_3$, 200 μM adriamycin; (D) 5 mM H_2O_2, 200 μM adriamycin; (F) 1 mM CH_2O, 200 μM adriamycin; (G) 1 mM CH_2O. All reactions were allowed to progress 96 h except lanes F and G, which were stopped after 24 h.

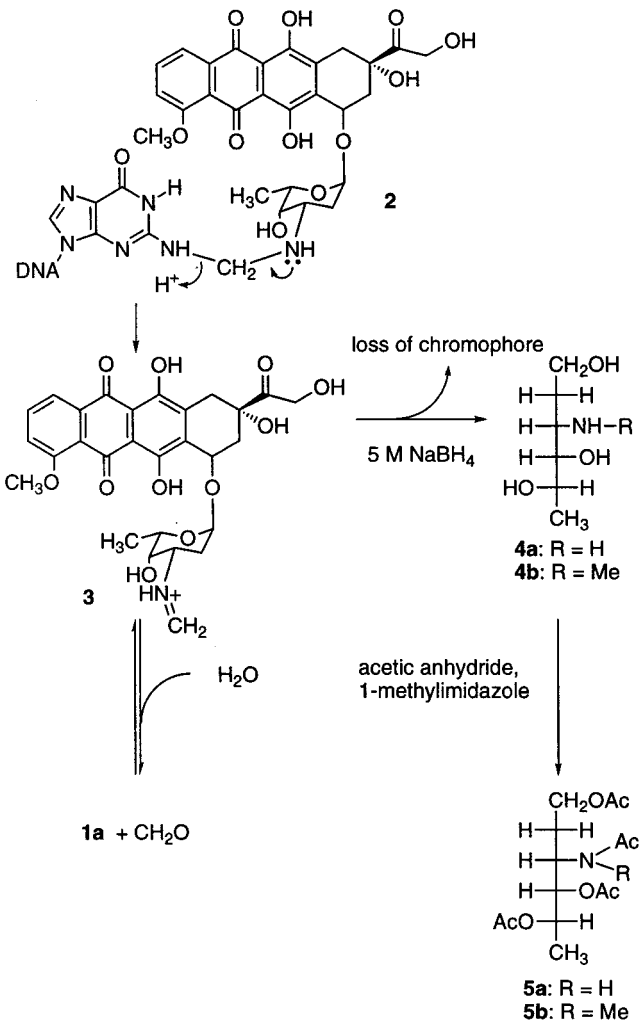
On reduction, DNA from the DS band (but not the SS band) afforded roughly the expected yield of **5b** determined by GC-MS (Figure 5 and Table 1). **5a** detected by GC-MS for the DS DNA presumably originates by a combination of **2** that has degraded during the elution from gel slices and adriamycin that remained noncovalently intercalated in the DNA duplex on the gel. Extremely low levels of **5a** and **5b** are detected in the SS band region. That virtually all of lesion **2** is in the DS DNA region and in levels which are nearly commensurate with the amount of DNA duplex I provides further evidence for this lesion being the cause of the DS DNA observed by DPAGE.

DISCUSSION

These results provide further strong support for the conclusion of Koch and co-workers (*20, 21*) that the DS DNA observed by Phillips and co-workers (*17*) is caused

[3] Other potential reasons for this phenomenon include: (i) the presence of Fe^{3+} affects the sequence specificity of the formation of lesion **2** or decreases the duplex DNA's ability to resist denaturation; (ii) adriamycin which has been oxidized in a Baeyer−Villiger fashion forms a lesion similar to **2**, but which is not as resistant as **2** to denaturation.

Scheme 1: Strategy for Capturing Iminium Decomposition Intermediate by $NaBH_4$



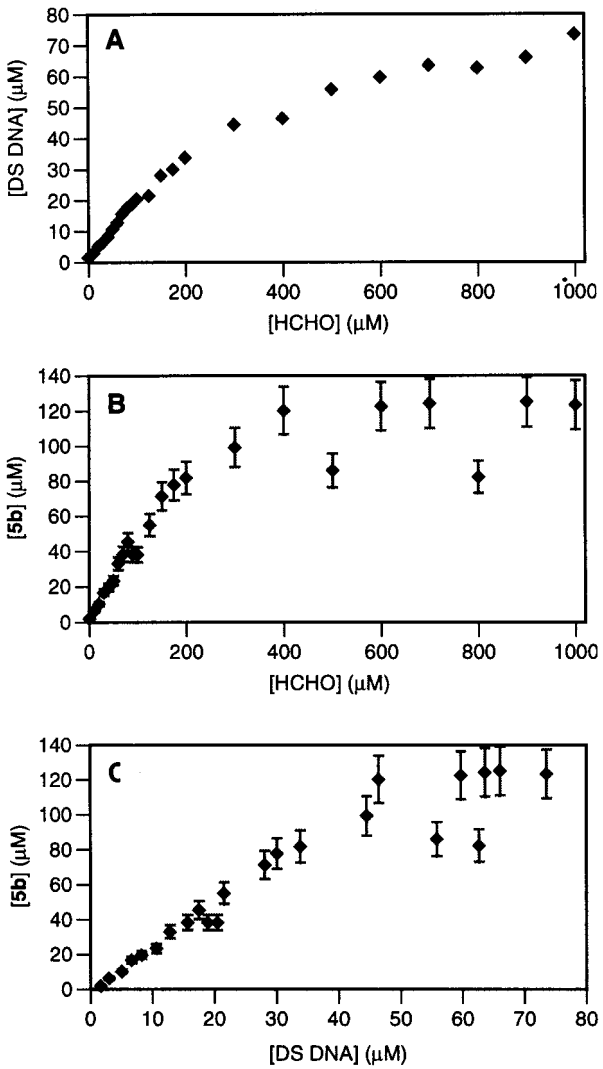


FIGURE 2: GC-MS and DPAGE analysis of CH_2O-activated lesion formation. Concentrations of DNA duplex I and adriamycin were 100 μM and 200 μM, respectively. (A) [DS DNA] determined by phosphorimaging vs [CH_2O] (μM). (B) Concentration of **5b** determined by GC-MS vs [CH_2O] (μM). (C) [**5b**] vs [DS DNA] for each sample.

by lesion **2**, and not a true covalent interstrand cross-link. First, it has been shown by DPAGE that activation of adriamycin by DTT/Fe^{3+}, H_2O_2, or CH_2O leads to DS DNA bands by DPAGE. Second, we provide further structural evidence for the presence of lesion **2** in these DNAs by detection of methylated adriamycin derivatives resulting from reductive trapping of an intermediate in its decomposition pathway. Third, we show linear correlations between the amounts of lesion **2** and DS DNA observed by DPAGE. Finally, we have indirectly demonstrated that the DS DNA band (but not the SS band) contains lesion **2** when isolated from the gel.

It seems reasonable to assume that formation of lesion **2** proceeds through a noncovalent complex of adriamycin and DNA. It has been shown that the preferred DNA sequence at which adriamycin binds noncovalently is either 5′-(A/T)-CG or 5′-(A/T)GC (*28*). The chromophore is intercalated between either the CpG or the GpC steps, and the daunosamine sugar lies in the minor groove next to the AT base pair. Neither of these two complexes, however, is competent to progress to lesion **2**, because the formaldehyde-mediated coupling can occur only when the daunosamine moiety is in close proximity to a dG residue. As such, we presume that these DNA sequences which exhibit higher affinity for noncovalent binding of adriamycin compete with the lower affinity sequences at which covalent binding is ultimately observed. The latter point has not been experimentally investigated.

We return now to the apparent contradiction described in the introduction of this paper, the strong preference for DNAs containing [5′-d(GC)]$_2$ to yield DS DNA bands on DPAGE, despite the observation that formaldehyde-mediated coupling of daunorubicin to guanine can occur in any sequence. Either one or both of two explanations could account for the requirement of [5′-d(GC)]$_2$ for the generation of DS bands on DPAGE, either (a) lesion **2** forms preferentially at the sequence [5′-d(GC)]$_2$ or (b) DNAs containing lesion **2** and the sequence [5′-d(GC)]$_2$ are more resistant to denaturation. Our data support the first explanation but provide no information on the second.

Although Chaires observed that the formaldehyde-mediated coupling of daunorubicin to guanine occurred in any sequence, the conditions for this coupling involved much higher concentrations of CH_2O (at least 500-fold) than were used in the experiments reported here. It seems possible that at such high concentrations the differences in reactivity of guanines in different sequence contexts would be masked.

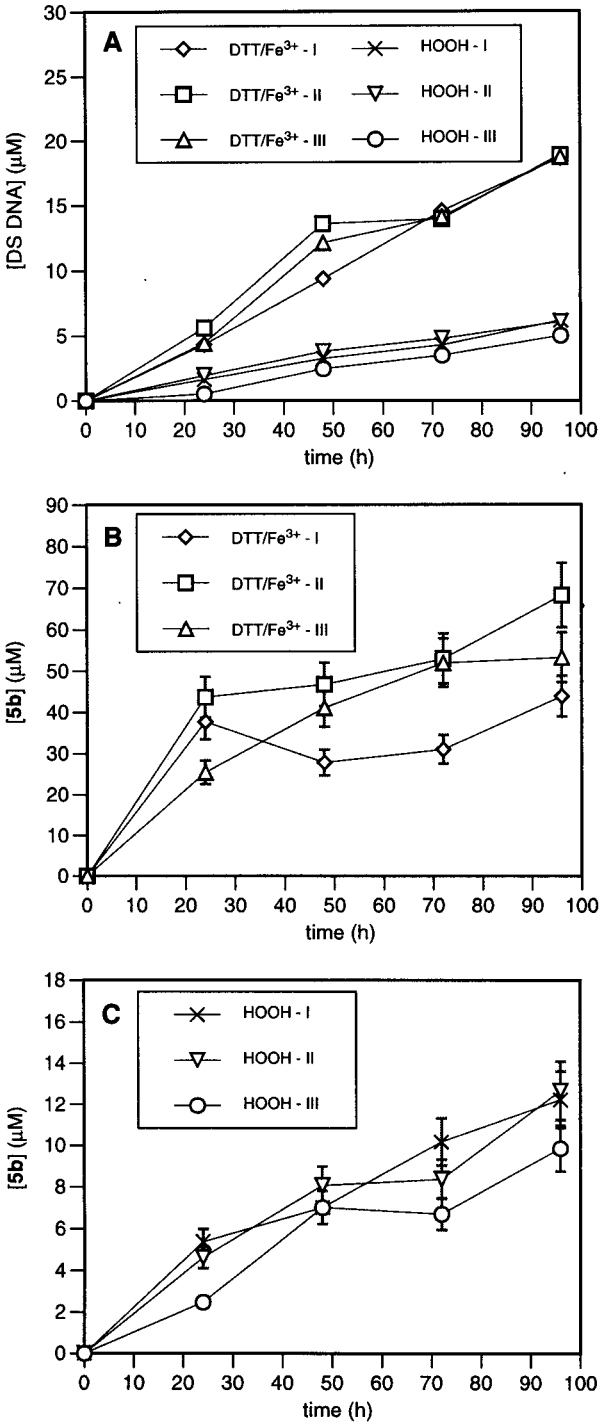


FIGURE 3: DPAGE and GC-MS analysis of DTT/Fe^{3+}- and H_2O_2-activated lesion formation. Concentrations of DTT and Fe^{3+} were 7 mM and 40 μM, respectively. The concentration of H_2O_2 was 5 mM. Each reaction was done in triplicate (I, II, and III), and aliquots were taken every 24 h. (A) [DS DNA] determined by phosphorimaging for both DTT/Fe^{3+} and H_2O_2 activation. (B) [**5b**] determined by GC-MS for DTT/Fe^{3+} activation. (C) [**5b**] determined by GC-MS for H_2O_2 activation.

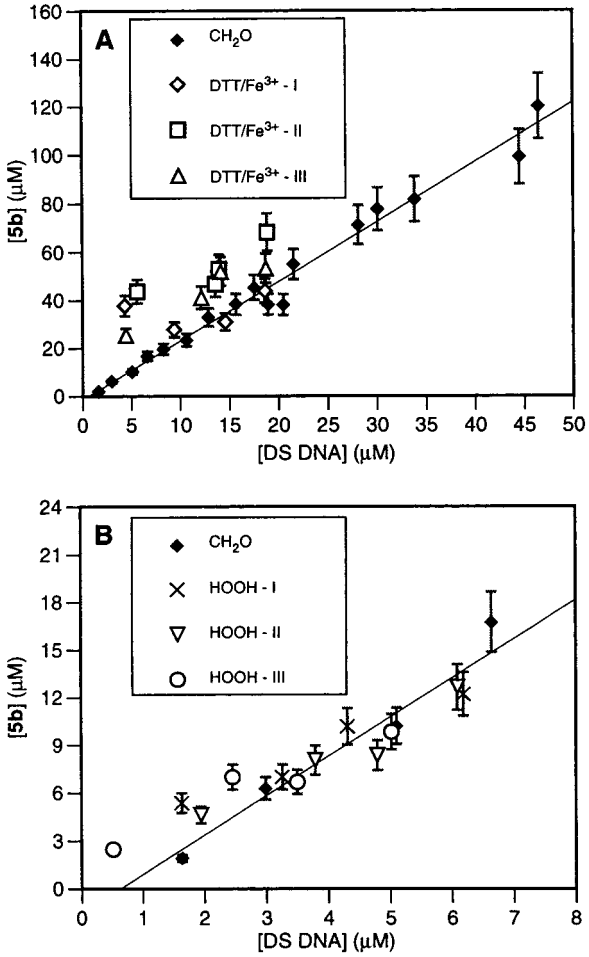


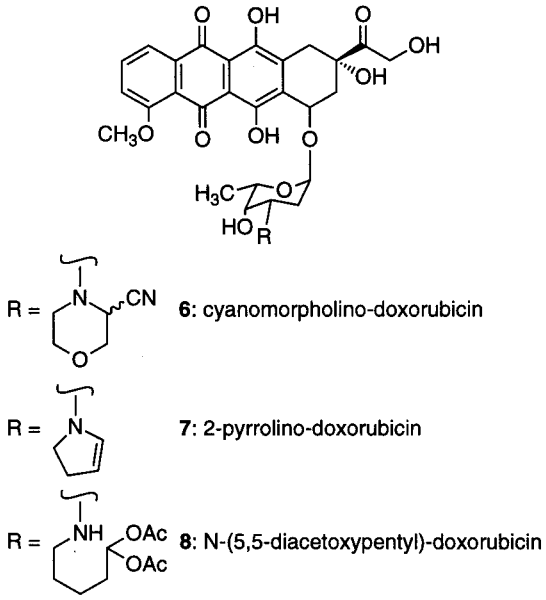
FIGURE 4: Correlation between DPAGE and GC-MS analyses. [**5b**] vs [DS DNA] for each time point was plotted for either DTT/Fe^{3+} or H_2O_2 activation and overlaid with the data from the different CH_2O concentrations. (A) DTT/Fe^{3+} activation. (B) H_2O_2 activation.

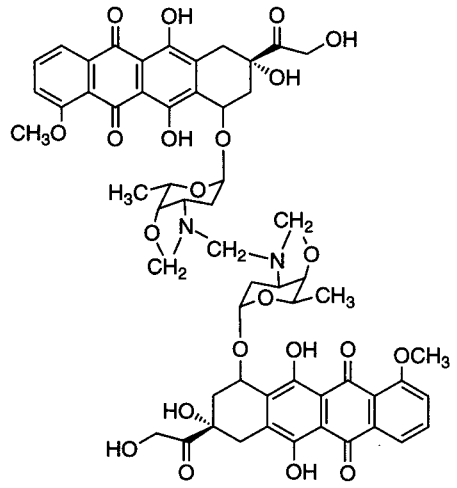
We have observed that small changes in DNA structure can affect the rate of formation of lesion **2**. GC-MS experiments reveal that lesion **2** (as detected through **5b**) is formed at least 50-fold more efficiently at the sequence [5′-d(GC)]$_2$ relative to either 5′-d(GC)·5′-d(IC) or [5′-d(CG)]$_2$ (data not shown). Although the possibility that borohydride trapping is less efficient in these other sequence contexts cannot be excluded, it seems likely that the rate of formation of lesion **2** is greatest at the dinucleotide sequence [5′-d(GC)]$_2$.

Koch has suggested that a hydrogen bond between the 9-OH of adriamycin and N2 of dG on the nonalkylated strand is required for the observation of the DS DNA bands, acting as a "virtual cross-link". Given the greatly diminished amounts of **5b** detected in the duplex 5′-d(GC)·5′-d(IC) compared to [5′-d(GC)]$_2$, it seems reasonable that this hydrogen bond is important for formation of lesion **2**. Whether or not this hydrogen bond is important for the resulting resistance of the duplex to denaturation is unclear. It has been determined, however, that 9-OH of doxorubicin contributes approximately 1 kcal/mol to the noncovalent binding of DNA, presumably due to hydrogen bonding (*29*).

This study demonstrates a serious and potentially general failing of DPAGE analysis as an indicator of covalent DNA interstrand cross-linking, particularly in cases where presumed thermal lability of the cross-link precludes vigorous denaturation of DNA samples. In this system, noncovalent interactions are evidently strong enough to leave the DNA duplex intact under the conditions of DPAGE.

Questions remain as to the biological relevance of the formaldehyde-mediated alkylation of DNA by adriamycin. Several adriamycin derivatives which are known to alkylate DNA have each been shown to be more cytotoxic than the





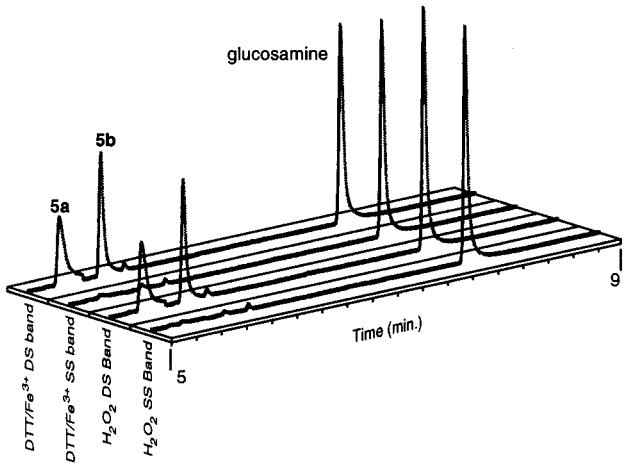


FIGURE 5: GC-MS chromatagrams of excised SS and DS bands for both DTT/Fe^{3+}- and H_2O_2 activated reactions between adriamycin and DNA. GC-MS analyses were done using single-ion monitoring (SIM), monitoring **5a**, **5b**, and glucosamine at 98.1, 112.1, and 84.1, respectively. Chromatagrams were normalized using the integrated area of the internal standard glucosamine.

Table 1: UV/Vis and GC-MS Analysis of Excised SS and DS Bands

sample	[adriamycin][a] (μM)	[DNA][a] (μM)	[**5b**][b] (μM)
H_2O_2 − DS band	244	220	176
H_2O_2 − SS band	<10	283	3.8
DTT/Fe^{3+} − DS band	272	198	185
DTT/Fe^{3+} − SS band	<10	237	5.3

[a] Determined by UV/Vis. [b] Determined by GC-MS.

parent adriamycin. These include cyanomorpholinodoxorubicin (**6**) (*30*), 2-pyrrolinodoxorubicin (**7**) (*31*), *N*-(5,5-diacetoxypentyl)doxorubicin (**8**) (*32*), and doxoform (**9**) (*33*). Each of these compounds has a masked aldehyde functionality through which it in principle could alkylate DNA, bypassing the need for formaldehyde. The greater potency of these derivatives relative to adriamycin suggests that formaldehyde-mediated alkylation or related reactions may be important. The GC-MS assay described herein opens avenues to investigate the sequence specificity with which **2** forms in DNA, as well as quantification of **2** in biological systems.

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