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Iminium Ion Chemistry of Mitosene DNA Alkylating Agents. Enriched ^{13}C NMR and Isolation Studies†

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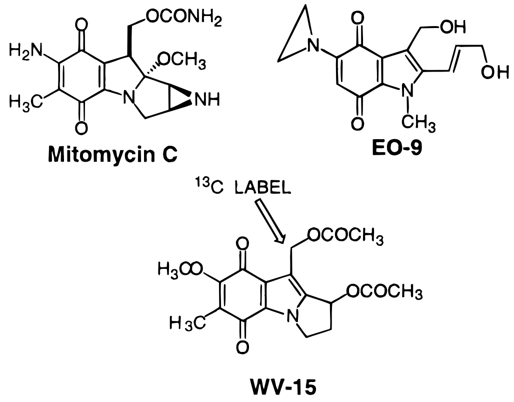
ABSTRACT: Described herein is a study of the reductive alkylation chemistry of mitosene antitumor agents. We employed a ^{13}C-enriched electrophilic center to probe the fate of the iminium ion resulting from reductive activation. The ^{13}C-labeled center permitted the identification of complex products resulting from alkylation reactions. In the case of DNA reductive alkylation, the type and number of alkylation sites were readily assessed by ^{13}C NMR. Althougth there has been much excellent work done in the area of mitosene chemistry and biochemistry, the present study provides a number of new findings: (1) The major fate of the iminium ion is head-to-tail polymerization, even in dilute solutions. (2) Dithionite reductive activation results in the formation of mitosene sulfite esters as well as the previously observed sulfonate adducts. (3) The mitosene iminium ion alkylates the adenosine 6-amino group as well as the guanosine 2-amino group. The identification of the latter adduct was greatly facilitated by the ^{13}C-label at the electrophilic center. (4) The mitosene iminium ion alkylates DNA at both nitrogen and oxygen centers without any apparent base selectivity. The complexity of mitosene reductive alkylation of DNA will require continued adduct isolation studies.

The mechanistic study of the mitomycin C and mitosene reductive alkylation of DNA has involved nearly three decades of work (*1*, *2*). The isolation and identification of reductive alkylation products has been carried out by a number of research groups and continues to this day (3−13). Considering the possible formation of multiple DNA alkylation products by mitosene analogues such as EO9 and WV-15 shown in Chart 1, as well as numerous natural products (*14*, *15*), the study of these interesting antibiotics could continue for decades to come (*1*, *2*, *16*−*19*, *20*).

The ongoing design of new DNA reductive alkylating agents requires a facile method of characterizing and inventorying alkylation sites as well as studying the chemistry of the reactive species formed upon reductive activation. The substitution of ^{13}C at the electrophilic center of the agent would allow for the rapid identification and isolation of reductive alkylation products and even the determination of the number and sites of DNA alkylation products. The use of ^{13}C labeling to study biological processes is well-known (*21*), and recently ^{13}C labeling studies of DNA alkylation have been carried out with the methylating agent *N*-methyl-*N*-nitrosourea (*22*). With a ^{13}C at the electrophilic center, oxygen alkylation ($\delta = 55-65$) can be readily distinguished from nitrogen alkylation (amino and N(7), $\delta = 35-40$). If a quantitative ^{13}C spectrum can be obtained, the relative amounts of adducts can be measured. Otherwise, the number of different adducts can be assessed by counting the resonances. The absence of a hydrolytic workup, which could decompose labile adducts, would ensure the detection of all adducts. While the eventual isolation of detected adducts is still a requirement, a ^{13}C NMR adduct inventory would assist in the isolation process. Initially, the number and the type (oxygen, nitrogen, hydrogen, and carbon substitution) of alkylation products can be determined with a ^{13}C NMR spectrum of the crude product mixture. These components can then be isolated, with the chemical shift of the ^{13}C electrophilic center acting as an identifier.

Chart 1



We chose ^{13}C-labeled WV-15 to illustrate this method because of the synthetic accessibility of the labeled center as well as for its importance in understanding mitosene and mitomycin chemistry. The study described herein provides insights into the fate of the mitosene iminium ion, the dithionite reductive activation process, and the reactivity of the mitosene iminium ion with DNA.

EXPERIMENTAL PROCEDURES

All analytically pure compounds were dried under high vacuum in a drying pistol over refluxing toluene. Elemental

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analyses were run at Atlantic Microlab, Inc., Norcross, GA. All TLCs were performed on silica gel plates using a variety of solvents and a fluorescent indicator for visualization. IR spectra were taken as thin films and the strongest absorbances reported. ^{1}H NMR and ^{13}C NMR spectra were obtained from 300 and 75 MHz spectrometers respectively, unless otherwise specified. All chemical shifts are reported relative that of to TMS.

The procedures for the preparation of the ^{13}C analogues of WV-15 and **14** are outlined below employing Cu^{13}CN or [*carbonyl*-^{13}C]dimethylformamide (Cambridge Isotopes). These procedures were adapted from the literature (*20*, *23*, *24*), and the reaction intermediates were characterized by ^{13}C and ^{1}H NMR spectra. Other spectral data matched those from the literature.

The reaction products of ^{13}C-labeled reactants (WV-15 and **14**) and were characterized by the isolation and purification or simply by a ^{13}C NMR of the crude reaction mixture. The reactions studied include reductive activation (catalytic or dithionite) and the reductive alkylation of nucleosides (dA and dG) and synthesized DNA hexamers (AAATTT and GGGCCC). Procedures for the reaction and isolation studies with the ^{13}C-labeled reactants are provided after the synthetic procedures.

*[10-^{13}C]-2,3-Dihydro-7-methoxy-6-methyl-1H-pyrrolo[1,2-a]indol-1-one-9-carbonitrile (**2**).* The procedure for preparing this intermediate was adapted directly from the literature,[24] but the workup was altered. The ferric chloride/HCl-treated reaction mixture was stirred for 2 h at room temperature, and then 200 mL of water was added followed by storage in a refrigerator for 96 h. The precipitated solid was filtered and dried, purified by flash chromatography on silica gel using chloroform as the eluant, and then further purified by decolorization with charcoal and recrystallized from chloroform/hexane: 39% vs 26% yield from the literature;[24] ^{1}H NMR (DMSO-d_6) δ 7.61 and 7.14 (2H, 2s, aromatic protons), 4.46 (2H, t, $J = 6$ Hz, methylene protons), 3.89 (3H, s, methoxy), 3.20 (2H, t, $J = 6$ Hz), 2.29 (3H, s, methyl); ^{13}C NMR (CDCl$_3$) δ 114.059 (^{13}CN); MS (EI mode) 241 *m*/*z* (^{13}C, M$^+$), 226, 218, 198, 170.

*[10-^{13}C]-2,3-Dihydro-1-hydroxy-7-methoxy-6-methyl-1H-pyrrolo[1,2-a]indole-9-carbonitrile (**3**).* The procedure outlined below has not been reported in the literature. To a solution of 400 mg (1.6 mmol) of **2** in 30 mL of methanol was added 1 g of NaBH$_4$, and the reaction was then stirred for 1.5 h. The completed reaction mixture was concentrated to dryness, and the residue was combined with a mixture of 20 mL of water and 5 mL of 2 N HCl. This mixture was extracted 3× with 30 mL portions of CH$_2$Cl$_2$, and the organic extracts were dried (Na$_2$SO$_4$) and concentrated to a solid, which was recrystallized from chloroform/hexane: 360 mg (90%) yield; ^{1}H NMR (CDCl$_3$) δ 7.08 and 7.05 (2H, 2s, aromatic protons), 5.53 (1H, m, methine proton), 4.29 and 4.08 (2H, 2m, methylene protons), 3.89 (3H, s, methoxy), 2.95 and 2.61 (2H, 2m, methylene protons), 2.32 (3H, s, methyl); ^{13}C NMR (CDCl$_3$) δ 116.23 (^{13}CN); MS (EI mode) *m*/*z* 243 (^{13}C, M$^+$), 228 (M$^+$ − CH$_3$), 215, 210, 200, 187.

*[10-^{13}C]-2,3-Dihydro-1-hydroxy-7-methoxy-6-methyl-1H-pyrrolo[1,2-a]indole-9-carboxaldehyde (**4**).* The procedure for preparing this intermediate from **3** was adapted directly from the literature:[20] 80% yield; ^{1}H NMR (CDCl$_3$) δ 10.05 (1H, d, $J = 178$ Hz, [^{13}C]aldehyde proton), 7.36 and 7.08 (2H, 2s, aromatic protons), 5.59 (1H, m, methine proton), 4.31 and 4.05 (2H, 2m, methylene protons), 3.92 (3H, s, methoxy), 3.05 and 2.63 (2H, 2m, methylene protons), 2.32 (3H, s, methyl); ^{13}C NMR (CDCl$_3$) δ 184.6 ([^{13}C]carbonyl).

*[10-^{13}C]-2,3-Dihydro-1-hydroxy-7-methoxy-6-methyl-8-nitro-1H-pyrrolo[1,2-a]indole-9-carboxaldehyde (**5**).* The procedure for preparing this intermediate from **4** was adapted directly from the literature,[20] except for the purification step. The crude product was purified with flash chromatography on silica gel using chloroform as the eluant and recrystallized from chloroform/hexane to afford a yellow crystalline solid: 74%; ^{1}H NMR (CDCl$_3$) δ 9.79 (1H, d, $J = 178$ Hz, [^{13}C]aldehyde proton), 7.28 and 7.26 (2H, 2s, aromatic protons), 5.55 (1H, m, methine proton), 4.35 and 4.10 (2H, 2m, methylene protons), 3.90 (3H, s, methoxy), 3.06 and 2.66 (2H, 2m, methylene protons), 2.44 (3H, s, methyl); ^{13}C NMR (CDCl$_3$) δ 186.17 ([^{13}C]carbonyl); MS (EI mode) *m*/*z* 291 (^{13}C, M$^+$), 274, 261, 256, 243, 229, 213, 201, 188, 185.

*[10a-^{13}C]-2,3-Dihydro-1-hydroxy-7-methoxy-6-methyl-5,8-dioxo-1H-pyrrolo[1,2-a]indole-9-carboxaldehyde (**6**).* The procedure for preparing this intermediate from **5** was adapted directly from the literature:[20] 78% yield; ^{1}H NMR (CDCl$_3$) δ 10.28 (1H, d, $J = 184$ Hz, [^{13}C]aldehyde proton), 5.42 (1H, m, methine proton), 4.54 and 4.22 (2H, 2m, methylene protons), 4.06 (3H, s, methoxy), 3.02 and 2.59 (2H, 2m, methylene protons), 1.99 (3H, s, methyl); ^{13}C NMR (CDCl$_3$) δ 188 ([^{13}C]carbonyl); MS (EI mode) 276 (^{13}C, M$^+$), 261, 230, 204.

*[10-^{13}C]-2,3-Dihydro-1-hydroxy-9-(hydroxymethyl)-7-methoxy-6-methyl-1H-pyrrolo[1,2-a]indole-5,8-dione (**7**).* The procedure for preparing this intermediate from **6** was adapted directly from the literature,[20] with changes made in the purification step. The crude product was purified by silica gel chromatography using chloroform/ethyl acetate (2:3) as the eluent, and recrystallized from chloroform/hexane: 87% yield; ^{1}H NMR (CDCl$_3$) δ 5.23 (1H, m, 1-methine proton), 4.84 (2H, doublet of triplets, $J = 144.4$ Hz and $J = 6$ Hz, 10-[^{13}C]methylene), 4.34 and 4.18 (2H, 2m, 3-methylene), 3.98 (3H, s, methoxy), 3.58 and 3.38 (2H, 2 br s, hydroxyls), 2.83 and 2.50 (2H, 2m, 2-methylene protons), 2.50 and 1.95 (6H, 2s, methyl; ^{13}C NMR (CDCl$_3$) δ 57.52 ([^{13}C]CH$_2$OH); MS (EI mode) *m*/*z* 278 (^{13}C, M$^+$), 260, 231, 217.

[10-^{13}C]-1-(Acetyloxy)-9-[(acetyloxy)methyl]-2,3-dihydro-7-methoxy-6-methyl-1H-pyrrolo[1,2-a]indole-5,8-dione ([10-^{13}C]WV-15). The procedure for preparing this product from **7** was adapted directly from the literature[20] with an improvement in yield: 128 mg (98%); ^{1}H NMR (CDCl$_3$) δ 6.15 (1H, dd, $J = 1.8$ and 6.6 Hz, methine proton), 5.28 (2H, d, $J = 151$ Hz, 10-[^{13}C]methylene protons), 4.32 (2H, m, 3-methylene protons), 4.01 (3H, s, methoxy), 2.94 and 2.54 (2H, 2m, 2-methylene protons), 2.06, 2.04, and 1.95 (9H, 3s, methyl); ^{13}C NMR (CDCl$_3$) δ 57.65 (^{13}CH$_2$OAc); MS (EI mode) *m*/*z* 362 (^{13}C, M$^+$), 320, 303, 276, 260, 243.

*[10-^{13}C]-2,3-Dihydro-7-methoxy-6-methyl-1H-pyrrolo[1,2-a]indole-9-carboxaldehyde (**9**).* The procedure for preparing this intermediate from **8** was adapted directly from the literature,[23] but with carbonyl-^{13}C-labeled DMF employed as starting material and the purification steps changed. The crude product was purified by silica gel chromatography employing chloroform as the eluent and the product recrystallized from chloroform/hexane: 147 mg (65%); ^{1}H NMR (CDCl$_3$) δ 9.89 (1H, d, $J = 166$ Hz, aldehyde), 7.64 and

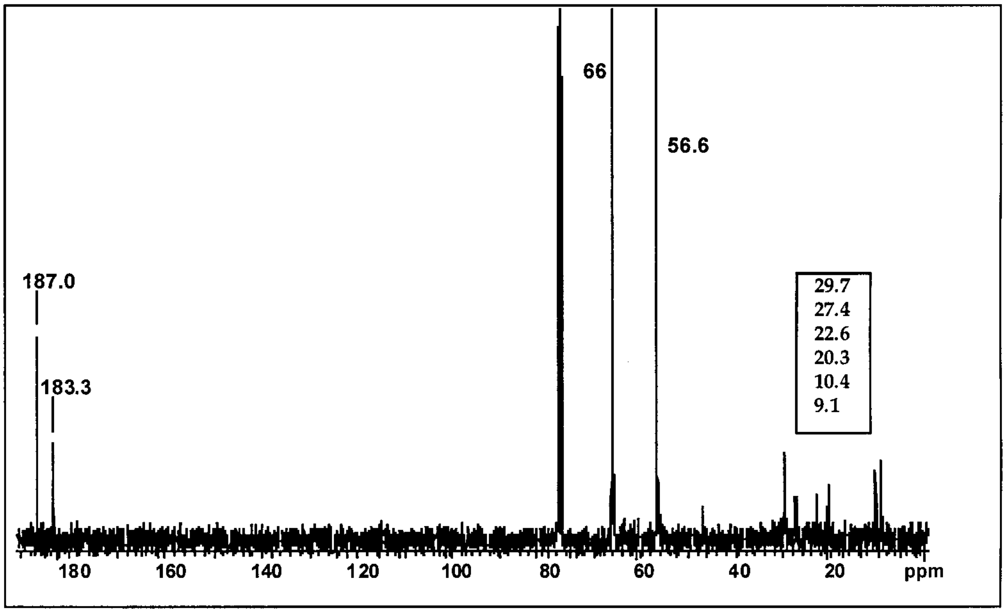


FIGURE 1: ^{13}C NMR spectrum of the mother liquor for **11** → **13**, after removal of the bulk of **13**. This mixture is largely **13** and the methoxy derivative **16** along with minor amounts of unreacted **12** and its hydroquinone. Shown in the box are the chemical shifts of three other minor products and their respective hydroquinones; the structures are shown in Scheme 3.

6.98 (2H, 2s, aromatic protons), 4.03 (2H, t, $J = 7.2$ Hz, methylene protons), 3.91 (3H, s, methoxy), 3.20 (2H, t, $J = 7.2$ Hz, methylene protons), 2.60 (2H, d, $J = 7.2$ Hz, methylene protons), 2.31 (3H, s, methyl); ^{13}C NMR ($CDCl_3$) δ 183.30 ([^{13}C]carbonyl).

*[10-^{13}C]-2,3-Dihydro-7-methoxy-6-methyl-8-nitro-1H-pyrrolo[1,2-a]indole-9-carboxaldehyde (**10**).* This procedure was adapted from the literature.[20] To a solution of 50 mg (0.22 mmol) of **9** in 5 mL of CH_2Cl_2 was added dropwise 0.06 mL of 65% HNO_3 at room temperature for 20 min. To the reaction mixture was added 3 mL of saturated $NaHCO_3$, and the mixture was shaken in a separatory funnel. The CH_2Cl_2 layer was dried (Na_2SO_4) and concentrated to a crude solid, which was purified with flash chromatography on silica gel using chloroform as the eluant. The isolated product was recrystallized from chloroform/hexane to afford **10** as a yellow crystalline solid: 55 mg (92%) yield; ^{1}H NMR ($CDCl_3$) δ 9.82 (1H, d, $J = 172$ Hz, [^{13}C]aldehyde), 7.20 (1H, s, aromatic proton), 4.13 (2H, t, $J = 7.2$ Hz, methylene protons), 3.88 (3H, s, methoxy), 3.33 (2H, t, $J = 7.8$ Hz, methylene protons), 2.73 (2H, d, $J = 7.2$ Hz, methylene protons), 2.43 (3H, s, methyl); ^{13}C NMR ($CDCl_3$) δ 182.91 ([^{13}C]carbonyl).

*[10-^{13}C]-2,3-Dihydro-7-methoxy-6-methyl-5,8-dioxo-1H-pyrrolo[1,2-a]indole-9-carboxaldehyde (**11**).* This procedure was adapted from the literature.[20] To a solution of 50 mg (0.18 mmol) of **10** in 8 mL of EtOH and 4 mL of 3 M HCl was added 150 mg of tin powder, and the resulting reaction mixture was stirred at room temperature for 4 h. Subsequently, a saturated $NaHCO_3$ solution was added to neutralize the reaction, and the mixture was extracted 3× with 20 mL portions of $CHCl_3$. The combined extracts were filtered through Celite, dried (Na_2SO_4), and concentrated to afford the crude amine.

To a solution of the amine in 5 mL of acetone was added a solution of 500 mg of KH_2PO_4 and 700 mg of Fremy salt in 25 mL of water, and the reaction was stirred for 2 h. The product was extracted with chloroform, dried (Na_2SO_4), and concentrated to a residue which was recrystallized from chloroform/hexane: 35 mg (75%) yield; ^{1}H NMR ($CDCl_3$) δ 10.36 (1H, d, $J = 181$ Hz, [^{13}C]aldehyde), 4.28 (2H, t, $J = 7.8$ Hz, methylene protons), 4.04 (3H, s, methoxy), 3.14 (2H, t, $J = 7.5$ Hz, methylene protons), 2.63 (2H, d, $J = 7.8$ Hz, methylene protons), 1.98 (3H, s, methyl); ^{13}C NMR ($CDCl_3$) δ 187 ([^{13}C]carbonyl).

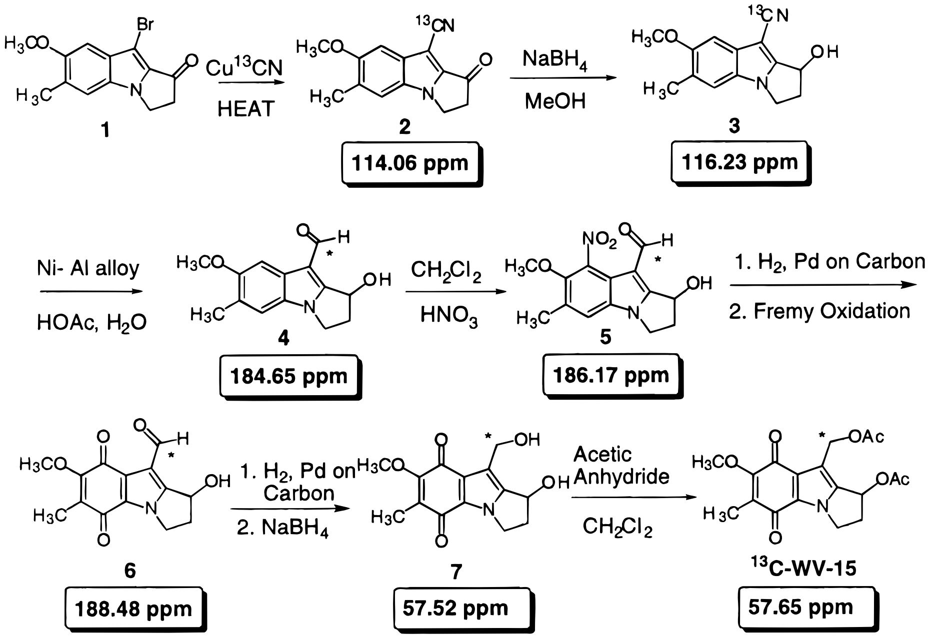
*[10-^{13}C]-2,3-Dihydro-9-(hydroxymethyl)-7-methoxy-6-methyl-1H-pyrrolo[1,2-a]indole-5,8-dione (**13**).* The procedure for preparing this intermediate from **11** was adapted directly from the literature:[23] 77% yield; ^{1}H NMR ($CDCl_3$) δ 4.58 (2H, dd, $J = 143$ and 7.2 Hz, 10-[^{13}C]methylene protons), 4.21 (2H, t, $J = 7.2$ Hz, methylene protons), 4.06 (1H, m, OH), 3.98 (3H, s, methoxy), 2.83 (2H, t, $J = 7.5$ Hz, methylene protons), and 2.56 (2H, d, $J = 7.8$ Hz, methylene protons), 1.96 (3H, s, methyl); ^{13}C NMR ($CDCl_3$) δ 56.59 ($^{13}CH_2OH$).

*]10-^{13}C]-9-(Acetyloxymethyl)-2,3-Dihydro-7-methoxy-6-methyl-1H-pyrrolo[1,2-a]indole-5,8-dione (**14**).* The procedure for preparing this intermediate from **13** was adapted directly from the literature:[20] 95% yield; ^{1}H NMR ($CDCl_3$) δ 5.22 (2H, d, $J = 149$ Hz, methylene protons), 4.22 (2H, t, $J = 7.2$ Hz, methylene protons), 4.00 (3H, s, methoxy), 2.90 (2H, t, $J = 7.5$ Hz, methylene protons), 2.56 (2H, d, $J = 7.8$ Hz, methylene protons), 2.07 and 1.96 (6H, 2s, methyl); ^{13}C NMR ($CDCl_3$) δ 57.76 ($^{13}CH_2OAc$).

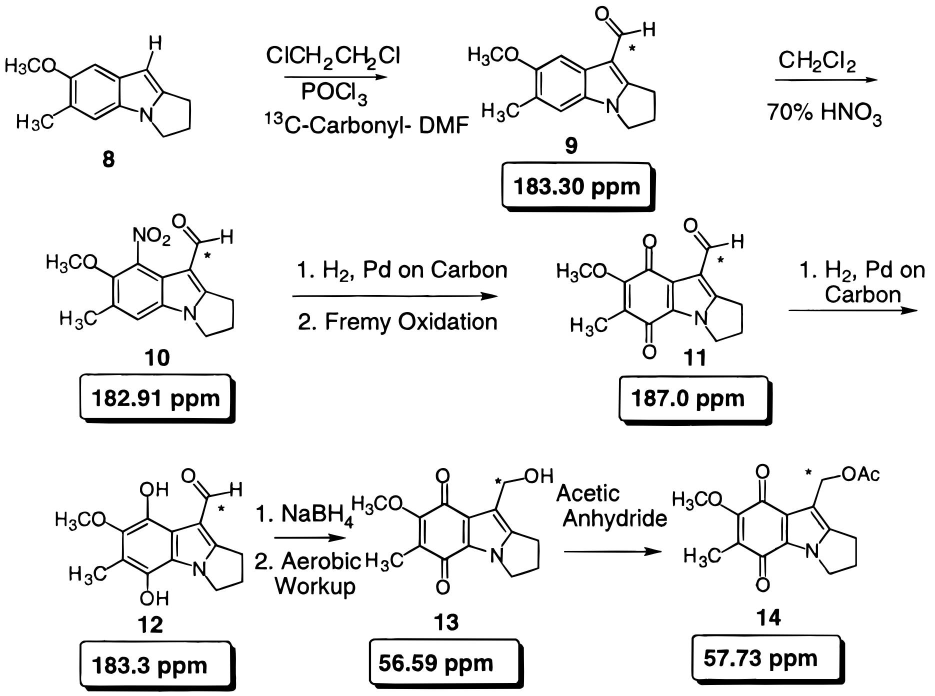
*Isolation and Identification of the Byproducts of the Conversion of **11** to **13**.* The mother liquor, from which **13** was crystallized, was concentrated to dryness and an ^{13}C NMR spectrum of the crude mixture obtained; see Figure 1. The crude mixture was then subjected to preparative silica gel thin-layer chromatography using chloroform/methanol (95:5) as the eluent. The physical properties of the separated products are provided below.

*[10-^{13}C]-2,3-Dihydro-7-methoxy-6,9-dimethyl-1H-pyrrolo-[1,2-a]indole-5,8-dione (**15**)*: 2.2 mg (1.1%) yield; ^{1}H NMR-($CDCl_3$) δ 4.20 (2H, t, $J = 6.9$ Hz, methylene protons), 3.98 (3H, s, methoxy), 2.76 (2H, t, $J = 7.5$ Hz, methylene), 2.53

Scheme 1



Scheme 2



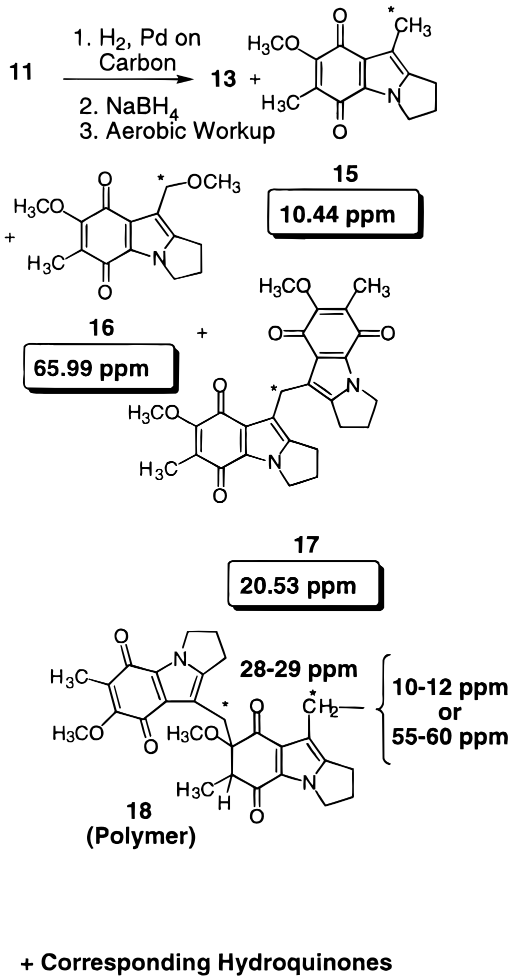
(2H, d, $J = 7.2$ Hz, methylene), 2.24 (3H, 2d, $J = 128$ Hz, [^{13}C]methyl), 1.94 (3H, s, methyl); MS (EI mode) *m/z* 246 (^{13}C, M$^+$), 231 (M$^+$ − CH_3), 217(M$^+$ − CH_2CH_3), 203 (M$^+$ − $CH_2CH_2CH_3$), 187, 175.

*[10-^{13}C]-2,3-Dihydro-7-methoxy-9-methoxymethyl-6-methyl-1H-pyrrolo[1,2-a]indole-5,8-dione (**16**)*: 2.8 mg (1.2%); ^{1}H NMR ($CDCl_3$) δ 4.60 (2H, d, $J = 143.4$ Hz, 10-[^{13}C]-methylene), 4.21 (2H, t, $J = 6.9$ Hz, 3-methylene), 3.98 (3H, s, 7-methoxy), 3.41 (3H, d, $J = 5.7$ Hz, 10a-methoxy split by ^{13}C), 2.89 (2H, $J = 7.5$ Hz, 1-methylene), 2.55 (2H, quintet, $J = 7.2$ Hz, 2-methylene), 1.95 (3H, s, methyl); MS (EI mode) *m/z* 276 (^{13}C, M$^+$), 261 (M$^+$ − CH_3), 245 (M$^+$ − CH_2OH), 229, 217.

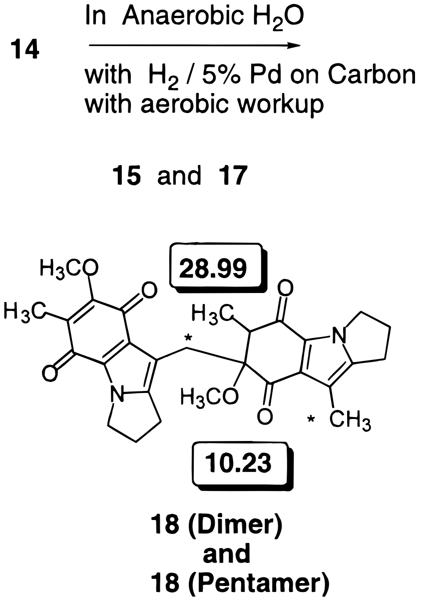
*Di(2,3-dihydro-7-methoxy-6-methyl-5,8-dioxo-1H-pyrrolo-[1,2-a]indol-9-yl)[^{13}C]methane (**17**)*: 3.1 mg (1%); ^{1}H NMR ($CDCl_3$) δ 4.21 (2H, d, $J = 130$ Hz, 10-[^{13}C]methylene), 4.17 (4H, t, $J = 6.9$ Hz, 3-methylenes of the dimer), 3.97 (6H, s, 7-methoxys of the dimer), 2.56 (4H, $J = 7.5$ Hz, 1-methylenes of the dimer), 2.45 (4H, quintet, $J = 7.2$ Hz, 2-methylenes of the dimer), 1.95 (6H, s, methyls of the dimer); MS (EI mode) *m/z* 475 (^{13}C, M$^+$), 432 (M$^+$ − $CH_2CH_2CH_3$), 245.

*Catalytic Hydrogenation of **14** in the Presence of Aqueous Methanol.* To a solution of 41 mg (mmol) of **14** in 7 mL of methanol was added water to afford a total volume of 40 mL. To this solution was added 35 mg of 5% Pd on C, and

Scheme 3



Scheme 4



the mixture was then deaerated with argon for 30 min, followed by bubbling with hydrogen gas for 15 min, and finally bubbling with argon for 30 min to remove the excess hydrogen. The reaction mixture was incubated at 30 °C for 24 h and then opened to the air. The catalyst was filtered off and the filtrate extracted 3× with 20 mL portions of chloroform. The extracts were dried (Na_2SO_4) and concentrated to a red solid, which was subjected to preparative silica gel thin-layer chromatographic separation using chloroform/methanol (95:5) as eluant. The yields and physical properties of the hydrolysis products are provided below.

*[10-^{13}C]-2,3-Dihydro-7-methoxy-6,9-dimethyl-1H-pyrrolo-[1,2-a]indole-5,8-dione (**15**)*: 1.8 mg (5.4%) yield; the physical properties were the same as reported above.

*[10-^{13}C]-2,3-Dihydro-7-methoxy-9-methoxymethyl-6-methyl-1H-pyrrolo[1,2-a]indole-5, 8-dione (**16**)*: 1.3 mg (3.5%) yield; the physical properties were the same as reported above.

*Di(2,3-dihydro-7-methoxy-6-methyl-5,8-dioxo-1H-pyrrolo-[1,2-a]indol-9-yl)[^{13}C]methane (**17**)*: trace; the physical properties were the same as reported above.

*Dimeric **18***: 7.8 mg (23%) yield; ^{1}H NMR($CDCl_3$, 500 MHz) δ 4.13 and 3.96 (4H, m, 3- and 3′-methylene), 3.95 (3H, s, 7-methoxy), 3.49 (2H, doublet of AB quartets, J = 13.5, 64.3, 130.5 Hz, 10-[^{13}C]methylene), 3.40 (3H, s, 7′-methoxy), 2.96 (1H, doublet of quartets, J = 2.5, 7 Hz, 6′-methine), 2.73 and 2.63 (4H, 2m, 1 and 1′-methylenes), 2.48 (4H, m, 2- and 2′-methylenes), 2.16 (3H, d, J = 127.5 Hz, 10-methyl), 1.86 (3H, s, 6-methyl), 1.15 (3H, d, J = 7 Hz, 6′-methyl); MS (EI mode) m/z, 492.4 (^{13}C, M$^+$), 460.4 ($M^+ - CH_3OH$), 246.5. Anal. Calcd for $C_{26}{}^{13}C_2H_{30}N_2O_6$: C, 68.68; H, 6.14; N, 5.69. Found: C, 68.71; H, 6.80; N, 5.26.

*Pentameric **18***: 3. 8 mg (11%) yield.

*^{13}C-Labeled Sulfite Diester **20***. To a solution of 31 mg (0.10 mmol) of **14** in a mixture of 20 mL of CH_2Cl_2/MeOH/H_2O (1:1:1), was added a solution of 44 mg (0.31 mmol) of $Na_2S_2O_4$ in 1 mL of water. After 10 min, the reduction reaction was complete, and reoxidation by air had taken place. The reaction mixture was extracted with 3 × 50 mL portions of $CHCl_3$, and the organic extracts were dried (Na_2SO_4) and concentrated to a red solid. This solid was subjected to preparative silica gel thin-layer chromatographic separation using chloroform/methanol (95:5) as the eluent. The physical properties of the isolated dithionite trapping product are provided below: 9 mg (16%) yield; ^{1}H NMR ($CDCl_3$) δ 4.55 (4H, doublet of triplets, J = 140.4, 3.3 Hz, 10-[^{13}C]-methylene), 4.24 (2H, t, J = 7.2 Hz, 3-methylene), 3.93 (3H, s, 7-methoxy), 2.98 (2H, t, J = 7.2 Hz, 1-methylene), 2.57 (2H, quintet, J = 6.9 Hz, 2-methylene), 1.92 (3H, s, 6-methyl); MS (EI mode) m/z 554 ($M^+ - 16$), 490 ($M^+ - SO_3$), 475, 259, 245. Anal. Calcd for $C_{26}{}^{13}C_2N_2O_9S$: C, 59.15; H, 4.96; N, 4.93. Found: C, 59.25; H, 4.93; N; 4.91.

Reduction of WV-15 with Sodium Dithionite in D_2O. A solution of 2.62 mg (0.0072 mmol) of ^{13}C-labeled WV-15 in 0.1 mL of DMSO and 1 mL of D_2O was purged with Ar and then placed in a N_2 glovebox. A solution of 6.2 mg (0.03 mmol) of $Na_2S_2O_4$ in 1 mL of D_2O (30 mM) was purged with argon and placed in the same N_2 glovebox as above. To the WV-15 solution was added 0.24 mL of the dithionite stock solution. The reaction mixture was transferred to a sealed NMR tube.

Hydrolysis of Reduced WV-15. A solution consisting of 5 mL of DMSO and 10 mg (0.028 mmol) of WV-15 was added to 20 mL of 0.05 M pH 7.4 Tris buffer containing 1 M KCl. To this solution was added 5 mg of 5% Pd on carbon, and the mixture was then deaerated with argon for 30 min, followed by bubbling with hydrogen gas for 15 min, and finally bubbling with argon for 30 min to remove the excess

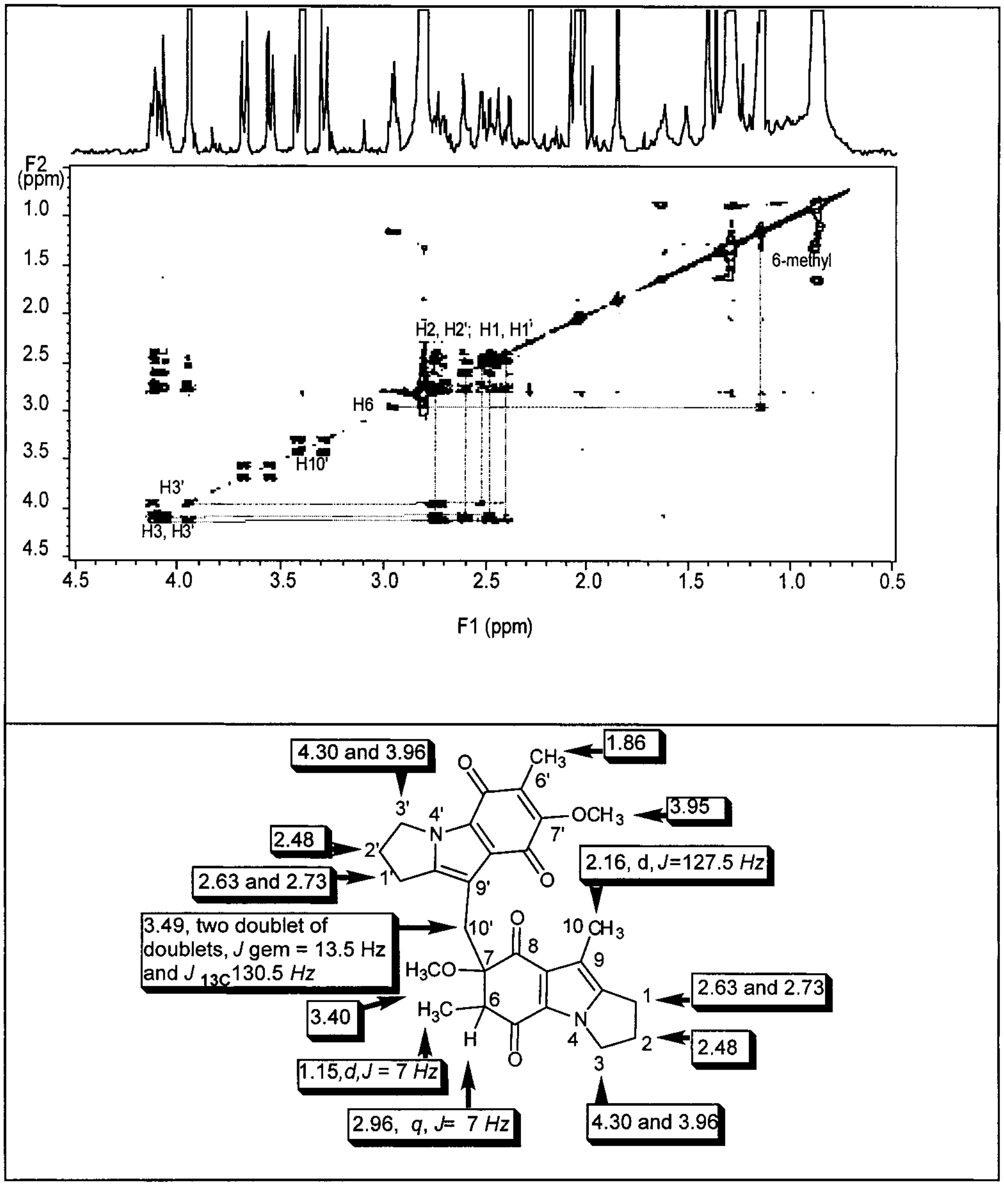


FIGURE 2: TOCSY NMR spectrum of purified dimeric **18** in $CDCl_3$ with chemical shift and coupling assignments.

hydrogen. The reaction mixture was incubated at 30 °C for 24 h and then opened to the air. The catalyst was filtered off and the filtrate extracted 3× with 20 mL portions of chloroform. The extracts were dried (Na_2SO_4) and concentrated to a red solid, which was subjected to preparative silica gel thin-layer chromatographic separation using chloroform/methanol (95:5) as eluant. The physical properties of the hydrolysis product are provided below.

*[10-^{13}C]-2,3-Dihydro-1-acetyloxy-9-(hydroxymethyl)-7−6-methyl-1H-pyrrolo[1,2-a]indole-5,8-dione (**23**)*: 1 mg (11%) yield; ^{1}H NMR ($CDCl_3$) δ 6.05 (1H, dd, $J = 6.6$, 1.5 Hz, 1-methide), 4.71 (2H, dd, $J = 6.9$, 1.8 Hz, 10-methylene), 4.34 (2H, m, 3-methylene), 4.09 (1H, t, $J = 7$ Hz, hydroxyl), 4.01 (3H, s, 7-methoxy), 2.88 and 2.59 (2H, 2m, 2-methylene), 2.06 (3H, s, methyl), 1.98 (3H, s, methyl); MS (EI mode) *m/z* 333 (M^+), 319, 301, 293, 273, 259, 244.

[10-^{13}C]WV-15: 0.7 mg (7%) yield.

*[10-^{13}C]-2,3-Dihydro-1-hydroxy-9-(hydroxymethyl)-7-methoxy-6-methyl-1H-pyrrolo[1,2-a]indole-5,8-dione (**7**)*: 1.7 mg (22%)yield.

*Isolation of ^{13}C-Labeled Deoxyadenosine Adduct **24**.* A mixture of 135 mg (0.5 mmol) of deoxyadenosine, 30 mg (0.085 mmol) of WV-15, and 10 mg of PtO_2, dissolved in 16 mL of pH 6.0 0.015 M phosphate buffer, was purged with argon for 15 min. Reduction was carried out by H_2 bubbling for 15 min, and then the reaction was purged again with argon for 15 min. The reaction mixture was incubated at 30 °C for 24 h and then opened to the air. The catalyst was filtered off and the filtrate extracted 3× with 20 mL portions of chloroform. The extracts were dried (Na_2SO_4) and concentrated to a red solid. A ^{13}C NMR spectrum of the resulting crude mixture was taken, Figure 6, and then the reaction mixture was subjected to preparative silica gel thin-layer chromatographic separation using chloroform/methanol (95:5) as eluent. Data for **24**: 4.8 mg (11%) yield; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.38 (1H, s, H(8) in dA), 8.25 (1H, s, H(2) in dA), 7.84 (1H, br s, 6-amino of dA), 6.36 (1H, t, $J = 6$ Hz, H(1′) in dA), 5.02 (1H, m, H(1″)-methide), 4.98 and 4.71 (2H, 2 br s, $J = 135$ Hz, 10″-[^{13}C]-methylene), 4.41 (1H, m, H(3′) of dA), 4.21 and 4.14 (2H,

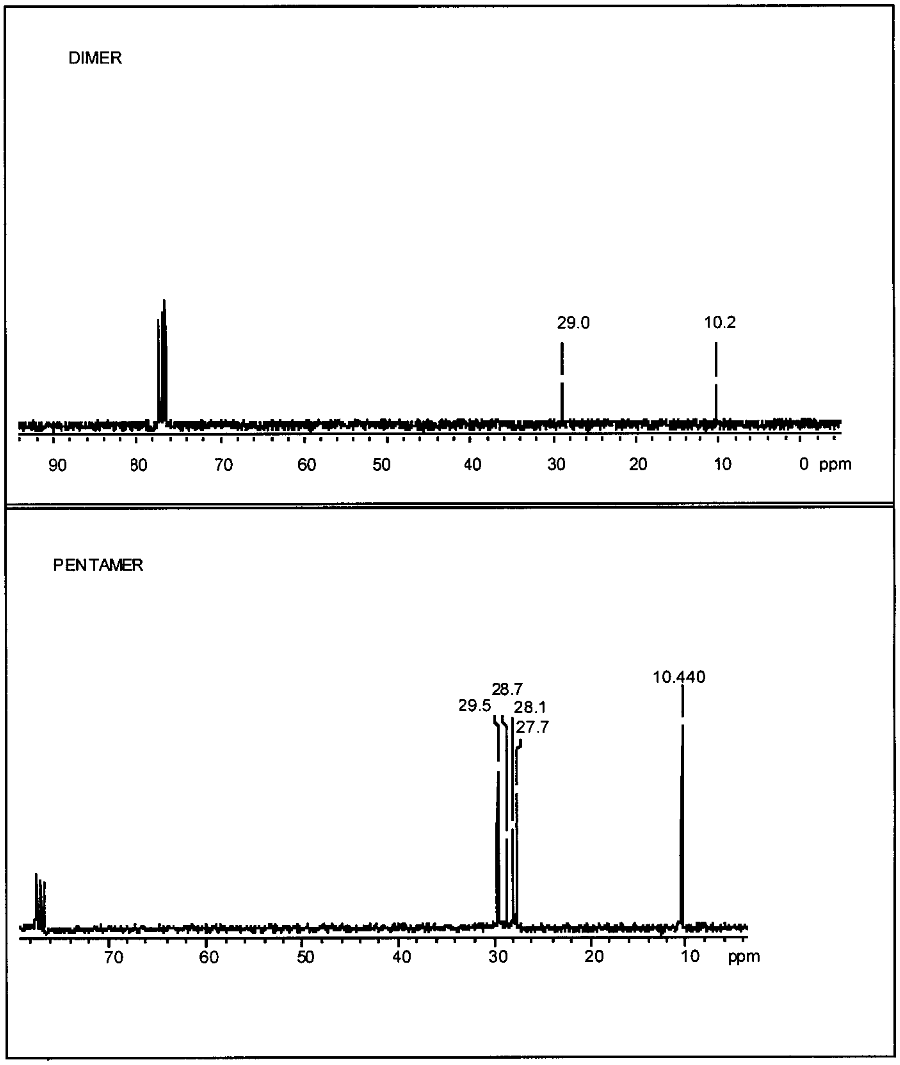


FIGURE 3: ^{13}C NMR spectra of the purified dimer and pentamer ra=un in $CDCl_3$ solvent. Both spectra show the terminal methyl at δ = 10 ppm and linking methylenes at δ = 27−29 ppm.

2m, 3″-methylene), 3.92 (3H, s, 7″-methoxy), 3.90 (1H, m, H(4′) of dA), 3.62 and 3.52 (2H, 2m, H(5′) of dA), 2.72 and 2.28 (4H, 2m, 2″- and 2′-methylenes), 1.86 (3H, s, 6″-methyl); MS *m*/*z* 394 (M^+ − 116), 276 (M^+ − 234), 251, 221.

^{13}C NMR Spectra of WV-15 Trapping Reactions of dA and dG. A solution consisting of 80 mg (0.28 mmol) of either 2-deoxyguanosine or 2-deoxyadenosine, 20 mg (0.055 mmol) of WV-15, and 20 mg of 5% Pd on charcoal in aqueous methanol (20 mL water/5 mL methanol) was deaerated, reduced with hydrogen, and purged with argon as described above. The reaction mixture was incubated at 30 °C for 24 h and then opened to the air. The catalyst was filtered off, and the products were extracted with 3 × 20 mL portions of chloroform. The dried (Na_2SO_4) extracts were then concentrated to a red solid, which was dissolved in DMSO-d_6 to obtain the ^{13}C NMR spectrum.

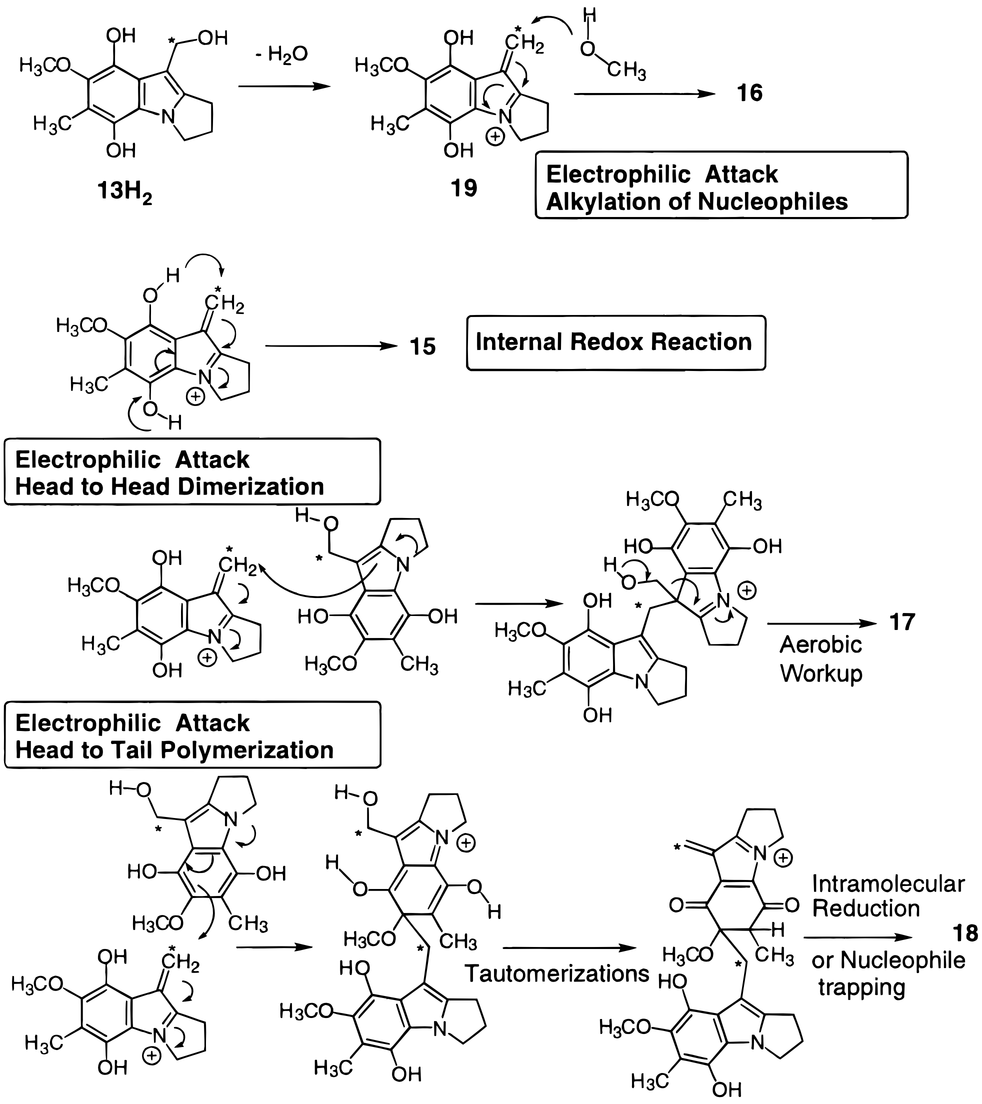
Reductive Alkylation of DNA Hexamers. To a solution of 10 mg of hexamer in 1 mL of 0.015 M pH 6.0 phosphate buffer was added a solution of 5.6 mg (0.015 mmol) of WV-15 in 1 mL of methanol along with 2 mg of 5% Pd on carbon. The mixture was purged with argon for 20 min and then with hydrogen for 10 min to facilitate reduction. Finally hydrogen gas was removed by a short purge with argon and the reaction mixture incubated at 25 °C for 12 h. The completed reaction was opened to the air and centrifuged at 12000*g* to remove the catalyst and any solids. The supernatant was extracted with chloroform and then diluted with two volumes of ethanol and chilled at 20 °C for 12 h, resulting in an orange precipitate. The precipitate was centrifuged (12000*g*, 20 min) to afford an orange pellet, which was redissolved in water and precipitated again. The yield upon drying was ∼6 mg.

RESULTS AND DISCUSSION

Synthesis. The preparation of WV-15 and its monoacetate analogue **13**, both labeled at the 10-position with ^{13}C, were carried out as illustrated in Schemes 1 and 2. The synthetic methodology is based on previously published procedures (*20*, *23*, *24*) or modifications thereof; see the Experimental Section. These schemes also show the ^{13}C NMR chemical shift values for the enriched center of each of the intermediates and both final products. These chemical shift values aided in the analysis of complex mixtures obtained in the course of this study.

Iminium Ion Fate Studied by ^{13}C NMR and Isolation. In this section we discuss the isolation and identification of the iminium ion derived products of reduced **13** and **14**. Both

Scheme 5



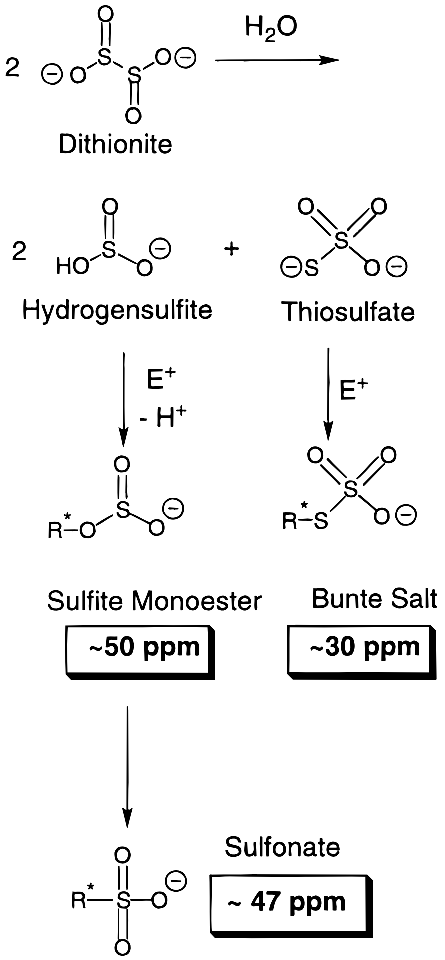
of these systems possess a single leaving group, hydroxyl or acetate, respectively, and afford relatively simple products compared to those obtained from reduced WV-15. The preparation of **13** illustrated in Scheme 2 involved the reduction of the aldehyde quinone **11** to afford reduced **13**, which was then air oxidized. Iminium ion formation from reduced **13** is minimal due to the poor hydroxide leaving group. However, bulk synthesis of **13** afforded a significant amount of liquor containing iminium ion derived products. Shown in Figure 1 is a ^{13}C NMR of this liquor containing the starting aldehyde **11** (187 ppm) and its hydroquinone **12** (183 ppm) along with the desired product **13** (56.6 ppm) and byproducts identified as **15**−**18** (Scheme 3). The minor resonances shown in the box of Figure 1 represent both the quinone and hydroquinone forms of the three products **15**, **17**, and **18**. Isolation and purification of **15** and **17** were carried out and the structures confirmed by analysis and spectroscopy. These same iminium ion derived products were obtained from the reduction of **14**, Scheme 4, except with the polymer products **18** present in significant quantities.

The unusual 28−29 ppm ^{13}C chemical shift range of polymeric products and the presence of these ^{13}C chemical shifts in all WV-15 reductive alkylation reaction mixtures prompted their isolation and identification. NMR spectroscopic data in Figures 2 and 3 of the purified dimer confirmed the assigned "head-to-tail" structure **18**. Identification of the dimeric and pentameric species is possible merely by counting the ^{13}C resonances of each spectrum of Figure 3. These spectra show the terminal [^{13}C]methyl at ∼10 ppm and the [^{13}C]methylene links at 27−29 ppm.

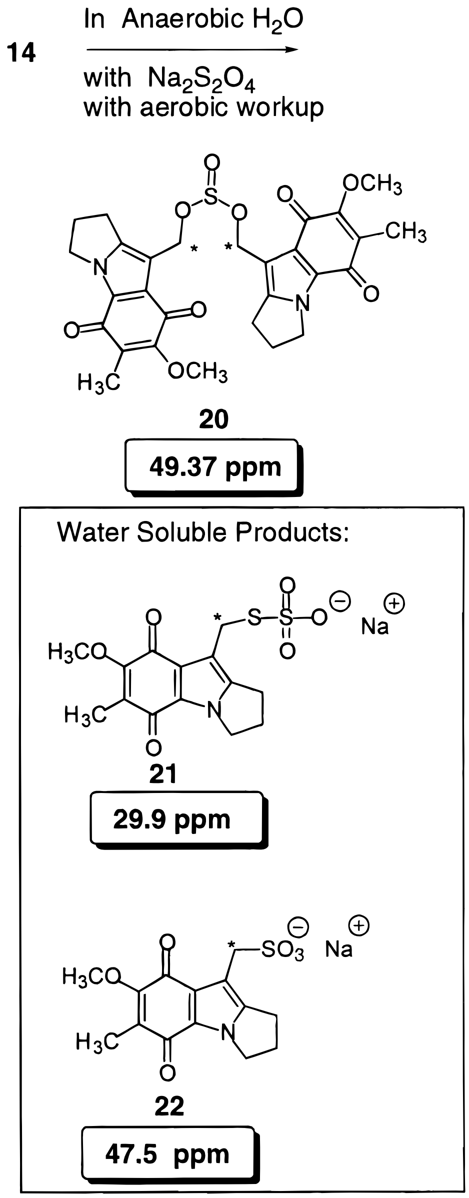
The dimer structure was further confirmed by the TOCSY NMR shown in Figure 2, which also shows the chemical shift assignments of dimer **18**. The [^{13}C]methylene linker (C-10′) bears diastereotopic protons, resulting in different chemical shifts for each proton as well as gem coupling ($J = 13.5$ Hz) with each other. The resulting doublet of doublets is split by the ^{13}C ($J = 130.5$ Hz) to afford two widely spaced doublets of doublets. The "boxes" for each doublet of doublets (one is labeled H10′) are centered at ∼3.5 ppm in Figure 2. The TOCSY also provides evidence of coupling between the H-6 and 6-methyl substituents, further confirming the dimer structure.

Illustrated in Scheme 5 are the mechanisms of iminium ion **19** fate, which involves either electrophilic attack or an internal redox reaction. Alkylation reactions by iminium ion species related to **19** are well-known in the mitomycin and mitosene literature (*1*, *20*, *25*−*27*) and is responsible for the cytotoxicity/antitumor activity of these compounds. The

Scheme 6



Scheme 7



electron-rich hydroquinone **$13H_2$** can also be attacked by the iminium ion, resulting in either head-to-head or head-to-tail coupling. The head to head coupling illustrated in Scheme 5 is followed by loss of formaldehyde to afford the coupled hydroquinone species, which oxidizes to **17** upon aerobic workup. Analogous dimerization processes have been documented in the indole literature (*28*−*30*), while the head-to-tail polymerization mechanism is unreported. Both mechanisms involve electrophilic attack, resulting in an iminium ion intermediate. To explain the observed dimer **18**, electrophilic attack must occur at the 7-position followed by tautomerization steps and finally reduction of the iminium ion with reducing equivalents originating from the hydroquinone ring. This iminium ion could also trap water to afford a 10-hydroxyl substituent. The mass balance of the reaction shown in Scheme 4 was only ∼20%, indicating the presence of other reaction products. Indeed a ^{13}C NMR of the crude reaction mixture showed a multitude of products; an envelope of resonances was present between 25 and 30 ppm, indicating a range of polymeric species. Electrophilic attack at the 6-position and/or the 7-position could give rise to many polymeric products.

The internal redox reaction shown in Scheme 5 involves proton trapping from the solvent or from the hydroquinone hydroxyl group as shown. This process has been documented for the mitomycin system (*31*) and also occurs in many quinone methide systems (*32*−*35*). Because the internal redox reaction gives rise to the quinone chromophore without aerobic workup, anaerobic reaction mixtures consisting of reduced **14** or WV-15 will become colored due to quinone buildup.

Reductive Activation with Dithionite. Dithionite-mediated reductive activation of mitomycin C has been employed in DNA alkylation reactions (*36*, *37*) and structural studies indicate the presence of a sulfonate ($-SO_3^-$) residue at electrophilic sites (*38*, *39*). However, the chemistry of dithionite suggests that sulfite esters ($-OSO_2^-$) and Bunte salts could be formed as well. The 10-^{13}C-labeled **14** and WV-15 were used to confirm that the sulfonate, sulfite diester, and only trace amounts of Bunte salt are formed upon dithionite-mediated reductive activation.

The dithionite species disproportionates in aqueous media to afford the hydrogen sulfite and thiosulfonate nucleophiles, Scheme 6 (*40*). Addition of electrophilic reagents to aqueous dithionite solutions is reported to afford S-substituted thiosulfates called "Bunte salts (*41*). Similarily sulfite monoesters and diesters are expected to result from the reaction of hydrogen sulfite with electrophiles. An anionic sulfite

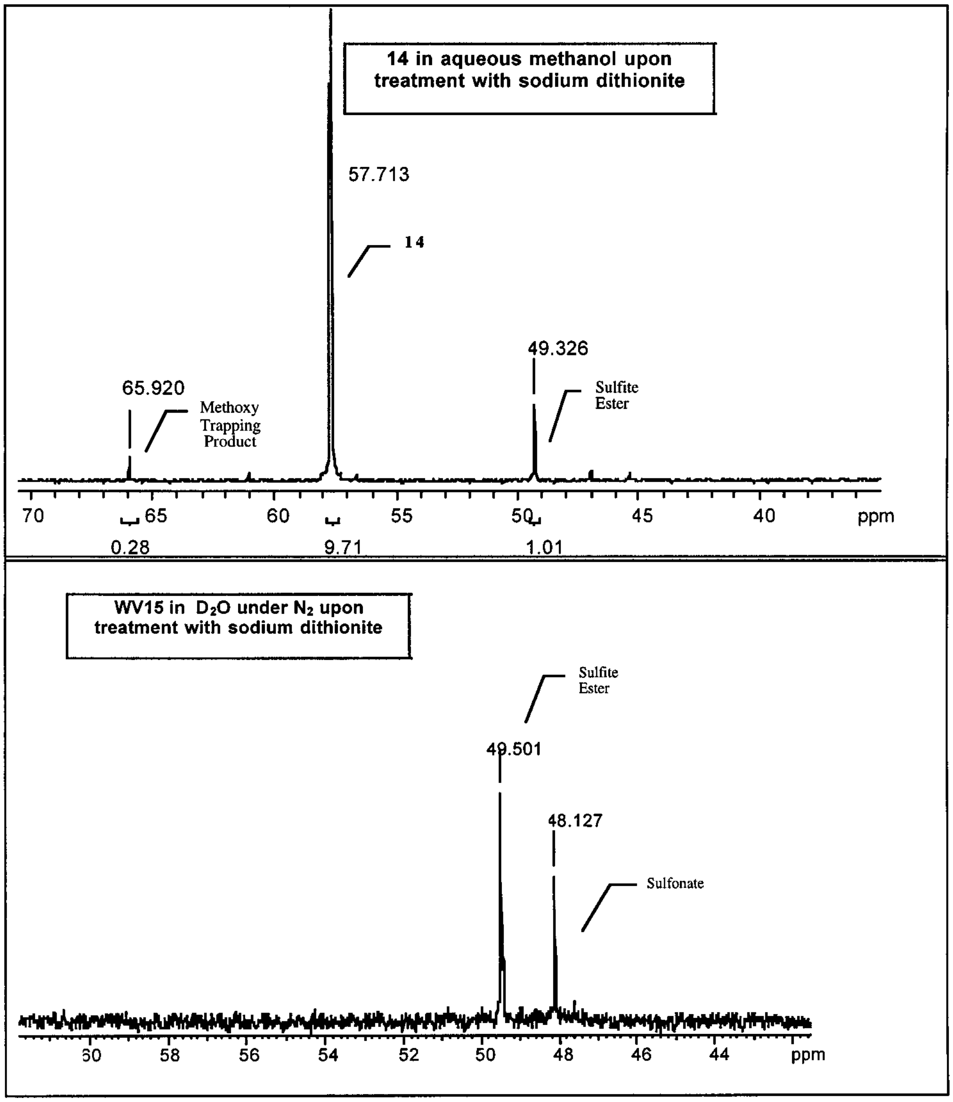


FIGURE 4: Quantitative ^{13}C NMR spectra of dithionite reduction reactions. The first spectrum shows the products obtained from the aerobic reduction of **14** by chloroform extraction. The second spectrum shows anaerobic reduction products of WV-15 in D_2O.

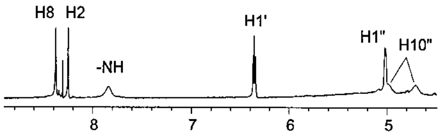


FIGURE 5: ^{1}H NMR spectrum of **24** showing the 10″-methylene split by the ^{13}C center into two broad singlets. Both of these singlets are coupled to the NH group of adenosine, whose anomeric (C1′) and aromatic protons are also shown.

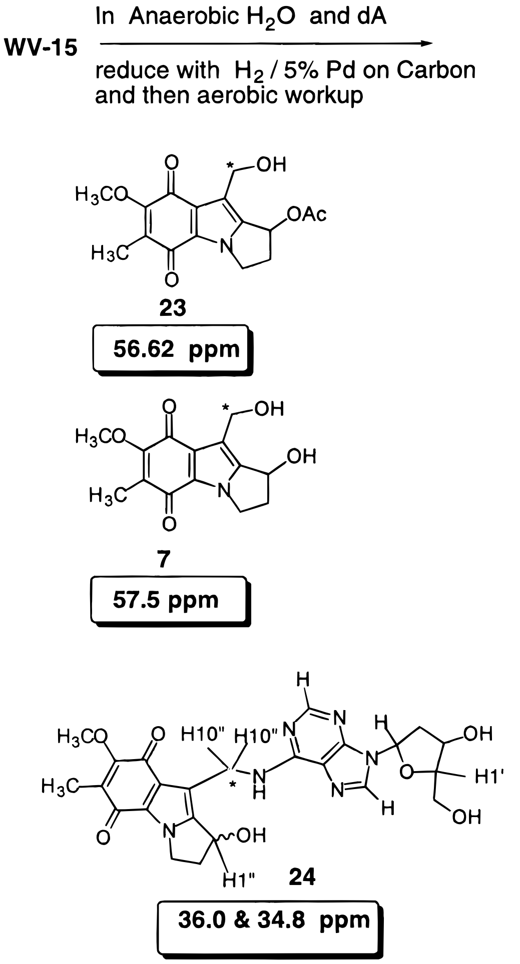
monoester ($-OSO_2^-$) can then rearrrange to the sulfonate ($-SO_3^-$) residue. A sulfite diethyl ester possesses a ^{13}C chemical shift of 58 ppm for the carbon attached to oxygen (*42*), while alkylsulfonates possess ^{13}C chemical shifts from 41 to as high as 49 ppm for the same carbon (*42*). Another way to distinguish sulfonate from sulfite is by conversion of the latter to a symmetric diester. In contrast, the sulfonate can only form a monoester.

Shown in Scheme 7 are the product structures resulting from the dithionite reduction of **14** under *aerobic* conditions; see the Experimental Section. Methylene chloride was added to the reaction mixture to extract the diester **20** and other nonionic species formed in the reaction. Shown in Figure 4 is the *quantitative* ^{13}C NMR spectrum of the methylene chloride extract, containing **20**, **14**, and methoxy trapping product **16**. This spectrum shows that most of the **14** was not reduced upon aerobic dithionite reduction. The isolation and characterization of the symmetric sulfite diester **20** confirms that a sulfite ester can form in dithionite reductive activation reactions and also provides the chemical shift for the 10-^{13}C center of a mitosene sulfite ester (49.37 ppm). In contrast, mitosene sulfonates possess a 10-^{13}C chemical shift of ∼47 ppm (*36*, *37*). The aqueous fraction of the reaction contained the mitosene sulfonate **22** and Bunte salt **21**, on the basis of their ^{13}C chemical shifts. The chemical shift of **22** (47.5 ppm) is consistent with previous reports (*36*, *37*). A reference for the ^{13}C chemical shift of the Bunte salt is not available, but a calculation indicates that the ^{13}C chemical shift should be ∼30 ppm.

Dithionite reduction of WV-15 was carried out in anaerobic D_2O, and the resulting products were evaluated by *quantitative* ^{13}C NMR spectroscopy immediately after the reduction, Figure 4. The spectrum indicates that the sulfite ester and the sulfonate are formed in a 60:40 ratio.

The above studies show that dithionite reduction of mitosenes results in formation of 10-substituted sulfite esters

Scheme 8



as well as sulfonates. The presence of the excellent sulfite leaving group at the mitosene 10-position suggests that alkylation reactions at this position could still occur. However, the subsequent rearrangement of sulfite to sulfonate results in observed loss of alkylation capability by this position (*36, 37*).

Reductive Activation of 10-^{13}C-Labeled WV-15. Reactions with Deoxyadenosine, Deoxyguanosine, and Buffer. Reductive activation of WV-15 was carried out in anaerobic pH 6.0 phosphate buffer in the presence of catalyst and hydrogen. We repeated the previously reported trapping of guanosine by reduced WV-15 (*27*) and also preformed a similarly designed adenosine trapping experiment; see the Experimental Section. 6-Amino adenosine adduct **24** (Scheme 8) was obtained in 11% yield. The ^{13}C NMR of the adduct revealed that the electrophilic carbon has chemical shifts of δ 36 0.0 and 34.8 consistent with the presence of nitrogen substitution at the electrophilic center and the formation of diastereomers. The 500 MHz ^{1}H NMR spectrum illustrated in Figure 5 shows the C-10-methylene protons split into a doublet by ^{13}C ($J = 148$ Hz) as well as the amino protons and other adduct protons. A COSY spectrum (not shown) revealed that the C-10-methylene doublet is coupled to the amino group proton.

This adduct is analogous to the previously isolated 2-amino guanosine adduct.[27] Both the adenosine and the guanosine trapping reactions produce a large number of products but very little of the amino adducts. TLCs of crude trapping reactions show a continuum of products with few discrete bands. However, the nature of these reaction products is evident from the ^{13}C NMR spectra in Figure 6 considering the chemical shift assignments obtained from the study of **14**.

The resonances at ~65 and ~68 ppm correspond to methanol trapping at the 10-center; substitution of DMSO for methanol in the reaction mixture results in loss of these resonances. Water trapping at the 10-position is not evident in the adenosine trapping reaction on the basis of the absence of resonances at ~56 ppm. However, water trapping is evident in the guanosine trapping reaction. The structures of water trapping products were assessed from a preparative hydrolysis study. The hydrolysis products of reduced WV-15 shown in Scheme 8 were identified as the diol **7** (22%) and the mono alcohol **23** (11%) along with unreacted WV-15 (7%), with the balance of products being head-to-tail polymers. The nitrogen-substituted 10-position is apparent in the adenosine trapping reaction (~35 ppm), but the guanosine trapping reaction shows very little of this product. Indeed, only trace amounts of the 2-amino adduct are obtained from trapping reactions (*27*). Both reactions formed considerable amounts of the head-to-tail polymer (28−29 ppm) and some head-to-head dimer (22.7 ppm).

Reductive activation of 10-^{13}C-Labeled WV-15. Reactions with DNA. For the DNA alkylation studies, we employed synthesized DNA hexamers d(AAATTT) and d(GGGCCC). These self-complementary hexamers permitted an assessment of WV-15 reductive alkylation of AT versus GC base pairs and also kept the number of unique adducts low. The use of long, noncomplementary pieces of DNA could dilute the ^{13}C label so as to make the labeled spectrum indistinguishable from the natural abundance spectrum.

The reductive alkylation reactions were carried out in anaerobic aqueous buffer at 25 °C, which is nearly equal to the hexamer melting point. Thus, there was duplex hexamer present in solution during alkylation reactions, ensuring the interaction of reduced WV-15 with either DNA groove. Reduced WV-15 did not react with nonduplex DNAs such as 2-mers. The alkylated DNAs were separated from WV-15 hydrolysis and polymerization products by two precipitations and then dried to afford orange pellets. The two precipitations are designed to remove any hydrolysis products from the DNA pellet. The hexameric DNA employed in these experiments does not entangle small molecules such as 600 bp strands, and the precipitations will readily remove any small molecules. Thus, the ^{13}C NMR chemical shifts of the alkylated DNA do not match those of the hydrolysis products. Indeed, the DNA spectra clearly show nitrogen and nitrogen/oxygen alkylation, while the hydrolysis spectra only show oxygen and carbon alkylation. The quinone chromophore ($\lambda_{max} = 450$ nm, $\epsilon = 1100$ M^{-1} cm^{-1}) permitted a determination of the percent alkylation by weight: 2% for d(AAATTT) and 2.4% for d(GGGCCC).

Shown in Figure 7 are the ^{13}C spectra of the WV-15 reductively alkylated hexamers measured in D_2O (4 mg sample/mL) with 1.6×10^{6} scans. Both spectra show iminium ion trapping of nitrogen, oxygen, and both nitrogen and oxygen (gem dialkylation). Possible nitrogen centers include the 2-amino of guanine bases, the 6-amino of adenine, and the N(7) centers. The reductive alkylation of the 2-amino center and possibly the N(7) center has been

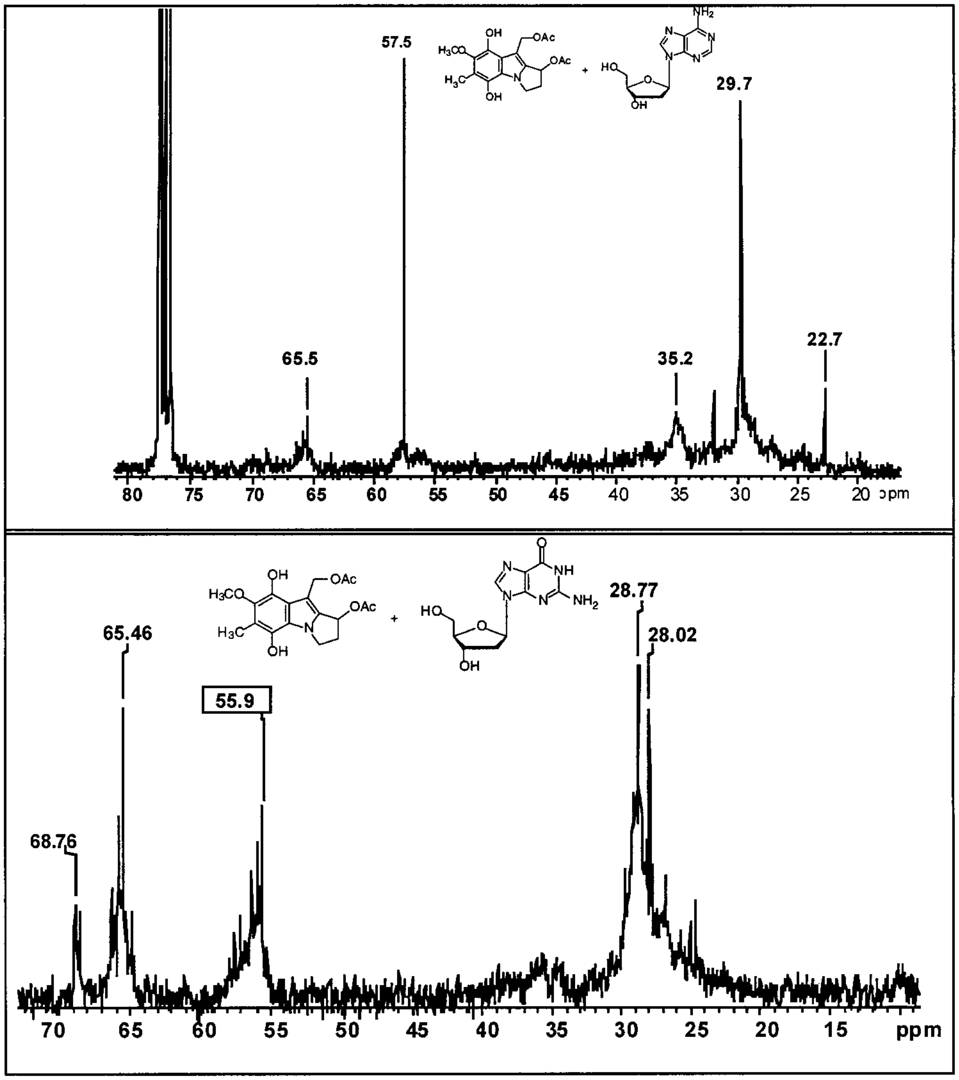


FIGURE 6: ^{13}C NMR spectra of crude chloroform extracts obtained from the reductive alkylation of adenosine and guanosine by WV-15. Both spectra reveal the polymerization and hydrolysis are the major pathways, with only trace amounts of the desired amino adducts present.

documented (*27*). Significantly, recent studies have confirmed that the 6-amino of adenine is reductively alkylated by mitomycin C (*12*). The ^{13}C-alkylated N(7) center has a chemical shift similar to those of alkylated amines,[22] and an assessment cannot be made as to which type of nitrogen is alkylated. The reductive alkylation of oxygen by WV-15 has not been documented; phosphate and guanine O(6) alkylations are likely. These are reasonable choices since quinone methides can alkylate phosphate oxygens (*43*), while cationic alkylating agents are known to alkylate phosphate oxygens and guanine O(6) centers (*22*). The resonances at δ 85−86 are best explained by both nitrogen and oxygen substitution at the electrophilic center. The gem dialkylation mechanism is as yet unknown, and further study is required.

CONCLUSIONS

The iminium ion chemistry of mitosenes was elucidated by placement of a ^{13}C at the 10-electrophilic center. ^{13}C NMR spectra of crude reaction mixtures readily reveal the number of products formed in the reaction as well as the atom attached to the electrophilic center (H, C, N, O, and S). These reaction products were then isolated with the ^{13}C chemical shift acting as an identifier. These studies revealed both known and yet unreported mitosene iminium ion chemistry.

Our findings show that mitosene iminium ions in anaerobic aqueous buffer undergo proton trapping, nucleophilic trapping, and head-to-head dimerization reactions in accord with previous reports (*1*, *20*, *25*−*30*). However, the new head-to-tail polymer **18** described in this paper represents the major product of the mitosene iminium ion. We have characterized both the dimeric and pentameric head-to-tail products **18**.

Our studies of the dithionite reduction of ^{13}C-labeled **14** and WV-15 revealed that sulfite esters and sulfonates are the reduction products. There was also spectral evidence of trace Bunte salt formation. Previously, the dithionite reduction products of mitosenes were considered to be only sulfonates (*38*, *39*). Evidence of sulfite ester formation was obtained from ^{13}C NMR spectra and the isolation and characterization of symmetric sulfite diester **20**. The chemistry of dithionite reductive activation of mitomycin C is very complex and depends on the rate and amount of dithionite addition (*37*). This complex chemistry will be amendable to study employing the spectral techniques described herein

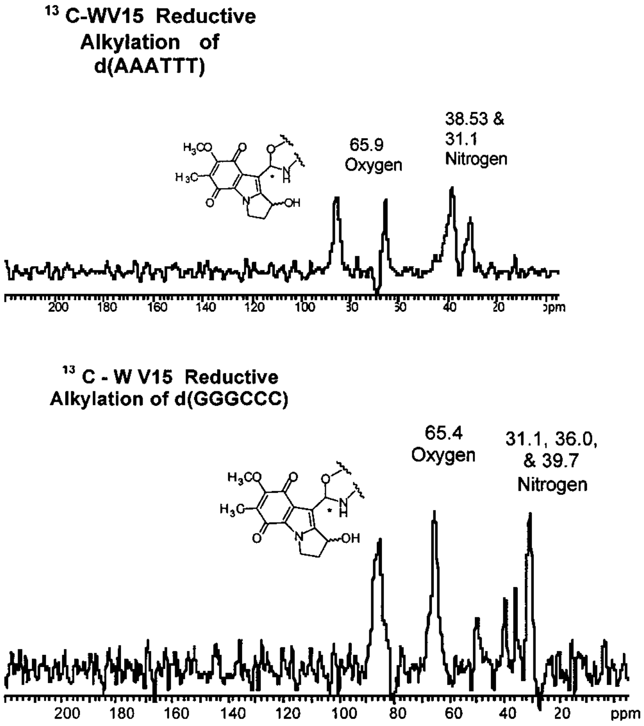


FIGURE 7: ^{13}C NMR spectra of the WV-15 reductive alkylation products of d(AAATTT) and of d(GGGCCC) in D_2O solvent. Both spectra show the presence of oxygen and nitrogen alkylation along with an as yet uncharacterized gem alkylation product.

with 10-^{13}C-labeled mitomycin analogues currently being prepared.

The reductive alkylation of deoxyguanosine and deoxyadenosine by WV-15 afforded a complex mixture of polymeric products along with trace quantities of the amino adducts, on the basis of ^{13}C NMR spectra of crude reaction mixtures (Figure 6). In fact, the reported reductive alkylation of deoxyguanosine by 150 mg of WV-15 afforded only ~4 mg of each enantiomer. Our reductive alkylation of deoxyadenosine by WV-15 likewise afforded a low yield of the amino adduct **24**. These preparative reactions are characterized by the presence of large amounts of head-to-tail polymer on the basis of the ^{13}C chemical shift of 27–29 ppm.

With the knowledge of the ^{13}C chemical shifts of 10-substituted WV-15, we were able to study the types of adducts formed with self-complementary DNA hexamers. This method for studying DNA alkylations rapidly provided an adduct inventory that supports evidence of nitrogen and oxygen alkylation reactions. In addition, evidence for the gem dialkylation of DNA at the 10-center of WV-15 was obtained from these studies. It would appear from our results that WV-15 reductively alkylates DNA in a nonselective fashion. These results indicate the need for further isolation studies of DNA WV-15 adducts.

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