Mitosene−DNA Adducts. Characterization of Two Major DNA Monoadducts Formed by 1,10-Bis(acetoxy)-7-methoxymitosene upon Reductive Activation†

Marc Maliepaard,*,‡ Nico J. de Mol,‡ Maria Tomasz,§ Dario Gargiulo,§ Lambert H. M. Janssen,‡ John P. M. van Duynhoven,|| Ewoud J. J. van Velzen,|| Willem Verboom,|| and David N. Reinhoudt||

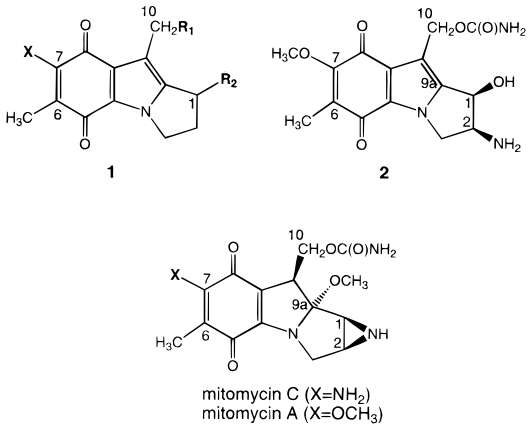
Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands, Department of Chemistry, Hunter College, City University of New York, New York, New York 10021, and Laboratory of Organic Chemistry, Faculty of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

Received January 13, 1997; Revised Manuscript Received April 16, 1997⊗

ABSTRACT: Reductive activation of racemic 1,10-bis(acetoxy)-7-methoxymitosene WV15 in the presence of DNA, followed by enzymatic digestion and HPLC analysis, revealed the formation of various DNA adducts. Reduction is a necessary event for adduct formation to occur. This reductive activation was performed under hypoxic conditions in various ways: (1) chemically, using a 2-fold excess of sodium dithionite ($Na_2S_2O_4$), (2) enzymatically using NADH-cytochrome *c* reductase, (3) electrochemically on a mercury pool working electrode, and (4) catalytically, using a H_2/PtO_2 system. Five different mitosene−DNA adducts were detected. These adducts were also present when poly(dG-dC) was used instead of DNA, but were absent with poly(dA-dT). All were shown to be adducts of guanine. Reduction of 1,-10-dihydroxymitosene WV14 in the presence of DNA did not result in detectable adduct formation, demonstrating the importance of good leaving groups for efficient adduct formation by these mitosenes. Finally, two of the adducts were isolated and their structures elucidated, using mass spectrometry, ^{1}H NMR and circular dichroism (CD). The structures were assigned as the diastereoisomers N^2-(1″-*n*-hydroxymitosen-10″-yl), 2′-deoxyguanosine ($n = \alpha$ or β). These type of adducts, in which the mitosene C-10 is covalently bonded to the N-2 of a guanosylic group, are different from the well-known mitomycin C 2′-deoxyguanosine monoadducts, that is linked via the mitomycin C C-1 position, demonstrating that the order of reactivity of the C-1 and C-10 in these mitosenes is reversed, as compared to mitomycin C. The 7-methoxy substituent of WV15 is a likely factor causing this switch. Evidence is presented that the 7-substituent of mitosenes also influences their DNA alkylation site. Adducts **4** and **5** represent the first isolated and structurally characterized covalent adducts of DNA and a synthetic mitosene.

Mitosenes (basic structure **1**) are derived from the clinically significant cytostatic agent mitomycin C (Chart 1), which is used in the treatment of solid tumors in the breast, lung, and the gastrointestinal tract (Remers & Dorr, 1988). Mitomycin C requires reductive activation to exert its antitumor activity (Doroshow, 1992). Several types of reduction, e.g., chemical (Iyer & Szybalski, 1964; Tomasz et al., 1986, 1988a; Hong & Kohn, 1991; Schiltz & Kohn

Chart 1



1992), electrochemical (Andrews et al., 1986; Kohn et al., 1987; Maliepaard et al., 1993), and enzymatic (Tomasz et al., 1981; Keyes et al, 1984; Pan et al., 1984, 1986; Gustafson & Pritsos, 1992; Siegel et al., 1992; Prakash et al., 1993; Hodnick & Sartorelli, 1993) have been used to activate mitomycin C *in vitro*. The activation mechanism of mitomycin C (Scheme 1) has been studied extensively and has been shown to proceed via reduced mitosene intermediates (Rao et al., 1977; Danishefski & Ciufolini, 1984; Danishefski & Egbertson, 1986; Peterson & Fisher, 1986, Andrews et

† This work is supported by a grant of the Dutch Cancer Soceity (NKB 90-05) to M.M., a grant (CA28681) from the National Cancer Institute and a Research Centers in Minority Institutions award (RR003037) from the Division of Research Resources, National Institutes of Health, U.S. (both to M.T.).

* Author to whom correspondence should be addressed. Present address: Department of Experimental Therapy, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

‡ Utrecht University.

§ Hunter College, City University of New York.

|| University of Twente.

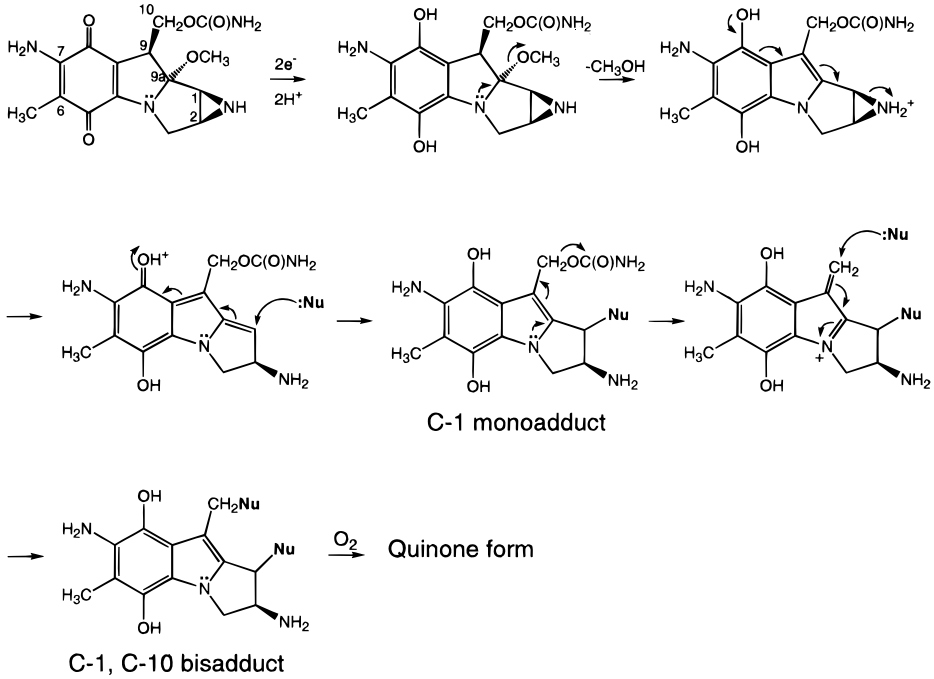
⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1997.

[1] Abbreviations: AcN, acetonitrile; CD, circular dichroism; CI, chemical ionization; dG, deoxyguanosine; EDTA, ethylenediaminetetraacetic acid; EI, electron impact; MeOH, methanol; NH_4Ac, ammonium acetate; SVD, snake venom diesterase; v/v, volume to volume ratio.

[2] The original definition of the trivial name mitosene included as invariant the C-10 carbamoyloxy substituent in structure **1** (Webb et al., 1962a). In this article, mitosene is redefined as structure **1**, i.e., R_1 is a variable substituent of mitosene.

S0006-2960(97)00068-8 CCC: $14.00 © 1997 American Chemical Society

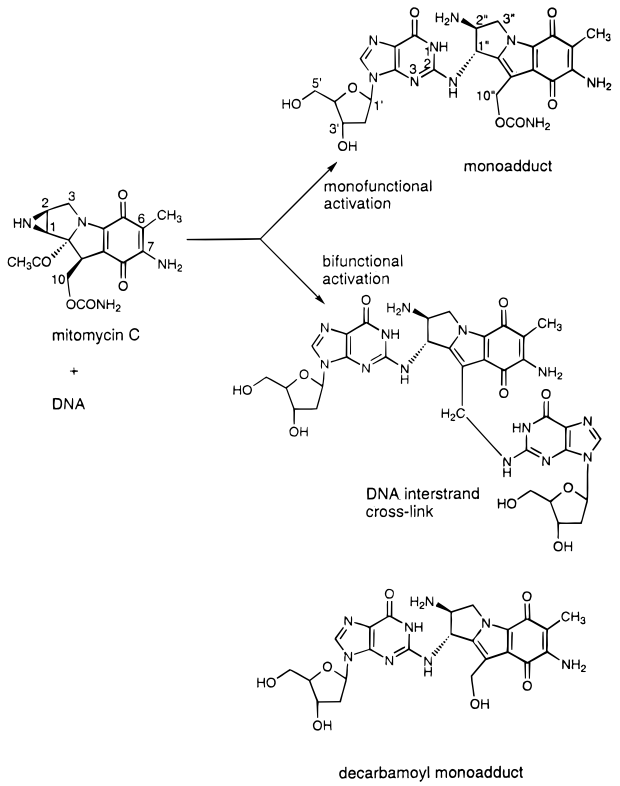
Scheme 1: Mechanism of Reductive Activation of the Electrophilic Centers C-1 and C-10 of Mitomycin C



al., 1986; Hoey et al., 1988; Schiltz & Kohn, 1993a,b). The C-1 of mitomycin C is the most reactive site following reductive activation [e.g., Remers and Dorr (1988) and Kohn and Hong (1990)]. Activation of the C-10 site of mitomycin C has only been detected after opening of the C-1 aziridine group [e.g., Iyengar et al. (1990) and Kohn and Hong (1990)]. In the absence of external nucleophiles, reductively activated mitomycin C preferentially acts as a trapping agent for electrophiles, whereas in the presence of nucleophiles (e.g., DNA) nucleophilic substitution reactions can take place at the C-1 and C-10 site of mitomycin C (Schiltz and Kohn, 1993b). Thus, following reductive activation, mitomycin C is capable of binding to the N-2 of a 2′-deoxyguanosyl residue in DNA via the C-1 site (Tomasz et al., 1986, 1988b; Kumar et al., 1992), or via both the C-1 and C-10 sites (Tomasz et al., 1987). In the latter case, DNA inter- and intrastrand cross-links can be formed (Bizanek et al., 1992) (Scheme 2). Interstrand cross-links can prevent (tumor) cell division and are therefore considered to be the ultimate cause for antitumor activity of mitomycin C (Zwelling et al., 1979; Kohn, 1983). However, results obtained by Basu et al. (1993), who noticed arrest of *in vitro* replication of DNA in the presence of mitomycin C monoadducts, suggest that also monoadducts may be relevant for antitumor activity of mitomycin C.

The unique mechanism of action of DNA cross-linking by mitomycin C has inspired numerous efforts to design synthetic analogs having the potential to undergo bioreductive activation to a bifunctional DNA alkylating agent. Most of such analogs belong to the class of mitosenes, based on the fact that mitosenes are intermediates in the reductive activation path of mitomycin C (Scheme 1). Examples include 1,10-functionalized mitosenes (Casner et al., 1985, Verboom et al., 1987), cyclopropamitosenes (Jones & Moody, 1989a,b; Cotterill et al., 1994), azamitosenes (PBI-A) (Islam et al., 1991; Skibo & Schulz, 1993) the indoloquinone EO9 (Oostveen & Speckamp, 1987; Hendriks et al., 1993) and nucleotide-mitosene conjugates (Iyengar et al., 1988). Many of these have been shown to exhibit significant

Scheme 2: Adducts of Mitomycin C and DNA Formed by Reductive Activation



antitumor activity. EO9 is currently under clinical trials (Hendriks et al., 1993, Pavlidis et al., 1996).

Like mitomycin C, mitosenes can be activated reductively. Chemical and enzymatic reduction of 7-methoxymitosenes have been shown to result in loss of leaving groups at C-1 and/or C-10 (Maliepaard et al., 1993; Maliepaard, 1994). Interestingly, upon reduction of 7-methoxymitosenes with two acetoxy groups attached to the C-1 and C-10 site, the acetoxy group at C-10 is expelled preferentially (Maliepaard et al., 1993) (Scheme 3). In the presence of DNA, this

Scheme 3: Mechanism of Sequential Reductive Activation of the Two Electrophilic Centers of WV15

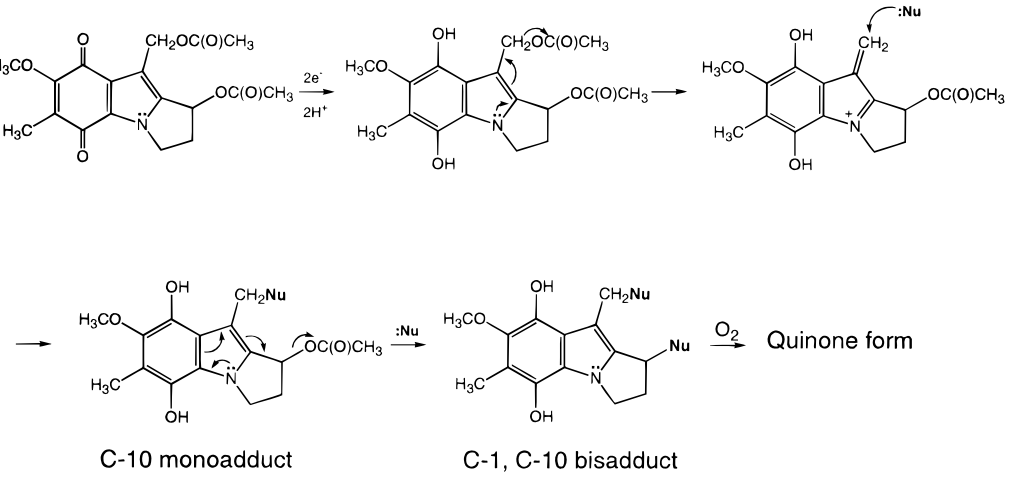


Table 1: Structures of Mitosene Compounds (**1**)

compound	X	R_1	R_2
WV14	OCH_3	−OH	compound
WV15	OCH_3	$-OC(O)CH_3$	$-OC(O)CH_3$
WV16	OCH_3	$-OC(O)N(H)C_2H_5$	−OH

reductive activation of mitosenes has been shown to result in the formation of DNA interstrand cross-links (Maliepaard et al., 1993, 1994). The presence of two leaving groups appears to be a prerequisite for *in vitro* antitumor activity of these mitosenes (Maliepaard et al., 1992), suggesting the importance of interstrand DNA cross-links for this antitumor activity.

Despite these advances, little has been known about the actual DNA alkylation chemistry of these designed mitosenes. No DNA adducts have been detected, isolated, and characterized with any mitosene. Therefore, the intended chemical analogy to the mono- and bifunctional alkylation of DNA by mitomycin C (Scheme 1) and mitomycin A (McGuinness et al., 1991) has remained conjectural for mitosenes.

This investigation was aimed at demonstrating the formation of DNA adducts upon reductive activation of a synthetic 7-methoxymitosene WV15 (Verboom et al., 1987). This compound shows promising activity in several *in vitro* and *in vivo* tumor models (Orlemans et al., 1989). Herein, we report the formation of several DNA adducts of this substance in cell-free activation systems. The structure of the major monoadducts was unambiguously determined, providing evidence for the alkylation of DNA and its chemical mechanism. The adduct structures revealed a difference in the activation paths of the mitosene WV15 and mitomycin C.

MATERIALS AND METHODS

Mitosenes were synthesized as described previously (Orlemans et al., 1989) and were kept as 100 mM stock solutions in *N*,*N*-dimethylformamide at 4 °C. The mitosenes used in this study are shown in Table 1. All mitosenes possess a chiral center at C-1 and have been used as racemic mixtures. In addition, WV15 was resolved into its (+) and (−) enantiomers, which were used separately in some experiments. Calf thymus DNA (type I, sonicated before use), *Micrococcus lysodeikticus* DNA (*Micrococcus luteus* DNA, type XI, sonicated before use), NADH-cytochrome c reductase, and deoxynucleosides were purchased from Sigma (St. Louis, MO). Snake venom diesterase (SVD,[2] phosphodiesterase I, from *Crotalus adamanteus*) and *Escherichia coli* alkaline phosphatase were from Worthington Biochemical Corporation (Freehold, NJ). Nuclease P_1, poly(deoxyadenosine-thymidine) [poly(dA-dT)] and poly(deoxyguanosine-deoxycytidine) [poly(dG-dC)] were from Pharmacia Biotech Inc. (Piscataway, NJ).

UV−vis measurements were performed on a Perkin-Elmer λ 5 and a Cary 3 spectrophotometer, both connected to a temperature controller. HPLC analyses were performed using a 250 × 4.5 mm Beckman ODS C-18 Ultrasphere reverse phase column. Products were eluted from the column using a gradient of 13−40% (v/v) AcN/0.02 M NH_4Ac in 20 min at a flow-rate of 1 mL/min and were detected using a Beckman 165A detector at 254 nm. Peak areas were quantitated with a Beckman 427M integrator. Preparative HPLC was carried out using a Rainin HPLX instrument equipped with a Dynamax C-18 column (21.4 × 250 mm) and employing 16/84 AcN/H_2O at 10 mL/min flow rate. ^1H NMR measurements in DMSO-d_6 were performed at 400 MHz on a Varian Unity 400WB spectrometer. TOCSY (MLEV17) (Bax & Davis, 1985) and DQF-COSY (Rance et al., 1983) experiments were conducted using standard Varian pulse programs. Mixing times of the TOCSY experiments ranged from 40 to 120 ms. NMR data were processed by the Varian VnmrS/VnmrX software packages.

Separation of Enantiomeric Forms of 1,10-Bis(acetoxy)-mitosene WV15. Mitosene WV15 was separated into its two enantiomeric forms using a 250 × 4.6 mm Chiralcel OD column (Chiral Technologies Inc., Exton, PA). WV15 was dissolved in 17/83 *n*-hexane/isopropyl alcohol (final concentration of WV15, 10 mM) by heating the solution at 50 °C for approximately 1 min. The enantiomers were eluted with 70/30 *n*-hexane/isopropyl alcohol at a flow rate of 1 mL/min and were detected at 254 nm. The (+) and (−) enantiomers of WV15 were collected separately. Finally, the pooled (+) and (−) fractions were dried using a rotavapor. Purity of the enantiomers was checked on the Chiralcel OD column as well as on the Spherisorb S5-ODS2 C_{18} column and was estimated to be >98%. The absolute configuration of (+) and (−) WV15 was determined using circular dichroism (CD), as described later for mitosene−

DNA monoadducts **4** and **5**. (+)-WV15 was assigned the (*R*), and (−)-WV15 the (*S*) configuration at the C-1 position.

Preparation of Mitosene−DNA Complexes. (1) Chemical Reduction. In a volume of 1 mL, 0.67 μmol of calf thymus or *M. lysodeikticus* DNA was mixed with 0.75 μmol of mitosene in 0.015 M phosphate buffer, pH 6.0. The reaction mixture was purged with argon for 10 min, and subsequently two 25 μL portions of 30 mM $Na_2S_2O_4$ were added at t = 0 and 15 min. The $Na_2S_2O_4$ solution was prepared freshly in argon-purged buffer. After 30 min of reduction, the solution was purged with air, and the reaction mixture was chromatographed immediately on a 56 × 2.5 cm Sephadex G-100 column, using 0.02 M NH_4HCO_3 as eluent. The DNA fraction, eluting in the void volume, was collected.

(2) Enzymatic Reduction. M. lysodeikticus DNA (0.67 μmol), 0.75 μmol of mitosene, and 1.5 units of NADH-cytochrome *c* reductase was dissolved in 1 mL of 0.015 M phosphate buffer, pH 6.0. The reaction mixture was purged with argon for 10 min, and the reaction was started by adding 4.5 μmol of NADH. After 3 min of reduction, the reaction mixture was flushed with air and immediately chromatographed on a Sephadex G-100 column as described above.

(3) Electrochemical Reduction. Mitosenes were reduced in an electrochemical cell, consisting of a mercury pool working electrode, an Ag/AgCl reference electrode and a platinum basket counter electrode. A potential of −510 mV vs Ag/AgCl was used for reduction of the mitosenes. A solution of 1.2 μmol of *M. lysodeikticus* DNA in 0.015 M phosphate buffer, pH 6.0, was purged with nitrogen for 10 min. Subsequently, the solution was reduced at −510 mV vs Ag/AgCl until the current through the solution was constant (after approximately 10 min). The reduction was stopped, and 2.0 μmol of mitosene was added. After purging with nitrogen for another 2 min, the solution was reduced at −510 mV for 30 min. The solution was chromatographed on a Sephadex G-100 column as described above.

(4) H_2/PtO_2 Activation. M. lysodeikticus DNA (0.66 μmol) of and 0.75 μmol of mitosene were mixed together with 90 μg of PtO_2. The solution was deaerated as described above, and hydrogenation was performed for 5 min, followed by purging with argon for 5 min. Further handling was as described above.

Digestion of Mitosene−DNA Complexes. After separation of the mitosene−DNA complexes from unbound mitosene on a Sephadex G-100 column, the mitosene−DNA complexes were digested using the nuclease P_1/SVD/alkaline phosphatase system (Borowy-Borowski et al., 1990). First, 2 units of nuclease P_1 was added to 4 OD_{260} of mitosene−DNA complex in dilute acetic acid, pH 5.5 (1 mL), and the reaction mixture was incubated at 55 °C for 2 h. Subsequently, the pH was adjusted to pH 8.2. Next, 0.9 mM $MgCl_2$ and 16 units of SVD were added, and the mixture was incubated at 37 °C for 1 h. After adding 6.4 units of alkaline phosphatase, the samples were incubated for another 2 h at 37 °C. The digested samples were analyzed on reversed phase HPLC as described above.

Bulk Preparation of Mitosene−Deoxyguanosine Monoadducts. Deoxyguanosine (dG, 670 mg, 2.47 mmol), 150 mg of mitosene WV15 (0.40 mmol), and 48 mg of PtO_2 (0.12 mg/μmol of WV15) were dissolved in 80 mL of 0.015 M phosphate buffer pH 6.0. The mixture was heated at 40 °C until all dG was dissolved. Next, the solution was purged with He for 15 min, followed by H_2 purging for 5.5 min. After purging with He and air (both 5 min), the reaction mixture was loaded in six fractions on a 5.0 × 56 cm Sephadex G-25 column and eluted with 0.02 M NH_4HCO_3. The fractions between 1890 and 2610 mL of the six Sephadex G-25 chromatography runs were pooled and concentrated on a rotavapor to a final volume of 35 mL. This solution was stored at 4 °C until chromatographing it on a preparative C-18 HPLC column.

^{1}H NMR Measurements. After concentrating the pooled fractions of the bulk preparation, the mixtures were injected on preparative HPLC. Using an isocratic 16/84 AcN/H_2O system, adducts **4** and **5** eluted after approximately 75 and 85 min, respectively. The fractions were collected separately, dried on rotavapor, dissolved again in 40% MeOH/H_2O, and the HPLC separation step was repeated. The total yield of this bulk preparation of adducts **4** and **5** was 3.9 and 3.6 mg, respectively. From analysis of the purified samples on HPLC the purity of the adducts was estimated to be >95%. After drying, the samples were used for ^{1}H NMR measurements without further purification, using TOCSY and ^{1}H NMR spectroscopy.

*Determination of CD spectra of 1,10-Bis(acetoxy)mitosene WV15 Enantiomers and Adducts **4** and **5**.* CD spectra were determined on a Jasco 720 spectropolarimeter. The solvent used was methanol. The extinction coefficient of apomitomycin A (**2**) at 346 nm is 3620 (Webb et al., 1962b), and the same value was used for adducts **4** and **5**. Apomitomycin A was prepared by acid hydrolysis of mitomycin A, as described (Taylor & Remers, 1975).

RESULTS

Formation of Adducts of M. lysodeikticus DNA and 7-Methoxymitosene WV15 under Various Activating Conditions. Mitosene−DNA complexes were prepared using either $Na_2S_2O_4$, NADH-cytochrome *c* reductase/NADH, electrochemical reduction, or H_2/PtO_2 as reductive activating agents. The complexes were digested to nucleosides and mitosene−nucleoside adducts. The reaction products were analyzed by HPLC. The HPLC pattern is shown in Figure 1. The unmodified deoxynucleosides are eluted early. Later-eluting peaks, marked 3−7, correspond to putative adducts. These were not detected in control experiments using WV15 without DNA or DNA without WV15. Several peaks, i.e., at t = 9, 10, 14.5, and 20 min (marked with asterisks in Figure 1), appeared to be present in the digest of control samples, originally containing all components, except compound WV15. Therefore, these peaks most likely are products of DNA or digesting enzymes themselves. These peaks were not detected in previously reported, similar experiments using mitomycin C or mitomycin A (Tomasz et al., 1987; McGuinness et al., 1991), probably due to a more polar eluent used in those studies. Use of the four different activating agents resulted in similar patterns, with the notable exceptions of $Na_2S_2O_4$, which failed to yield adducts **6** and **7**, and NADH-cytochrome *c* reductase, which induced an additional component preceding adduct **6**. This product was not futher characterized.

As is evident from Figure 1, the relative amounts of putative adducts 3−5 depend on the type of reduction. In the case of $Na_2S_2O_4$ reduction, relatively more of adduct **3** is formed, while using the other types of reduction, adducts

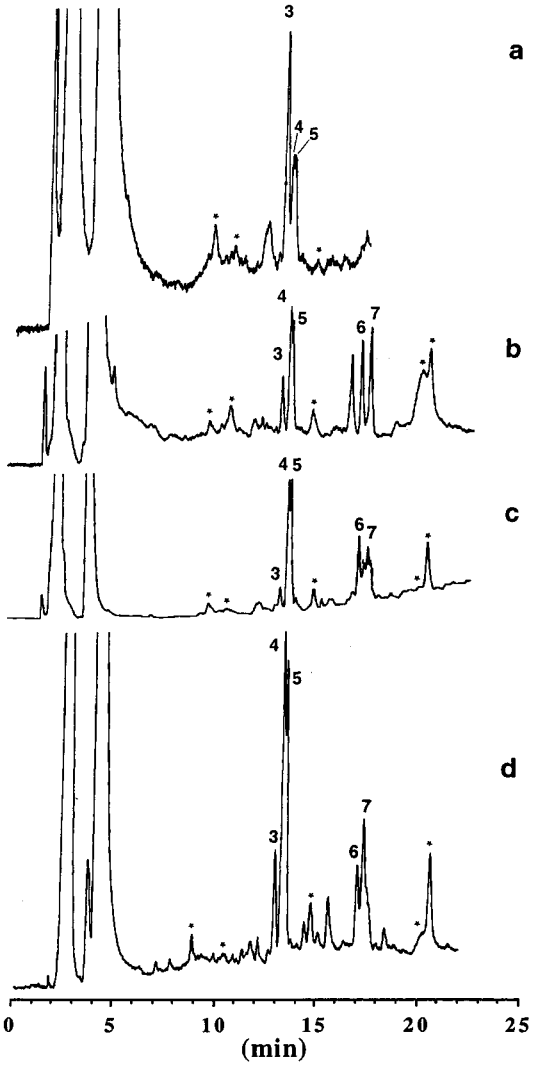


FIGURE 1: HPLC profile, resulting from digestion of WV15-DNA reaction mixtures following various ways of reduction. WV15 (0.075 μmol) was reductively activated in 0.015 M phosphate buffer, pH 6.0, under anaerobic conditions. The following activation methods were used: (a) 2-fold excess $Na_2S_2O_4$; (b) 1.5 units of NADH-cytochrome *c* reductase/4.5 μmol of NADH; (c) electrochemical reduction on a mercury pool working electrode at −510 mV vs Ag/AgCl; (d) H_2/PtO_2 activation (90 μg PtO_2). Digestion of the WV15-DNA mixtures was performed using nuclease P_1/SVD/alkaline phosphatase. Details are described in Materials and Methods.

4 and **5** are more abundant. However, the total amount of adducts 3−5 that was formed upon $Na_2S_2O_4$ reduction was lower than that using the other types of reduction. This reduced yield is likely to be caused by trapping of reduced mitosenes by the dithionite nucleophile (Schiltz & Kohn, 1993a,b). Upon dialysis of the mitosene−DNA complexes against 0.015 M phosphate/1 mM EDTA buffer, pH 7.4, adducts 3−5 appeared to be more stable than adducts **6** and **7**. Because of the abundance (except when using $Na_2S_2O_4$ reduction) and the stability of adducts **4** and **5**, we mainly focused on these two adducts in further experiments.

Previously, DNA cross-linking by mitosenes following chemical reduction has been shown to be pH dependent, with enhanced DNA cross-linking at lower pH (Maliepaard et al., 1993). However, no such pH dependence was observed for the formation of adducts 3−5. Digestion and HPLC analysis of WV15−DNA adduct preparations using $Na_2S_2O_4$ reduction, performed at pH 7.4 and 6.0, yielded identical adduct patterns. At both pH values, comparable amounts of these adducts were formed (not shown).

Requirement of Reduction of the Mitosene for DNA Adduct Formation. In order to study the importance of reduction for DNA adduct formation by WV15, reactions were performed without reduction of the mitosene, both at pH 6.0 and 7.4. These incubation mixtures were kept at 25 °C for 4 days, after which the DNA was separated from unreacted mitosenes on a Sephadex G-100 column and subsequently digested. HPLC analysis of the mitosene-containing fractions from the Sephadex G-100 column showed the presence of various reaction products of WV15. The two main products found were 1-hydroxy-10-acetoxy-7-methoxymitosene and 1,10-dihydroxy-7-methoxymitosene WV14. Also some 1-acetoxy-10-hydroxy-7-methoxymitosene could be detected (not shown). The relatively high amount of C-1 deacetylated product without reduction is in agreement with previously reported observations (Maliepaard et al., 1993): without reduction, loss of the acetoxy group was shown to occur mainly at the C-1 site of WV15, whereas upon reduction, the C-10 acetoxy group is more eager to react (Scheme 3). Upon HPLC analysis of the digested DNA fractions of these reactions, no DNA adduct peaks were detected, thus demonstrating that in the case of WV15, reduction is a prerequisite for adduct formation, both at pH 6.0 and 7.4.

Requirement of Good Leaving Groups at C-1 and/or C-10 for DNA Adduct Formation. Earlier experiments showed the necessity of good leaving groups for DNA cross-link formation following reductive activation with $Na_2S_2O_4$ (Maliepaard et al., 1994). Therefore, we used WV14, which has two poor leaving OH groups at C-1 and C-10 to verify the importance of leaving groups at C-1 and C-10 for the formation of adducts 3−7. In these DNA alkylation experiments, WV14 was reduced at pH 6.0 using $Na_2S_2O_4$ or NADH-cytochrome *c* reductase. No formation of adducts 3−7 was observed in chromatograms of the digestion mixtures (not shown). In addition, the reaction has been monitored without reduction at pH 6.0 and 7.4, analogously as described above for WV15, and again these reactions did not reveal any DNA adduct peaks.

Adducts Formed with Synthetic Polynucleotides. Additional information concerning the identity of the adducts 3−7 was obtained from experiments using polynucleotides instead of DNA in the adduct preparations. Both poly(dA-dT) and poly(dC-dG) were used, and WV15 was activated using the H_2/PtO_2 system or NADH-cytochrome *c* reductase as described in Materials and Methods. Adducts 3−7 were only present when poly(dC-dG) was used (not shown). From this, it was concluded that the adducts that were detected in the presence of DNA are likely to be mitosene-2′-deoxyguanosine adducts.

*The Structures of Adducts **4** and **5**.* The UV spectra of adducts **4** and **5** were identical (Figure 2a) and exhibited characteristic mitosene absorption maxima at 235, 282, and 361 nm, together with the 2′-deoxyguanosine absorption maximum at 256 nm. These spectra are very similar to the UV−vis spectrum that was obtained from a 1:1 mixture of 1,10-dihydroxy-7-methoxymitosene WV14 and 2′-deoxyguanosine [Figure 2b, mitosene absorption maxima at 236, 278, and 355 nm, 2′-deoxyguanosine absorption maximum (shoulder) at 253 nm]. UV−vis spectra of mixtures of WV14 with

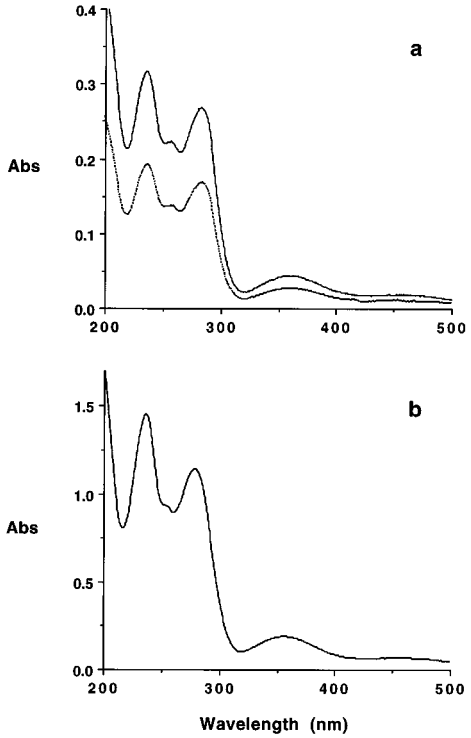


FIGURE 2: UV−vis spectra, recorded in 0.015 M phosphate buffer pH 7.4: (a) adduct **4** (—) and adduct **5** (---); (b) 1:1 mixture of dihydroxymitosene WV14/2′-deoxyguanosine.

other nucleosides yielded different UV−vis spectra (not shown).

Attempts to characterize adducts **4** and **5** with EI and CI mass spectrometry were not successful. However, conclusive evidence for the involvement of 2′-deoxyguanosine in the DNA adduct formation was obtained from positive ion electrospray mass spectrometry. The mixture of adducts **4** and **5** yielded only one mass peak, at $m/z = 527.63$. The calculated mass of adduct **4** or **5** is 526.181 ($C_{24}H_{26}N_6O_8$). The m/z value found corresponds to the $[M + H^+]$of this adduct. The relative stability of adducts **4** and **5** upon heating indicated that these adducts are unlikely to be N^7-2′-deoxyguanosine adducts. After 30 min of heating at 100 °C in 0.015 M phosphate buffer, pH 7.4, only 12% of adducts **4** and **5** was destroyed, as measured from the peak areas on the chromatograms (not shown). Under these heating conditions, N^7-2′-deoxyguanosine adducts are known to be unstable, undergoing complete hydrolysis of the glycosidic linkage (Lawley & Brookes, 1963).

*Role of C-10 as the Active Site for Forming Adducts **4** and **5**.* DNA was reacted with 1-hydroxy-10-ethylcarbamoylmitosene WV16 (Table 1). Due to the presence of a good leaving group only at the C-10 site of this molecule, it was expected to bind to DNA exclusively at that site upon reductive activation. Analysis of the digestion mixture of this reaction indeed revealed the presence of adducts **4** and **5**, and this supports the hypothesis that binding to 2′-deoxyguanosine in these adducts occurs via the mitosene C-10 site. Moreover, the presence of both adducts **4** and **5** in the WV16/DNA digestion mixture suggested that the double peak at 13.5 min (marked **4** and **5**) represents two stereoisomeric forms of the same adduct, due to the chiral atom at C-1, common to both WV15 and WV16. This possibility was verified as follows.

Table 2: Selected ^{1}H NMR Data of Products **4** and **5**

	δ (ppm) in DMSO-d_6	
^{1}H resonance	adduct **4**	adduct **5**
1-N*H*	10.70	10.73
8-C*H*	7.93	7.93
2-N*H*CH_2	6.64	6.68
1″-CHO*H*	5.53	5.61
1″-C*H*OH	5.13	5.13
10″-CH_2NH	4.45, 4.66	4.47, 4.62

*Diastereoisomeric Relationship between Adducts **4** and **5**.* The two adducts have nearly mirror image CD spectra in the 220−300 nm spectral region (Figure 3a). Interestingly, their most intense Cotton effect, at 237 nm, coincides with the most intense Cotton effect of the two enantiomeric forms of the parent drug, (+)- and (−)-WV15 (positive at 235 nm and negative at 237 nm, respectively; Figure 3b). This suggested that monoadduct **4** (positive Cotton effect at 237 nm) and **5** (negative Cotton effect at 237 nm) were derived from (+)- and (−)-WV15, respectively. This was experimentally confirmed by comparing the HPLC patterns of adducts obtained using either racemic (+)- or (-)-WV15 to alkylate DNA (Figure 4). Using a single enantiomer resulted in the elimination of two of the five adducts. The relationship observed between a single WV15 enantiomer and its resulting adduct **4** or **5** was the same as predicted from the CD: (+)-WV15 gave **4** and (−)-WV15 gave **5**. These results strongly suggested that adducts **4** and **5** were diastereoisomers. An analogous relationship was observed for adducts **6** and **7**, but adduct **3** was formed from either WV15 enantiomers (Figure 4).

^{1}H NMR of adducts **4** and **5** confirmed the above conclusions. For the NMR analysis, the adducts were prepared from deoxyguanosine on larger scale, as described in Materials and Methods, in order to obtain sufficient amounts of these adducts. The presence of adducts **4** and **5** in the Sephadex G-25 fractions between 1890 and 2610 mL was demonstrated by HPLC coinjection with original samples from the analytical scale pure preparations of **4** and **5** from DNA and by comparison of UV−vis spectra. No compound **3**, **6**, or **7** was detected in these bulk preparation fractions. The ^{1}H NMR spectrum of adduct **5**, and TOCSY spectra of adducts **4** and **5** are shown in Figure 5. ^{1}H NMR data of the two adducts are summarized in Table 2.

In both ^{1}H NMR spectra of adducts **4** and **5** the 2′-deoxy-β-guanosine characteristics were present [see Wüthrich (1990)]. Importantly, the C-8 proton signal of the guanosine moiety was observed at 7.93 ppm. Therefore, binding of the mitosene to the N-7 position of guanine can be excluded for both adducts **4** and **5**, since the C-8 proton of 7-alkylated guanosines is shifted downfield to above 9 ppm (Hurd & Reid, 1979). Moreover, in the TOCSY spectrum (see Figure 5), cross-peaks were detected between the guanine-N^2 proton signal (at 6.64 ppm for adduct **4**, and at 6.68 ppm for adduct **5**) and the mitosene 10″-CH_α and 10″-CH_β signals (at 4.45/4.66 ppm for adduct **4** and at 4.47/4.62 ppm for adduct **5**). This clearly indicates that the guanine moiety is bonded to the mitosene via a -CH_2-NH-bridge, and that bonding thus occurs via the mitosene C-10″ site. The chemical shifts and coupling pattern of these protons are essentially identical to the analogous protons in the mitomycin C cross-link adduct (Tomasz et al., 1987), and to the 2,7-diaminomitosene−

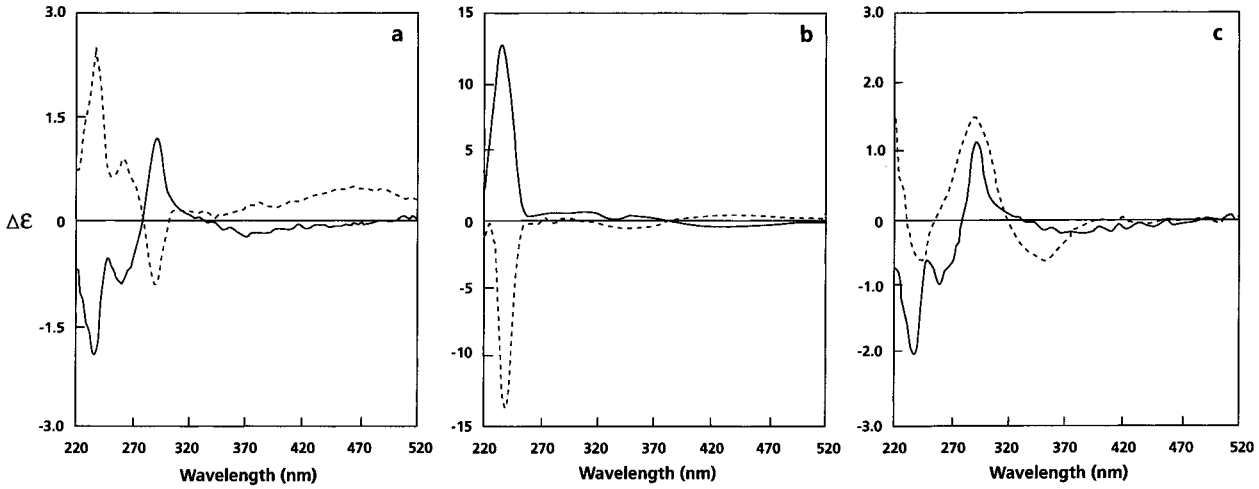
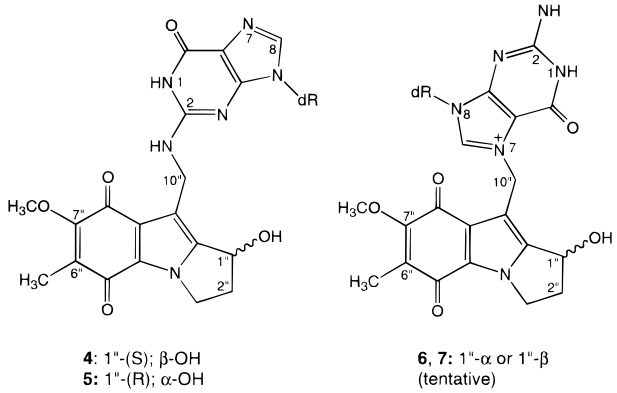


FIGURE 3: CD-spectra: (a) adducts **4** (---) and **5** (—); (b) (+)-WV15 (—) and (−)-WV15 (---); (c) apomitomycin A (---) and adduct **4** (—).

Chart 2 [a]



[a] dR = 2′-deoxyribos-1′-yl.

deoxyguanosine adduct (Iyengar et al., 1990), which contain the same C-10″-guanine-N^2 linkage. The C-1″ site of both adducts **4** and **5** appeared to be hydroxylated, with the 1″-proton signals of adducts **4** and **5** at 5.13 ppm. This value for the 1″-proton signal is close to the value obtained for the C-1 deacetylated product of WV15 (5.26 ppm), as characterized previously (Maliepaard et al., 1993). The 1″-OH proton signal was detected at 5.53 ppm for adduct **4** and at 5.61 ppm for adduct **5**. From these results, adducts **4** and **5** were conclusively characterized as the diastereoisomers N^2-(1″-*n*-hydroxymitosen-10″-yl), 2′-deoxyguanosine ($n = \alpha$ or β, Chart 2). However, the absolute configuration of the C-1″ hydroxyl groups in adduct **4** or **5** could not be determined from these measurements.

In order to determine this absolute configuration, CD measurements were performed. This technique has been used successfully in the determination of the absolute configuration of mitomycin C−DNA adducts (Tomasz et al., 1984). The CD spectra of adducts **4** and **5** (Figure 3a) show an approximate mirror image relationship, despite the fact that **4** and **5** are diastereoisomers. This indicates that the chirality of the C-1″ stereocenter predominantly determines the direction of the Cotton effects, although the deoxyguanosine moiety undoubtedly contributes to the overall shape of the spectrum. An entirely analogous, quasi-mirror image relationship of the CD exists between the C-1″α- and C-1″β-deoxyguanosyl monoadducts of mitomycin C (Gargiulo et al, 1995). In general, CD studies in the mitomycin C-type mitosene series indicated that the presence and chirality of

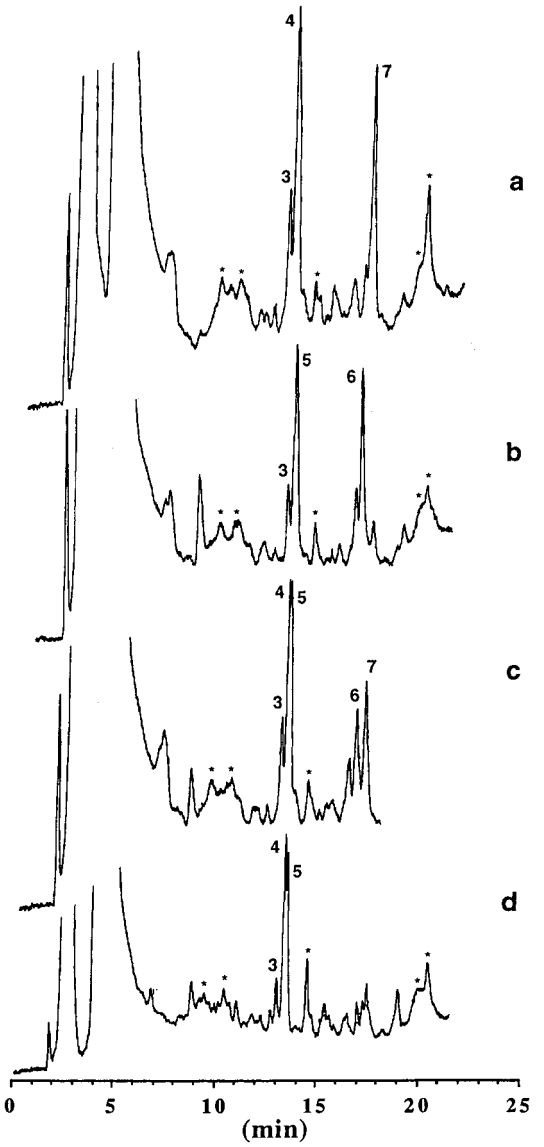


FIGURE 4: HPLC profiles of WV15-DNA digests. WV15 enantiomers were reductively activated using NADH-cytochrome *c* reductase under anaerobic conditions. Digestion of the WV15-DNA mixtures was performed using nuclease P_1/SVD/alkaline phosphatase. (a) (+)-WV15-DNA digest; (b) (−)-WV15-DNA digest; (c) racemic WV15-DNA digest; (d) racemic WV15-DNA digest, following heating for 20 min at 100 °C. Details are described in Materials and methods.

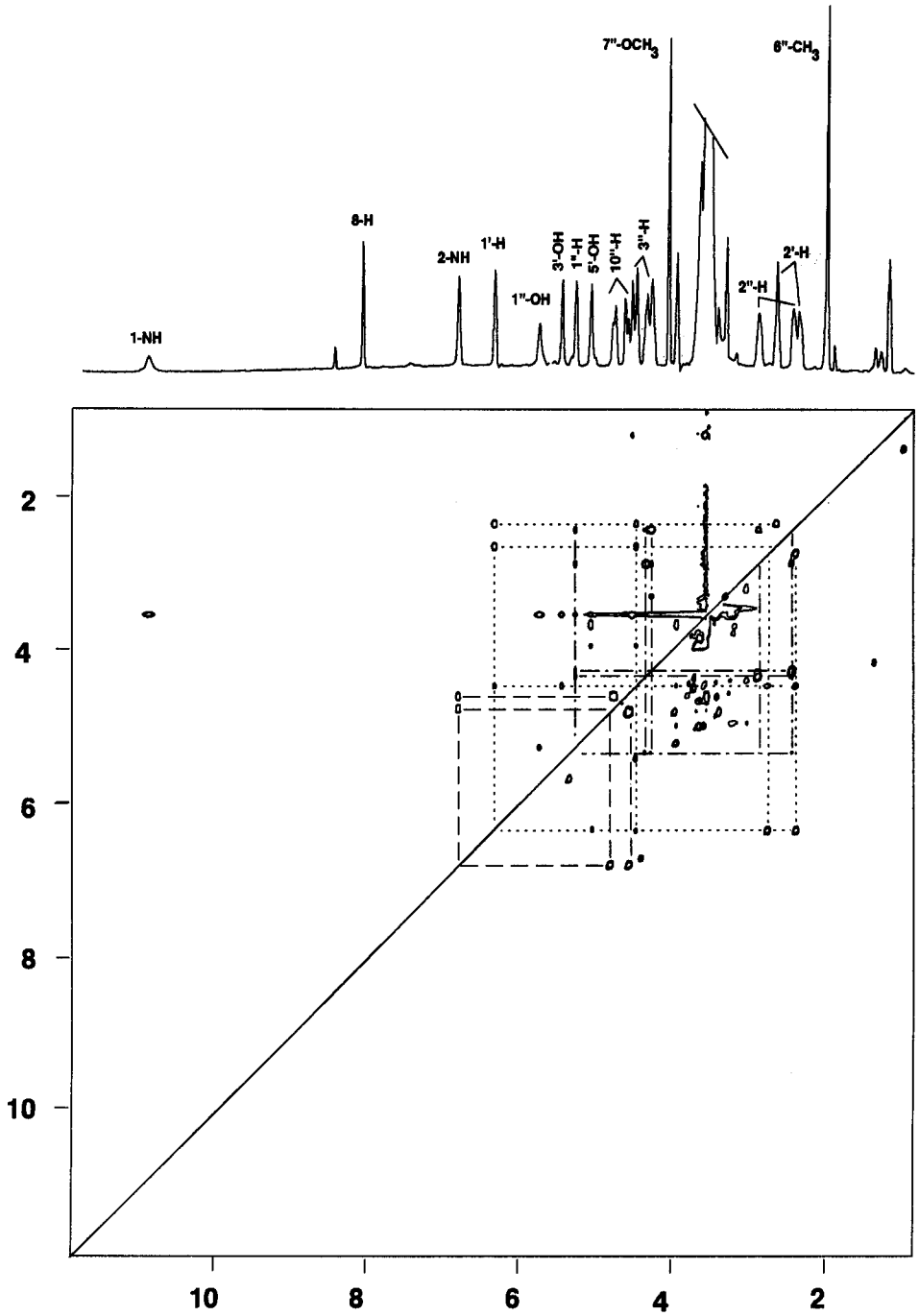


FIGURE 5: ^{1}H NMR spectrum of adduct **5**, and TOCSY spectra of adducts **4** and **5** in DMSO-d_6. TOCSY spectra are combined in one figure. Right lower part, **4**; left upper part, **5**.

a C-2 (same as C-2″ in DNA adducts) substituent does not influence the CD spectrum which, however, responds sensitively to the chirality of the C-1 (C-1″ in DNA adducts) substituent (Tomasz et al., 1984). This is apparent in the comparison of the present, 7-methoxy-mitosene C-1″ enantiomers **4** and **5** also. Deoxyguanosine alone has negligible CD above 280 nm (not shown).

Apomitomycin A (Chart 1, **2**) which has the same mitosene chromophore as well as the same hydroxy substituent in the chiral C-1 position as **4** and **5** displays a prominent positive Cotton effect at 289 nm ($\Delta\epsilon = 1.45$, Figure 3c). We find that this 289 nm Cotton effect of apomitomycin A has the same sign and magnitude as that of adduct **4** (290 nm, $\Delta\epsilon = 1.20$), while it is opposite to that of adduct **5**. On the basis of all these findings, we conclude with confidence that adduct **4** has the same C-1 configuration [(*S*); C-1″β-OH] as apomitomycin A (Taylor & Remers, 1975) while adduct **5** has the (*R*), i.e., C-1″α-OH configuration.

*Structure of Adducts **6** and **7**.* By using the separated enantiomers of WV15, additional information concerning the identity of adducts **6** and **7** was obtained. Experiments with (+)- and (−)-WV15 demonstrated that only one of these two adducts is formed when enantiomerically pure WV15 is used (see Figure 4, panels a and b). Upon heating of the mitosene−DNA complexes at 100 °C for 20 min, adducts **6** and **7** could no longer be detected (see Figure 4d). From these results, two possible structures of adducts **6** and **7** can be hypothesized: (a) heat labile deoxyguanosine-N-7 adducts, or (b) heat labile C-1″ acetoxylated precursors of

adducts **4** and **5.** The latter possibility is based on the fact that heating of a solution of WV15 at 100 °C for 20 min in 0.015 M phosphate buffer, pH 7.4, results in complete loss of acetyl groups (data not shown). However, we tentatively favor deoxyguanosine-N-7 adduct structures for **6** and **7** (Chart 2) on theoretical grounds (see Discussion).

DISCUSSION

The results demonstrate that 1,10-bis(acetoxy)-7-methoxymitosene WV15 is capable of alkylating DNA following reductive activation and that monoadduct formation takes place via the C-10 site of the mitosene which becomes linked to the 2-amino group of a guanine. Since WV15 is a racemic mixture due to the C-1 chiral center, its adduction to deoxyguanosine in DNA resulted in two diastereoisomers **4** and **5**. Monomeric deoxyguanosine is also alkylated by WV15 to give the same adducts **4** and **5**. In addition to the two diastereoisomers of this guanine-N^2 adduct three other guanine−mitosene adducts were detected, **3**, **6**, and **7**. Since these were not formed with deoxyguanosine as substrate, we could not obtain them in sufficient quantities to determine their structures. It is likely that some of them correspond to bis(guanine) adducts since reduced WV15 is known to cross-link DNA (Maliepaard et al., 1993). However, since adducts **6** and **7** are heat-labile, this rules out their being bis(guanine-N^2) adducts in analogy to the mitomycin C bis(guanine-N^2) adduct (Scheme 2). The latter is stable to heat, as guanine-N^2 adducts are in general. Therefore, the diastereoisomeric mitosene adducts **6** and **7** may be guanine-N-7-substituted products which are heat-labile (Chart 2). As precedent, the reduced mitomycin C metabolite 2,7-diaminomitosene was recently shown to alkylate DNA at guanine-N7 (Suresh Kumar et al., 1996). The heat-stable adduct **3**, however, may correspond to a bis(guanine-N^2) cross-link adduct.

Isolation and elucidation of the structure of the WV15 monoadducts **4** and **5** now validates the design of WV15 as bioreductive DNA-alkylating agent, which mimics the action of the prototype mitomycin C. However, there is an interesting difference: **4** and **5** are C-10-substituted monoadducts, in contrast to the monoadduct of mitomycin C, which is formed exclusively at the C-1 (aziridine) position (Tomasz et al., 1988a). Clearly, the relative reactivity of the two alkylating functions at C-1 and C-10 is reversed in WV15 compared to mitomycin C. This was also observed in model reductive hydrolysis experiments (Maliepaard et al., 1993; Scheme 3).

What may be the causes of this reversal? First, the C-1 acetoxy groups of WV15 are less reactive than the aziridine of mitomycin C. In addition, there is substantial evidence that in 7-methoxymitomycins (e.g., mitomycin A; Chart 1), the C-10 position is more reactive than that in the 7-aminomitomycin series (McGuinness et al., 1991; Subramanian & Kohn, 1993). Comparison in the mitosene series is also available. The C-10 carbamate group of various 7-aminomitosenes was hydrolytically stable upon reduction (Sharma & Tomasz, 1994; Suresh Kumar et al., 1996). In contrast, the same group in 7-methoxymitosenes underwent rapid hydrolysis at C-10 when reduced (Maliepaard et al., 1993). These findings provide a rationale for formation of C-10-linked monoadducts of the 7-methoxymitosene WV15, unlike the C-1-linked monoadducts of mitomycin C.

Increased C-10 reactivity of 7-methoxymitosenes as compared to 7-aminomitosenes may also explain the apparent paradox that 2,7-diaminomitosene alkylates DNA to give exclusively a deoxyguanosine-N-7 monoadduct (Suresh Kumar et al., submitted; Prakash et al., 1993) while the 7-methoxymitosene WV15 yields a deoxyguanosine-N^2 monoadduct in major proportion. We propose that this difference reflects S_N2-type reactivity of the C-10 center of the 7-aminomitosene versus S_N1-type reactivity of that of the 7-methoxymitosene. It is well-known that relatively weak, i.e., S_N2-type, alkylators are selective for the strongest nucleophile in DNA, i.e., deoxyguanosine-N-7, while the more reactive, S_N1-type, alkylators are able to react with the less nucleophilic N^2-atom of guanine, among others (Lawley, 1984). It is possible that the unknown, heat-labile adducts of 7-methoxymitosene WV15, **6** and **7** are deoxyguanosine-N-7 adducts. This would indicate a mixed S_N1/S_N2 reactivity of WV15. Elucidation of the structures of these unknown adducts of WV15 would be useful for substantiating the proposed mechanism.

To summarize the results of the comparison between adducts of 7-methoxy and 7-aminomitosenes, it appears that the 7-substituent in the quinone ring influences both the relative reactivity of the C-1 and C-10 alkylating centers and the site of DNA alkylation by these mitomycin analogs. This relationship is relevant for mitomycin analog drug design.

REFERENCES

Andrews, P. A., Pan, S.-S., & Bachur, N. R. (1986) *J. Am. Chem. Soc. 108*, 4158−4166.

Basu, A. K., Hanrahan, C. J., Malia, S. A., Kumar, S., Bizanek, R., & Tomasz, M. (1993) *Biochemistry 32*, 4708−4718.

Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 65*, 355−366.

Bizanek, R., McGuinness, B. F., Nakanishi, K., & Tomasz, M. (1992) *Biochemistry 31*, 3084−3091.

Borowy-Borowski, H., Lipman, R., Chowdary, D., & Tomasz, M. (1990) *Biochemistry 29*, 2992−2999.

Casner, M. L., Remers, W. A., & Bradner, W. T. (1985) *J. Med. Chem. 28*, 921−926.

Cotterill, A. S. Moody, C. J., Mortimer R. J., Norton, C. L., O'Sullivan, N., Stephens, M. A., Stradiotto, N. R., Swann, E., & Stratford, I. J. (1994) *J. Med. Chem. 37*, 3834−3843.

Danishefski, S. J., & Ciufolini, M. (1984) *J. Am. Chem. Soc. 106*, 6424−6425.

Danishefski, S. J., & Egbertson, M. (1986) *J. Am. Chem. Soc. 108*, 4648−4650.

Doroshow, J. H. (1992) *J. Natl. Cancer Inst. 84*, 1138−1139.

Gargiulo, D. Musser, S. S., Yang, L., Fukuyama, T., & Tomasz, M. (1995) *J. Am. Chem. Soc. 117*, 9388−9398.

Gustafson, D. L., & Pritsos, C. A. (1992) *J. Natl. Cancer Inst. 84*, 1180−1185.

Hendriks, H. R., Pizao, P. E., Berger, D. P., Kooistra, K. L., Bibby, M. C., Boven, E., Dreef-van der Meulen, H. C., Henrar, R. E. C., Fiebig, H. H., Double, J. A., Hornstra, H. W., Pinedo, H. M., Workman, P., & Schwartsmann, G. (1993) *Eur. J. Cancer 29A*, 897−906.

Hodnick, W. F., & Sartorelli, A. C. (1993) *Cancer Res. 53*, 4907−4912.

Hoey, B. M., Butler, J., & Swallow, A. J. (1988) *Biochemistry 27*, 2608−2614.

Hong, Y. P., & Kohn, H. (1991) *J. Am. Chem. Soc. 113*, 4634−4644.

Hurd, R. E., & Reid, B. R. (1979) *Biochemistry 18*, 4017−4024.

Islam, I., Skibo, E. B., Dorr, R. T., & Alberts, D. S. (1991) *J. Med. Chem. 34*, 2954−2961.

Iyengar, B. S., Dorr, R. T., Remers, W. A., & Kowal, C. D. (1988) *J. Med. Chem. 31*, 1579−1585.

Iyengar, B. S., Dorr, R. T., Shipp, N. G., & Remers, W. A. (1990) *J. Med. Chem. 33*, 253−257.

Iyer, V. N., Szybalski, W. (1964) *Science 145*, 55−58.
Jones, G. B., & Moody, C. J. (1989a) *J. Chem. Soc., Perkin Trans. 1*, 2449−2454.
Jones, G. B., & Moody, C. J. (1989b) *J. Chem. Soc., Perkin Trans. 1*, 2455−2462.
Keyes, S. R., Fracasso, P. M., Heimbrook, D. C., Rockwell, S., Sligar, S. G., & Sartorelli, A. C. (1984) *Cancer Res. 44*, 5638−5643.
Kohn, H., Zein, N., Lin, X. Q., Ding, J. Q., & Kadish, K. M. (1987) *J. Am. Chem. Soc. 109*, 1833−1840.
Kohn, H., & Hong, Y. P. (1990) *J. Am. Chem. Soc. 112*, 4596−4598.
Kohn, K. W. (1983) in *Molecular Aspects of Anti-Cancer Drug Action* (Neidle, S., & Waring, M. J., Eds.) pp 315−361, Verlag Chemie, Weinheim.
Kumar, S., Lipman, R., & Tomasz, M. (1992) *Biochemistry 31*, 1399−1407.
Lawley, P. D., & Brookes, P. (1963) *Biochem. J. 89*, 127−138.
Lawley, P. D. (1984) in *Chemical Carcinogenesis, ACS monograph 182* (Searle, C. E., Ed.) pp 325−484, American Chemical Society, Washington DC.
Maliepaard, M., de Mol, N. J., Janssen, L. H. M., van der Neut, W., Verboom, W., & Reinhoudt, D. N. (1992) *Anti-Cancer Drug Des. 7*, 415−425.
Maliepaard, M., de Mol N. J., Janssen, L. H. M., Hoogvliet, J. C., van der Neut, W., Verboom, W., & Reinhoudt, D. N. (1993) *J. Med. Chem. 36*, 2091−2097.
Maliepaard, M., Sitters, C. A. M. C., de Mol, N. J., Janssen, L. H. M., Stratford, I. J., Stephens, M., Verboom, W., & Reinhoudt, D. N. (1994) *Biochem. Pharmacol. 48*, 1371−1377.
Maliepaard, M. (1994). Ph.D. Thesis, Utrecht University.
McGuinness, B. F., Lipman, R., Goldstein, J., Nakanishi, K., & Tomasz, M. (1991) *Biochemistry 30*, 6444−6453.
Oostveen, E. A., & Speckamp, W. N. (1987) *Tetrahedron 43*, 255−262.
Orlemans, E. O. M., Verboom, W., Scheltinga, M. W., Reinhoudt, D. N., Lelieveld, P., Fiebig, H. H., Winterhalter, B. R., Double, J. A., & Bibby, M. C. (1989) *J. Med. Chem. 32*, 1612−1620.
Pan, S.-S., Andrews, P. A., Glover, C. J., & Bachur, N. R. (1984) *J. Biol. Chem. 259*, 959−966.
Pan, S.-S., Iracki, T., & Bachur, N. R. (1986) *Mol. Pharm. 29*, 622−628.
Pavlidis, N., Hanauske, A. R., Gamucci, T., Smyth, J., Lehnert, M., te Velde, A., Lan, J., & Verweij, J. (1996) *Ann. Oncol. 7*, 529−531.
Prakash, A. S., Beall, H., Ross, D., & Gibson, N. W. (1993) *Biochemistry 32*, 5518−5525.
Peterson, D. M., & Fisher, J. (1986) *Biochemistry 25*, 4077−4084.
Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479−485.
Rao, G. M., Begleiter, A., Lown, J. W., & Plambeck, J. A. (1977) *J. Electrochem. Soc. 124*, 199−202.
Remers, W. A., & Dorr, R. T. (1988) in *Alkaloids: Chemical and Biological Perspectives 6* (Pelletiers, S. W., Ed.) pp 1−74, Wiley, New York.
Schiltz, P., & Kohn, H. (1992) *Tetrahedron Lett. 33*, 4709−4712.
Schiltz, P., & Kohn, H. (1993a) *J. Am. Chem. Soc. 115*, 10497−10509.
Schiltz, P., & Kohn, H. (1993b) *J. Am. Chem. Soc. 115*, 10510−10518.
Sharma, M., & Tomasz, M. (1994) *Chem. Res. Toxicol. 7*, 390−400.
Siegel, D., Beall, H., Senekowitsch, C., Kasai, M., Arai, H., Gibson, N. W., & Ross, D. (1992) *Biochemistry 31*, 7879−7885.
Skibo, E. B., & Schulz, W. G. (1993) *J. Med. Chem. 36*, 3050−3055.
Subramanian, S., Kohn, H. (1993) *J. Am. Chem. Soc. 115*, 10519−10526.
Suresh Kumar, G., Musser, S. M., Cummings, J., & Tomasz, M. (1996) *J. Am. Chem. Soc. 118*, 9209−9217.
Taylor, W. G., & Remers, W. A. (1975) *J. Med. Chem. 18*, 307−311.
Tomasz, M., & Lipman, R. (1981) *Biochemistry 20*, 5056−5061.
Tomasz, M., Jung, M., Verdine, G. L., & Nakanishi, K. (1984) *J. Am. Chem. Soc. 106*, 7367−7370.
Tomasz, M., Chowdary, D., Lipman, R., Shimotakahara, S., Veiro. D., Walker, V., & Verdine, G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6702−6706.
Tomasz, M., Lipman, R., Chowdary, D., Pawlak, J., Verdine, G. L., & Nakanishi, K. (1987) *Science 235*, 1204−1208.
Tomasz, M., Chawla, A. K., & Lipman, R. (1988a) *Biochemistry 27*, 3182−3187.
Tomasz, M., Lipman, R., McGuinness, B. F., & Nakanishi, K. (1988b) *J. Am. Chem. Soc. 110*, 5892−5896.
Verboom, W., Reinhoudt, D. N., Lammerink, B. H. M., Orlemans, E. O. M., van Veggel, F. C. J. M., Lelieveld, P. (1987) *Anti-Cancer Drug Des. 2*, 271−277.
Webb, J. S., Cosulich, D. B., Mowat, J. H., Patrick, J. B., Broschard, R. W., Meyer, W.E., Williams, R. P., Wolf, C. F., Fulmor, W., Pidacks, C., & Lancaster, J. E. (1962a) *J. Am. Chem. Soc. 84*, 3185−3187.
Webb, J. S., Cosulich, D. B., Mowat, J. H., Patrick, J. B., Broschard, R. W., Meyer, W. E., Williams, R. P., Wolf, C. F., Fulmor, W., Pidacks, C., & Lancaster, J. E. (1962b) *J. Am. Chem. Soc. 84*, 3187−3188.
Wüthrich, K. (1990) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
Zwelling, L. A., Anderson, T., & Kohn, K. W. (1979) *Cancer Res. 39*, 365−369.

BI9700680