Reductive Activation of Potential Antitumor Mitosene Compounds

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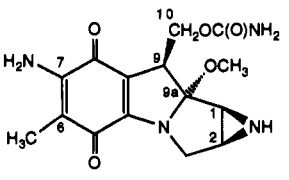
The reductive activation of mitosene compounds was studied with cyclic voltammetry and HPLC analysis. Reduction of mitosenes, possessing good leaving groups at C-1 and C-10, was shown to result in loss of these groups at pH 7.0 and pH 6.0. The loss of leaving groups from mitosenes occurred faster at lower pH. Mitosenes without good leaving groups were found to be stable upon reduction. In the presence of acetoxy groups at C-1 and C-10, the C-10 site is the most reactive site upon reductive activation. This is opposite to the case of mitomycin C, where the C-1 site is the first to react upon reduction. At pH 6.0 without reduction, acid degradation also caused the loss of leaving groups of mitosenes, although at a very slow rate. In contrast to reductive activation, upon acid degradation of a diacetoxymitosene the C-1 group appeared to be lost faster. Electrochemical as well as dithionite reduction of a bifunctional (diacetoxy) mitosene compound in the presence of calf thymus DNA at pH 5.5 resulted in the formation of DNA interstrand cross-links. Depending on activation method, this diacetoxymitosene was at least as efficient in DNA cross-linking as mitomycin C under comparable conditions.

Introduction

Mitomycin C (1) is a representative of the group of bioreductive alkylating species.[1] Reduction of mitomycin C has been shown to result in the formation of reactive species, which are able to form DNA adducts.[2] The bioreductive activation proceeds via reduced mitosene structures, formed after cleavage of the C-9a methoxy group.[3-5] After this cleavage, first the C-1 site of the reduced intermediate becomes accessible for electrophilic or nucleophilic substitution (dependent on the pH and the presence of external nucleophiles),[5] followed by the C-10 site. In the presence of nucleophiles like DNA, nucleophilic attack predominates.[5] Acid-promoted aziridine ring opening at pH 7 is slow enough to permit reoxidation to the C-9a demethoxylated mitosene structure, which can undergo aziridine ring opening by nucleophilic attack.[5] This reaction pattern results in DNA adducts with the mitomycin C C-1, or both C-1 and C-10, covalently linked to the N-2 of a guanosyl residue.[6-8] In the latter case DNA cross-links are formed, which are believed to be important for the antitumor effect of mitomycin C.[9] Apart from reductive activation, also mild acidic conditions can activate mitomycin C to an alkylating species. However, in this case mitomycin C preferentially binds to the guanosyl N-7.[10,11]



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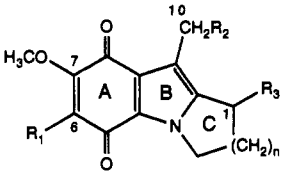
Because of the toxicity of mitomycin C, much attention has been given to the development of analogues with enhanced therapeutic index.[12-14] Various mitosene analogues of mitomycin C have been prepared and tested.[15-20] These studies have shown the necessity of leaving groups at C-1 and C-10 for antitumor activity of mitosenes. Furthermore the effect of C-6 substituents[18] and C-7 substituents,[17,19] together with the size of ring C (see 2), have been evaluated.[20]



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The series of mitosenes, used in the study of Orlemans et al.[20] showed large variation in activity. Some of these compounds showed better *in vitro* activity than the lead compound mitomycin C. Furthermore, some mitosenes appeared to be highly active *in vivo* in a human tumor xenograft.[20] Previous studies showed that the antitumor effect of these mitosene compounds *in vitro* is controlled by lipophilicity.[21] In the same series of mitosenes, no significant correlation was obtained between the half-wave reduction potentials and the *in vitro* antitumor effect.[21] However, this may be partly due to the rather narrow range of half-wave reduction potentials within this series. Earlier work suggested that for mitosane and mitosene analogues cytotoxic effects are dependent on reduction potential.[15,16,22] Compounds with very low or high reduction potentials may not be reductively activated or activated to fast, respectively. Reductive activation is expected to be necessary for DNA alkylation, and cross-link formation may be an important factor governing the antitumor effect of mitosenes.[21] Therefore the activation mechanism of mitosene compounds upon electrochemical reduction was studied, using cyclic voltammetry and HPLC analysis. With cyclic voltammetry measurements, information could be obtained about the stability of mitosenes upon reduction, while various reaction products of mi-

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Table I. Structures of Mitosene Compounds

compd	code[a]	n	R_1	R_2	R_3
3	WV15	1	CH_3	$OC(O)CH_3$	$OC(O)CH_3$
4	WV1	2	H	OH	OH
5	WV8	1	H	OH	OH
6	WV14	1	CH_3	OH	OH
7	WV21	1	CH_3	H	H
8	WV17	1	CH_3	$OC(O)CH_3$	H
9	WV16	1	CH_3	$OC(O)N(H)C_2H_5$	OH
10	WV22	1	CH_3	H	$OC(O)CH_3$
11	WV2	2	H	$OC(O)CH_3$	$OC(O)CH_3$
12	WV4	2	H	$OC(O)CH_3$	OH
13	WV5	2	H	$OC(O)N(H)C_2H_5$	OH
14	WV7	1	H	$OC(O)CH_3$	$OC(O)CH_3$
15	WV10	1	Br	$OC(O)CH_3$	$OC(O)CH_3$
16	WV18[b]	1	CH_3	$OC(O)CH_3$	$OC(O)CH_3$

[a] Refers to internal coding. [b] Aziridinyl group at C-7.

tosenes could be detected with HPLC analysis. The DNA alkylating activity of mitosene nucleophiles was evaluated by measuring the formation of DNA cross-links by the bifunctional compound 3 (Table I) in comparison with mitomycin C.

Results and Discussion

The structures of the mitosenes (basic structure 2) used in this study are listed in Table I. These mitosenes generally possess one or two good leaving groups (e.g. acetoxy or ethyl carbamate groups) at C-1 and/or C-10, and are therefore regarded as either monofunctional or bifunctional alkylating agents. In this series, some mitosenes were present which lack good leaving groups at both the R_2 and R_3 sites (e.g. compounds 4, 5, 6, and 7). All compounds except 7 and 8 contain a chiral center at C-1. AM1 calculations showed the pyrrole–indole ring system to be almost flat (data not shown), resulting in the existence of two enantiomeric forms for most mitosenes used in this study. The experiments described in this paper have been performed using a racemic mixture of these two enantiomeric forms. For reasons of clarity, mitosene compounds possessing a 6-membered ring C are numbered in the same way as those with a 5-membered ring with respect to the C-1, C-6, C-7, and C-10 (see 2).

Cyclic Voltammetry. Cyclic voltammetry (CV) measurements were used to study the fate of mitosenes upon reduction. Results are summarized in Table II. An example of the cyclic voltammogram of a dihydroxylmitosene, i.e. 6 at pH 7.4 at a scan rate of 100 mV/s, is shown in Figure 1. This cyclic voltammogram of 6 displayed a cathodic peak at −315 mV and an anodic peak at −284 mV. The cathodic current (i_{pc}) was approximately equal to the anodic current (i_{pa}) and the difference in peak potentials (ΔE_p) of 31 mV is in reasonable agreement with the theoretical value of $\Delta E_p \approx 59/n$ mV (n = number of electrons involved) for a reversible two-electron reduction at room temperature (21 °C).[23] With multicycle scanning, no new peaks developed, while also variation of the scan rate in a range from 20 to 500 mV/s did not influence the characteristics of the cyclic voltammogram of 6 (Table II), indicating the stability of this compound upon reduction. For the other two dihydroxyl compounds 4 and 5 and the unsubstituted 7 (at a scan rate of 500 mV/s) comparable results were obtained (Table II).

For mitosene compounds possessing good leaving groups R_2 and/or R_3 the cyclic voltammograms became more complex, as is shown in Figure 2 for ethyl carbamate 9. At a scan rate of 500 mV/s (Figure 2a, Table II), a cathodic peak developed at −305 mV with a poorly resolved second cathodic peak at a potential approximately 30 mV more negative. The anodic peak appeared at −303 mV. Futhermore two (small) cathodic peaks were present at −1153 and −1265 mV. Change of the switching potential from −1.4 to −0.7 V resulted in a slight shift of the anodic peak from −303 to −291 mV (Figure 2a). At lower scan rates (Figures 2b and c), the relative height of the second cathodic peak at −335 mV increased markedly. The development of a second cathodic peak clearly indicates the conversion upon reduction of 9 into a compound with reduction characteristics different from 9. The same effects were detected for all mono- or bifunctional mitosene compounds used in this assay (Table II). With multicycle scanning the cathodic and anodic peaks in the cyclic voltammograms of these compounds start drifting apart (data not shown), which indicates that the new product formed in this assay is not likely to be the related dihydroxylmitosene. The possible formation and detection of phosphate adducts was checked by recording a cyclic voltammogram of 9 in 0.1 M Tris-HCl buffer pH 7.4 instead of phosphate buffer. This resulted in comparable characteristics of the cyclic voltammogram. Therefore phosphate adduct formation is unlikely to be responsible for the detection of a new product upon reduction and for

Table II. Cathodic Peak Potentials (E_{pc}) and Anodic Peak Potentials (E_{pa}) in Cyclic Voltammograms between 0 and −1400 mV vs Ag/AgCl Reference of Mitosene Compounds in 0.1 M Phosphate Buffer, pH 7.4, at Scan Rates of 500, 100, and 20 mV/s

	$-E_{pc}$ (mV)/$-E_{pa}$ (mV)		
compd	500 mV/s	100 mV/s	20 mV/s
3	299 sh[a]/310	296 sh/301	297–340[b]/nd[c]
4	362/332	361/330	364/330
5	334/304	341/312	343/312
6	315/285	315/284	316/283
7	348/320	nm[d]	nm
8	312–369[b]/345	293–366[b]/346	278–361[b]/327
9	305 sh/303	305–335[b]/303	sh 335/303
10	351/327	351 sh/327	366 sh/322
11	336/336	nm	344/nd
12	322–390[b]/336	326–380[b]/322	317–405[b]/322
13	294–361[b]/309	sh 356/282	sh 366/271
14	nm	338 sh/289	332–381[b]/268
15	225–298[b]/nd	234–317[b]/254	sh 322/234
16	356/424	356 sh/444	nm

[a] sh, shoulder. [b] Two peaks present as shown in Figure 2b. [c] nd, not detectable. [d] nm, not measured.

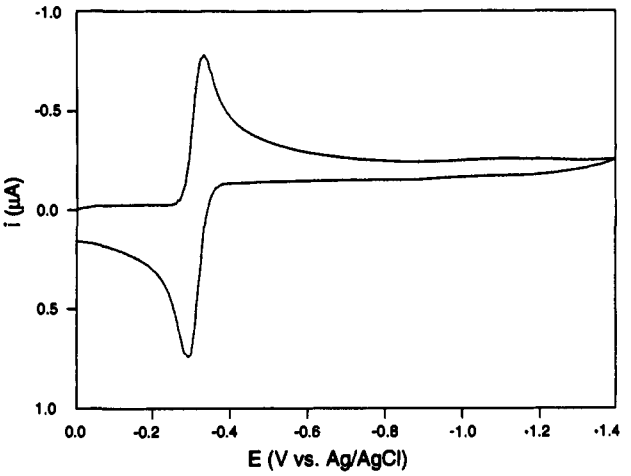


Figure 1. Cyclic voltammogram of 100 μM 6 in 0.1 M phosphate buffer, pH 7.4, at a scan rate of 100 mV/s between 0 and −1.4 V vs Ag/AgCl.

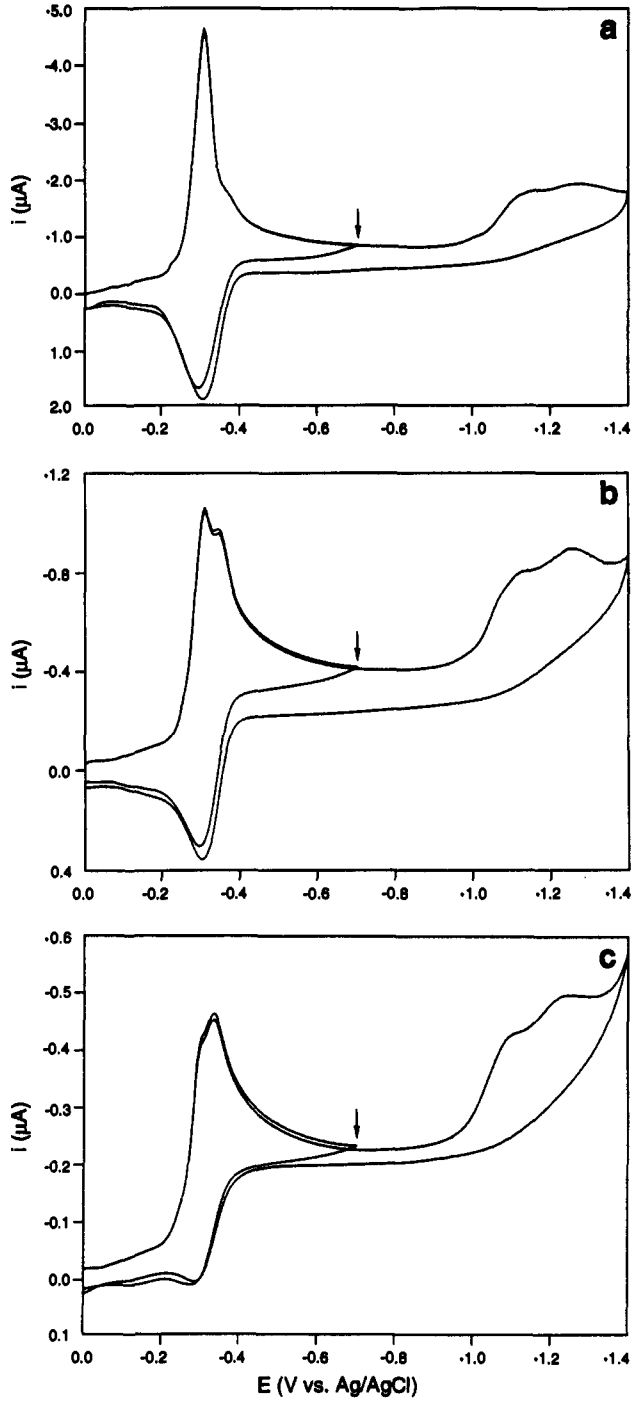


Figure 2. Cyclic voltammograms of 100 μM **9** in 0.1 M phosphate buffer, pH 7.4, between 0 and −0.7 or −1.4 V vs Ag/AgCl: ↓, switching potential, −0.7 V; a, scan rate, 500 mV/s; b, scan rate, 100 mV/s; c, scan rate, 20 mV/s.

drifting apart of the cathodic and anodic peaks in multicycle scanning experiments. These phenomena can most probably be explained by adsorption of a mitosene intermediate at the working mercury electrode surface. This adsorption at the mercury surface only takes place with mitosenes possessing good leaving groups at C-1 and/or C-10. Also compounds **8** and **10**, which are unsubstituted at C-1 or C-10, respectively, displayed this effect (Table II). Therefore it is plausible to state that the C-1 and C-10 sites are involved in reactions leading to the observed changes in the cyclic voltammogram of mono- and bifunctional mitosenes and that these changes are a qualitative indication for reactivity of the various compounds. The identity of the −335 mV compound formed upon reduction of **9** could not be determined from CV measurements. Possibly an intermediate mitosene product is detected, which would normally be subject to further reaction, but under these conditions is captured at the mercury surface.

Table III. Cathodic Peak Potentials (E_{pc}) and Anodic Peak Potentials (E_{pa}) in Cyclic Voltammograms between 0 and −1400 mV vs Ag/AgCl Reference of Mitosene Compounds in 0.1 M Phosphate Buffer, pH 6.0

	$-E_{pc}$ (mV)/$-E_{pa}$ (mV)	
compd	500 mV/s	100 mV/s
3	223 sh[a]/219	214 sh/209
6	228/204	228/202
9	225 sh/215	226–265[b]/210

[a] sh, shoulder. [b] Two peaks present.

Effect of pH. The pH of a tumor cell may be different from the pH of the surrounding tissue. In some tumor cells the internal pH may be lowered,[24] although this lower pH may be restricted to the extracellular fluid.[25] The effect of lowering the pH from 7.4 to 6.0 on the cyclic voltammogram is shown in Table III. The pH change to pH 6.0 resulted in roughly 85 mV less negative E_{pc} and E_{pa} values than at pH 7.4 (Table II) for the three mitosenes studied (**3**, **6**, and **9**). These differences match nicely with the expected pH dependence of the half-wave reduction potential of about 60 mV/pH unit for a two-electron, two-proton process. Lowering the pH did not affect the relative values of the cathodic (i_{pc}) and the anodic current (i_{pa}). Lowering the scan rate from 500 to 100 mV/s at pH 6.0 resulted in comparable changes of the cyclic voltammogram of compounds **3** and **9** as in the case of the pH 7.4 measurements, i.e. increase in relative height of the second cathodic peak at lower scan rate. Also at pH 6.0 multicycle scans and variation of scan rates (from 100 to 500 mV/s) had no influence on the CV characteristics of **6**, while peaks start drifting apart upon multicycle scanning for the other two compounds, tested at pH 6.0 (**3** and **9**). Therefore, CV measurements revealed that no significant other chemical processes occurred upon reduction of mitosenes at pH 6.0 than at pH 7.4. However, lower pH leads to less negative half-wave reduction potentials, as indicated by the higher E_{pc} and E_{pa} values, and therefore the reductive activation of mitosene compounds is expected to be enhanced at pH 6.0.

HPLC Analysis. In order to study the product formation in more detail, reduction of various mitosene compounds was followed by HPLC analysis. With this method conversion of mitosenes and eventually formed reduction products can be detected. Progress of the reduction process was followed by recording the current through the solution as a function of time (chronoamperometry). This current indicates the electron flow to the mitosene and will exponentially drop until reduction is completed.

Conversion rates upon reduction of a series of mitosenes were determined using the HPLC assay by analyzing samples at various time intervals, while keeping the potential at a constant value of −510 mV. In agreement with results obtained from CV measurements, the two dihydroxyl compounds used in this assay (**4** and **6**) were shown to be stable under reduction at both pH 7.4 and 6.0, while mitosenes possessing good leaving groups R_2 and/or R_3 were converted upon reduction. The initial conversion rate of mitosene compounds with good leaving groups R_2 and/or R_3 in this assay at pH 7.4 varied considerably, as

Table IV. Initial Conversion Rate of Various Mitosene Compounds at a Concentration of 100 μM upon Electrochemical Reduction under Nitrogen in 0.1 M Phosphate buffer, pH 6.0 and 7.4, at −510 mV vs Ag/AgCl at 25 °C[a]

compd	conversion rate (nmol/min)	
	pH 6.0	pH 7.4
3	126.2 ± 2.8[a]	56.2 ± 14.0
4	9.2 ± 0.0	0.0
6	2.2 ± 0.0	0.0
9	109.9 ± 9.2	49.4 ± 1.9
11	43.2 ± 4.9	17.7 ± 6.7
12	36.4 ± 2.0	9.8 ± 0.1
14	56.4 ± 13.8	20.2 ± 0.4
15	nd	157.1 ± 37.1

[a] Values are means ± SD from at least three experiments.

shown in Table IV. The conversion rate at pH 7.4 for these mono- and bifunctional mitosenes is significantly correlated with the half-wave reduction potential ($E_{1/2}$), as determined previously:[21]

$$\text{conversion rate (nmol/min)} = 433 + 1.29E_{1/2}\,\text{(mV)} \quad \text{(1a)}$$

$$n = 6, r^2 = 0.95, s = 13.0, F = 79.75$$

Changing the pH from 7.4 to 6.0 resulted in more than doubled conversion rates upon reduction at −510 mV (Table IV). This is in agreement with the less negative E_{pc} and E_{pa}, and therefore higher $E_{1/2}$, observed in CV measurements at lower pH, as mentioned above.

Reduction of mitosene compounds with good leaving groups at R_2 and/or R_3 was shown to result in the formation of various products. A typical series of HPLC chromatograms, following the reduction of the diacetoxy compound **3** at −510 mV vs Ag/AgCl at pH 6.0 as a function of time, is shown in Figure 3. Two main products were formed upon reduction of **3**, denoted **I** and **II**, together with some early eluting products. The early eluting products were not characterized, but, since these peaks are absent in 0.1 M Tris-HCl buffer pH 7.4, these are most likely mitosene phosphate adducts. These early eluting phosphate adducts have also been reported for mitomycin C.[26,27] Product **I** was isolated on a milligram scale as described in the Experimental Section and was subsequently characterized by ^{1}H-NMR measurements [selected ^{1}H-NMR data ($CDCl_3$): δ 6.05 (dd, J = 1.7 and 6.5 Hz, C*H*CO_2CH_3), 4.71 (AB q, 2H, J = 14.2 Hz, C*H*$_2$OH), 4.02 (s, 3H, OCH_3), 2.07 (s, 3H, CO_2CH_3), 1.98 (s, 3H, CH_3)]. This ^{1}H-NMR spectrum was compared with the ^{1}H-NMR spectrum of **3**.[20] The C-1 proton signal of **3** at 6.05 ppm was also present in the NMR spectrum of product **I**. However, the C-10 two-proton signal of **3** at 5.29 ppm was shifted to 4.71 ppm, and only one three-proton signal was present at 2.07 ppm (six protons involved in the case of **3**), indicating the replacement of the C-10 acetoxy group by a hydroxyl group. The other ^{1}H-NMR signals exhibited by product **I** were also present in the ^{1}H-NMR spectrum of **3**. Reduction of diacetoxy mitosene **3** in 0.1 M phosphate buffer therefore results in deacetylation at the C-10 position first. Upon further reduction, this C-10 deacetylated compound is converted into product **II**. Product **II** has first roughly been characterized by means of retention times of the authentic dihydroxylmitosene as compound **6**. This characterization was confirmed by ^{1}H-NMR measurements: The acetylic proton signals of compound **3** around 2.07 ppm were completely absent, and the presence of a one- and two-proton signal at 5.26 and 4.71 ppm, respectively, indicates the presence of hydroxyl groups at both the C-1 and C-10 position of the mitosene. Finally, compound **II** was characterized by CV measurements. For this purpose a solution of reduced **9** at pH 6.0, which was totally converted into **II** as appeared by HPLC analysis, was used to record a cyclic voltammogram. This voltammogram was identical with that of dihydroxyl **6** (data not shown).

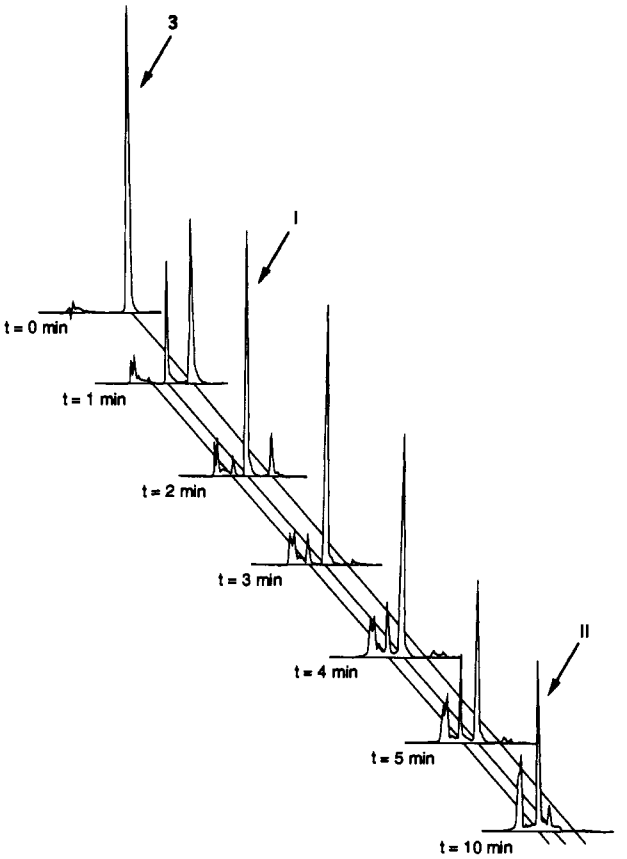


Figure 3. HPLC analysis as a function of time upon reduction of 100 μM **3** at −510 mV vs Ag/AgCl in 0.1 M phosphate buffer, pH 6.0.

For all mitosenes studied, the only detectable reduction products were the corresponding hydroxyl compounds and the phosphate adducts. It is evident that both leaving groups do not split off at the same time, but that the C-10 is the most reactive site upon reduction of the mitosene compounds studied. This reaction pattern is opposite to that found for mitomycin C, where the C-1 is the most reactive site of the molecule.[6]

The loss of leaving groups R_2 and R_3 obviously is dependent on reduction. Without reduction no formation of metabolites was detected within 1 h at both pH 7.4 and 6.0. However, upon incubation at pH 6.0 for longer periods (±1 day) some degradation products could be detected. HPLC analysis suggested that from the diacetoxy compound **3** again a deacetylated mitosene was formed under these mild acidic conditions, but retention times were not completely equal to that of product **I**, formed upon reduction of **3**. ^{1}H-NMR characterization of this acid degradation product as described in the Experimental Section [selected ^{1}H-NMR data ($CDCl_3$): β 5.29 (AB q, 2H, J = 12.2 Hz, C*H*$_2CO_2CH_3$), 5.26 (dd, 1H, J = 1.8 and 6.4 Hz, C*H*OH), 4.02 (s, 3H, OCH_3), 3.79 (s, 1H, OH), 2.09 (s, 3H, CO_2CH_3), 1.96 (s, 3H, CH_3)] revealed that instead of the C-10 acetyl group the C-1 acetyl group was split off, resulting in the corresponding 1-hydroxy-10-acetoxymi-

Table V. Formation Rate of Dihydroxyl Compound 6 upon Electrochemical Reduction of 100 μM 3 and 9 and Formation Rate of Dihydroxyl Compound 4 upon Reduction of 100 μM and 11 and 12 under Nitrogen in 0.1 M Phosphate buffer, pH 6.0 and 7.4, at −510 mV vs Ag/AgCl at 25 °C[a]

	formation rate (nmol/min)	
original compd	pH 6.0	pH 7.4
3	2.6 ± 0.7[a]	0.7 ± 0.0
9	97.4 ± 3.3	5.3 ± 1.5
11	7.0 ± 0.0	0.0
12	21.8 ± 0.4	0.8 ± 0.0

[a] Values are means ± SD from at least three experiments.

Table VI. Percent DNA Cross-Linking of Diacetoxymitosene 3 and Mitomycn C upon Electrochemical Reduction at −510 mV vs Ag/AgCl and $Na_2S_2O_4$ Reduction (2-fold excess) in 30 mM Tris-Acetate Buffer, pH 5.5, at 25 °C

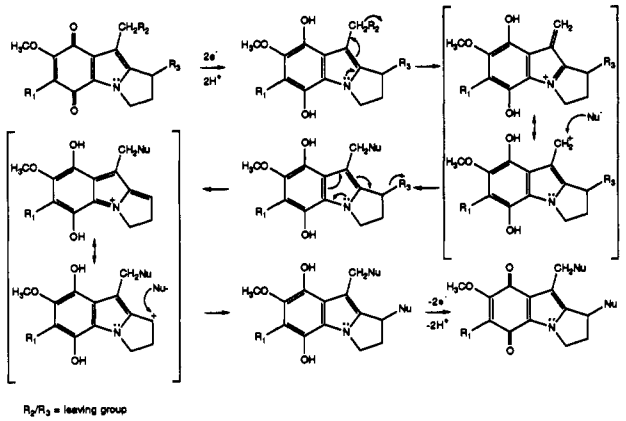
	% cross-links	
compd	electrochemical	$Na_2S_2O_4$
3	83.7 ± 3.7	44.0 ± 9.0
MC	35.0 ± 3.0	55.3 ± 7.7

tosene. This structure was evident from the shift of the C-1 proton signal to 5.26 ppm (6.05 ppm for 3), and the presence of the C-10 two-proton signal at 5.29 ppm, as found in the ^{1}H-NMR spectrum of 3. Again, as with reduction product I, only one acetylic three-proton signal was present, at 2.09 ppm. Furthermore, the C-1 hydroxyl proton signal appeared at 3.79 ppm. The dihydroxylmitosene 6 was formed as the eventual degradation product of 3 without reduction at pH 6.0, but at a very slow rate.

Reduction at pH 6.0 not only resulted in increased conversion rates of the mitosenes but also in a different product distribution. Comparison of Tables IV and V demonstrates that at lower pH relatively more dihydroxyl products are formed upon reduction of all mono- or bifunctional mitosenes. For example, at pH 6.0 9 is almost completely converted (±90%) into 6, whereas at pH 7.4 only approximately 10% of the original 9 ends as 6 after reduction.

DNA-Adduct Formation. The results of the CV measurements and HPLC analyses following bulk reduction support the hypothesis that reduction of mitosene compounds leads to the formation of reactive intermediates. Nucleotide derivatives of 2,7-diaminomitosene have been shown to be able to alkylate DNA (using catalytic reduction in a palladium/H_2 system), and the ability of these mitosenes to form DNA cross-links was suggested.[28] Diacetoxy 3, as a bifunctional mitosene, could be capable of cross-linking DNA upon reduction. The alkylating ability of this compound 3 was tested in the ethidium bromide DNA cross-linking assay with electrochemical as well as dithionite activation (Table VI). Indeed the assay of DNA cross-links revealed that electrochemical reduction of 3 in the presence of calf thymus DNA under anaerobic conditions resulted in a strongly accelerated return of ethidium bromide fluorescence after denaturation of the DNA, indicating the formation of interstrand DNA cross-links. Mitomycin C, which is an established DNA cross-linking agent,[29] resulted under these circumstances in a lower amount of DNA cross-links. However, after 30 min of electrochemical reduction at −510 mV vs Ag/AgCl, only 70% of mitomycin C was converted, in contrast to 3, which was totally converted within approximately 5 min. This difference in conversion, however, seems not enough to explain the large difference in the amount of cross-links formed by the two compounds upon electrochemical reduction. Formation of DNA cross-links by mitomycin C has been shown to be strongly dependent on activation methods.[7,30] Interestingly, using a 2-fold excess of $Na_2S_2O_4$, an increased amount of cross-links formed by mitomycin C, relative to that of 3, was noticed (Table VI) (Under these circumstances, both 3 and mitomycin C were almost completely converted after 30 min of reduction.) Apparently the presence of $Na_2S_2O_4$ in the reaction mixture has a positive effect on DNA-adduct formation by mitomycin C, indicating a possible contribution of mitomycin C sulfonate adducts in this process, as suggested by Schiltz.[31] The combined results demonstrate that 3 is an efficient DNA cross-linking agent under anaerobic conditions and illustrate the formation of DNA-alkylating species upon reduction fo mono- or bifunctional mitosene compounds.

Scheme I



Conclusions

HPLC and CV results allow us to propose a mechanism for reductive activation of these mitosene compounds in the presence of external nucleophiles (Scheme I), which differs from that for mitomycin C[32] with respect to the order of nucleophilic attack at C-1 and C-10. After reduction of the mitosene compound and following delocalization of the nitrogen lone pair electrons, the loss of leaving group R_2 is facilitated. This leaves the C-10 site accessible for nucleophilic attack. Activation of the C-1 site can subsequently take place in an analogous way. After reoxidation a mitosene, at two sites linked to a nucleophile, results. The scheme suggests a mitosene C-10 linked DNA monoadduct first to be formed upon reduction in the presence of DNA. These types of mitosene–DNA adducts have never been isolated, except for a 2,7-diaminomitosene and a 2,7-diamino-1-hydroxymitosene, derived from mitomycin C, lacking the C-1 functionality.[33] Probably 3, and also other diacetoxymitosenes, are capable of forming these C-10 monoadducts in the presence of a functional group at C-1. The different reaction pattern results in a different active species, which could possibly have implications for the sequence preference of these mitosenes. This preference may be different from the 5′-CG preference of mitomycin C,[29,34,35] which is possibly determined already at the monoalkylation step.[36-38] AM1 calculations demonstrate that the mitosenes, without an aziridinyl group at C-1 and C-2, are planar molecules (data not shown). In analogy with mitomycin C, these flat mitosene structures could fit snugly into the minor groove,[39] thereby possibly entering the DNA in the righ position for DNA cross-linking.

The bifunctional mitosene 3 has been shown to be highly active in various *in vitro* tumor models.[21] The observed DNA cross-linking, resulting from the interaction of reductively activated 3 and calf thymus DNA, supports the hypothesis that DNA cross-links are important for antitumor activity of mitosenes. Studies are under way to investigate the DNA bis-adduct formation in relation to the DNA damaging effects of these mitosene compounds in more detail.

In conclusion, reduction of mitosenes leads to the formation of alkylating intermediates. Reactive intermediates, derived from bifunctional mitosenes, are able to form DNA interstrand cross-links. Although the *in vitro* activity of these bifunctional mitosenes was shown to be controlled by lipophilicity of the compounds,[21] the DNA cross-link formation may be a crucial factor determining the eventual tumor cell death caused by mitosenes.

Experimental Section

Mitosenes were synthesized as described.[20,21] Purity of all compounds, as checked with HPLC, was >96%. Mitomycin C was obtained from Bristol-Myers Co. *N,N*-Dimethylformamide (DMF) p.a. was obtained from Baker (Deventer, The Netherlands). Methanol (HPLC quality) was purchased from Westburg (Leusden, The Netherlands). Calf thymus DNA was from Sigma (Amsterdam, The Netherlands). Stock solutions of mitomycin C or mitosene compounds in DMF (10 mM) were used in all experiments, unless stated otherwise. These stock solutions were stored at 4 °C in the dark. No degradation of compounds was detected in a month time.

UV–vis spectra were recorded on a Perkin-Elmer Lambda 5 spectrophotometer. Analyses were performed with a semiautomatic HPLC system using UV detection. Separations were carried out on a Spherisorb S5-ODS2 column (Phase Separations, Waddinxveen, The Netherlands), typically at a flow rate of 1 mL/min, using various mixtures of MeOH/H_2O (70/30 to 90/10 v/v), to which 1% 0.5 M phosphate buffer, pH 7.4, was added. ^{1}H-NMR measurements were performed with a Bruker AC 250F spectrometer.

Electrochemical Measurements. Cyclic voltammetry (CV) experiments were performed at room temperature with an electrochemical cell consisting of an EG&G 303 hanging mercury working electrode, an Ag/AgCl (saturated KCl) reference electrode, and a platinum wire auxiliary electrode. A computer-controlled potentiostat (Autolab, Eco Chemie B.V., Utrecht, The Netherlands) with the GPES 2.2 software package was used. Cyclic voltammograms of 100 μM mitosene solutions in 0.1 M phosphate buffer pH 7.4 or 6.0 were recorded after purging with nitrogen for 10 min. Nitrogen used in electrochemical experiments was passed through a methyl viologen/proflavine photochemical system (modified from ref 40) in order to remove traces of oxygen. Reduction/oxidation cycles were performed between 0 and −1.4 V vs Ag/AgCl at various scan rates, typically 20, 100, and 500 mV/s. The solutions remained under nitrogen atmosphere during scanning.

Electrochemical Activation of Mitosene Compounds. Bulk reduction of mitosene compounds was carried out at 25 °C. A mercury-pool working electrode, an Ag/AgCl (saturated KCl) reference electrode, and a platinum wire auxiliary electrode were used in this assay. The extent of reduction of the mitosenes was checked by chronoamperometrical measurements. Prior to reduction, 2970 μL of 0.1 M phosphate buffer was purged with oxygen-free nitrogen for 10 min. The potential of the working electrode was subsequently kept at −510 mV vs Ag/AgCl until the current was constant. The cell was switched off and 30 μL of 10 mM mitosene stock solution in DMF (final concentration of mitosene, 100 μM) was added. After purging with nitrogen for another 2 min, mitosene compounds were reduced at −510 mV vs Ag/AgCl, again with simultaneously nitrogen purging. Samples were taken at various time intervals and were analyzed with HPLC after allowing reoxidation under air.

Isolation and Identification of Deacetylated Reaction Products of 3. Compound 3 (15 mg, 41.5 μmol) was dissolved in DMF (300 μL) and added to 0.1 M phosphate buffer (5 mL), pH 6.0. This solution was reduced at −510 mV vs Ag/AgCl as described above. After 60 min of reduction, approximately 30% of 3 was converted into the monodeacetylated product, as estimated from HPLC analysis. The final reaction mixture was lyophilized, which was repeated after desalting by addition of MeOH. The various products were dissolved in CH_2Cl_2 and separated on a 25-cm SiO_2 column, using a 1:1 CH_2Cl_2/EtOAc mixture as eluent. The monodeacetylated product was collected and characterized by ^{1}H-NMR spectroscopy.

The monodeacetylated acid degradation product of 3 was obtained by keeping 3 (10 mg, 28 μmol) at pH 6.0 for 5 days. A yield of approximately 40% monodeacetylated product was obtained. Purification and identification was performed as described for the reduction product of 3.

DNA Interstrand Cross-Linking of Mitomycin C and 3. A. Electrochemical Reduction. 30 mM Tris-acetate buffer, pH 5.5 (3 mL), containing 350 μg/mL calf thymus DNA was purged with nitrogen for 10 min, after which the solution was reduced at −510 mV vs Ag/AgCl at the mercury-pool working electrode until the current was constant. Reduction was stopped, and 3 or mitomycin C stock solution in DMF was added (final concentration mitosene or mitomycin C, 150 μM). After purging with nitrogen for 2 min, this solution was reduced for 30 min at −510 mV vs Ag/AgCl. After stopping the reduction, unbound mitosene was removed by spun-column chromatography[41] (centrifugal molecular sieve filtration) over Sephadex G-25 coarse, followed by ethanol precipitation of DNA. The precipitated DNA was rehydrated overnight in water (200 μL) at 4 °C. From this solution 80 μL was mixed in a cuvette with ethidium bromide measuring solution (3 mL, containing 3 μg/mL ethidium bromide and 0.4 mM EDTA in 20 mM phosphate buffer pH 12), after which fluorescence was measured at 600 nm (excitation wavelength 525 nm). Next the DNA was denaturated at 95 °C for 3 min and allowed to cool at 20 °C for exactly 10 min. After this the fluorescence wsa measured immediately. The ratios between the fluorescence after and before denaturation of the samples $((F_a/F_b)_a)$ and a blank DNA sample $((F_a/F_b)_b)$ were used to calculate the extent of cross-link formation with use of the following formula:[42]

$$\% \text{ cross-links} = \frac{(F_a/F_b)_a - (F_a/F_b)_b}{1 - (F_a/F_b)_b} \times 100\%$$

B. Dithionite Reduction. A solution of 150 μM mitomycin C or 3 and calf thymus DNA (350 μg) in 30 mM Tris-acetate buffer, pH 5.5 (1 mL), was purged with nitrogen for 10 min, after which reduction was started by the addition of 6 mM $Na_2S_2O_4$ solution in water (50 μL, final concentration 300 μM). The $Na_2S_2O_4$ solution was prepared in nitrogen-purged water directly before use. After 30 min of reduction, the reaction was stopped by flushing with air. Samples were treated as described in the electrochemical activation procedure A.

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