Reassignment of the Guanine-Binding Mode of Reduced Mitomycin C†

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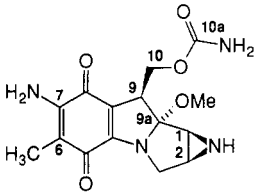
ABSTRACT: Mitomycin C (**1**) is a clinically used antitumor antibiotic that binds covalently to deoxyribonucleic acid under reductive or acidic catalysis. We have determined the structures of the adducts resulting from attack of reductively activated **1** on the dinucleoside phosphate d(GpC) to be N^2-(2″β,7″-diaminomitosen-1″α-yl)-2′-deoxyguanosine (**2**) and its 1″β-isomer (**3**). This represents a revision of the previously reported structures for these adducts in that the mitomycin residue is linked to the N^2- rather than O^6-position of 2′-deoxyguanosine. This revision is the result of applying to the mitomycin case a newly developed general method that leads to unambiguous assignment of the linkage position in complex alkylated guanosines. The method as described here takes advantage of the resolution enhancement gained by calculation of the second derivatives of absorbance Fourier transform infrared spectra. In addition, we present ^{1}H NMR data that corroborate the assigned structures of **2** and **3** and that should serve as a useful reference for future investigations into the binding of mitomycin C to DNA. The convenient synthesis of adducts **2** and **3** from deoxyguanosine and mitomycin C reported here should facilitate such investigations as well. Furthermore, we demonstrate a useful acetylation procedure for adducts and metabolites of mitomycin C that furnishes spectroscopically superior chemical derivatives (e.g., triacetates **4** and **5**, derived from acetylation of adducts **2** and **3**).

A large number of chemical agents originating from both man-made and biotic sources bind covalently to deoxyribonucleic acid (DNA). Most of these electrophiles attack preferentially the heterocyclic bases of DNA to form covalent alkylator–base binary complexes (Singer & Grunberger, 1983). While the structurally simple alkylating agents such as dimethyl sulfate and N-mustards show considerable nonselectivity with regard to the target base, more functionalized organic electrophiles often show overwhelming base specificity for the nucleophilic heteroatoms of guanine. Thus, diol epoxide metabolites of polyaromatic hydrocarbons, *N*-acetoxy-*N*-acetyl-2-aminofluorene, aflatoxin B_1 oxide (Singer & Grunberger, 1983), and mitomycin C (MC,[1] **1**) (vide infra) attack

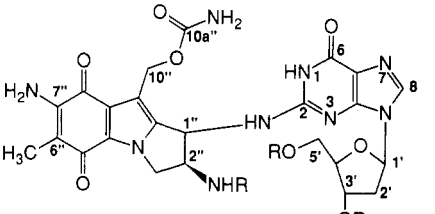


MC, 1

guanine residues in DNA with high preference.

Considerable interest exists in developing sensitive and reliable micromethods for use in determination of the site of attachment of functionalized alkylators to guanine residues in DNA. Previous studies (Kasai et al., 1978; Tomasz et al., 1983) have taken advantage of the characteristic Fourier transform infrared (FTIR) frequencies of differentially substituted guanosines (Miles, 1971) in order to assign the alkylation position. While this method as described (Kasai et al., 1978; Tomasz et al., 1983) has the distinct advantages of superb sensitivity and ease of measurement, it suffers from a critical drawback in that ambiguous results can be obtained if extreme care is not taken to minimize crystalline orientational effects in the solid sample prior to measurement.

We report here that FTIR spectra of highly polar DNA adducts as exemplified by **2–5** can be reproducibly obtained



2: 1″α-, R = H
3: 1″β-, R = H
4: 1″α-, R = Ac
5: 1″β-, R = Ac

free of spectral artifacts when the spectra are measured in Me_2SO-d_6 solution with a cylindrical internal reflectance (CIRCLE) cell.[2] Furthermore, we demonstrate the utility of second-derivative FTIR (SEDIR) measurements (Susi & Byler, 1983; Lee et al., 1985; Verdine & Nakanishi, 1985a) in performing weighted spectral subtraction involving poorly resolved composite FTIR spectra.

This protocol of using solution SEDIR measurements (Verdine & Nakanishi, 1985a) has been applied here to a structural reinvestigation of the adducts resulting from

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[1] Abbreviations: MC, mitomycin C; M, 2β,7-diaminomitosen-1-yl (cf. footnote 4); G, guanosin-N^2-yl; FTIR, Fourier transform infrared; Me_2SO, dimethyl sulfoxide; SEDIR, second-derivative infrared; DMAP, 4-(dimethylamino)pyridine.

[2] Commercially available from Barnes Analytical Division, Spectra-Tech, Inc., Stamford, CT 06906.

0006-2960/86/0425-4337$01.50/0

treatment of the dinucleoside phosphate d(GpC) and DNA with the reductively activated form of the antitumor antibiotic mitomycin C (MC, **1**).[3] MC is a clinically important chemotherapeutic agent that, in combination with other drugs, is widely used in the treatment of breast, gastrointestinal, and cranial tumors (Crooke & Bradner, 1976). One of the key biologically relevant features of MC is its requirement for chemical activation via either (i) reduction of the diaminoquinone function ("reductive activation"; Iyer & Szybalski, 1964; Lin et al., 1976) or (ii) protonation of the 9a-methoxyl oxygen ("acidic activation"; Lown et al., 1976; Tomasz & Lipman, 1979; McClelland & Lam, 1985). Each activation mechanism produces a high-energy intermediate (here termed MC^{*H} or MC^{**}, respectively), which is efficient at alkylating DNA. The structures of the adducts resulting from treatment of d(GpC) with acid-activated MC were recently shown to be derived from attack of MC^{**} at the N-7 position of guanine residues (Verdine & Nakanishi, 1985a; Tomasz et al., 1985). MC^{*H}, on the other hand, was reported to attack primarily at the O^6-position of guanine in the reaction with d(GpC) (Tomasz et al., 1983, 1984). Very recently, the latter conclusion became suspect in light of the finding that extensive modification of DNA by this adduct results in stabilization of secondary structure in the modified biopolymer (Tomasz & Lipman, 1985). This finding is in striking contrast to the loss of stability in DNA secondary structure that is generally observed upon alkylation at the O^6-position of guanine residues in DNA (Kuzmich et al., 1983). This anomalous finding prompted a thorough structural reappraisal of the published adduct structures (Tomasz et al., 1983, 1984). On the basis of the newly developed FTIR methods described here in conjunction with ^1H NMR measurements, we have determined the position of guanine attachment in these adducts (**2** and **3**, a 1″,2″-cis,trans diastereomeric pair) to be at N^2 and not at O^6 as previously reported. In addition, we report a simplified procedure for the facile synthesis of adducts **2** and **3** that makes these adducts readily accessible for further study.

This revision of the binding mode of MC to d(GpC) under reductive activation is especially significant in light of the recent finding that the same major adduct (**2**) is obtained from analogous reaction of MC with DNA (Tomasz et al., 1986).

Materials and Methods

Adducts. Adducts **2** and **3** were generated by reacting d(GpC) with reductively activated MC (H_2/PtO_2) according to the published procedure (Tomasz et al., 1983, 1984) or from 2′-deoxyguanosine as follows:

MC (92.0 mg) and 2′-deoxyguanosine (336 mg) were mixed in 34 mL of H_2O, and the resulting suspension was heated at 40 °C until complete dissolution was effected (5–10 min). PtO_2 (32 mg) was added, and the following gases were sequentially bubbled (via syringe needle) through the solution: helium, 20 min (to deaerate); hydrogen, 8 min (reductive activation); helium, 20 min (to displace hydrogen); air, 15 min (to reoxidize mitosene hydroquinones to the quinone form). The solids were subsequently removed by filtration, and the crude reaction mixture was chromatographed on a Sephadex G-25 (fine) column (5 × 112 cm) with 0.02 M NH_4HCO_3 as eluent as previously described (Tomasz & Lipman, 1979). The fraction eluting between 6300 and 7420 mL was lyophilized and subjected to preparative HPLC (Whatman Magnum 20 ODS-3, 250 × 22 mm; 8.0 mL/min; 12% CH_3CN–88% 0.03 M aqueous ammonium acetate). **2** and **3** eluted at 35 and 59 min, respectively. In addition of **2** and **3**, several other compounds (resulting from attack of water on the MC aziridine function) were obtained; i.e., the 1α- and 1β-hydroxy isomers of 2β,7-diaminomitosene. The maximum loading capacity with this system is 20 mg; however, the more polar eluent 6% CH_3CN–94% 0.03 M aqueous ammonium acetate allows loading of up to 60 mg of sample per injection. The adduct fractions were pooled and were concentrated and desalted by extensive lyophilization. Samples of **2** and **3** obtained in these experiments were in all respects identical with those described earlier (Tomasz et al., 1983, 1984). Adduct **2** can also be obtained from MC-modified DNA (Tomasz et al., 1986). The yields of **2** and **3** obtained were 5.4 and 4.5 mg, respectively. The availability of these adducts in such large quantities allowed the determination of more accurate molar extinction coefficients than those reported previously (Tomasz et al., 1983, 1984): $\epsilon_{312}(H_2O) = 10\,590 \pm 30$; $\epsilon_{252}(H_2O) = 24\,360 \pm 70$. Adducts **2** and **3** were acetylated (acetic anhydride/pyridine/DMAP, 25 °C, 30 min) and purified by preparative TLC (Analtech 500u, 80:20 $CHCl_3$/MeOH saturated with H_2O) to give triacetates **4** and **5**, respectively.

Model Guanine Ribosides. 1-, N^2-, and 7-methylguanosines were obtained from Sigma. O^6-Methyl-2′-deoxyguanosine was synthesized by a known procedure (Farmer et al., 1973). The model compounds were acetylated in order to remove the varying amounts of hydrated water molecules and also to facilitate spectroscopic measurements. The FTIR and NMR spectra of all model compounds were recorded both before and after acetylation (same acetylation conditions as above) to ensure that acetylation did not occur on the guanine base moiety.

FTIR Spectroscopy. All FTIR spectra were measured on an IBM IR85 instrument equipped with MCT detector, operating at a peak-resolution setting of 2 cm^{-1}. Samples (250 μg–2 mg) were dissolved in 50 μL of Me_2SO-d_6 (Aldrich, minimum isotopic purity of 99.96%) and charged (25-μL capacity) into the cylindrical internal reflectance (CIRCLE) microcell (ZnSe crystal). All spectra were initially measured as single-beam interferograms (2500 transients) and subsequently Fourier transformed to provide frequency-domain single-beam spectra. The single-beam spectrum of the argon-charged cell was subtracted from all simple and solvent spectra (weighting factor 1:1) to generate absorbance FTIR spectra. An absorbance spectrum of ambient water vapor and carbon dioxide was obtained by 1:1 subtraction of single-beam spectra obtained on the argon-charged cell at low and high levels of sample compartment purge (purging gas, dry N_2). All absorbance sample spectra were corrected for solvent and ambient H_2O/CO_2 absorptions by absorbance-mode spectral subtraction with the IBM SAM option. Other weighted spectral subtraction described further under Results and under Discussion were performed similarly. Second-derivative FTIR (SEDIR) spectra were generated by performing two sequential first-order derivative calculations on finalized absorbance spectra with a wavelength interval of 5 cm^{-1} (IBM parameter SMF = −5).

NMR Spectroscopy. ^1H NMR spectra reproduced in this paper were recorded in Me_2SO-d_6 solvent on a Bruker WM-250 instrument (250 MHz). Additional NMR measurements

[3] Recent X-ray studies (Hirayama & Shirahata, 1983) have shown that the absolute configuration of MC should be reversed from the previously accepted one. This conclusion has been independently verified on the basis of circular dichroic measurements (Verdine & Nakanishi, 1985b). All of the structures given in this paper depict the revised and correct absolute configuration of MC. It should be noted, therefore, that virtually all publications prior to 1984 reflect the wrong, opposite absolute configuration for MC and its derivatives.

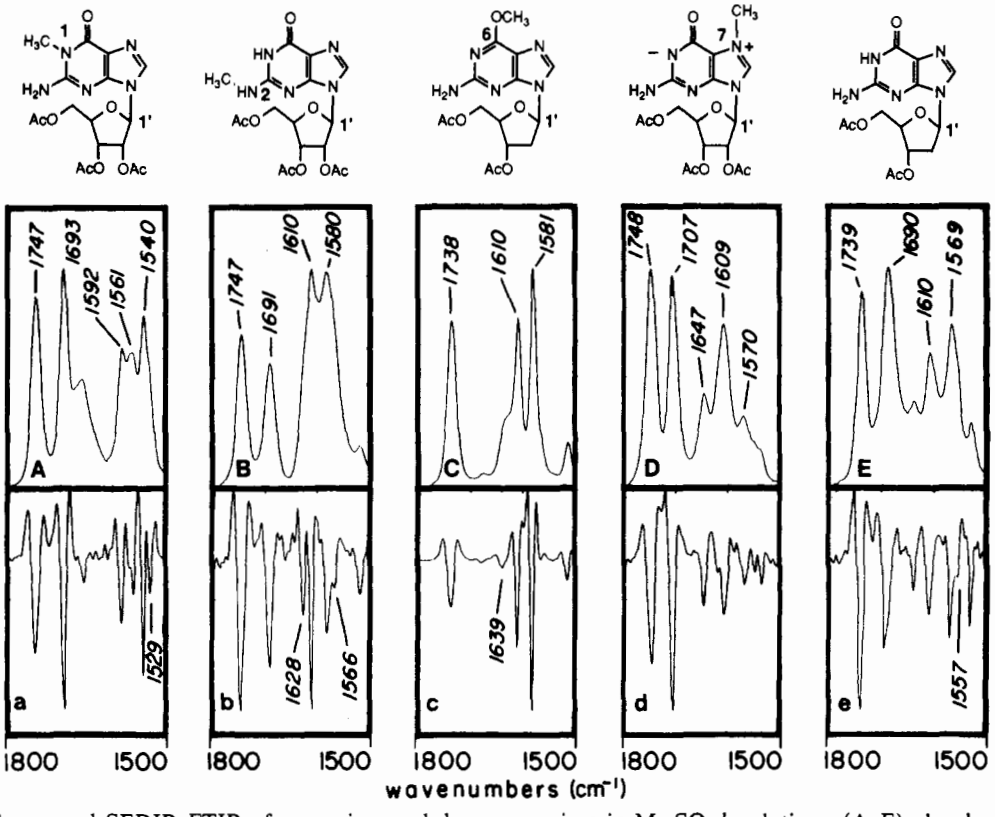

FIGURE 1: Absorbance and SEDIR FTIR of guanosines and deoxyguanosines in Me_2SO-d_6 solution: (A–E) absorbance spectra; (a–e) second-derivative (SEDIR) spectra. Characteristic peaks in (A–E) are denoted; those denoted in (a–e) are additional peaks that emerge upon second derivatization.

were conducted on a Jeol GX-400 spectrometer (400 MHz). All chemical shift values are recorded in ppm downfield from tetramethylsilane. Resolution-enhanced spectra, where indicated in text, were generated by zero filling of the spectrum from 16 to 32 K and Gaussian modification of the FID (LB = −3.0, GB = 0.3) prior to Fourier transformation.

Results

Model Compounds. Figure 1A–E shows the 1500–1800-cm^{-1} region FTIR absorbance spectra of differentially substituted guanine riboside acetates recorded in Me_2SO-d_6 solution with a CIRCLE cell. The spectra sufficiently differ from one another such that, in each case, the combined shapes and positions of major absorption bands make up a spectral "fingerprint" that is unique to only that heterosubstitution site (numbered peaks in Figure 1 denote those that are most characteristic). The spectra obtained in this study agree well with previously reported infrared spectra of methylated guanosines obtained under different conditions from those described here (Miles, 1971). However, due to the inherently broad nature of FTIR peaks obtained in solution (solid-state spectra suffer the same limitation), the spectra in Figure 1A–E exhibit only a fraction of the total information contained therein. Shouldered peaks can readily be resolved by calculation of the second derivative of the original digitized absorbance spectrum (Figure 1a–e). The power of this simple routine is demonstrated by a comparison of the second-derivative FTIR (SEDIR) spectra in Figure 1a–e with their respective precursor absorbance spectra in Figure 1A–E (denoted peaks in Figure 1a–e are significant absorption maxima that emerge upon second-derivative calculation). In all cases, additional spectral features are present in the SEDIR spectra that will clearly be of diagnostic value. A particularly striking example of this resolution enhancement is seen in the emergence of the 1628- and 1566-cm^{-1} peaks in Figure 1b, which are present as unclear shoulders in Figure 1B. It is worthy of note that the conventional absorbance FTIR spectra for N^2- and O^6-substitutions (Figure 1B,C) are quite similar in the 1650–1550-cm^{-1} region. The corresponding SEDIR spectra (spectra b and c of Figure 1, respectively) for these two substitutions, however, are readily differentiated, owing to the emergence of additional peaks in Figure 1b upon second derivatization. Thus, the highly resolved SEDIR spectra exhibit bands that are of considerable importance in obtaining unambiguous structural assignment.

Adducts. The FTIR of adducts **2** and **3** (not shown) and corresponding adduct triacetates **4** and **5** (Figure 2A; **4** and **5** exhibiit virtually identical FTIR spectra) are a composite of both "mitosene"-derived[4] (M) and substituted guanosine-related (G) absorption bands. In order to obtain an FTIR that is comprised of only G-derived bands, it is necessary to perform a weighted spectral subtraction of the M bands from the composite adduct spectrum. In practice, this is readily accomplished by subtraction of the FTIR spectrum of a simple model mitosene (i.e. **6**) from that of adduct **4**. Usage of this protocol is justified only if the mitosene-related FTIR absorptions in a mitosene–guanosine adduct are not significantly perturbed by the presence of the attached guanosine moiety. In this case, this routine is allowed because the region of interest (1800–1500 cm^{-1}) consists of mostly C=C and C=O stretching-mode absorptions; adduct **4** and model **6** have essentially identical mitosene C=C and C=O bonding motifs, and thus, the M-related FTIR absorptions of **4** and **6** in the 1800–1500-cm^{-1} region are expected to be almost identical. The foregoing assumption has been experimentally verified by careful peak-matching analysis of the second-derivative

[4] The term "mitosene" refers to the structure as in **6** without substituents in the 1-, 2-, and 7-positions (Webb et al., 1962).

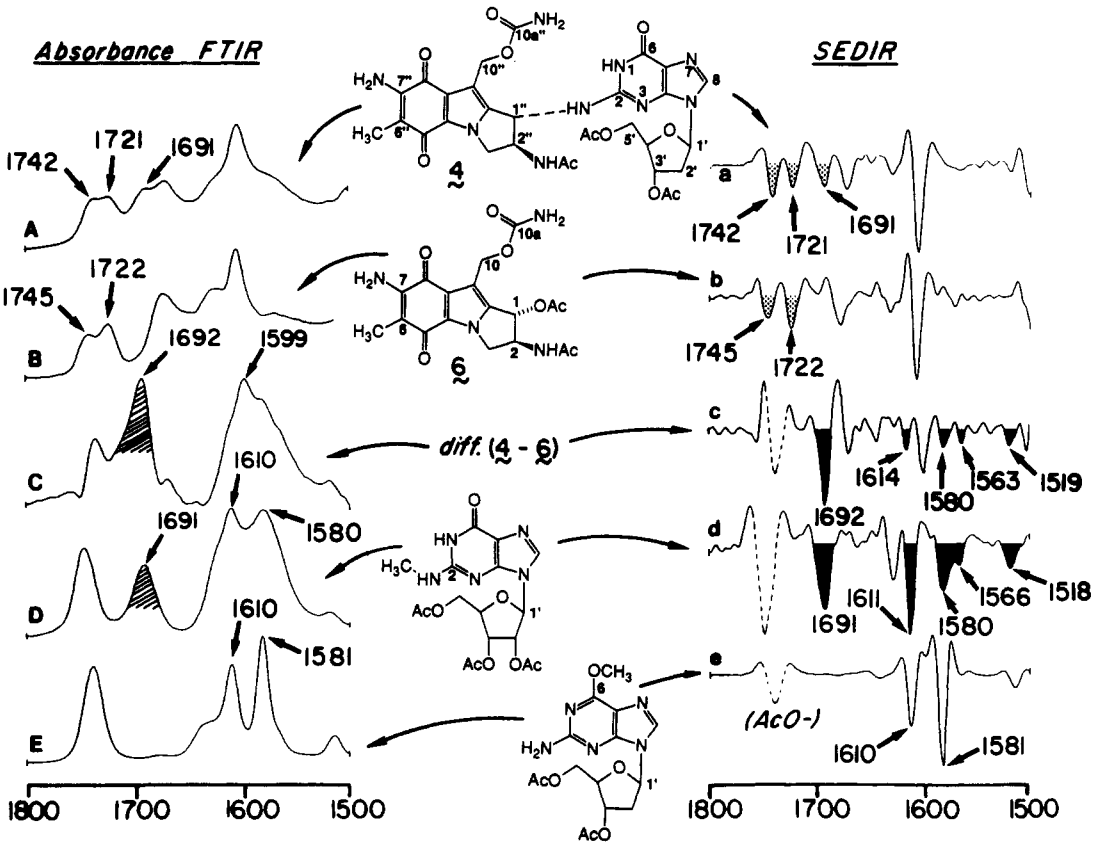

FIGURE 2: Absorbance and SEDIR FTIR of adduct triacetate **4** (A and a), mitosene **6** (B and b), difference **4** – **6** (C and c), and reference compounds N^2-methylguanosine triacetate (D and d) and O^6-methyldeoxyguanosine diacetate (E and e). Shaded peaks in (a and b) are resolved acetate, carbamate, and G-skeletal peaks; the carbamate peak is used for nullification to produce difference spectrum c. Dashed bands in (c–e) are acetate peaks that are not relevant to discussion of substituted-guanosine FTIR absorptions. Blacked-out bands in (c and d) are bands that are common to adduct difference spectrum and reference spectrum. Hatched bands in (C and D) are prominent, characteristic bands for N^2-alkylguanosines.

FTIR spectra of **4** and **6** (apparent in expanded-scale spectra; not shown) prior to the subtraction routine. Even in such a dipolar solvent as Me_2SO, mitosenes that bear an ammonium group at C-2 tend to aggregate and thus sometimes give rise to complex spectra. We have found that the acetylated derivatives of these compounds (as exemplified by **4**–**6**) exhibit superior spectral reproducibility and thus are ideal for purposes of spectral subtraction.

Shown in Figure 2A is the FTIR of adduct triacetate **4**; Figure 2B is that of mitosene diacetate **6**. Previous difference FTIR studies involving MC adducts (Tomasz et al., 1983; Verdine & Nakanishi, 1985a) have utilized the mitosene 1722-cm^{-1} carbamate band as the peak to be nulled during weighted spectral subtraction, since this region is devoid of G-related IR bands. In this case, this is not possible due to extensive overlap of the 1745-(acetate) and 1691-cm^{-1} (G, C=O) IR bands with the crucial 1722-cm^{-1} absorption (Figure 2A). Furthermore, since **4** is a triacetate and **6** is a diacetate, it is not possible to simultaneously null both the carbamate 1722- and acetate 1745-cm^{-1} IR bands (i.e., the carbamate:acetate ratio is different in the two compounds; Figure 2A,B). Increased isolation of the crucial 1722-cm^{-1} spectral gauge is readily accomplished, however, in the corresponding SEDIR spectra as shown in Figure 2a,b (shaded peaks). Thus, weighted subtraction in this case can be performed effectively in the SEDIR mode because it accomplishes the requisite isolation of the 1722-cm^{-1} weighting marker. Weighted subtraction of the SEDIR spectrum of mitosene **6** (Figure 2b) from that of adduct triacetate **4** (Figure 2a) with the common 1722-cm^{-1} band produces the difference second-derivative spectrum in Figure 2c.[5] In turn, the weighting factor determined from the SEDIR subtraction routine can then be used to weight- subtract the original absorbance spectra, thus giving rise to the difference FTIR curve in Figure 2C.

Comparison of both adduct difference spectra (Figure 2C,c) with those of the model guanosines in Figure 1 reveals that the adduct difference spectra most closely resemble that of N^2-methylguanosine triacetate (Figure 1B,b). To facilitate further comparison, the FTIR spectra of N^2- and O^6-methylguanosines have been reproduced in expanded form in Figure 2 (D and d and E and e, respectively). Upon inspection of curves C–E, it is clear that the differential absorbance spectrum of adduct triacetate **4** much more closely resembles an N^2-substituted guanosine (C vs. D) than an O^6-substituted guanosine (C vs. E). Particularly striking is the presence of the strong 1691-cm^{-1} C=O band in both adduct and reference spectra (Figure 2C,D, shaded peaks). Unfortunately, however, the peak resolution in the area 1650–1550 cm^{-1} of curves C and D is not sufficient to judge the extent of correspondence between the two spectra in this region. The respective SEDIR spectra, however, are sufficiently well resolved as to permit a direct peak-matching analysis over the entire 1800–1500-cm^{-1} spectral range (Figure 2c,d, blacked-out peaks denote

[5] Second-derivative curves of absorbance spectra have been theoretically (Cahill, 1979; Kauppinen, 1981; Maddams & Southen, 1982; Maddams & Tooke, 1982) and experimentally (Cahill & Padera, 1980; Verdine & Nakanishi, 1985a) shown to exhibit peak intensities that are linearly related to the concentration of the solution in which the solute is measured; i.e., second-derivative spectra follow Beer's law (despite the fact the intensity is in units cm^{-2}). This being the case, it is permissible to use multiplicative concentration-adjustment factors in order to perform weighted subtraction of SEDIR spectra.

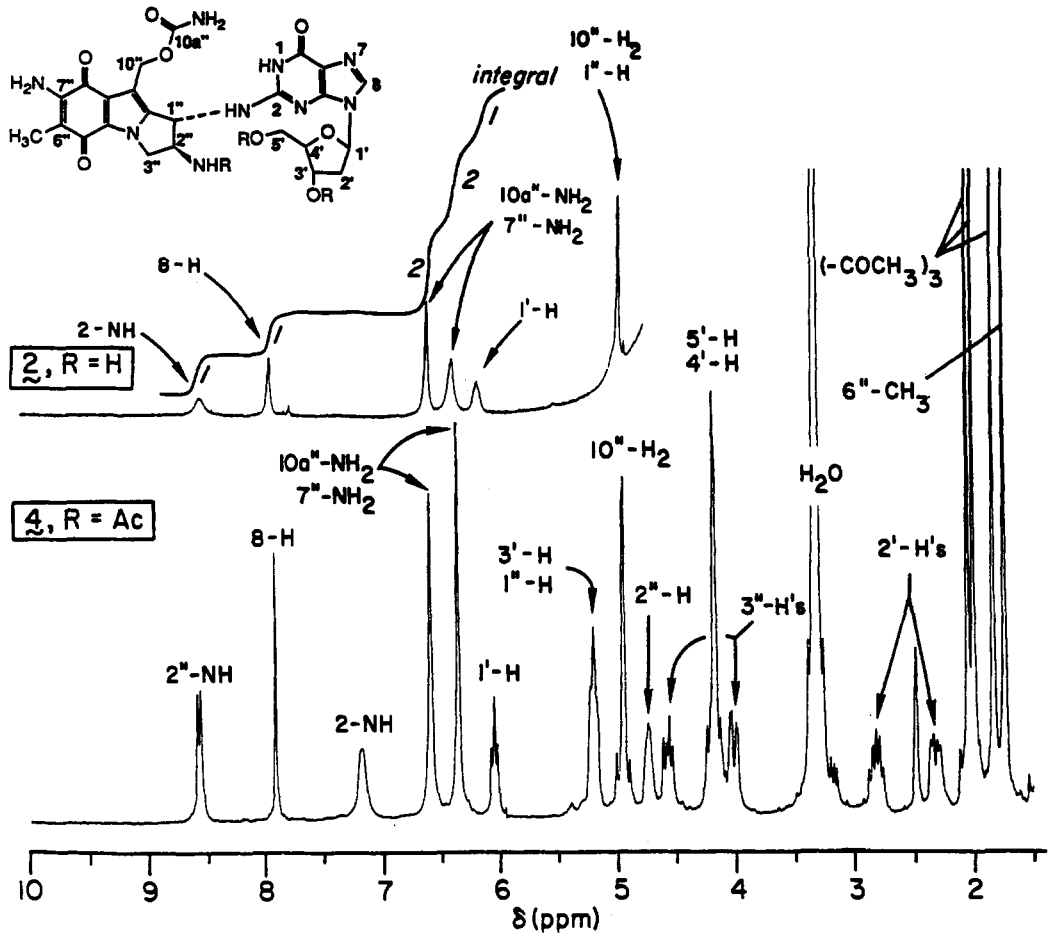

FIGURE 3: Proton magnetic resonance spectra of adduct **2** and **2**-triacetate **4**, in Me_2SO-d_6, 250 MHz.

those that are common to both adduct and reference spectra). It should be noted, however, that the strong 1610-cm^{-1} absorption exhibited by N^2-methylguanosine triacetate (Figure 2D,d) is apparently shifted to 1599 cm^{-1} (11-cm^{-1} shift) in the adduct difference spectrum (Figure 2C,c). This 11-cm^{-1} shift is reproducibly observed in difference spectra arising from both the unacetylated adducts **2** and **3** (data not shown) and triacetates **4** and **5**; furthermore, it is observed regardless of the mitosene model compound employed in the subtraction routine. We ascribe this anomolous frequency shift to a substituent effect in the N^2-alkylguanosine moiety. In adduct **4**, the N^2-substituent is allylic to the mitosene indoloquinone moiety, while in N^2-methylguanosine triacetate the attached alkyl group is a simple methyl substituent. The presence of an allylic N^2-substituent in **4** thus presumably gives rise to an electronically induced frequency shift in one of its guanine absorption bands. Due to the unavailability of a suitable N^2-substituted guanosine model compound (e.g., N^2-benzylguanosine), we were unable to experimentally confirm this hypothesis. The shift observed in this one band, however, does not affect the overall conclusion derived from comparison of Figure 2C,c with Figure 1; the adduct difference spectra in Figure 2C,c most closely match those in Figure 1B,b. From the foregoing results, it is clear that adduct **4** possesses an N^2-substituted guanosine moiety and not the previously reported O^6-substitution site (see Discussion).

NMR Spectroscopy. Figure 3 shows the downfield region of the ^1H NMR spectrum of adduct **2** (upper trace) and the entire spectrum of adduct triacetate **4** (lower trace) in which all proton resonances are assigned. The 2-NH_2 of guanosine (not shown) appears at 6.50 ppm, and mitosene **6** exhibits a broad four-proton signal at 6.3–6.7 ppm arising from carbamate NH_2 and 7-NH_2 groups (not shown). Thus, an MC–deoxyguanosine adduct with a free 2-NH_2 group would be expected to exhibit proton resonances corresponding to three NH_2 groups (six protons) in the region ca. 6–7 ppm. Adduct **2**, however, has signals integrating to only four protons in this region (Figure 3, upper trace), thus indicating that the adduct does not posess a free guanine 2-NH_2 group. The only remaining downfield proton signal in **2** (other than 8-H) is a broad, exchangeable, one-proton absorption at 8.50 ppm, which is attributable to a monosubstituted guanosine-type 2-NH group.

As previously mentioned, acetylation of these mitosene–deoxyguanosine adducts furnishes derivatives that exhibit superior spectral quality to the original underivatized adducts. The ^1H NMR of adduct triacetate **4** (Figure 3, lower trace) is sufficiently sharp to permit assignment of all proton resonances after extensive decoupling experiments (not shown). Figure 4A shows the expanded downfield portion (5.4–4.8 ppm) of the ^1H NMR of adduct triacetate **4** (partial structure **4a**) in which proton assignments are indicated; Figure 4B is the spectrum recorded after irradiation of the broad 2-NH signal at 7.19 ppm (not shown in Figure 4; see Figure 3, lower trace, for full-scale spectrum). Upon decoupling of this amino proton, there is an apparent sharpening of the mitosene 1″-H (circled H in partial structure **4a**) signal centered at 5.18 ppm; unfortunately, this sharpening effect is not sufficiently dramatic to constitute unambiguous proof of NH–CH coupling. In contrast, this decoupling effect is clearly seen upon Gaussian resolution enhancement of the spectra in Figure 4A,B to spectra in Figure 4C,D. Thus, resolution of the mitosene 1″-H signal in Figure 4C,D is sufficiently high that collapse of a significant proton–proton coupling can be clearly observed

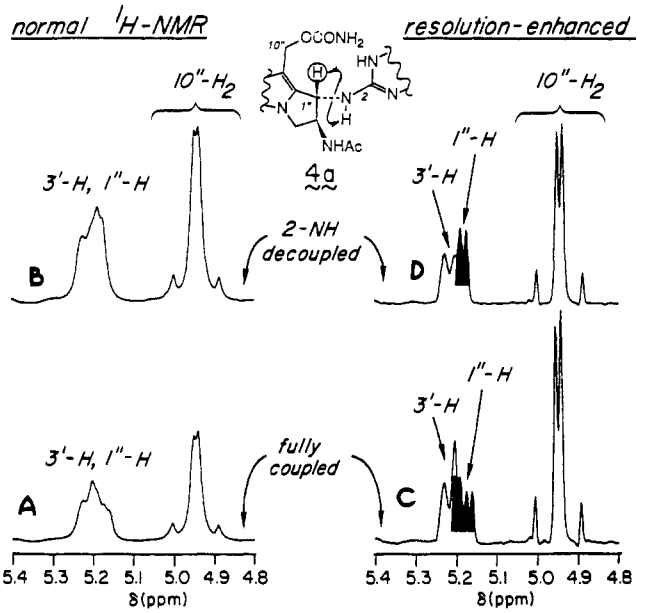

FIGURE 4: Proton magnetic resonance spectra of adduct triacetate **4**, before (A and C) and after (B and D) decoupling of 2-NH (not shown), in Me_2SO-d_6, 250 MHz. Spectra in (A and B) were resolution-enhanced to give (C and D), respectively. The 1"-H peak is highlighted in black.

upon irradiation of the 7.19 ppm 2-NH signal. These results unambiguously indicate that the mitosene moiety in adduct triacetate **4** is linked to the N^2-position of the deoxyguanosine base, as indicated by the FTIR evidence given above.

Analogous ^1H NMR results with respect to proton signal integration and 2-NH/C-1"-H coupling are obtained from the cis adduct **3** and its corresponding triacetate **5**, as can be seen in Figure 5. Thus, **3** shows exchangeable signals integrating to only four protons in the 6–7 ppm region and a broad, exchangeable one-proton signal at 8.50 ppm, again indicative of monosubstitution at the guanosine 2-NH_2 position (upper trace). Furthermore, in the ^1H NMR of triacetate **5**, the mitosene 1" proton signal appears as a triplet at 5.44 ppm, indicating that it is coupled to more than just the 2" mitosene proton. Indeed, irradiation of the 2-NH signal at 8.16 ppm or proton exchange with MeOH-d_4 effects the collapse of the mitosene 1" signal to a doublet (not shown).

Discussion

The results presented here demonstrate the power of second-derivative FTIR measurements in discerning the position of attachment of alkylating agents to guanine residues. Though the method described here has focused specifically on alkylated guanines, it should be applicable to modified forms of the other DNA bases as well. The resolution enhancement gained by calculation of second derivatives of FTIR spectra should be generally useful in any case in which crucial spectral bands are obscured or overlapped by proximal peaks. Although other resolution-enhancement techniques such as interferogram deconvolution are available, they require an FTIR instrument by their very nature. In addition, it is not clear that substraction of deconvolved FTIR spectra will lead to reliable results. SEDIR spectra, on the other hand, can be generated by mathematical second-derivative calculation on any digitized infrared absorbance spectrum. Several commercially available computer-slaved IR instruments now carry the software option required to perform this operation. When analyzing SEDIR spectra, it should be kept in mind that second-derivative spectra tend to exaggerate the intensity of peaks that are sharp in the normal absorbance spectrum; for this reason, it is generally inadvisable to ascribe quantitative

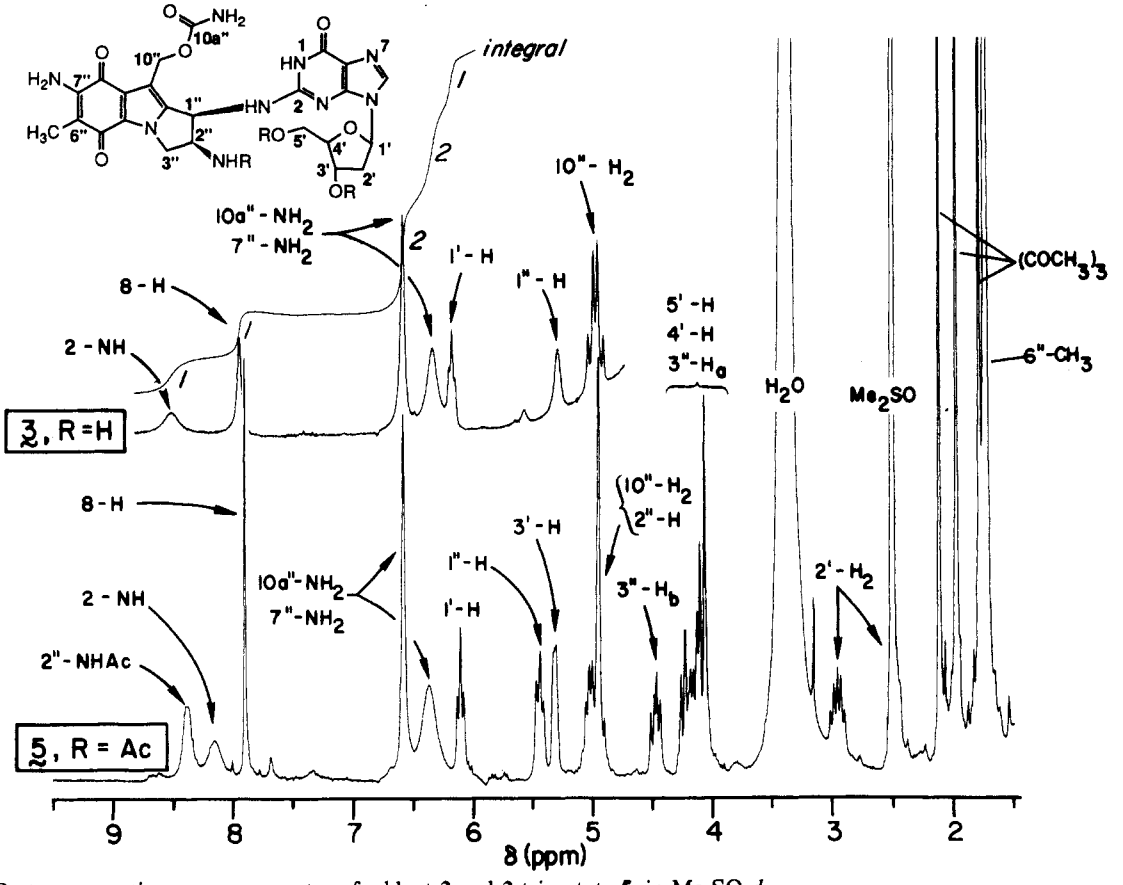

FIGURE 5: Proton magnetic resonance spectra of adduct **3** and **3**-triacetate **5**, in Me_2SO-d_6.

values to relative peak intensities within a single SEDIR spectrum. However, since SEDIR spectra do follow Beer's law, it is permissible to use them for purposes of weighted subtraction.[5]

Through a combination of SEDIR and ^{1}H NMR spectroscopies, we have clarified the bonding mode of reductively activated mitomycin C to the dinucleoside phosphate d(GpC). Thus, MC^{*H} binds in vitro largely to N^2 of the guanine moiety in d(GpC), rather than to O^6 as originally reported. The original, erroneous determination of the deoxyguanosine attachment site in adduct **2** (Tomasz et al., 1983) was derived largely from the results of difference FTIR measurements; in light of this fact, several comments must be made concerning the origin of the error.

(1) The absorbance FTIR spectra of N^2- and O^6-substituted guanosine acetates are quite similar except for the 1691-cm^{-1} peak, which is present in the former case and absent in the latter. Apparently, the error in assignment of substitution site arose through oversight of an apparent weak shoulder at around 1680 cm^{-1} in the original difference FTIR spectrum. However, the obscureness of the 1691-cm^{-1} peak was probably due to the fact that the original work employed difference spectra obtained in KBr disks, and not in Me_2SO-d_6 solution, the latter being used in the present work. We feel that although due caution must be exercised with respect to use of this difference FTIR method on absorbance FTIR spectra, usage of the corresponding second-derivative spectra would make it virtually impossible to overlook peaks obscured by shouldering.

(2) Employing solution FTIR in the strongly dipolar solvent Me_2SO-d_6 in this work instead of the previously used aqueous KBr dissolution/lyophilization technique (Kasai et al., 1977) led to superior resolution and reproducibility of the FTIR spectra of these adducts. In our previous studies on MC^{*H} adducts (Tomasz et al., 1983), difference FTIR studies were carried out on underivatized compounds in lyophilized KBr rather than on the acetates in Me_2SO-d_6 solution; the propensity of these polar unacetylated compounds to aggregate probably led to an enhancement of undesired solid-state effects in the resultant FTIR spectra. Highly polar and self-aggregating molecules such as MC–DNA adducts are apparently more reliably measured as a solution in a strongly dipolar solvent, thus ensuring the removal of solid orientational effects.

^{1}H NMR spectroscopy is not a generally definitive tool for deducing nucleic acid base–adduct structures, owing to the paucity of usable proton signals on the heterocyclic bases. However, in the case of N^2-substitution on guanine, NMR spectroscopy can be useful in that the resultant adduct is expected to contain vicinal N–H/C–H coupling at the point of attachment; coupling of adduct protons to those in the base portion can occur *only* in this heterosubstitution site. We have taken advantage of this diagnostic coupling phenomenon in order to corroborate the results obtained from FTIR measurements. Additionally, we have shown that Gaussian resolution enhancement of inherently broad ^{1}H NMR can be extremely powerful in such cases where the critical coupling pattern cannot be unambiguously ascertained from the conventional ^{1}H NMR spectra.

A perpetual problem that has been encountered in structural assessment of mitosenes is excessive broadening of ^{1}H NMR signals. Often, the broadening phenomenon is so severe as to preclude any interpretation of the spectra. We have circumvented this problem by acetylation of adducts **2** and **3** to give the corresponding triacetates **4** and **5**. This mild chemical modification procedure can be performed on adducts such as **2** and **3** in virtually quantitative yield and leads to excellent proton NMR spectra. An additional effect of this treatment is the apparent removal of hydrogen-bonded water molecules from the adduct, thus making solvent suppression unnecessary. An additional feature of the acetylated mitosene derivatives (including DNA adducts) is that they are extremely well suited for mass spectrometry by the fast atom bombardment technique (unpublished results); obtaining mass spectra on mitosenes has previously been troublesome. We have since used this acetylation protocol for several other unwieldy mitosene derivatives (unpublished results).

This work unambiguously establishes the correct structures for the adducts obtained in the reaction of reductively activated MC with d(GpC) and thus provides an essential point of reference for identifying MC adducts originating from corresponding treatment of DNA with MC^{*H} both in vitro and in vivo. The simplified method of synthesis of **2** and **3** described here using deoxyguanosine as starting material makes these adducts readily available for further studies. The recent finding (Tomasz et al., 1986) that **2** is overwhelmingly the major adduct resulting from attack of MC^{*H} on DNA in vitro indicates that the binding mode of MC to DNA results in a much simpler adduct distribution than previously reported (Hashimoto et al., 1982, 1983, 1984).

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Production of Dihydrothymidine Stereoisomers in DNA by γ-Irradiation†

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ABSTRACT: 5,6-Dihydrothymidine (dDHT) is a derivative of thymidine formed during γ-irradiation. This paper demonstrates the conditions under which dDHT is formed in solutions of DNA and that dDHT is produced in the DNA of HeLa cells during γ-irradiation. The product of dDHT by γ-irradiation of either thymidine or DNA has been quantitated by a sensitive and specific high-pressure liquid chromatography method. dDHT is a major product of the anoxic irradiation of thymidine (*G* value 0.5) but is produced in substantially smaller amounts in DNA irradiated under the same conditions (*G* value 0.026). The presence of oxygen reduces the yield of dDHT by at least 25-fold for both irradiation substrates. In HeLa cells, ^{60}Co irradiation under anoxia produces $(6.2 \pm 0.2) \times 10^{-8}$ mol of the *R* isomer of dDHT per mole of cell deoxynucleotide per gray (*G* value 0.11). γ-Irradiation of thymidine produces equal quantities of the *R* and *S* stereoisomers of dDHT. Irradiation of DNA produces significantly more (69%) (*R*)- than (*S*)-dDHT. DNA isolated from cultured human cells following γ-irradiation also contains more of the *R* than the *S* form of dDHT. The conformation of double-stranded DNA favors a stereospecific production of the *R* isomer. Among products of γ-irradiation of DNA, dDHT is unique in its strict requirement for anoxia during irradiation and the preferential production of a particular stereoisomer.

γ-Irradiation of DNA produces a variety of alterations in both the phosphodiester backbone and the component bases (Ward, 1981). Several of the altered bases produced by γ-irradiation in DNA, such as thymidine glycols (dTG)[1] and (hydroxymethyl)deoxyuridine (dHMU) have been quantitated following the irradiation of cultured cell lines (Cerutti, 1976; Frenkel et al., 1981b, 1985; Teebor et al., 1982, 1984). 5,6-Dihydrothymidine (dDHT), a product of the anoxic irradiation of thymidine, has been reported to occur in irradiated DNA (Teoule et al., 1978; Dizdaroglu, 1985). However, the exact amount of dDHT formed under various conditions and its production in irradiated cells has not been reported. Here we report a method for the quantitation of the radiation product dDHT in ^{3}H-labeled DNA. dDHT is quantitated by HPLC following enzymatic digestion of the DNA to deoxynucleosides. The method has been used to quantitate each of the two stereoisomers of dDHT (Figure 1) in DNA following γ-irradiation under a variety of irradiation conditions or following γ-irradiation of intact cultured cells. It will be demonstrated that dDHT production occurs at substantial levels only under strictly anoxic conditions and that, in DNA, the *R* stereoisomer is formed preferentially.

Experimental Procedures

Materials. Thymine, thymidine, 5-(hydroxymethyl)uracil, 5-(hydroxymethyl)deoxyuridine, 5,6-dihydrothymine, and 5,6-dihydrothymidine were obtained from Sigma Chemical Co., St. Louis, MO. [*methyl*-^{3}H]thymidine (76 Ci/mmol) and [6-^{3}H]thymidine (18.2 Ci/mmol) were obtained from New England Nuclear (Boston, MA). All solvents were HPLC grade (Burdick and Jackson, Muskegon, MI).

Preparation of dTG, TG, (R)-dDHT, and (S)-dDHT. *cis*-5,6-Dihydroxy-5,6-dihydrothymine and *cis*-5,6-dihydroxy-5,6-dihydrothymidine were prepared according to the method of Iida and Hayatsu (1970, 1971) as modified by

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[1] Abbreviations: dDHT, 5,6-dihydrothymidine; dTG, 5,6-dihydroxy-5,6-dihydrothymidine; DHT, 5,6-dihydrothymine; TG, 5,6-dihydroxy-5,6-dihydrothymine; dHMU, 5-(hydroxymethyl)-2′-deoxyuridine; HMU, 5-(hydroxymethyl)uracil; HPLC, high-pressure liquid chromatography; dT, thymidine; T, thymine.

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