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THE UNIVERSITY OF ALBERTA

STUDIES RELATED TO THE ANTITUMOR ANTIBIOTIC MITOMYCIN C AND ANALOGS

by

ASHER BEGLEITER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

SPRING, 1976

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADCATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled .....
STUDIES RELATED TO THE ANTITUMOR ANTIBIOTIC MITOMYCIN C AND ANALOGS .....

.....

submitted by ASHER BEGLEITER .....

in partial fulfilment of the requirements for the degree of Doctor of PHILOSOPHY .....

.....
Supervisor

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External Examiner

Date .....

To Gertie

ABSTRACT

Various aspects of the physical and chemical properties of mitomycin C have been studied in order to gain information about the mode of action of this clinically important antibiotic and antitumor agent. The ^{13}C nmr spectra of mitomycin C and the structurally related streptonigrin were analyzed and the peaks assigned. The strong conjugative interaction between the N-4 nitrogen and the quinone group of mitomycin C, which has been proposed to account for the stability of the unreduced form of the antibiotic, was confirmed.

The interaction of mitomycin C with DNA was examined using rapid and convenient ethidium fluorescence assays. This led to the development of an assay for measuring the ability of various agents to covalently alkylate DNA. Studies on the pH dependence of covalent cross-linking and alkylation of DNA by mitomycin C, as well as work with mitomycin derivatives confirmed, for the first time, the involvement of both the aziridine and carbamate groups in the covalent attachment to DNA. The step-wise nature of covalent cross-linking by mitomycin C was demonstrated and the reaction sequence involved has been determined. Possible mitomycin C metabolites were shown to have the ability to cross-link and alkylate DNA, raising the possibility of their involvement in the action of mitomycin C.

It was found that mitomycin C induces single strand

scission in DNA by the generation of superoxide and hydroxyl radicals. Evidence was obtained for the operation of a proximity effect in the scission process arising from the covalent interaction with the DNA.

Electroanalytical experiments confirmed the short life-time of the semiquinone of mitomycin C. Quinone analogs of mitomycin C were prepared. Studies of the interaction of these analogs with DNA supported the proposed preferential interaction of the aziridine group with DNA. The covalent cross-linking of DNA by aziridinoquinones was confirmed and evidence was obtained for preferential alkylation on guanine. The ability to cross-link DNA was found to correlate fairly well with antitumor activity.

Potential bioreductive alkylating agents were prepared and structure-cross-linking ability correlations were examined.

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

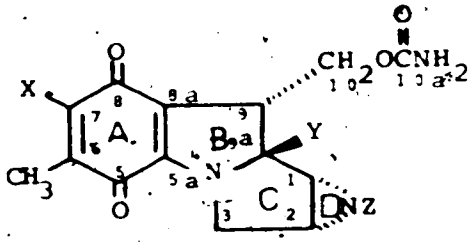
LIST OF FIGURES

LIST OF FIGURES (continued)

CHAPTER I

INTRODUCTION

The class of antitumor antibiotics known as the mitosanes was discovered by Hata[1] in 1956 and mitomycin C, one component of the culture broth, was isolated by Wakaki and coworkers[2] from *Streptomyces caespitosus* in 1958. Webb et al[3] showed that the mitosane structure was a fused four ring system containing both an indoloquinone and an aziridine ring. This structure was confirmed by x-ray analysis, which also revealed the relative stereochemistry of the four asymmetric centres.[4]

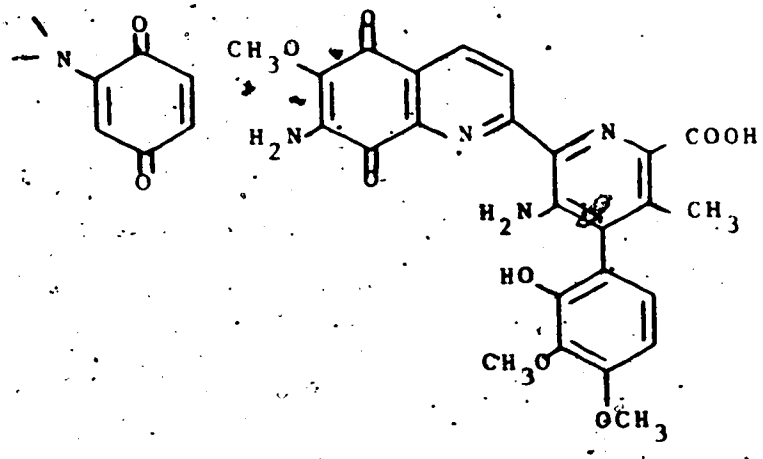


1

	X	Y	Z
Mitomycin A	OCH_3	OCH_3	H
Mitomycin B	OCH_3	OH	CH_3
Mitomycin C	NH_2	OCH_3	H
Porfiromycin	NH_2	OCH_3	CH_3

The mitosanes are antibiotics effective against both gram positive and gram negative bacteria, as well as against some viruses.[5] They exhibit useful activity against a variety of tumor cells including Erlich ascites tumor, Glioma 26 and Friend virus leukemia.[6] Mitomycin C and porfiromycin appear to be the most active in this regard and are now in clinical use in Japan. They have been found to be most effective against breast, stomach, and lung cancer, and cancer of the colon.[7] Mitomycin C is toxic with tolerated doses of approximately 40 mg per course of treatment.

The presence of an actual or potential aminoquinone moiety in mitomycin C, a common structural feature of other antibiotics and antitumor agents such as streptonigrin 2, actinomycin 3, rifamycin 4, and aziridinoquinones like tremimon 5, increases the interest in the mitosanes.



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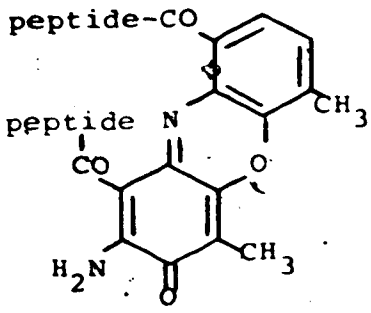

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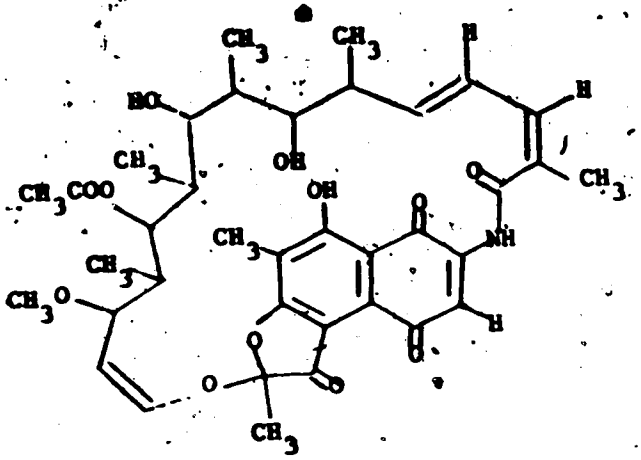

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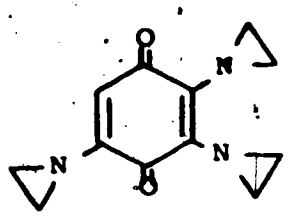

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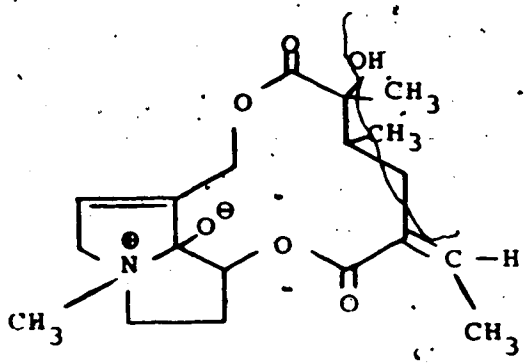
Other structural similarities to the pyrrolizidine alkaloids (e.g.) senkirkine 6 which also exhibit antitumor activity, provides additional impetus for studying this class



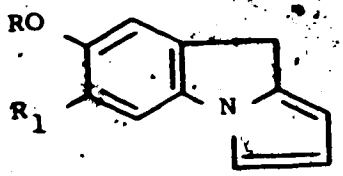
6

of compounds

Studies on the Synthesis of the Mitosanes

Despite the considerable efforts of a number of research groups in both North America and Japan, no total synthesis of the mitosane structure has been reported to date.

Weiss and his coworkers at Lederle Laboratories began their synthetic studies in 1962, and in a series of papers[8] they described a stepwise approach to the synthesis of the four ring system. This is illustrated in Scheme 1. Numerous attempts were made by these workers to introduce the aziridine ring. Conversion of 7 to the 1,2-hydroxyamine and cyclization was unsuccessful. Attempts to introduce a double bond between C-1 and C-2, so that other procedures for forming the D ring could be tried, failed due to rapid isomerization to 8. A final attempt to prepare the fused aziridine ring via a nitrene insertion was similarly unsuccessful. As a result, this approach to the total syn-

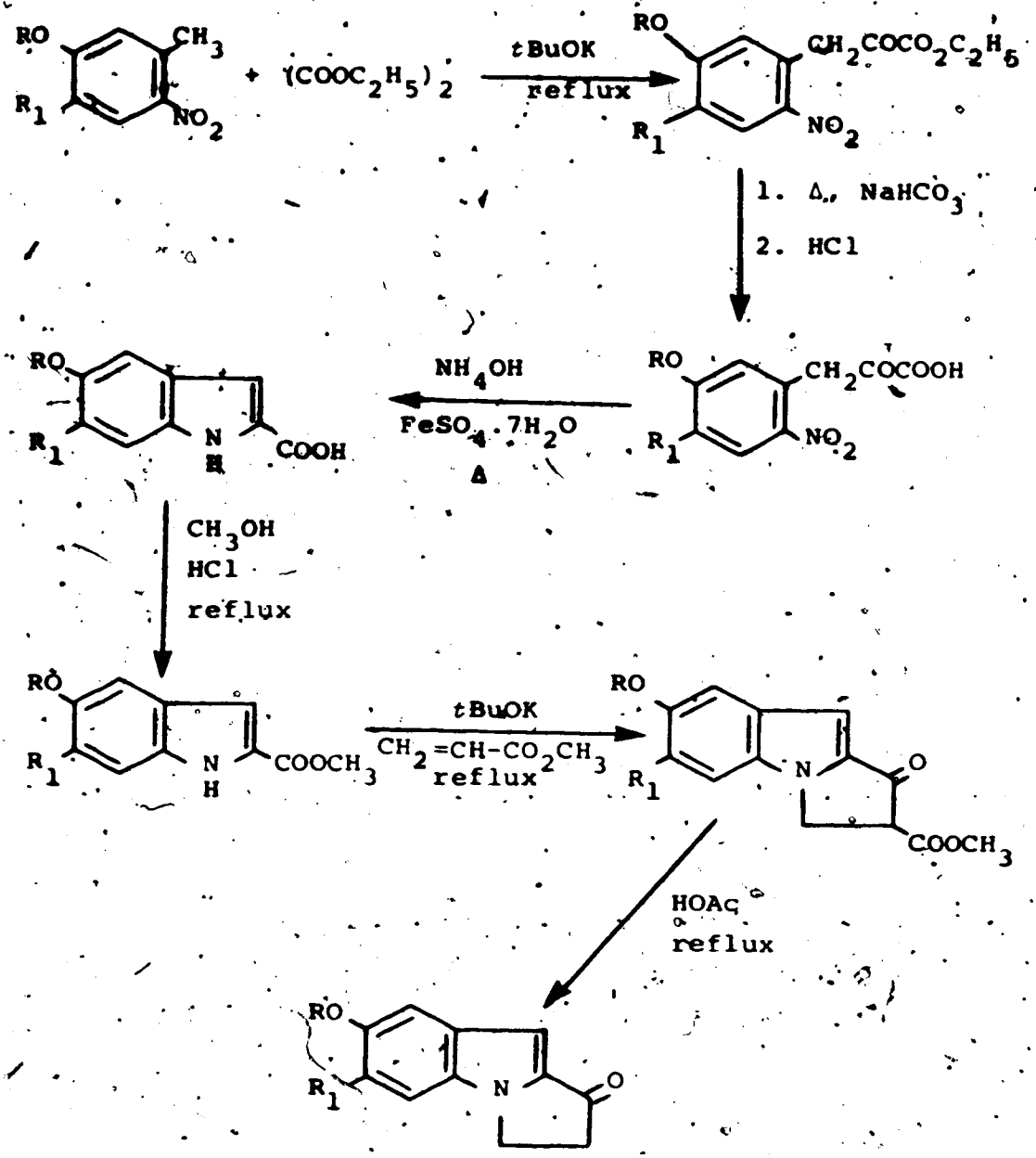


8

thesis of the mitosanes had to be abandoned.

However, in related work Weiss and coworkers[8] did

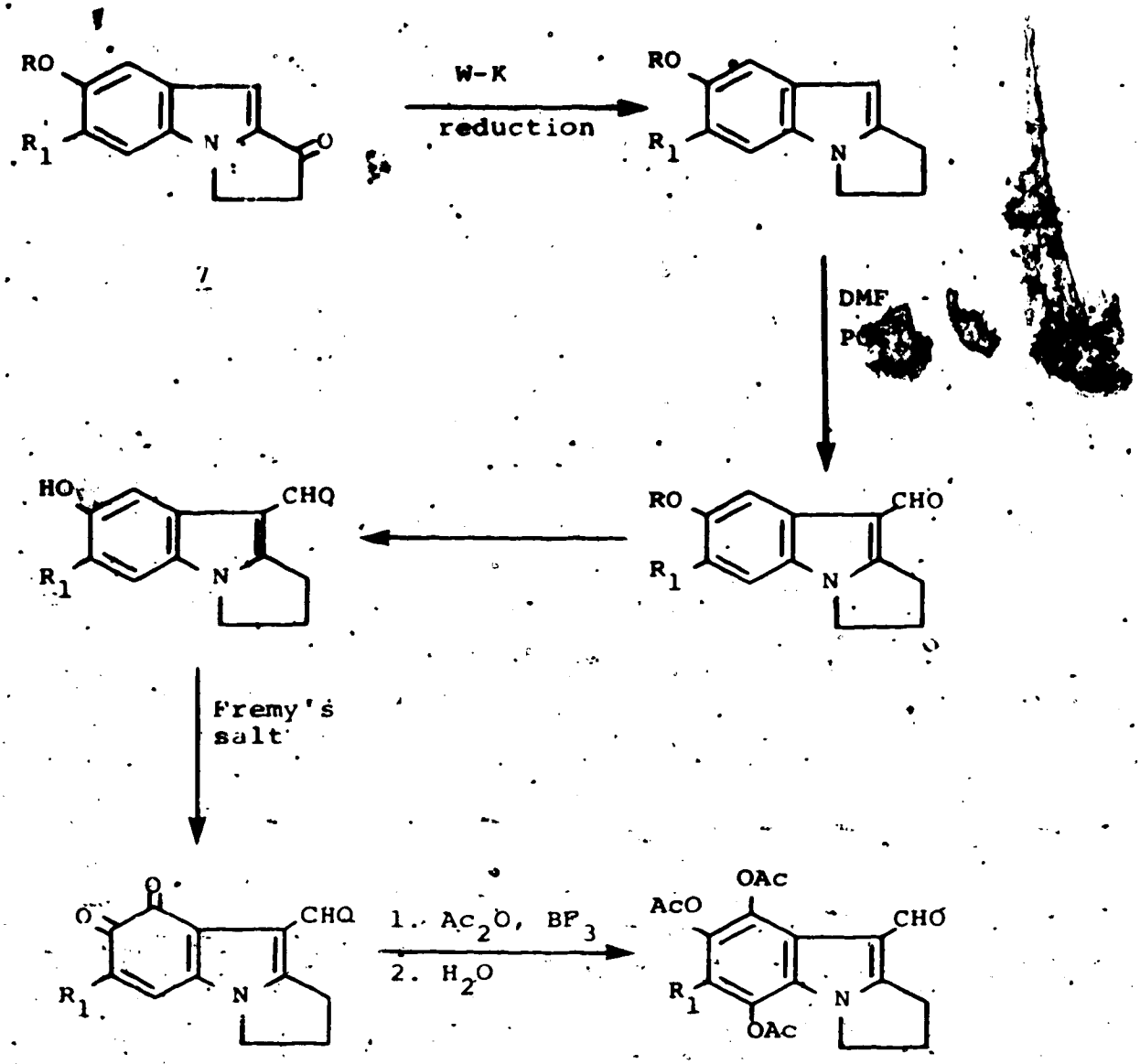
Scheme 1


RO
CH3
R1
NO2
+ (COOC2H5)2
tBuOK
reflux
CH2COCO2C2H5
1. Δ, NaHCO3
2. HCl
CH2COCOOH
NH4OH
FeSO4 . 7H2O
Δ
COOH
CH3OH
HCl
reflux
COOCH3
tBuOK
CH2=CH-CO2CH3
reflux
O
HOAc
reflux

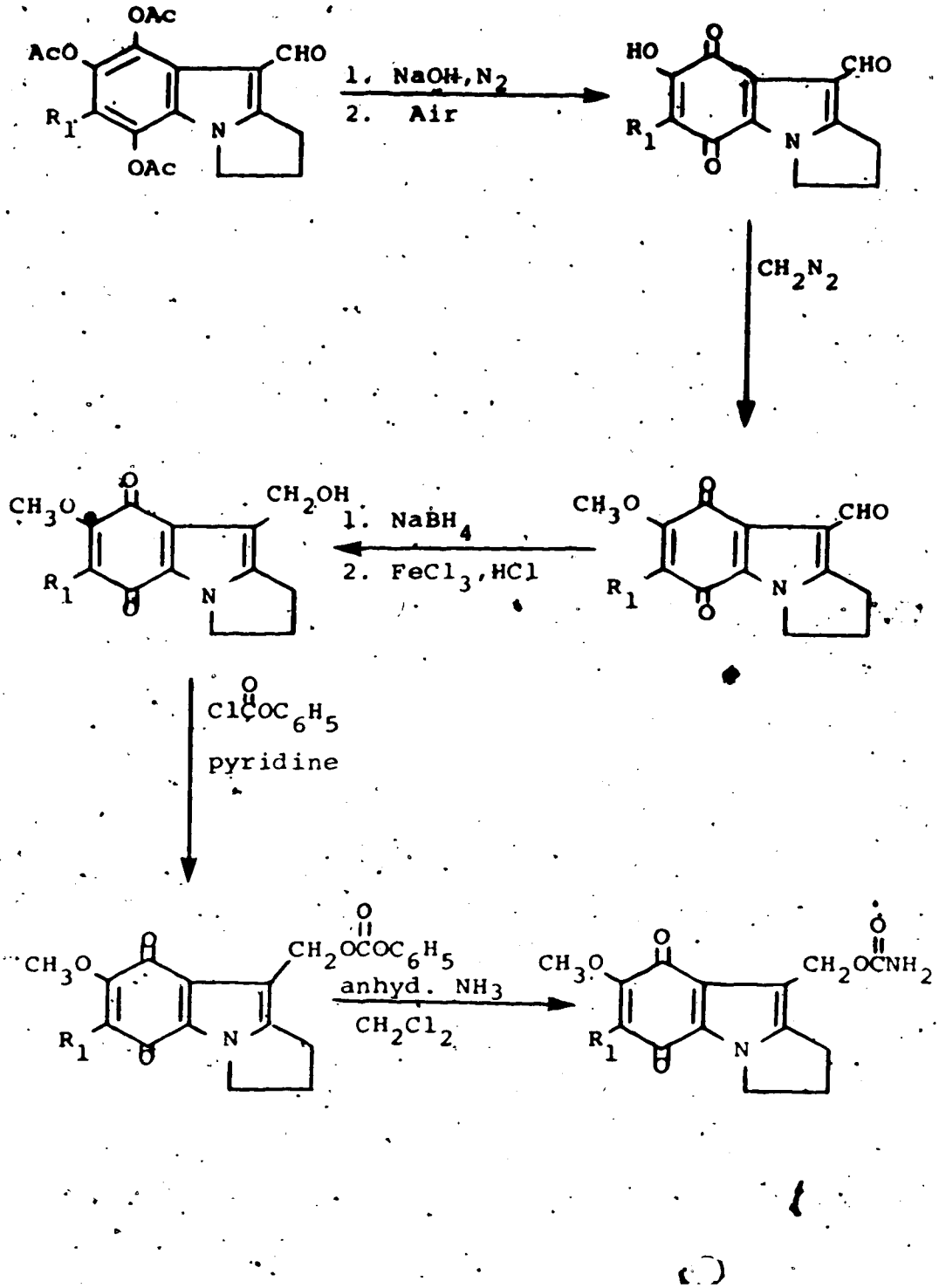
develop a number of useful methods for introducing the other important functional groups of the mitosanes. They were able to combine these methods in a compatible manner to introduce both the quinone and the carbamate side chain (Scheme 2). This scheme was later modified by Remers[9] to allow for the introduction of the carbamate group at C-10 while still maintaining functionality at C-1 (Scheme 3). The product obtained, 7-methoxy-1-oxomitosene 9, is a degradation product of mitomycin A and represents the first link between the synthetic and degradation studies on the mitosanes.

Takada and his coworkers[10] in Japan took an entirely different approach. They planned to introduce the C ring and possibly the D ring intact into a suitably substituted A ring, and then close the C-9, C-9a bond to give the mitomycin skeleton. This approach is illustrated in Scheme 4. The final ring closure to give the tricyclic product involved the generation of a carbene at C-9 from the corresponding tosylhydrazone 10 and insertion at the appropriate carbon of the pyrrolidine ring. Completion of the synthesis of mitomycin C by this route would require the introduction of the correct functionality at C-7 and C-10 and the preparation of the fused aziridine ring system prior to introduction of ring C. Work having a bearing on this last point is described in Chapter IV as a part of a study on the preparation of mitomycin analogs.

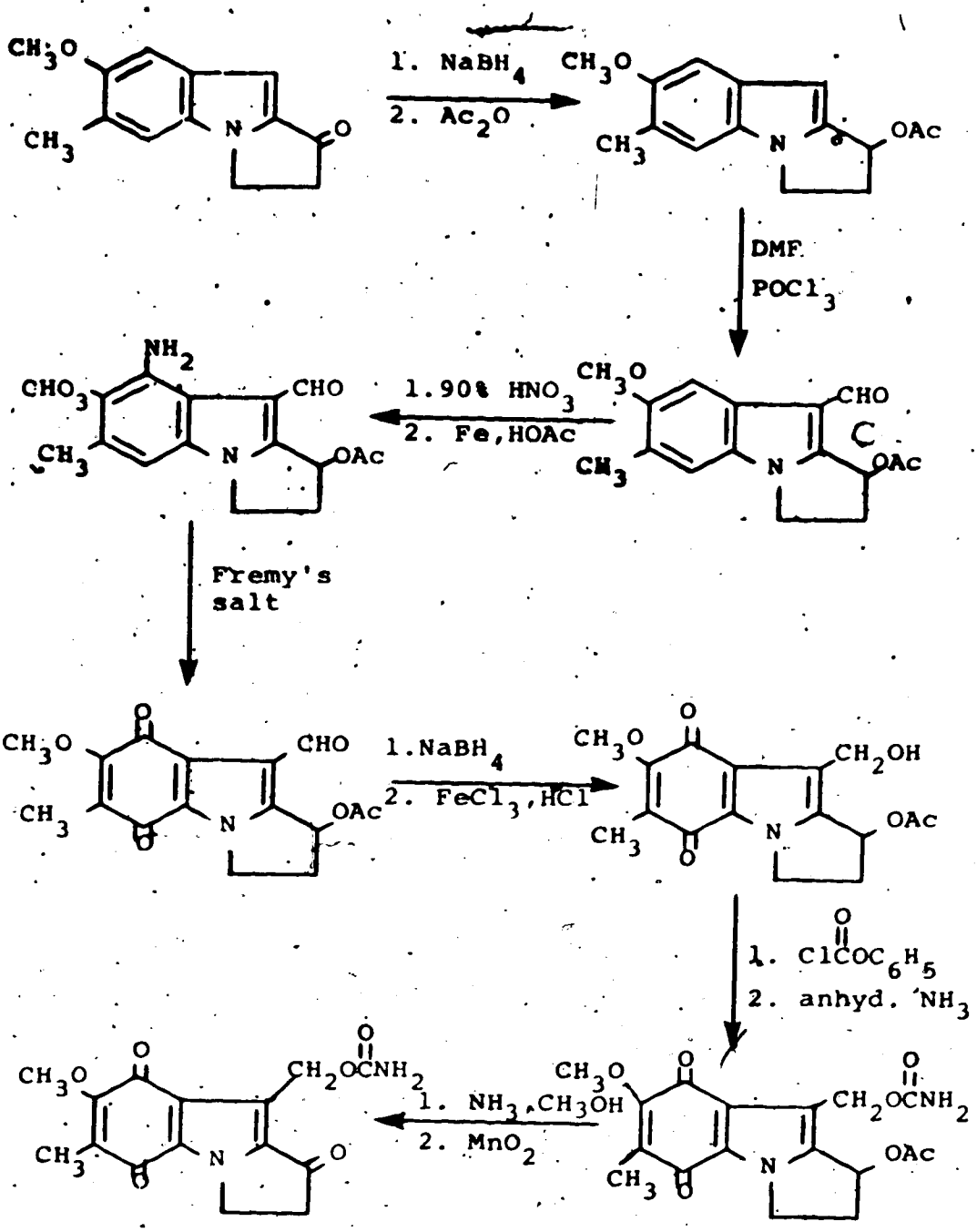
Scheme 2


RO
R1
N
O
W-K
reduction
DMF
HO
CHO
Premy's
salt
1. Ac2O, BF3
2. H2O
AcO
OAc

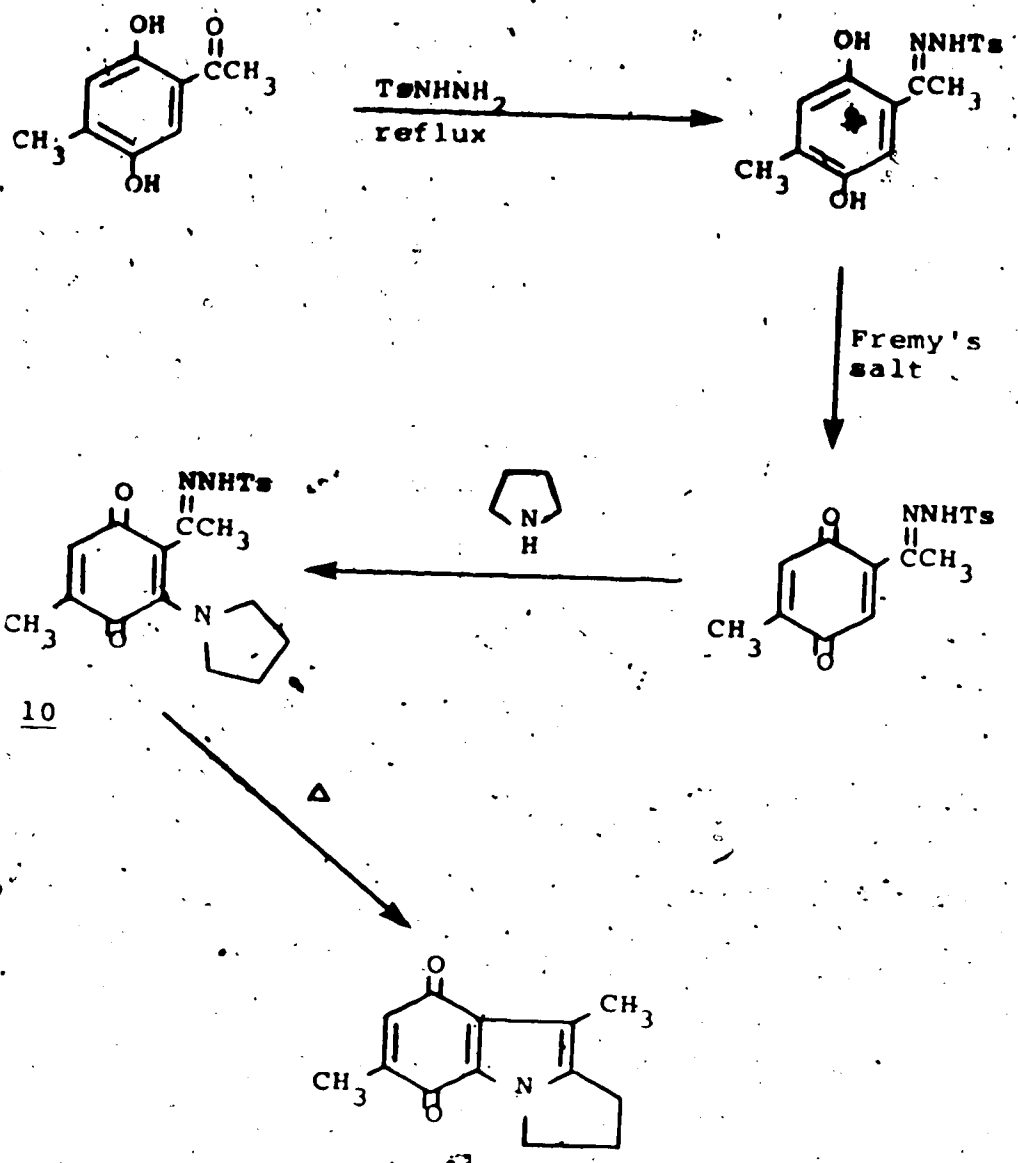
Scheme 2 (continued)



Scheme 3


1. NaBH4
2. Ac2O
DMF
POCl3
1.90% HNO3
2. Fe,HOAc
Fremy's salt
1.NaBH4
2. FeCl3,HCl
1. ClCOC6H5
2. anhyd. NH3
1. NH3,CH3OH
2. MnO2

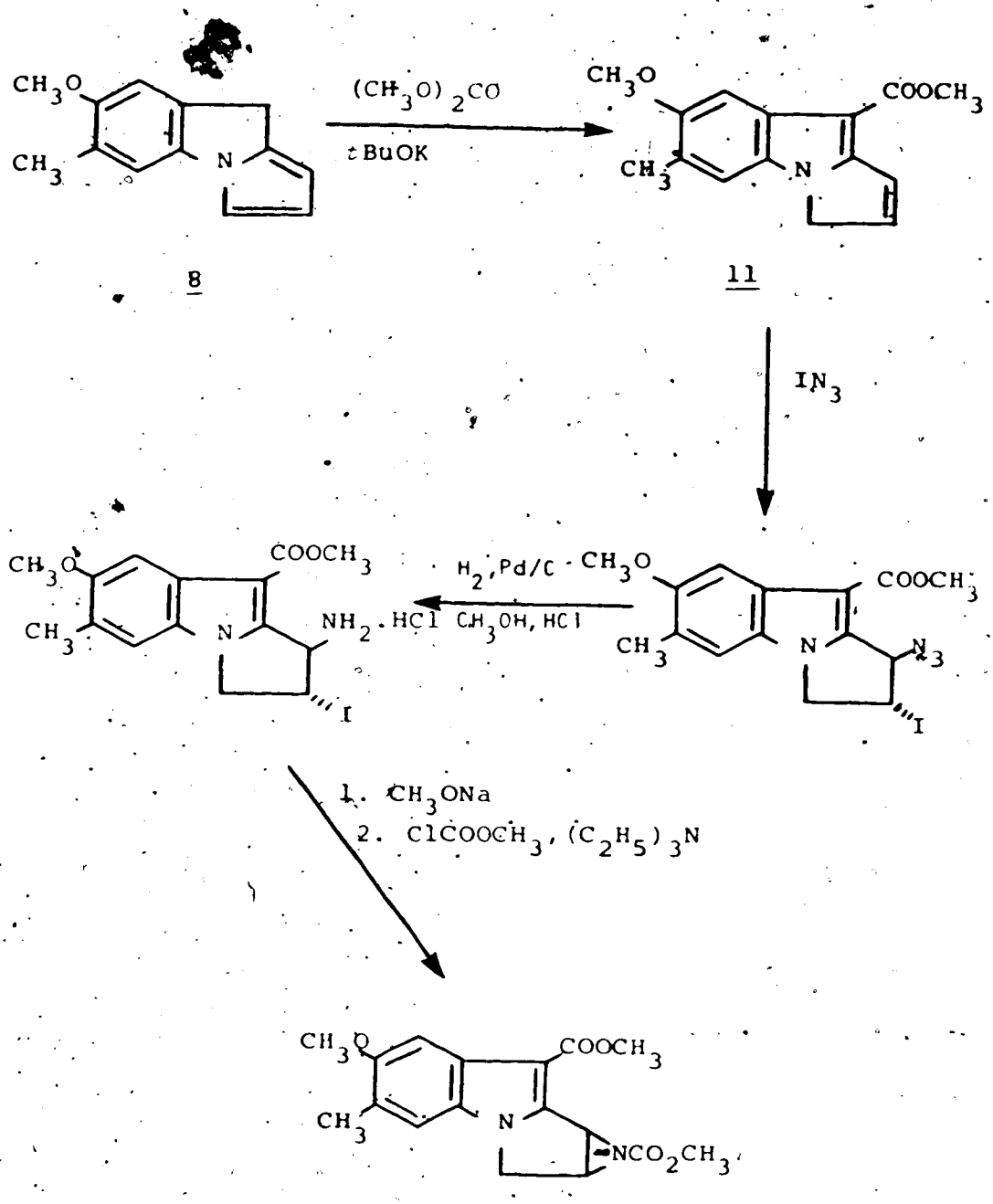
Scheme 4


OH
O
CCH3
CH3
OH
TsNHNH2
reflux
OH
NNHTs
CCH3
CH3
OH
Fremy's
salt
O
NNHTs
CCH3
CH3
O
N
H
O
NNHTs
CCH3
CH3
O
N
10
Δ
O
CH3
CH3
O
N

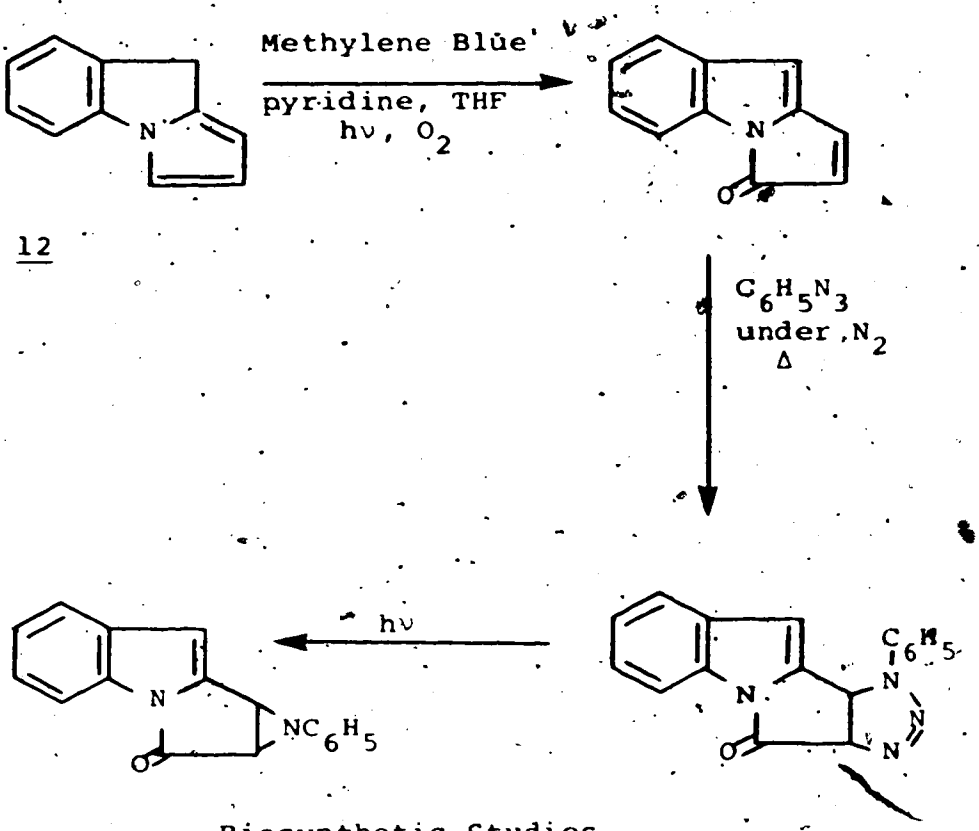
A Japanese group reported the synthesis of the tetracyclic ring system of the mitosanes in 1969.[11] They made use of the tricyclic pyrrolo compound 8 previously obtained by Weiss[8] (Scheme 5). They were able to isomerize it to 11 by the introduction of an ester group at C-9. The aziridine was then introduced by iodine azide addition to the double bond. This reaction sequence appears to hold great promise since a procedure has been devised for converting similar compounds to the corresponding quinones.[9]

Franck and Auerbach[12] also succeeded in preparing the four ring mitomycin skeleton (Scheme 6). Their synthesis began with a similar tricyclic compound 12. Singlet oxygen oxidation introduced a ketone at C-3. The double bond in ring C was then activated for 1,3-dipolar cycloaddition with phenylazide. Subsequent photolysis produced the fused aziridine system.

Scheme 5


CH3O
CH3
N
(CH3O)2CO
tBuOK
COOCH3
8
11
IN3
H2,Pd/C
CH3OH, HCl
NH2 . HCl
I
1. CH3ONa
2. ClCOOCH3, (C2H5)3N
NCO2CH3

Scheme.6

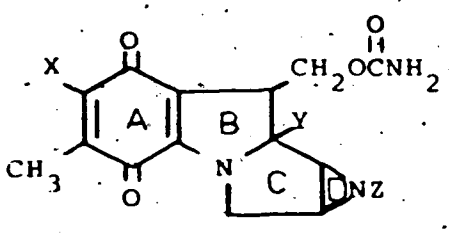


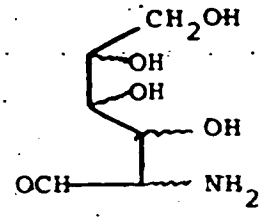
Biosynthetic Studies

In studies dealing with the biosynthesis of the mitosanes, Wakaki[2] found that the relative amounts of the different mitomycins produced by the organism could be altered by changing the culture conditions. Kirsch and his coworkers[13] developed a chemically defined medium which supported production of mitosanes by a wild culture of *Streptomyces verticillatus*. This allowed them to undertake a systematic study of the effect of various organic metabolites on mitomycin production.

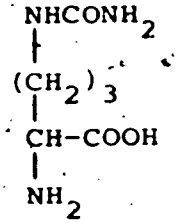
The presence of an indole-like nucleus in the mitosane structure suggested a biosynthetic pathway involving the amino-acid tryptophan. However, the addition to the culture medium of compounds structurally or biosynthetically related to the indole nucleus had no effect on antibiotic yield. Similarly, addition of ^{14}C-labelled tyrosine or tryptophan produced no label incorporation.[13,14] On the other hand, ^{14}C-label was incorporated when labelled L-methionine was supplied to the culture medium. This, along with the inhibitory effect of ethionine, a known antagonist of biological transmethylation by methionine, suggests that L-methionine is involved in the methylation of sites 7 and 9a.[15]

Work by Hornemann and coworkers[14,16] has shown that label from D-glucose and from D-ribose appears in the methylbenzoquinone moiety (ring A), which is thus probably derived from these sugars. A second study with multiple-labelled D-glucosamine 13 suggested that this sugar was incorporated intact in the formation of rings B, C, and D.





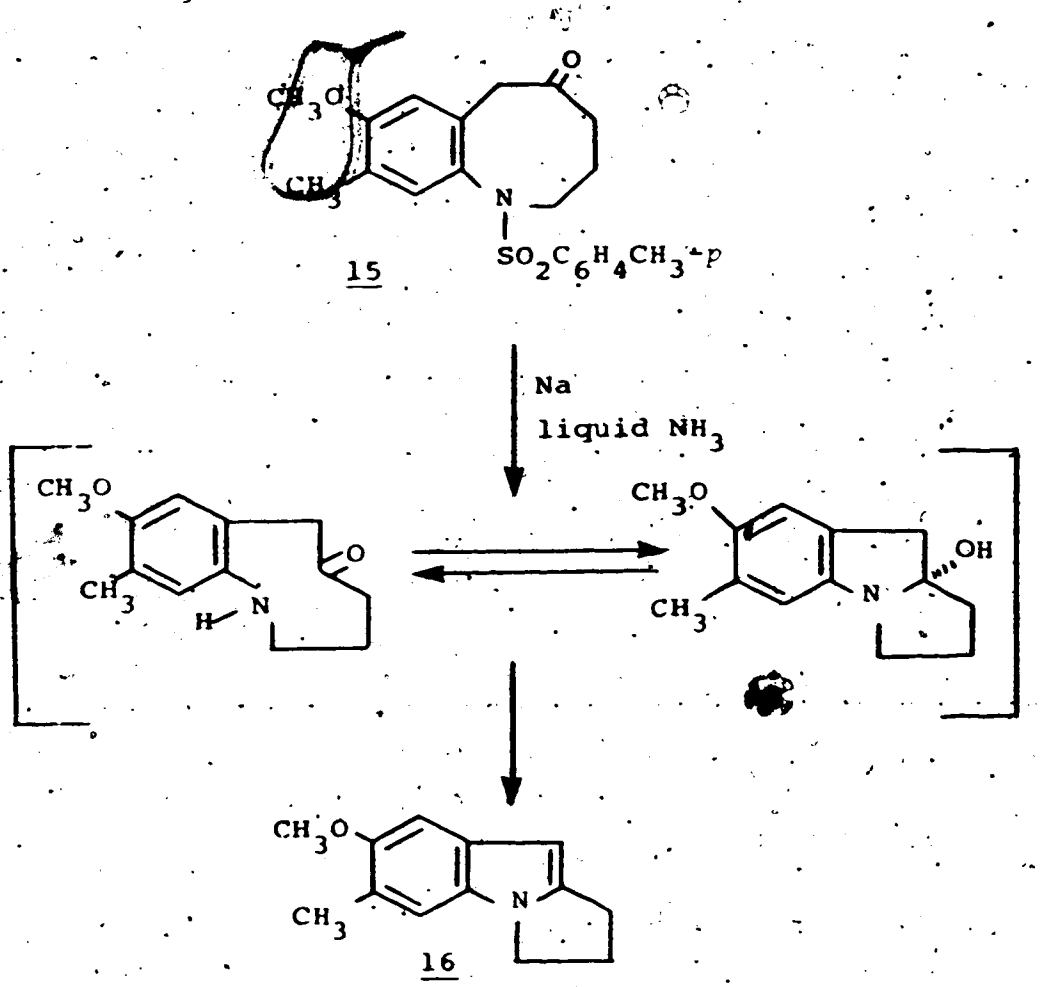
13



14

In addition, L-citrulline 14 was shown to be an intermediate in the insertion of the carbamoyl side chain of mitomycin.

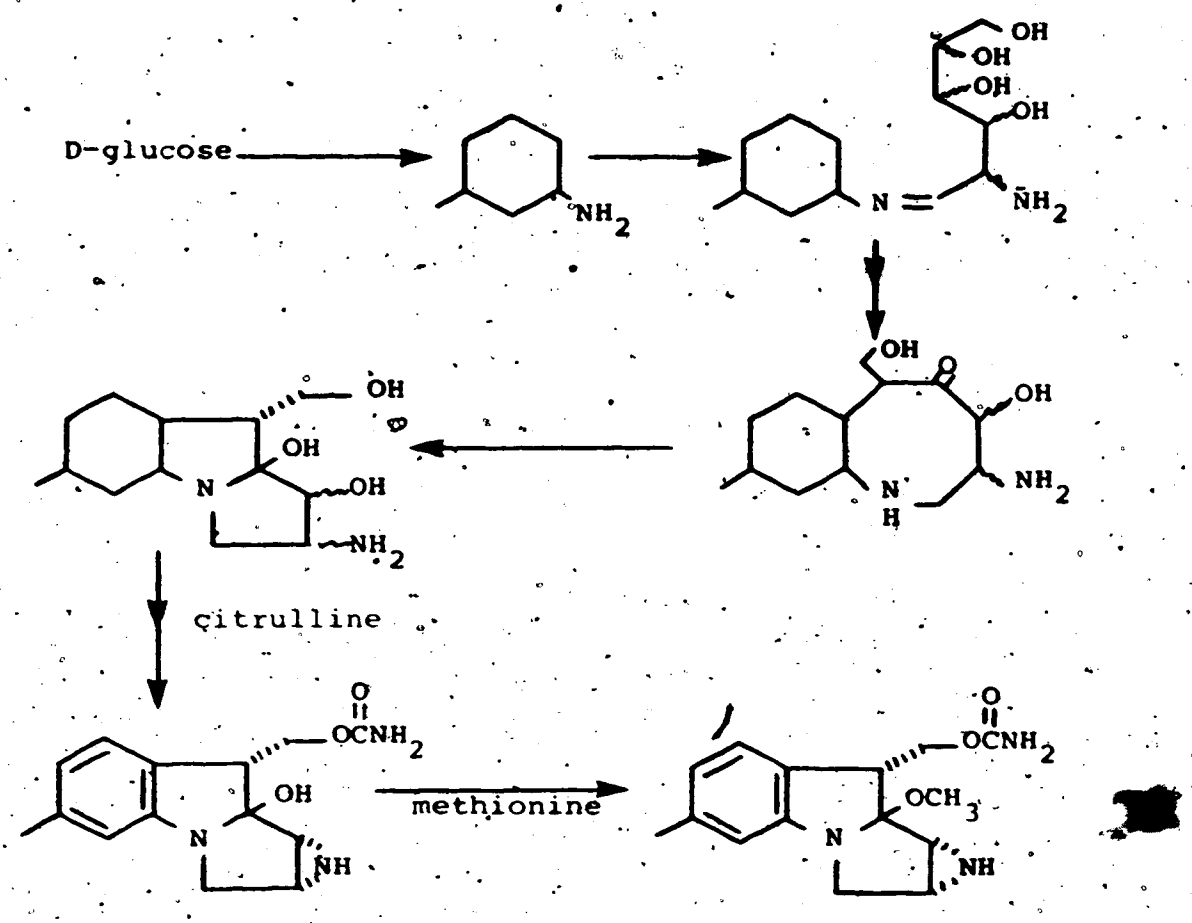
Recent work by Lown and Itoh[17] described the synthesis of a hexahydro-2,3-benzazocin-5-one 15 and its conversion via transannular interaction to a 2,3-dihydro-1H-pyrrolo[1,2-a]indole 16, the A,B,C parent ring systems of the mitosanes. This result led them to suggest the following biosynthetic scheme involving a similar transannular ring closure (Scheme 7):



15

16

Scheme 7



As is readily apparent, considerable work remains to be done before the biosynthesis of mitomycin is fully elucidated. In Chapter II the analysis of the ^{13}C nmr spectrum of mitomycin C is presented. This information should prove useful for further biosynthetic incorporation studies involving ^{13}C-labelled compounds, thus giving a more detailed picture of the metabolic precursors from which the structure of the mitosanes is derived.

Studies on the Mechanism of Action of Mitomycin C

Mitomycin C exhibits strong bactericidal and antineoplastic activity. In addition, it also shows mutagenic action against both bacteria and Drosophila, causing giant cell formation and other abnormal growth such as filament formation. It produces extensive DNA breakdown and fragmentation of chromosomes and is instrumental in the stimulation of chromosomal exchanges and crossing-over. Mitomycin C produces selective inhibition of DNA synthesis and inhibits the synthesis of induced enzymes.[5] Thus its activity seems primarily to be directed toward the DNA of the cell. The evidence for the interaction of mitomycin C with DNA is summarized in Table 1.

Table 1

Evidence for Interaction of Mitomycin C with DNA

1. Bacteria exposed to mitomycin C exhibit an initial first order decline in viability that implies a single hit mechanism. This points to the bacterial chromosome as the site of action.
2. Produces selective inhibition of monitored DNA synthesis in bacteria.
3. Extensive degradation of DNA accompanies administration of mitomycin C.
4. Chromosome fragmentation is a result of DNA breakdown.
5. Mitomycin C is mutagenic for both bacteria and drosophila.

Iyer and Szybalski[18] carried out a series of experiments in which they isolated the DNA from bacterial cells that had been treated with mitomycin C. They found that on heat denaturation of the isolated DNA, followed by rapid cooling and density-gradient centrifugation, most of the DNA appeared at the density characteristic of renatured DNA rather than in the position normally occupied by denatured DNA. They concluded from this that the complementary strands of the DNA were being covalently cross-linked.

The different behavior of denatured cross-linked and uncross-linked DNA on density-gradient centrifugation arises because the uncross-linked DNA does not renature to double stranded DNA on rapid cooling (Figure 1). This is because the re-registering of the separated complementary strands is a slow and temperature dependent process. On the other hand, in covalently cross-linked DNA, also called covalently linked complementary DNA (CLC-DNA), the complementary strands cannot completely separate on heat denaturation and the cross-link may serve as a nucleation point for rapid renaturation to double stranded DNA. Studies have shown that once such a nucleation point is present, the propogation of the helix proceeds at 10^7 - 10^8 base pairs per second.[19]

Shearing experiments with the cross-linked DNA produced a decrease in the amount of double stranded DNA found after heat denaturation (Figure 1). This indicated that the

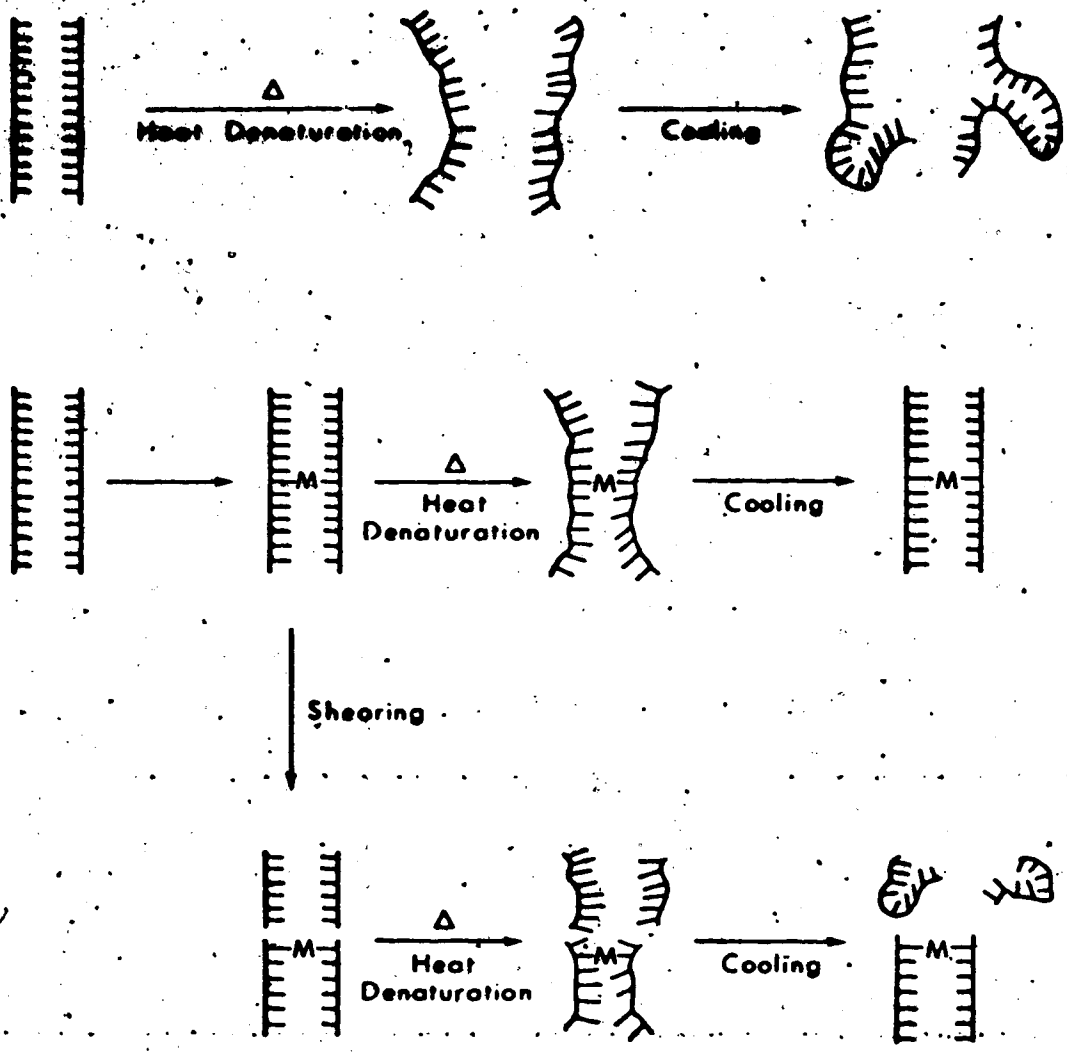


Figure 1. Behavior of DNA on heat denaturation.

mitomycin cross-links were infrequent. It has been estimated that mitomycin forms one cross-link per 20,000 base pairs.[18]

The rate of covalent cross-linking was found to be dependent on mitomycin concentration, temperature, and time.[18] Evidence that cross-linking is the primary mechanism producing the lethal effects of mitomycin C in bacteria was supplied by Szybalski and Iyer[18c] who showed that the rate of cell death correlated well with the degree of DNA cross-linking. Although mitomycin C also produces extensive DNA breakdown, this process is slow compared to the rapid lethal effect on bacteria. The work of Iyer and Szybalski indicated that inactivation of all cell nuclei by one cross-link per genome should result in cell death. This is no doubt due to the inability of the complementary strands of the cross-linked DNA to fully separate thus making it impossible for the DNA to replicate unless some repair mechanism intervenes.

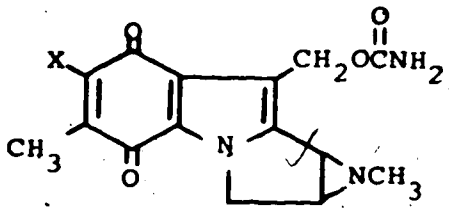
Interestingly, the transforming ability of mitomycin cross-linked DNA is only gradually lost.[18a] This provides further evidence for the infrequent formation of cross-links. Since normally only small DNA fragments are involved in the transforming activity, the few and widely separated cross-links would probably not prevent the localized denaturation required for the transforming activity of DNA, unless a cross-link occurred close to the DNA fragment in-

volved.

The mitomycin nucleus contains several reactive groups which could serve as the active sites for cross-linking:

1. the 7-amino or 7-methoxy group
2. the 9a-methoxy or hydroxy group
3. the aziridine ring
4. the carbamate side chain

Changing the 7-amino or 7-methoxy group had only a small effect on the lethal and cross-linking activities of the mitosanes.[20] It has been suggested[20c,d] that the C-7 substituent may modify the redox potential of the quinone and thus effect the biological activity. Replacement of methoxy with hydroxyl at position 9a produced a decrease in activity, while replacement with hydrogen produced an inactive compound. However, the mitosene derivative 17 in which the methoxy group has been eliminated is still a very active



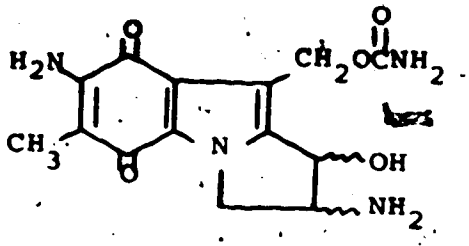
17

compound. Thus the 9a-position is also not involved in the

alkylation process; and in fact, the substituent at this position is eliminated during activation of the mitomycin.[18b] Mitomycin derivatives in which the aziridine nitrogen was alkylated retained their activity, while N-acylated derivatives, in which protonation of the aziridine nitrogen is prevented, showed decreased or no activity. Furthermore, aziridine ring-opened compounds showed no cross-linking ability. Thus the strained aziridine ring is implicated as one of the alkylating sites. Derivatives in which the carbamate is replaced by hydroxyl showed decreased biological activity. Methylation of the alcohol to give the corresponding methyl ether, produced a complete loss of activity. Further evidence for the involvement of the carbamate is provided by the demonstrated ability of ethyl carbamate to alkylate DNA *in vivo*.[21]

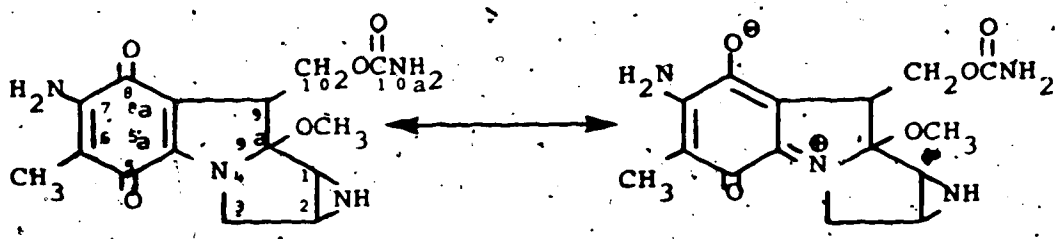
Attempts to induce cross-linking in purified DNA by mitomycin *in vitro* were unsuccessful. However, if a cell-free lysate from mitomycin sensitive bacteria was added, efficient cross-linking was observed.[18b] This indicated that mitomycin must be activated prior to cross-linking. Further investigation suggested that the activation process was an NADPH-dependent enzymatic reduction of mitomycin to its hydroquinone. Cross-linking of DNA by mitomycin could also be induced by chemical reduction with sodium borohydride, sodium dithionite, or by catalytic reduction by hydrogen. The reduced species thus formed is

quite unstable, since for cross-linking to be observed, DNA had to be added within seconds after the completion of the chemical reduction. Chemical reduction of mitomycin C followed by reoxidation gave the aziridine ring-opened derivative 18, which showed no cross-linking ability.



18

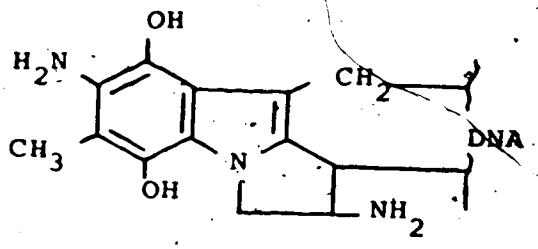
The stability of the unreduced mitomycins is related to the resonance between forms A and B which results



A B

in partial withdrawal of electrons from N-4 into the quinone ring and consequent stabilization of the 9a-methoxy

substituent, an otherwise good leaving group. Iyer and Szybalski[18b] have proposed that when mitomycin is reduced the conjugative interaction of the indole nitrogen with the six-membered ring is lost. The lone pair electrons on the N-4 nitrogen can then assist the elimination of the methoxy followed by formation of the fully aromatic indole system (Scheme 8). This proposal is supported by the facts that methanol was isolated quantitatively from reduction of mitomycin C[18b] and that the mitomycin derivative 17 shows activity comparable with that of mitomycin itself. Further activation at C-1 and C-10 can then lead to the covalently linked complex with DNA envisioned as 19.



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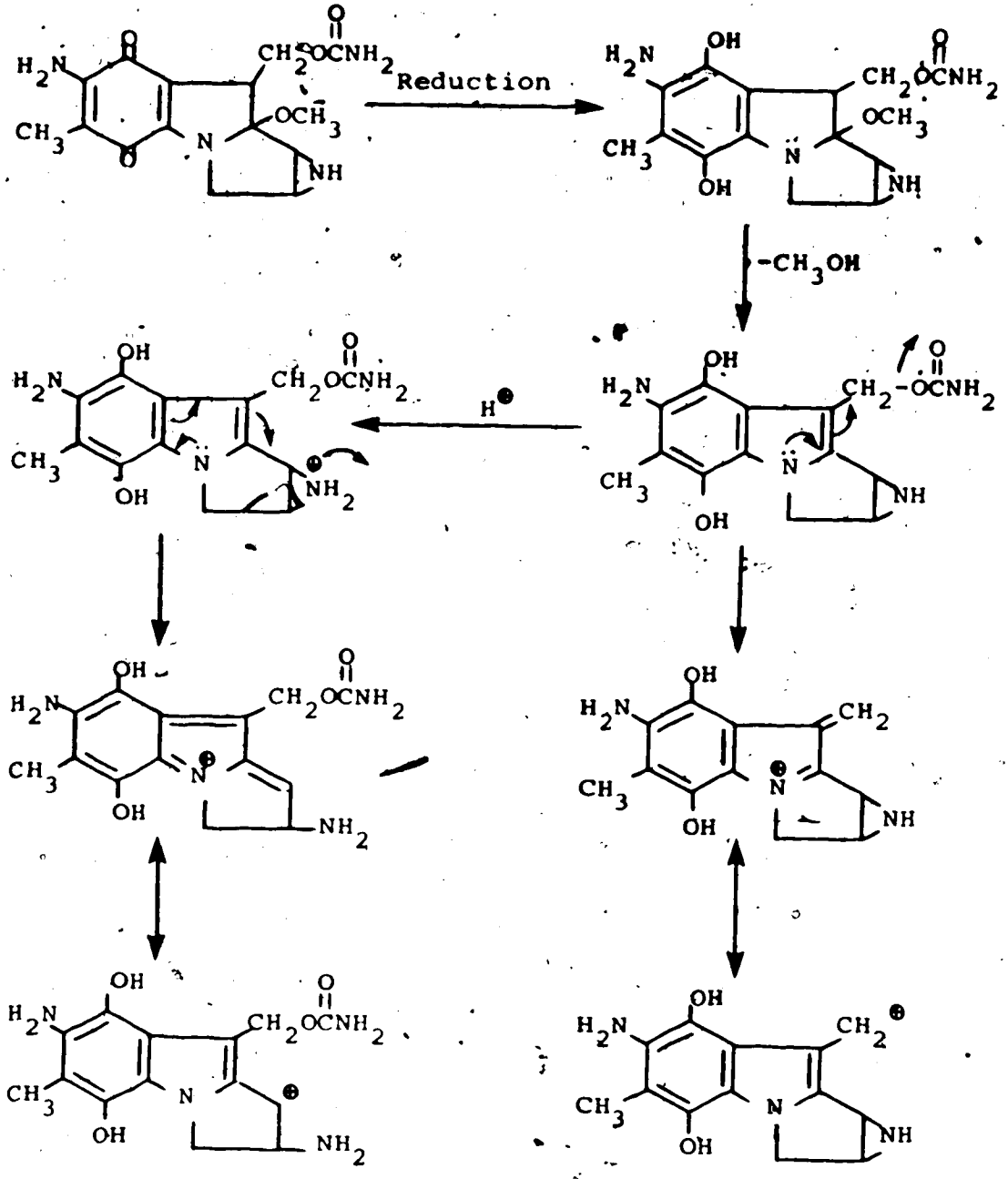
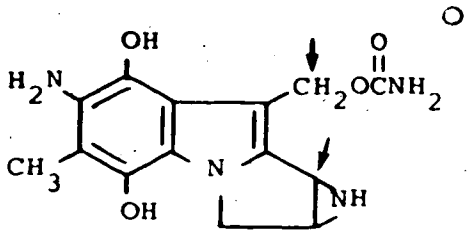
The activated form of mitomycin 20 shows a striking similarity to pyrrole analogs of the pyrrolizidine alkaloids *e.g.* dehydrosenecionine 21. Both are bifunctional alkylating agents with similarly located linkage points. There is evidence suggesting that senecionine activity may involve covalent cross-linking of DNA.[22] The alkyl-

Scheme 8



ating ability of structures such as 21 may be associated with the possibility of alkyl-oxygen cleavage of the allylic ester linkages.



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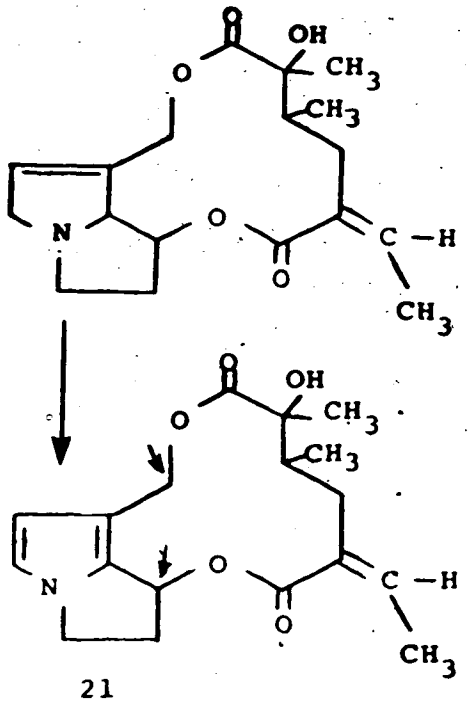


→ alkylating sites

21

Attempts to determine the sites of alkylation on the DNA have been made difficult by the low frequency of cross-links, not exceeding one per 1,000 base pairs.[18a] Iyer and Szybalski have shown that the degree of cross-linking increases with increasing (G+C) content of the DNA,[18b] indicating that either guanine and/or cytosine are the most favored points of alkylation. Studies by Lipsett and

Weiss[23] on the alkylation of homopolymers with ^{14}C-labelled porfiromycin, showed that guanosine polymers were alkylated to an extent at least four times higher than other nucleoside polymers. Attempts to alkylate 5'-AMP, 2'-UMP, and 2'-CMP with reduced mitomycin gave no alkylated nucleotides. However, guanine, guanosine, and 5'-GMP all underwent alkylation to give two products, guanyl and di-guanyl-mitomycin. The alkylation of tritiated s-RNA with ^{14}C-labelled porfiromycin was examined. Following alkaline hydrolysis of the RNA, paper chromatography revealed two radio-labelled products corresponding to monoalkylated guanine and diguanyl-mitomycin. There was no evidence for the involvement of C-C or G-C binding sites. More recent work by Tomasz[24] has cast some doubt on the reported chromatographic separation of the mitomycin-guanine complexes, however, the guanine residue still appears to be the most likely site of alkylation.

The N-7 and O-6 positions of guanine are the most sensitive to alkylation in DNA. Cross-links between the N-7 positions of the guanines on the opposite DNA strands has been postulated for cross-linking by nitrogen and sulfur mustards.[25] However, examination of space filling models of DNA and of mitomycin, revealed that the short (4.3 Å) four carbon span between C-1 and C-10 of mitomycin severely limits the choice of potential binding sites on the DNA.[18c] The best fit with the models was obtained for

links between the O-6 positions of the guanines.

Further evidence against the involvement of the N-7 position of guanine in the alkylation by mitomycin C was presented by Tomasz[26] who described a new assay to measure 7-alkylation of guanine residues in DNA. This assay is based on the finding that alkylation of the N-7 position of guanine derivatives makes the C-8 hydrogen extremely labile, resulting in rapid exchange of the C-8 hydrogen with the solvent under physiological conditions. When DNA labelled with tritium at the C-8 position of guanine residues was exposed to mitomycin C under the optimal conditions for covalent binding to DNA *in vitro*, no loss of tritium from the DNA was detected, thus indicating that mitomycin C did not alkylate at the N-7 position of guanine residues in DNA.

A recent paper by Tomasz and coworkers[24] proposes an important role for the semiquinone of mitomycin C. They suggest that the semiquinone combines with DNA in a non-covalent manner in a rate determining step. This interaction with DNA, which is proposed as intercalative in nature is then followed by further reduction to the hydroquinone and covalent binding to the DNA.

Weissbach and Lisio[27] used tritiated mitomycin C and ^{14}C-labelled porfiromycin to study the alkylation of DNA. They found that as many as one drug molecule per 1,000 base pairs was attached to the DNA. This is a much larger

figure than the one obtained by Iyer and Szybalski in their estimate of the extent of cross-linking. Furthermore, heat denatured, single stranded DNA also showed alkylation by labelled mitomycin. This indicated that only a small proportion, approximately 1 in 10, of the mitomycin molecules that alkylate DNA is actually involved in cross-linking. The other molecules must either alkylate the DNA with only one of the active sites on the mitomycin or must alkylate twice on the same DNA strand. This type of mechanism, which will henceforth be called alkylation of DNA as opposed to cross-linking of DNA, may have some importance in the mode of action of the mitosanes, since some mitomycin derivatives in which the aziridine ring has been opened retain their biological activity, although it is greatly reduced.[20b,c] Similarly, other monofunctional alkylating agents are known which also exhibit biological activity.[28] For the most part, however, monofunctional alkylating agents tend to be less active than the corresponding bifunctional compounds. Thus alkylation of DNA, although it does not appear to be a primary mechanism must be considered as a possible secondary mode of action.

It has been reported that mitomycin C produces extensive DNA breakdown.[29] Although this process is relatively slow compared to the rapid lethal effects of mitomycin C on bacterial cells, it has been suggested as a possible mode of action for the drug. A similar mechanism

has been proposed for other antitumor agents.[22,30] The DNA breakdown has been explained as being related to the excision phenomenon connected with the repair of mitomycin-alkylated DNA, owing to the stimulated production of exonucleases.[31]

Thus the mode of action of mitomycin C appears to be two-fold:

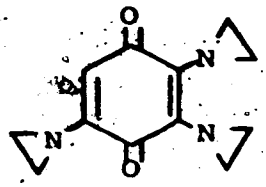
1. cross-linking and alkylation of DNA
2. degradation of DNA

As a part of our work on mitomycin C, the cross-linking, alkylation and degradation of DNA by this antitumor antibiotic was examined by a rapid and convenient ethidium bromide fluorescence assay. The cross-linking and alkylation of DNA by mitomycin C and some of its derivatives was studied as a function of pH in order to obtain information about the sequence of the cross-linking event. The preference for alkylation at guanine bases was examined by a study of cross-linking with DNAs of different (G+C) content. An electrochemical study of the reduction of mitomycin C was carried out and the redox potentials obtained were related to the proposed mode of action of the antitumor agent. Estimated lifetimes of the intermediates in the reduction of mitomycin C were determined from cyclic voltammetry and related to the proposed involvement of the semiquinone in the mechanism of action. The degradation of DNA by mitomycin C was explored by examining the induc-

tion of single strand scissions in covalently-closed circular DNA (CCC-DNA) by the ethidium fluorescence assay. The details of these studies, the results obtained, and their relation to the mode of action of mitomycin C are described in Chapter III of this work.

Studies on Mitomycin Analogs

Despite changes involving the substituents on the mitomycin skeleton, many mitomycin derivatives still show antibiotic and antitumor activity.[20] All of these compounds seem to have one common feature, the quinone ring system. Other benzoquinone derivatives have been shown to be good antibiotics and antineoplastic agents.[32] Many of these, including the clinically important antitumor agent, trenimon, 22, have other features in common with the mito-



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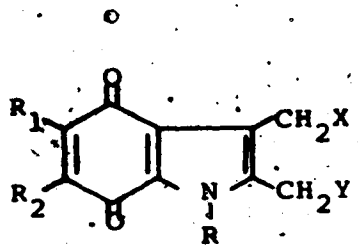
mycins, such as aziridine and carbamate groups. For the most part, their mode of action has been proposed to involve alkylation of DNA.

A series of amino and hydroxy benzoquinones have been synthesized and their antitumor activity tested.[33] More recently, Nakao and Arakawa[34] have prepared a series of quinones containing both aziridine and carbamate substituents in a configuration closely resembling that of mitomycin C. Tests revealed that many of these compounds were very active antitumor agents and in several cases much more active than mitomycin C itself. Since it seemed likely that the mechanism of action of these compounds involved cross-linking or alkylation of DNA, we undertook to synthesize some of them and to study their interaction with DNA using the ethidium fluorescence assay. Other aziridinoquinones that had exhibited antineoplastic activity were also prepared and studied. An effort was made to correlate the biological activity of these compounds with their structure. Results of these experiments are reported in Chapter IV.

Because of the relatively short span (4.3 Å) between the alkylating sites of mitomycin C, the number of possible alkylating sites on DNA is limited. In order to provide greater conformational flexibility between the alkylating centres, a series of mitomycin analogs in which the C-9, C-9a bond had been broken were prepared, and their alkylating and antitumor activities were investigated. These studies are also described in Chapter IV.

Recently, Sartorelli and coworkers have begun a study

on potential bioreductive alkylating agents related to the mitosanes.[28,35] It had previously been proposed that the portions of mitomycin essential for biological activity could be represented as 23.[20d] Sartorelli suggested that charge delocalization of the corresponding hydroquinone could result in *o*-quinone methide-like intermediates which



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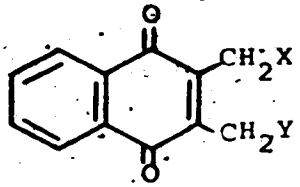
could act as the alkylating agent (Scheme 9). Consequently, they prepared a series of substituted naphthoquinones 24 and structurally similar *p*-benzoquinones 25 which on reduc-



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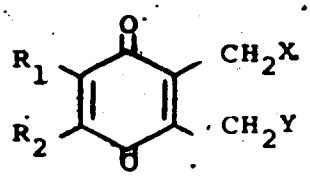

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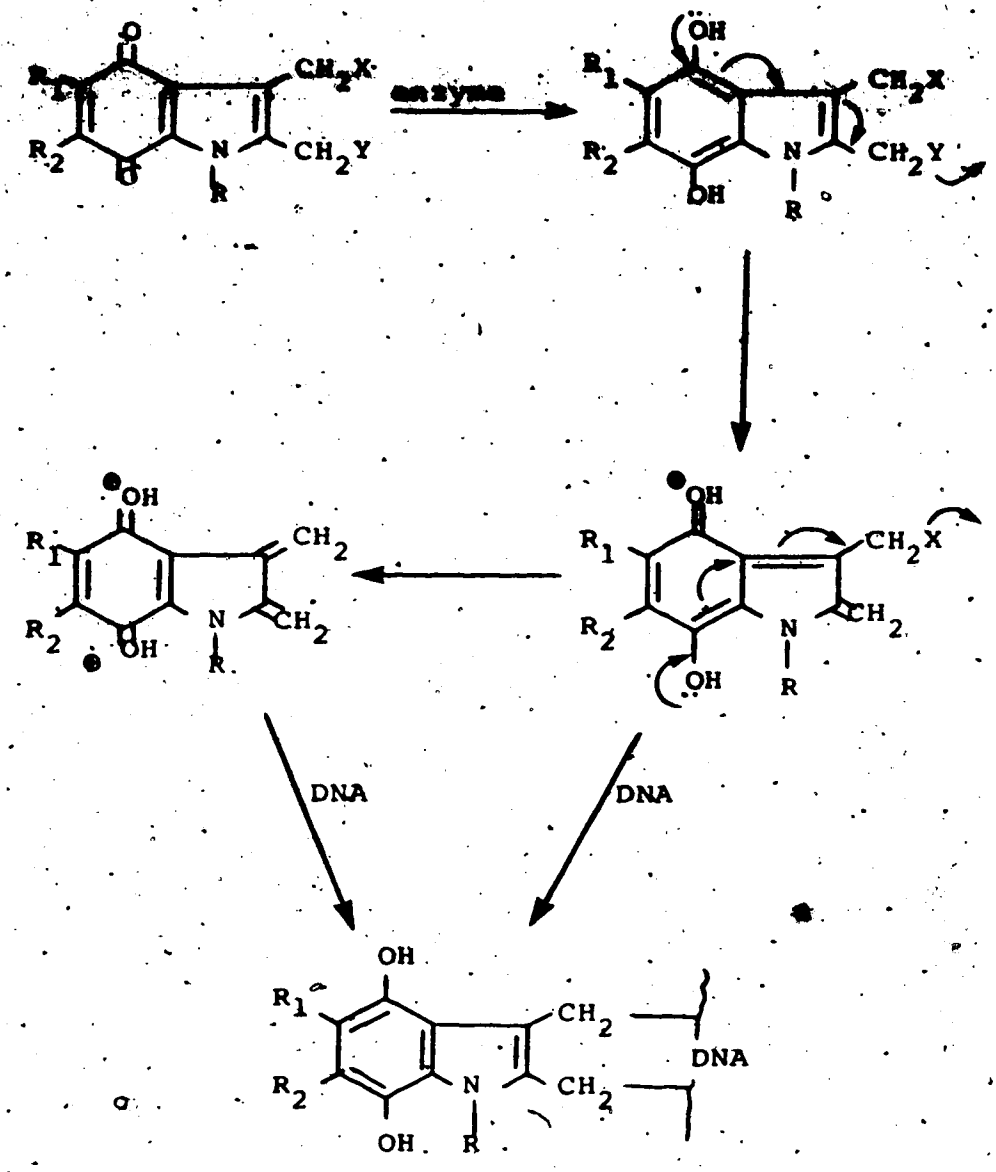
tion could form quinone methides and thus would have the

Scheme 9



potential to alkylate DNA. These compounds were tested for antitumor activity both *in vitro* and *in vivo* and many were found to show significant activity on reduction.

Compounds of this type, especially those having carbamate side chains at X and Y, were of interest to us since a study of their ability to alkylate DNA could provide useful information about the alkylating ability of the carbamate group in mitomycin C. As a result, a series of isomeric benzoquinones was prepared and tested for cross-linking and alkylating ability. With the collaboration of Dr. Sartorelli, these compounds are being tested for antitumor activity and possible structure-activity relationships will be examined. These experiments are presented in Chapter IV.

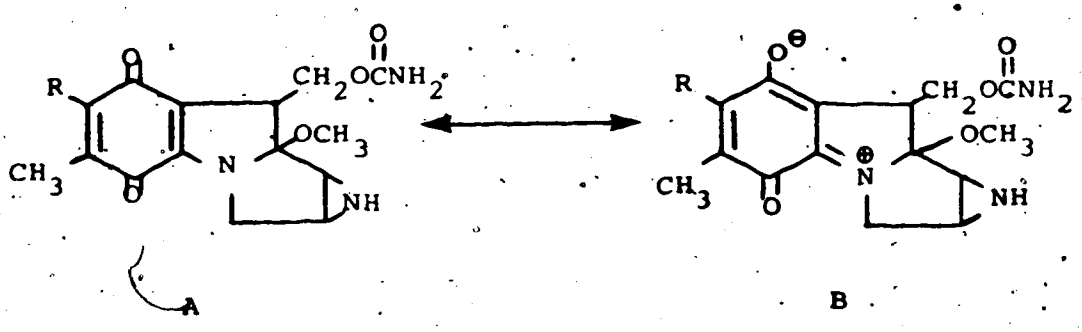
CHAPTER II

THE ^{13}C AND ^{1}H NUCLEAR MAGNETIC RESONANCE SPECTRA OF MITOMYCIN C AND STREPTONIGRIN

Mitomycin C and other related mitosanes are potent antibiotics[5] and are presently in clinical use in Japan as antitumor agents.[6,7] Considerable interest has been shown in their biosynthesis[13-17] and in their electronic structure and conformation as it relates to their mode of action.[18]

We undertook to carry out spectroscopic studies, including the natural abundance ^{13}C spectrum of mitomycin C and the structurally related streptonigrin.[36] It was hoped that the position of the quinone resonances would provide information about the proposed stabilization of mitomycin C *in vivo*. In addition a full analysis of the ^{13}C spectra of these antitumor agents could possibly assist in further biosynthetic studies employing incorporation of ^{13}C labelled substrates and in characterizing synthetic analogs.

The stability of the oxidized form of the mitosanes has been attributed to resonance between forms A and B



which results in partial withdrawal of electrons from N-4 into the quinone ring and consequent stabilization of the 9a methoxy, an otherwise good leaving group.[18b] Such conjugative interactions would be expected to shift the position of the quinone carbonyls to higher field as compared to unconjugated quinones. The spin decoupled ^1H nmr spectrum of mitomycin C was also obtained to gain information about the conformation of the antibiotic in solution.

The ^1H Nuclear Magnetic Resonance Spectrum of Mitomycin C

A 100 MHz pmr spectrum of mitomycin C in pyridine-d_5 was obtained (Figure 2). Double irradiation experiments were carried out in order to assign all of the proton signals and to determine the proton-proton coupling constants (Table 2). The double irradiation experiments

Table 2

Double Irradiation Experiments on Mitomycin C at 100 MHz - Proton Spectrum

Proton Irradiated	Decoupling Frequency (Hz)	Lines Collapsed: Original Form	Lines Collapsed: Final Form	Measured Remaining Coupling (Hz)
$H_{10'}$	538	d of d, 3.96 (H_9)	d	$J_{9,10} = 10.5$
H_{10}	502	d of d, 3.96 (H_9)	d	$J_{9,10'} = 4.5$
H_3	453	d of d, 3.56 ($H_{3'}$)	d	$J_{2,3} = 2.5$
H_9	400	d of d, 5.36 ($H_{10'}$)	d	$J_{10},J_{10'} = 10.5$
H_9	400	t, 5.00 (H_{10})	d	$J_{10,10'} = 10.5$
$H_{3'}$	358	d, 4.50 (H_3)	s	$J_{33'} = 13$
$H_{3'}$	358	d of d, 2.68 (H_2)	d	$J_{12} = 4.5$
H_2	270	d of d, 3.56 ($H_{3'}$)	d	$J_{33'} = 13$
H_2	270	d, 3.08 (H_1)	s	$J_{12} = 4.5$

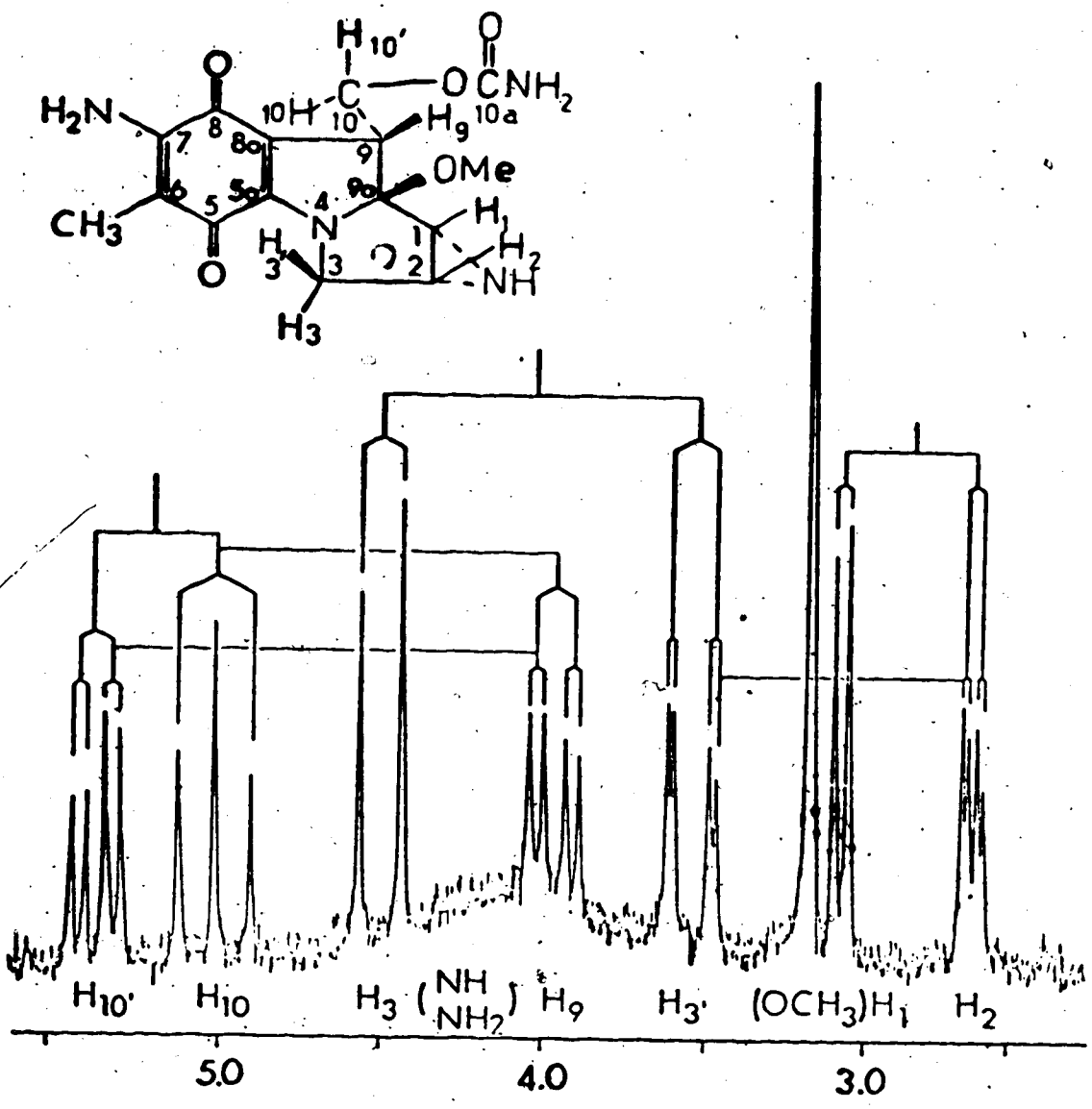


Figure 2. 100 MHz pmr spectrum of mitomycin C in pyridine-d_5.

reveal that the C-10 methylene protons at δ 5.00 and 5.36 are diastereotopic with unequal couplings of 10.5 and 4.5 Hz to the C-9 methine proton. In addition, the dihedral angle of $H\text{-}C_2\text{-}C_3\text{-}H_3$ is 90° since the vicinal coupling constant, $J_{23} = 0$, in agreement with the molecular geometry for the solid phase as indicated by x-ray analysis of the N-brosyl derivative of mitomycin A.[4] The observed value of $J_{12} = 4.5$ Hz is also consistent with *cis*-coupled aziridine ring protons. The broad signals in the region δ 3.7-4.6 (3H) were exchangeable with deuterium oxide and are assigned to NH and NH_2 protons.

This information on the conformation of mitomycin C in solution is of interest in connection with its proposed mode of antibacterial action which as discussed above is believed to involve cross-linking of the complementary strands of DNA by bifunctional alkylation at the reactive centres C-1 and C-10.

The ^{13}C NMR Spectrum of Mitomycin C

A natural abundance ^{13}C noise decoupled nmr spectrum was obtained at 25.15 MHz on a 0.224 M solution of mitomycin C in pyridine-d_5. All fifteen carbons are clearly distinguishable and appear as singlets. The spectrum (Figure 3) can be divided into three regions, low field carbonyl carbons, middle range quinone ring and quaternary carbons, and saturated carbons at high field.

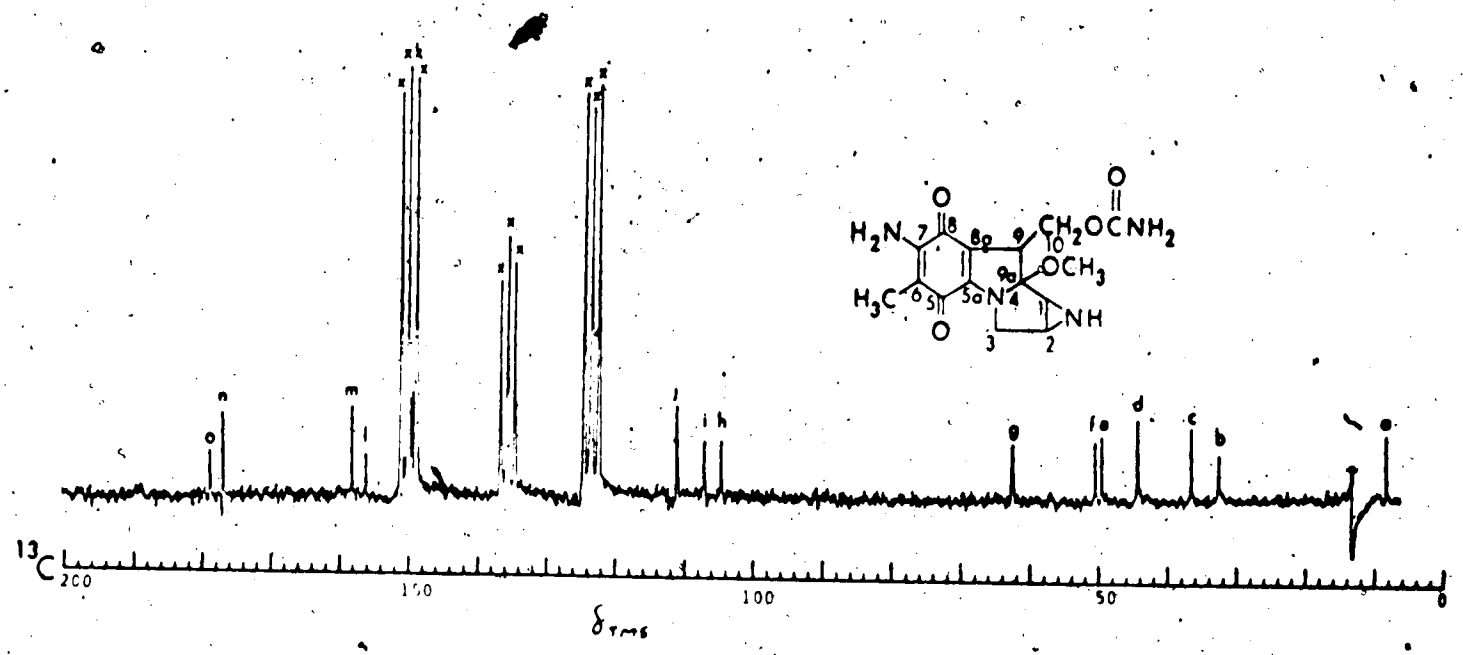


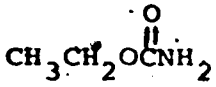
Figure 3. ^{13}C nmr spectrum of 0.224 M solutions of mitomycin C in pyridine-d_5 at 25.15 MHz.

a 6-CH_3 b C-1 c C-2 d C-9 e OCH_3 f C-3 g C-10

h C-6 or C-8a i C-6 or C-8a j C-9a k C-7 l C-5a

m C-10a n C-5 o C-8 x solvent

The following carbons could be readily assigned by comparison with commercially available ethyl carbamate 26: δ 158.0 (C-10a, carbamate carbonyl), δ 62.7 (C-10, CH_2). In contrast, the poor agreement for the quinone ring carbon



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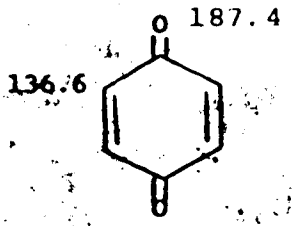
signals at δ 104.5, 107.0, 150.0, and 156.0 and for the carbonyl resonances at δ 178.6 and 176.8 with those of the quinone models 27, 28, and 29 signified an unusual structure for the quinone moiety of mitomycin C. Resonance



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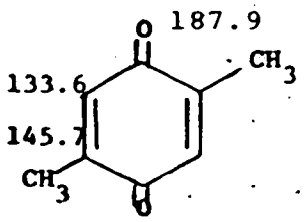

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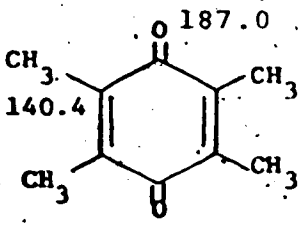

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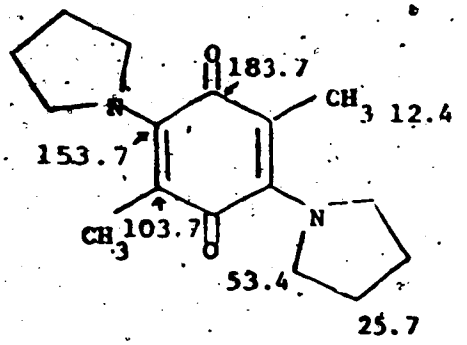
interaction of the type shown in A and B could explain the poor agreement with the simple quinone models.

Pullman and Pullman have pointed out the strong conjugative interaction of groups directly attached to the

quinone ring.[37] Since similar conjugative interaction has been established in simple aminoquinones by other spectroscopic means,[38] we sought information on this (A ↔ B) interaction and confirmation of the ^{13}C signals of mitomycin C by examining compounds 30 - 34.



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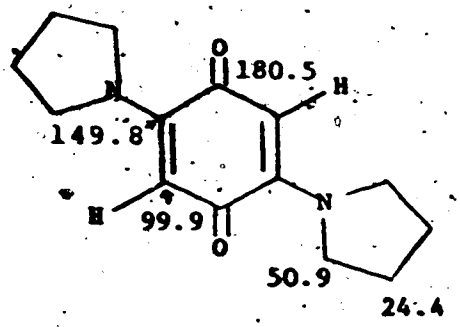

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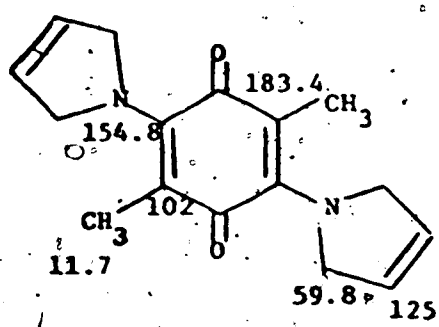

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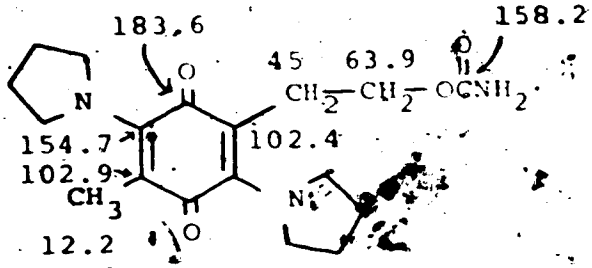

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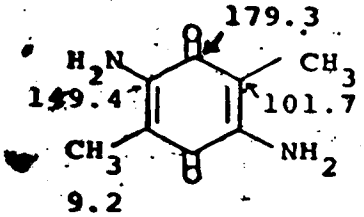

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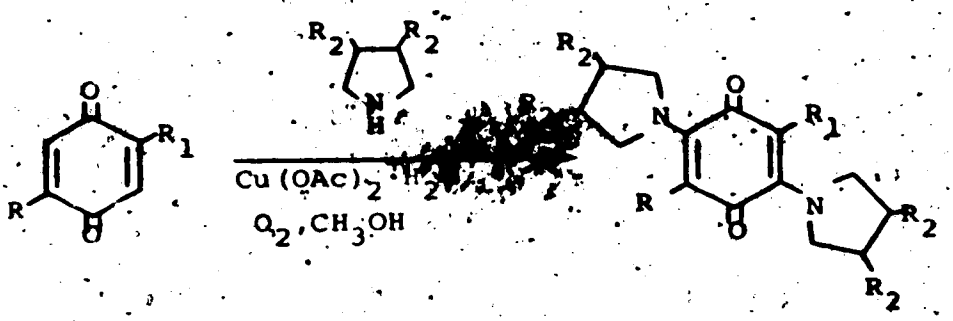
The pyrrolidine and pyrroline quinone models 30 - 33 were prepared by reaction of the five-membered ring heterocycle with the appropriate quinone under oxidative conditions.[39] The introduction of the 2,5 nitrogen substituents,



larly the amino group in 34 produced much better ment for the C-8 and C-5 carbonyls at δ 178.6 and .8 respectrvely. Model 34 also permits the assignment δ 150.0 to C-7, δ 156.0 to C-5a, and δ 104.5 and 107.0 to C-6 and C-8a, although at the present the latter pair cannot be discriminated further.

In order to observe the peak multiplicities, we also recorded the off-resonance decoupled ^{13}C nmr spectrum of mitomycin C (Table 3). The 62.7 ppm absorption became a triplet under these conditions confirming its assignment as C-10. The only other triplet signal at 50.7 ppm is therefore unambiguously assigned to C-3, consistent with

Table 3

^{13}C Chemical Shifts in ppm from TMS of Mitomycin C as 0.224 M Solution in Pyridine-d_5 at 75.15 MHz.†

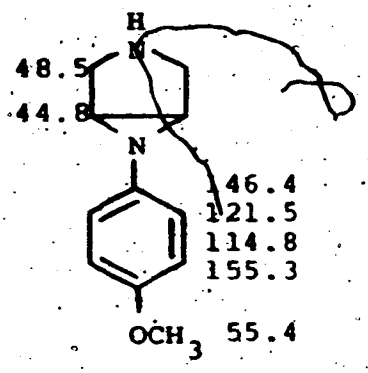
Chemical Shift	Multiplicity*	Carbon Assignment
178.6	s	8
176.8	s	5
158.0	s	10a
156.0	s	5a
150.0	s	7
111.0	s	9a
107.0	s	6
104.5	s	8a
62.7	t	10
50.7	t	3
49.7	(q)	9a-OCH_3
44.5	d	9
36.8	d	2
32.8	d	1
8.7	q	6-CH_3

* Determined by off-resonance decoupling

† Lock signal pyridine-d_5. Chemical shift data using a 4K data set are accurate to ±0.05 ppm.

models 30, 31, and 33. The absorption at δ 8.7 became a quartet under off-resonance decoupling and the signal at δ 49.7 appeared as a doublet but may well have been a quartet. On the basis of this information and by comparison with 34, the 6 - CH_3 carbon was assigned to δ 8.7. Therefore the δ 49.7 peak was ascribed to the 9a-methoxy carbon. The line position is also in excellent agreement with literature values of 51.0 ppm for methoxy groups.[40] Eight signals appeared as singlets. All except the line at 111.0 ppm have previously been assigned, allowing its assignment to C-9a.

Three signals appeared as clean doublets on off-resonance decoupling, at 44.5, 36.8, and 32.8 ppm, corresponding to the three methines in mitomycin C. Models 35 and 36 indicate an upfield shift of the aziridine carbons



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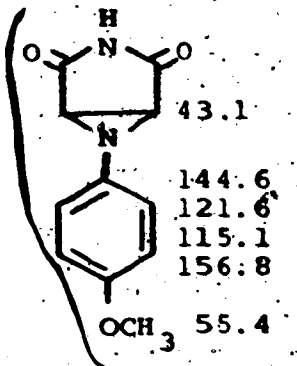

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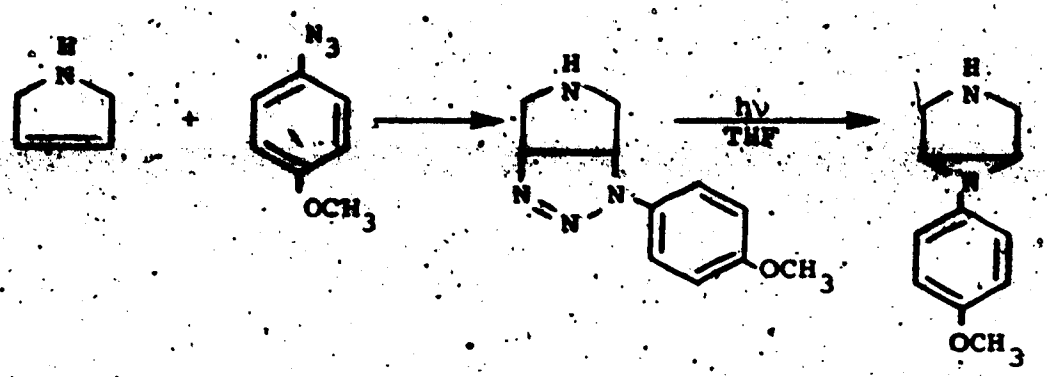
due to an adjacent oxygen function. On this basis C-1 and

C-2 were tentatively assigned to 32.8 and 36.8 ppm respectively, permitting the assignment of C-9 at 44.5 ppm. The bicycloaziridines were prepared by 1,3-dipolar cycloaddition followed by thermal or photochemical decomposition of the intermediate triazolines.



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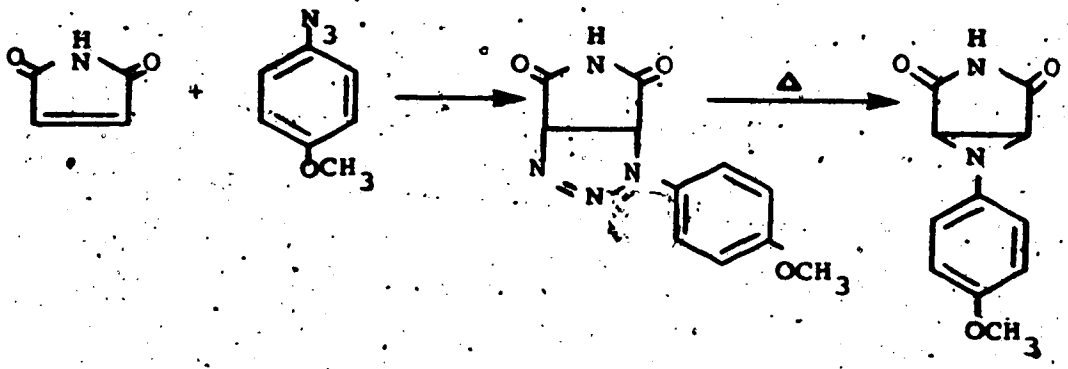

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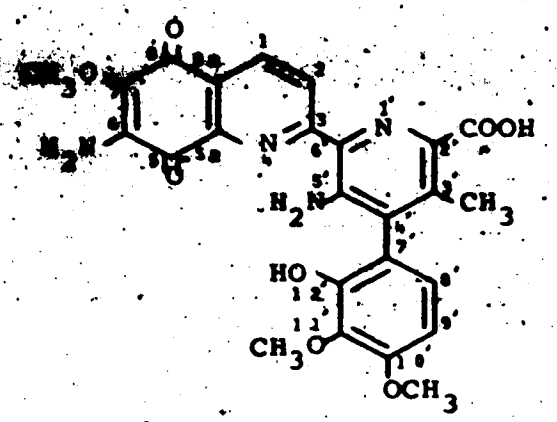
The ^{13}C NMR Spectrum of Streptonigrin

Streptonigrin 2 is an antibiotic and antineoplastic agent derived from *Streptomyces Flocculus*.[41] Streptonigrin and mitomycin C are similar in a number of respects:[42,43] (a) both contain the aminoquinone moiety (b) both selec-



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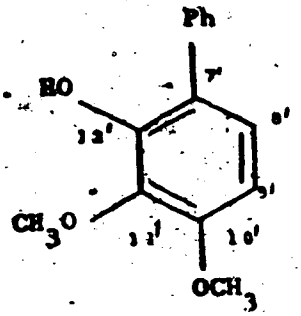
tively inhibit DNA synthesis in bacterial cells (c) both initiate bacterial DNA degradation (d) the primary cytotoxic activity of both appears to be connected with inhibition of DNA synthesis[30,45] (e) both must be activated by reduction to the hydroquinone form. This points to the biological significance of the common aminoquinone moiety in these drugs, so a comparison of the ^{13}C spectrum with that of mitomycin is appropriate to see if a similar conjugative interaction obtains for streptonigrin. Therefore,

A ^{13}C noise decoupled nmr spectrum of a 0.150 M solution of streptonigrin in pyridine-d_5 was obtained (Figure 4). In order to aid in the assignment of peaks, an off-resonance decoupled spectrum was also obtained to determine the signal multiplicities.

All twenty-five carbons are clearly visible and several firm assignments could be readily made (Table 4). The four high field signals at 60.5, 60.1, 56.1, and 17.8 ppm were all quartets on off-resonance decoupling. The latter was assigned to the C-3' methyl group by comparison with the quinone models. The remaining three quartets were assigned to the three OCH_3 groups.

Four signals became doublets on off-resonance decoupling as required by the structure of streptonigrin, δ 133.5, 126.2, 125.4, and 105.1. Of these, the latter two are tentatively ascribed to C-8' and C-9' respectively on the basis of substituent shift predictions for model 37[40a] (Table 5).



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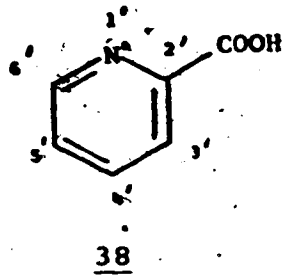

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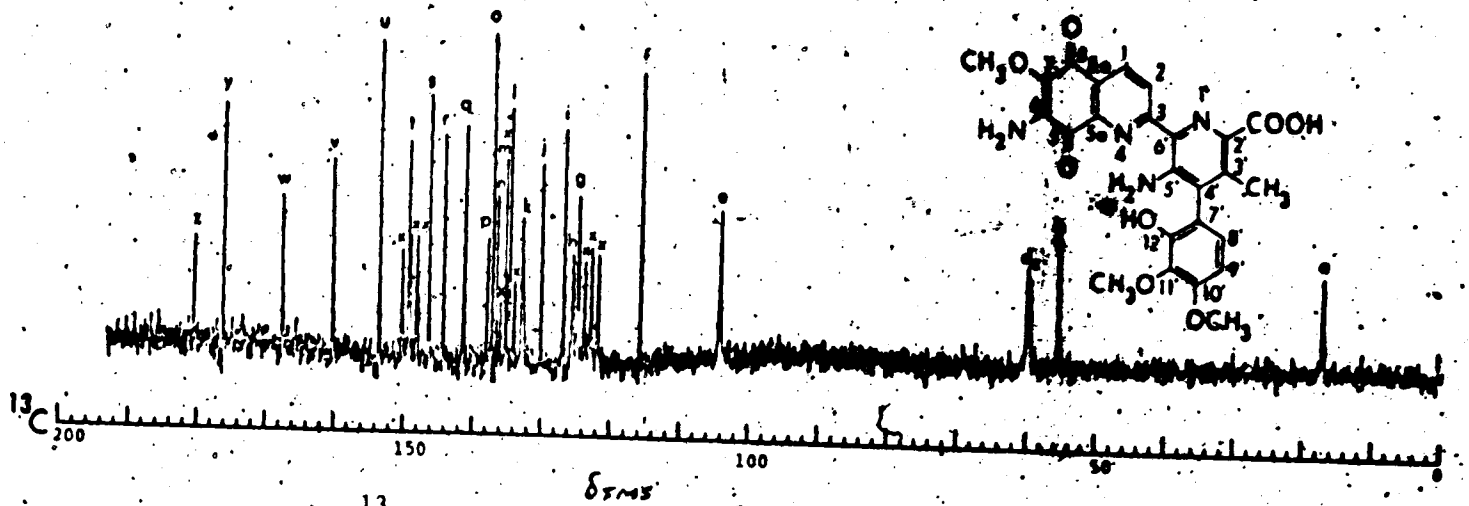

Figure 4. ^{13}C nmr spectrum of a 0.15 M solution of streptonigrin in pyridine-d_5 at 25.15 MHz.

a CH$_3$	b OCH$_3$	c OCH$_3$	d OCH$_3$	e C-9′	f C-7′	g C-8′
h C-2	i C-8a	j C-11′	k C-1	l C-3′	m C-6	n C-4′
o C-12′	p C-2′	q C-7	r C-10′	s C-5′	t C-6′	u C-5a
v C-3	w CO_2H	y C-8	z C-5	x solvent		

Table 4

^{13}C Chemical Shifts in ppm from TMS of Streptonigrin as 0.15 M Solution in Pyridine-d_5 at 25.15 MHz†

Chemical Shift	Multiplicity*	Carbon Assignment
181.1	s	5
176.9	s	8
168.3	s	CO_2H
161.0	s	3
154.2	s	5a
150.0	s	6'
147.1	s	5'
144.9	s	10'
141.9	s	7
138.6	s	2'
137.8	s	12'
137.1	s	4'
135.9	s	6
135.3	s	3'
133.5	d	1
130.7	s	11'
127.4	s	8a
126.2	d	2

(cont'd.)

Table 4 (cont'd.)

Chemical Shift	Multiplicity*	Carbon Assignment
125.4	d	8'
116.4	s	7
105.1	d	9'
60.5	q	OCH_3
60.1	q	
56.1	q	
17.8	q	CH_3

* Determined by off-resonance decoupling

† Lock signal pyridine-d_5. Chemical shift data using a 4K data set are accurate to ±0.05 ppm.

Table 5

Predicted Substituent Shifts on ^{13}C absorptions of 37

Carbon	1-OCH_3	2-OCH_3	3-OH	4-Ph	Σ	Predicted Position
10'	+30.2	-15.5	+1.8	-1.2	+15.3	144.0
11'	-15.5	+30.2	-12.6	+0.4	+2.5	131.2
12'	0.0	15.5	+26.9	-1.1	-10.3	139.0
7'	-8.9	0.0	-12.6	+13.6	-8.4	120.3
8'	0.0	-8.9	+1.8	-1.1	-8.2	120.5
9'	-15.5	0.0	-7.9	+0.4	-23.0	105.7

Base position for benzene taken as 128.7

The ^{13}C spectrum of picolinic acid (Table 6) served to establish the position of the carboxy carbon of streptonigrin (at 168.3 in good agreement with that in picolinic acid at 167.8), and also provided a base for substituent shift predictions. The remaining low field peaks at 181.1 and 176.9 were assigned to the quinone carbonyls, C-5 and C-8 respectively by analogy with mitomycin C.

Table 6

^{13}C Chemical Shifts in ppm from TMS of Picolinic Acid as 0.2 M Solution in Pyridine-d_5 @ 25.15 MHz

Chemical Shift	Multiplicity	Carbon Assignment
167.8	s	CO_2H
150.3	s	2'
149.7	d	6'
137.2	d	3'
126.7	d	5'
125.1	d	4'

The ^{13}C spectrum of the streptonigrin analog 39 (Table 7) provided valuable information about the chemical shifts of the quinolinoquinone carbons. It allowed the assignment of the remaining two doublets at δ 133.5 and 126.2 to C-1 and C-2 respectively. Further comparison with model 39 led to the assignment of the signals at 141.9,

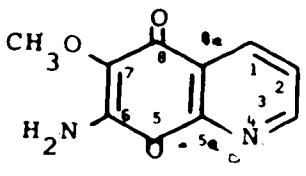

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Table 7

^{13}C Chemical Shifts in ppm from TMS of 39 as 0.25 M Solution in $(CD_3)_2SO$ at 22.6 MHz

Chemical Shift	Multiplicity	Carbon Assignment
180.3	s	5
175.8	s	8
152.2	d	3
146.3	s	5a
141.7	s	7
135.7	s	6
133.0	d	1
129.1	s	8a
128.1	d	2
59.6	q	OCH_3

135.9, and 127.4 to C-7, C-6, and C-8a. Peaks at 161.0 and 154.2 were tentatively ascribed to C-3 and C-5a.

Further assignments were made possible on the basis of substituent shift predictions from model 37 (Table 5) and model 40 (Table 8). It is recognized that the additivity relationships for four substituents probably have severe limitations so that Tables 6 and 9 are used only as a guide in the line assignments.

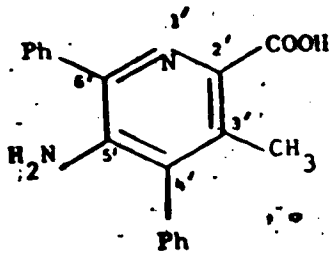

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Table 8

Predicted Substituent Shifts on ^{13}C Absorptions of 40

Carbon	Base	3-CH_3[d]	4-Ph[e]	5-NH_2[d]	6-Ph[e]	1-CO_2H[e]	$\sum$	Predicted Position
2'	150.4[a]	-0.1	+0.4	-9.5	+0.4	-	-8.8	141.6
3'	133.8[b]	-	-1.1	+1.3	-1.2	+1.5	+0.5	134.3
4'	136.4[c]	+0.7	+13.1	-12.7	+0.4	0.0	+1.5	137.9
5'	124.5[c]	-0.4	-1.1	+19.2	-1.1	+5.1	+21.7	146.2
6'	150.6[c]	-2.8	+0.4	-12.4	+13.1	0.0	-1.7	148.9

a Base is C_2 of α-picolinic acid

b Base is C_3 of β-picoline

c Base is corresponding carbon of pyridine

d Reference 40a

e Reference 40b.

The chemical shifts of the quinone carbonyl carbons in mitomycin C, streptonigrin, and the aminoquinone models all show a pronounced upfield shift compared to simple benzoquinones. This result is consistent with conjugative interaction of the nitrogens with the carbonyl groups,

strongly supporting the proposed explanation for the stability of the oxidized form of mitomycin C. It also suggests that a similar stabilizing effect may be occurring in streptonigrin. The ^{13}C peak assignments for mitomycin C and streptonigrin should prove valuable for further biosynthetic studies employing the incorporation of ^{13}C labelled substrates and in characterizing synthetic analogs.

Experimental

Throughout this work melting points were determined on a Fisher-Johns apparatus and along with boiling points are uncorrected. Infrared spectra were recorded on a Perkin-Elmer model 421 spectrophotometer and only the principal, sharply defined peaks are reported. Absorption spectra were measured in 'spectro'-grade solvents on a Beckman DB spectrophotometer. Proton magnetic resonance spectra (pmr) were recorded on Varian A-60 and HA-100 analytical spectrometers. The spectra were measured on approximately 10-15% (w/v) solutions in appropriate deuterated solvents with tetramethylsilane as reference. Line positions are reported in parts per million from the reference. Mass spectra were determined with an Associated Electrical Industries MS9 double-focusing high-resolution mass spectrometer. The ionization energy, in general, was 70 eV. Peak measurements were made by comparison with perfluorotributylamine at a resolving power of 15,000. Elemental

microanalysis was carried out by Mrs. D. Mahlow of this department. In the work-up procedure reported for the various syntheses described, solvents were removed with a rotary evaporator under reduced pressure unless otherwise stated.

The ^{13}C nmr spectra in natural abundance were obtained at 25.15 MHz in 12 mm spinning tubes in deuterated solvents on a Varian HA-100-15 instrument using tetramethylsilane as reference. Typically for mitomycin C a total of 4000 scans were made for multiscan averaging and accumulated with an interfaced Digitab FTS/NMR 3 data system. Additional ^{13}C spectra were determined at 22.63 MHz in 10 mm spinning tubes in the Fourier mode using a Bruker HFX-90 spectrometer in conjunction with a Nicolet 1085, 20K memory computer. The spectrometer features a deuterium lock system, a BSV-2 random noise (800 Hz band-width) proton decoupler, and a BSV-2 pulse generator-amplifier.

Materials

Commercial mitomycin C (Calbiochem Inc.) was used and characterized spectroscopically as follows:
Infrared spectrum ν_{max} (nujol): 1595 (quinone C=O); 1700; 1720 ($OCONH_2$); 3260, 3300, 3420 cm^{-1} (NH, NH_2).
Absorption spectrum λ_{max} (CH_3CN): 540 nm (log ε 3.43); 359 nm (log ε 5.37); 350 nm (log ε 5.32); 240 nm (sh) (log ε 5.03).

Mass Spectrum: Base peak 302 $(M-CH_3OH)^+$.

Pure streptonigrin was kindly supplied by Dr. H.B. Wood, Division of Cancer Treatment, National Institutes of Health.

The purity of the following commercially available compounds was established by ^1H nmr spectroscopy and by comparison of melting points with literature values.

Ethyl Carbamate 25

The ^{13}C nmr spectra: δ_{TMS} (HA-100-15, pyridine-d_5): 14.8 ($\underline{C}H_3$); 60.4 ($\underline{C}H_2$); 158.4 ($\underline{C}$=O). δ_{TMS} ($CDCl_3$)[46]: 14.5 ($\underline{C}H_3$); 60.9 ($\underline{C}H_2$); 157.8 ($\underline{C}$=O).

1,4-Benzoquinone 27

The ^{13}C nmr spectra: δ_{TMS} (HA-100-15, pyridine-d_5): 136.6 ($\underline{C}H$); 187.4 ($\underline{C}$=O). δ_{TMS} ($CDCl_3$)[46]: 136.4 ($\underline{C}H$); 187.1 ($\underline{C}$=O).

2,5-Dimethyl-1,4-benzoquinone 28

The ^{13}C nmr spectra: δ_{TMS} (HA-100-15, pyridine-d_5): 15.2 ($\underline{C}H_3$); 133.6 ($\underline{C}$-H); 145.7 ($\underline{C}$-CH_3); 187.9 ($\underline{C}$=O). δ_{TMS} (ext) (HFX-90, dimethyl sulfoxide-d_6): 15.7 ($\underline{C}H_3$); 133.8 ($\underline{C}$-H); 146.2 ($\underline{C}$-CH_3); 188.5 ($\underline{C}$=O).

Tetramethyl-1,4-benzoquinone 29

The ^{13}C nmr spectrum δ_{TMS} (HA-100-15, pyridine-d_5): 12.2 ($\underline{C}H_3$); 140.4 ($\underline{C}$-CH_3); 187.0 ($\underline{C}$=O).

2,5-Dimethyl-3,6-bis(pyrrolidino)-1,4-benzoquinone 30

This compound was prepared in 17% yield by the method of Crosby and Lutz,[39] m.p. 149-150.5° (lit. m.p. 149-150°).[47]

The ^{13}C nmr spectrum δ_{TMS} (HFX-90, pyridine-d_5): 12.4 ($\underline{C}H_3$); 25.7 ($\underline{C}H_2$); 53.4 ($\underline{C}H_2$-N); 103.7 ($\underline{C}$-CH_3); 153.7 ($\underline{C}$-N); 183.7 ($\underline{C}$=O).

2,5-Bis(pyrrolidino)-1,4-benzoquinone 31

This compound was prepared in 62% yield by the procedure of Crosby and Lutz,[39] m.p. 245-250° (dec.) (lit. m.p. 238-240°).[48]

The ^{13}C nmr spectrum δ_{TMS} (HFX-90, $CDCl_3$): 24.4 ($\underline{C}H_2$); 50.9 ($\underline{C}H_2$-N); 99.9 ($\underline{C}$-H); 149.8 ($\underline{C}$-N); 180.5 ($\underline{C}$=O).

2,5-Dimethyl-3,6-bis(3-pyrrolino)-1,4-benzoquinone 32

This compound was prepared in 70% yield by the method of Crosby and Lutz.[39] Upon heating it changed from a dark purple to a white solid between 164-172° and melted at 242.5-244.5°.

Anal. Calod. for $C_{16}H_{18}N_2O_2$ [mol. wt. 270.1368]: C, 71.09; H, 6.71; N, 10.36. Found [(mass spectrum) 270.1360]: C, 70.89; H, 6.48; N, 9.96.

The infrared spectrum ν_{max}($CHCl_3$): 1610 cm^{-1} ($\underline{C}$=O).

The ^1H spectrum δ_{TMS}($CDCl_3$): 2.02 (s, 6H, CH_3); 4.54 (s, 8H, methylenes); 5.85 (s, 4H, vinyl).

The ^{13}C nmr spectrum δ_{TMS} (HA-100-15; pyridine-d_5): 11.7 ($\underline{C}H_3$); 59.8 ($\underline{C}H_2$-N); 102.0 ($\underline{C}$-CH_3); 125.6 (vinyl); 154.8 ($\underline{C}$-N); 183.4 ($\underline{C}$=O).

2-(β-Carbamoyloxyethyl)-5-methyl-3,6-bis(pyrrolidino)-1,4-benzoquinone 33

A solution of 0.40 g (2 mmole) of freshly crushed cupric acetate monohydrate and 0.85 g (12 mmole) of pyrrolidine in 20 ml of methanol was purged with oxygen. While bubbling oxygen through the reaction mixture, a solution of 0.42 g (2 mmole) of 2-(β-carbamoyloxyethyl)-5-methyl-1,4-benzoquinone[34a] in 75 ml of methanol was added at such a rate that the temperature remained between 20-30°. Oxygenation was continued for 1 hour after all the quinone had been added. The solution was concentrated *in vacuo* to *ca.* 5 ml and chromatographed on a column of neutral alumina (Woelm), eluting with methanol. The first fraction was collected and evaporated affording 0.367 g (53% yield) of 33 as an oil.

Anal. Calcd. for $C_{18}H_{25}N_3O_4$ [mol. wt. 347.1845]. Found [(mass spectrum) 347.1847].

The ^1H nmr spectrum $\delta_{TMS}(CDCl_3)$: 1.94 (s, 3H, CH_3); 1.89 and 2.04 (2 multiplets, 4H each, H_3 and H_4 of pyrrolidine rings); 2.85 (t, 2H, H_A, J_{AB} = 7.5 Hz); 3.36 and 3.74 (2 multiplets, 4H each, H_2 and H_5 of pyrrolidine rings); 3.95 (t, 2H, H_B).

The ^{13}C nmr spectrum δ_{TMS} (HFX-90, pyridine-d_5): 12.2 ($\underline{C}H_3$); 24.6 and 25.6 (C_3 and C_4 of pyrrolidine rings); 45.0 ($\underline{C}H_2$-quinone); 53.2 and 53.5 (C_2 and C_5 of pyrrolidine rings); 63.9 ($\underline{C}H_2$-O); 102.4 and 102.9 ($\underline{C}$-CH_2 and $\underline{C}$-CH_3); 154.7 (2 $\underline{C}$=N); 158.2 (carbamate $\underline{C}$=O); 183.6 (quinone $\underline{C}$=O).

2,5-Diamino-3,6-dimethyl-1,4-benzoquinone 34

This compound was prepared by the procedure of Zee-Cheng and Cheng,[33c] m.p. 309-310° (lit. m.p. 310-312°).[33c]

The ^{13}C nmr spectrum $\delta_{TMS(ext)}$ (HFX-90, dimethyl sulfoxide-d_6): 9.2 ($\underline{C}H_3$); 101.7 ($\underline{C}$-CH_3); 149.4 ($\underline{C}$-N); 179.3 ($\underline{C}$=O).

4-(*p*-Methoxyphenyl)-2,3,4,7-tetraazabicyclo[3.3.0]oct-2-ene

A mixture of 2.2 g (15 mmole) of *p*-methoxyphenyl-azide and 1.4 g (15 mmole) of 3-pyrroline (75% pure) was set aside in the dark at ambient temperature for 3 weeks. The resulting precipitate was collected, washed with light petroleum and recrystallized from ethyl acetate: petroleum ether to give 1.1 g (30% yield) of a white crystalline solid m.p. 108.5-109°. This preparation is a modification of a literature procedure.[49]

Anal. Calcd. for $C_{11}H_{14}N_4O$ [M-N_2 190.1106]: C, 60.53; H, 6.47; N, 25.67. Found [(mass spectrum) 190.1107]: C, 60.52; H, 6.42; N, 25.19.

The infrared spectrum ν_{max} ($CHCl_3$): 3305 (NH); 1580 cm^{-1} (N=N).

The ^{1}H nmr spectrum δ_{TMS}($CDCl_3$): 1.46 (broad, 1H, NH); 2.69-3.62 (multiplet, 4H, CH_2); 3.79 (s, 3H, OCH_3); 4.35 (dd, 1H, H_1, J_{15} = 10 Hz, J_{18} = 4 Hz); 5.15 (dd, 1H, H_5, J_{56} = 6.5 Hz); 6.80-7.33 (multiplet, 4H, aryl protons).

6-(*p*-Methoxyphenyl)-3,6-diazabicyclo[3.1.0]hexane 35

A solution of 0.906 g (4 mmole) of 4-(*p*-methoxyphenyl)-2,3,4,7-tetraazabicyclo[3.3.0]oct-2-ene in 120 ml of tetrahydrofuran under nitrogen was irradiated for 6 hours with a Hanovia high pressure mercury lamp (200 W, fitted with a Pyrex filter) with stirring and cooling. The solvent was evaporated and the residue was treated with 100 ml of ether. The resulting precipitate was filtered. The filtrate was evaporated and the resulting residue was crystallized from benzene: light petroleum affording 0.742 g (98% yield) of 35 as an off-white hygroscopic solid m.p. 36-38°. This preparation is a modification of a literature procedure.[49]

Anal. Calcd. for $C_{11}H_{14}N_2O$ [mol. wt. 190.1106]: C, 69.45; H, 7.42; N, 14.72. Found [(mass spectrum) 190.1105]: C, 68.90; H, 7.57; N, 14.67.

The ^{1}H nmr spectrum δ_{TMS} (dimethyl sulfoxide-d_6): 2.79 (s, 2H, methine); 3.70 (s, 3H, OCH_3); 2.82 (AB quartet, 4H, methylenes, J = 12.5 Hz); 6.41-7.08 (multiplet, 4H, aryl protons).

The absorption spectrum λ_{max}(CH_3CN): 238 nm (log ε 4.21); 295 nm (log ε 3.30).

The ^{13}C nmr spectrum δ_{TMS} (HFX-90, pyridine-d_5): 44.8 (C_1 and C_5); 48.5 (C_2 and C_4); 55.4 (OCH_3); 114.8 ($C_{3'}$); 121.5 ($C_{2'}$); 146.4 ($C_{1'}$); 155.3 ($C_{4'}$).

6-(*p*-Methoxyphenyl)-3,6-diazabicyclo[3.1.0]hexa-2,4-dione 36

This compound was prepared in 63% yield by the procedure of Davis and Rondesveldt,[50] m.p. 243-245° (lit. m.p. 245°).[50]

The ^{13}C nmr spectrum δ_{TMS} (HA-100-15, pyridine-d_5): 43.1 (C_1 and C_5); 55.8 ($O\underline{C}H_3$); 115.1 ($C_{3'}$); 121.6 ($C_{2'}$); 144.6 ($C_{1'}$); 156.8 ($C_{4'}$); 168.2 and 169.3 ($\underline{C}$=O).

6-Amino-7-methoxy-5,8-quinolinedione 39

This compound was prepared in our laboratories by Dr. S.K. Sim according to the method of Liao and Cheng,[51] m.p. 202-204° (dec.) [lit. m.p. 202-203° (dec.)].[51]

The ^{13}C nmr spectrum is reported in Table 7.

CHAPTER III

STUDIES RELATED TO THE MECHANISM OF ACTION OF MITOMYCIN C

Mitomycin C is a potent and useful antibiotic and antitumor agent. Extensive studies on its mode of action have been carried out,[5,18,23,24,26,27,29,31] however, many aspects still remain unclear. From the review of the mechanistic studies presented in Chapter I it is evident that the primary site of action of the mitosanes is the DNA of the cell. The action of mitomycin C appears to be two-fold:

1. cross-linking and alkylation of DNA
2. degradation of DNA

We undertook to investigate various aspects of the interaction of mitomycin C and other mitosanes with the genetic material of the cell. These studies were carried out using rapid and convenient fluorescence assays based on the enhancement of fluorescence which occurs when the trypanocidal dye, ethidium bromide interacts specifically with double stranded DNA. The assays have been exploited to estimate:

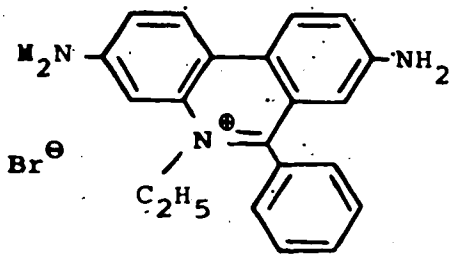
1. direct cross-linking of DNA
2. sequential cross-linking of DNA
3. alkylation of DNA without necessarily cross-linking

4. induction of single strand scission in covalently closed circular DNA (CCC-DNA)
5. simultaneous cross-linking and strand scission

Electrochemical studies were carried out to gain information about the mechanism of the reduction of mitomycin C and its relation to the mode of action of this biologically active compound.

The Ethidium Bromide Fluorescence Assay

Ethidium bromide 41 is a trypanocidal dye that in-



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teracts with DNA. Le Pecq and Paoletti[52], observed a very marked increase in the fluorescence of the dye when it was bound to bihelical nucleic acids. Their studies with the homopolymer rA_n established that ethidium bromide showed no fluorescence enhancement with this polymer and they concluded that only bihelical polynucleotides produce the enhanced fluorescence. This conclusion has been substantiated by Morgan and Paetkau.[53] Le Pecq and Paoletti[52]

further concluded from their work that the ethidium cation binds to the double-stranded polynucleotides by intercalation between the base planes. There is approximately one specific binding site for ethidium bromide per five nucleotides. This suggestion was also consistent with previous x-ray diffraction data.[54] They proposed that the fluorescence enhancement is due to the occlusion of the ethidium bromide, by intercalation, into the hydrophobic region of the nucleic acids where it is protected against quenching by the aqueous solvent. This view was supported by experiments that showed that the fluorescence of ethidium bromide increased when it was measured in alcohols of decreasing hydrophilic character, ranging from ethylene glycol to octanol.

Morgan and Paetkau[53] found that for an ethidium bromide concentration of 0.5 µg/ml a linear response of fluorescence with double stranded DNA concentration was observed for DNA concentrations up to 0.02 $O.D._{260}$ units/ml. Thus, in this concentration range the observed fluorescence is directly proportional to the amount of double stranded DNA present in solution. This result led to the development of a convenient assay for the detection and estimation of the relative amount of covalently linked complementary DNA, (CLC-DNA).[53,55]

Using the ethidium fluorescence assay, aliquots of cross-linked DNA are analyzed for CLC-sequences by diluting

them in a solution of ethidium bromide buffered to pH 11.8. The fluorescence of the DNA-ethidium bromide solution is measured to obtain an estimate of the total DNA concentration. The solution is heat denatured and cooled quickly. The fluorescence of the solution is again measured. Under the conditions used, separable DNA strands do not reanneal. Only CLC-sequences can reanneal to give ethidium fluorescence enhancement since the cross-link may serve as a nucleation point for rapid renaturation. The ratio of the fluorescence after heating to the fluorescence before heat denaturation is then a measure of the extent of covalent cross-linking. The assay is conducted at pH 11.8 to prevent spontaneous formation of short intrastrand bihelical structures after heating and cooling of separated single strands of DNA. Such structures are thermally unstable when compared with those formed by CLC-DNA and are due to a certain amount of self-complementarity within strands of naturally occurring DNAs.[53,55a] The assay is illustrated in Figure 3.

The fluorescence assay has been extended to detect single strand cleavage of DNA by making use of a covalently closed circular DNA (CCC-DNA). The amount of ethidium bromide taken up by CCC-DNA is restricted because of topological restraints. If the DNA is cleaved in one or more places, there is a release of topological constraints and the open circular (OC) form allows the intercalation

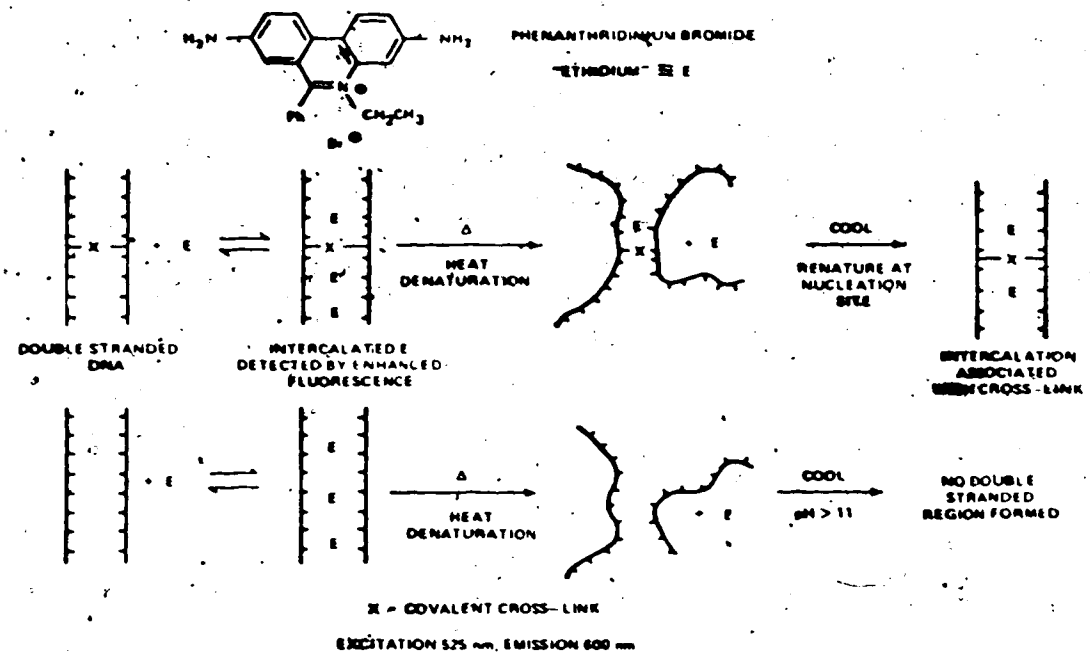


Figure 5. Fluorescence assay for detecting covalent cross-linking of DNA.

ethidium with an increase in the observed fluorescence of about 30% for PM2 DNA.[55] On heating and cooling of CCC-DNA at pH 11.8, there is complete reannealing, resulting in a 100% return of fluorescence. In contrast the OC-DNA is denatured into one circular strand and one linear strand which do not bind ethidium at pH's ~ 11.5 and the fluorescence falls to zero. This is illustrated in Figure 6(a) and (b).

The assay for single strand scission is complicated when the scission agent also cross-links or alkylates the DNA. If the scission agent cross-links the DNA, the loss of fluorescence that normally occurs after heating the nicked DNA is not observed due to the cross-linking and a full return of fluorescence after heating is seen (Figure 6(c)). However, the observed increase in fluorescence before heating can only be accounted for by cleavage of the CCC-DNA. Cross-linking without strand scission gives rise to the situation shown in Figure 6(d).

If the scission agent alkylates the DNA but does not cross-link it, again an increase in fluorescence due to nicking is observed. During denaturation of the DNA there is a heat induced depurination resulting from the covalent attachment of the alkylating agent to the CCC-DNA. The observed fluorescence after heating drops to zero and this situation shows a fluorescence behavior identical to that of simple single strand scission (Figure 6(b)). However,

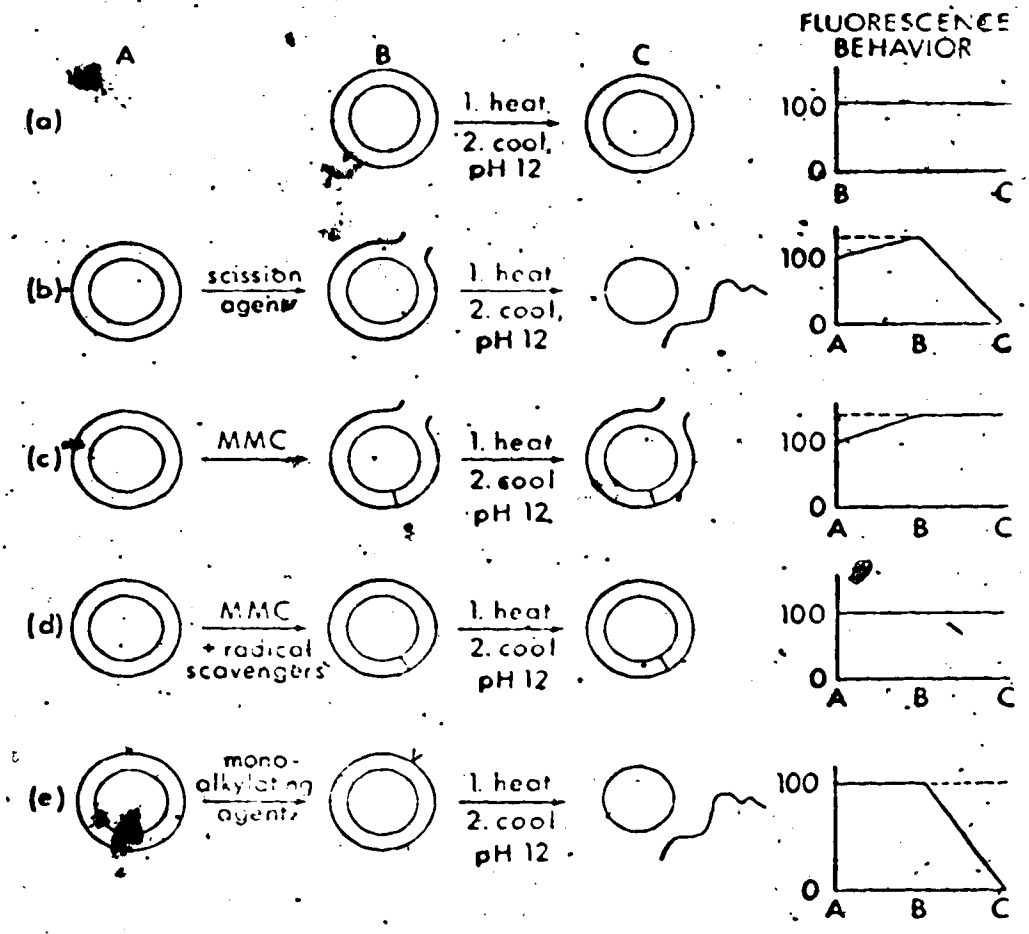


Figure 6. Fluorescence assay for detecting single strand scission, cross-linking and alkylation of CCC-DNA.

if the alkylating agent produces no single strand scission, no rise in fluorescence before heating is observed. After heating and cooling the fluorescence again drops to zero as a result of heat induced depurination (Figure 6(e)).

Studies on the Covalent Interaction of Mitomycin C with DNA

Detection of Covalent Cross-Linking of DNA by Mitomycin C by the Ethidium Fluorescence Assay

Covalent cross-linking of λ-phage DNA occurred on incubating the DNA with mitomycin C at room temperature, at pH 7.2 in the presence of sodium borohydride. Aliquots were removed at intervals and the extent and progress of covalent cross-linking was obtained by the ethidium fluorescence assay. Mitomycin C produced very efficient and rapid cross-linking with more than 80% of the DNA being cross-linked in 5 minutes (Table 9). These results obtained by the fluorescence enhancement technique are therefore in agreement with the demonstration of cross-linking of DNA by mitomycin C by Iyer and Szybalski[18] and by others.[24,56] It was considered desirable to investigate a well documented reaction of mitomycin C with DNA prior to the application of the technique to other studies of the interaction of the antibiotic with DNA.

Reductive activation of mitomycin C and efficient cross-linking of λ-DNA was also achieved with sodium

Table 9

% Cross-linking of λ DNA by Mitomycin C vs pH

Conc Mit C x 10^{-4} M	Conc $NaBH_4$ x 10^{-3} M	pH	% Cross-linking / Time to max.
0.6	1.3	5.0	93%/5min
		6.0	84%/5min
		7.2	79%/5min
		8.7	72%/10min
		10.3	65%/35min
1.2	2.3	5.0	100%/5min
		6.0	91%/5min
		7.2	84%/5min
		8.7	78%/5min
		10.3	75%/35min
2.0	3.1	5.0	100%/5min
		6.0	93%/5min
		7.2	84%/5min
		8.7	78%/5min
		10.3	76%/30min

dithionite. However, in contrast to the behavior of streptonigrin,[30] neither NADH nor model dihydropyridines such as Hantzsch ester were effective in activating the antitumor agent.

Confirmation of Covalent Cross-Linking of DNA by Mitomycin C Employing an S_1-Endonuclease Assay

Experiments were performed with the enzyme S_1-endonuclease to confirm that the fluorescence assay procedure detects the formation of CLC-DNA formed as a result of chemical cross-linking. S_1-endonuclease specifically cleaves single stranded DNA.[57,58] The technique is illustrated in Figure 7 and makes use of the enhancement of ethidium fluorescence at pH 8.0 to detect double stranded DNA. Since the time required for the S_1-endonuclease assay is long enough to allow renaturation of denatured λ DNA, *E. coli* DNA was used instead since it requires a relatively long time to reanneal after heat denaturation.

The DNA treated with mitomycin C and sodium borohydride was dialyzed to remove excess inorganic salts and decomposed mitomycin C prior to treatment with the enzyme. The results, summarized in Table 10 confirm the formation of covalent cross-links with mitomycin C. The lower absolute values for the percentage of cross-linked DNA reflects the use of an *E. coli* DNA which has a much lower molecular weight than the λ DNA. However, as is seen from

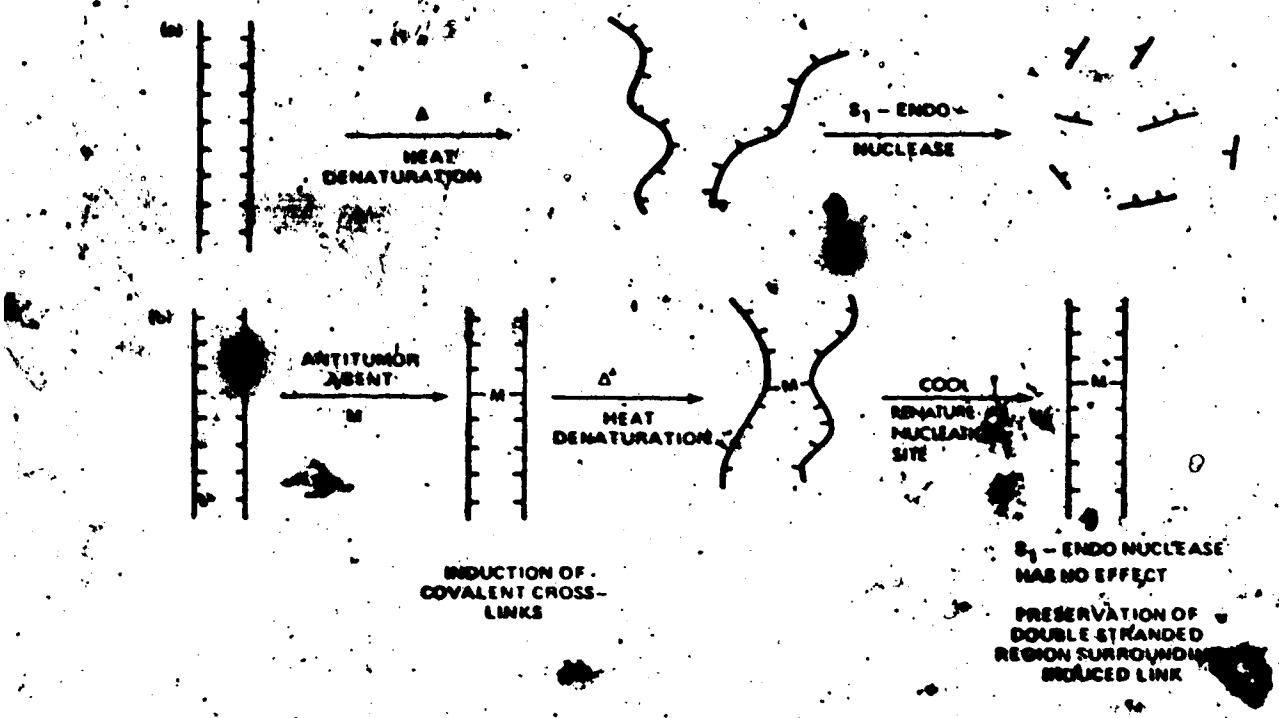


Figure 7. Confirmation of induction of covalent cross-linking of DNA by antitumor agents using S_1-endonuclease.

Table 10

Comparison of the Cross-linking of *E. coli* DNA Assayed by Ethidium Fluorescence and S_1-Endonuclease Sensitivity

Assay	% Cross-linked DNA		
Run No.	1[a]	2[b]	3[c]
Ethidium fluorescence - Before dialysis	34	48	[illegible]
After dialysis	[illegible]	[illegible]	60
S_1 Endonuclease - After dialysis	32	51	44

[a] 0.6×10^{-4} M mitomycin C

[b] 1.5×10^{-4} M mitomycin C

[c] 2.0×10^{-4} M mitomycin C

Table 10 there is a good correlation between the results obtained by the two independent assays. During dialysis there is no depurination or strand scission of the DNA as is indicated by the values for % cross-linked DNA before and after dialysis.

Detection of Alkylation of DNA by Mitomycin C

A significant observation in the study of cross-linking of DNA by mitomycin C was that as the concentration of mitomycin C in the reaction solution was increased

a progressive decrease in the fluorescence was observed. Table 11 indicates the extent of fluorescence loss with increasing antibiotic concentration. Three explanations for this observation seemed plausible:

(a) Alkylation of the DNA bases reduces the number of ethidium bromide intercalation sites either by steric hindrance or charge repulsion due to the positive charge on the ethidium ion and the positive charge on the alkylated bases.

(b) Alkylation followed by depurination or depyrimidation destroying intercalation sites.

(c) Cleavage and degradation of the DNA by reactive free radicals such as superoxide and hydroxyl radicals similar to the action of streptonigrin.[30] It has been reported that mitomycin C also degrades DNA.[29]

Experiments were carried out which allowed us to distinguish between these three possibilities.

Points (b) and (c) were tested by allowing activated mitomycin C to react with a labelled synthetic polynucleotide, poly dG·dC with ^{14}C labelled guanine and ^{3}H labelled cytosine. The labelled polynucleotide was incubated with progressively increasing concentrations of mitomycin C in parallel with *E. coli* DNA. There was a progressive decrease in the ethidium fluorescence. An acid insolubility assay was used, which would selectively remove bases pro-

Table 11

% Loss of Fluorescence in Cross-linking of Mitomycin C with λDNA vs pH

Conc Mit C $\times 10^{-5}$ M	Conc $NaBH_4$ $\times 10^{-3}$ M	pH	% Loss of Fluorescence
0.6	1.3	5.0	19%
		6.0	15%
		7.2	11%
		8.7	4.5%
		10.3	0%
1.2	2.2	5.0	47%
		6.0	30%
		7.2	15.5%
		8.7	6%
		10.3	0%
2.0	3.1	5.0	66%
		6.0	44%
		7.2	25%
		8.7	14%
		10.3	0%
4.0	5.3	5.0	77%
		6.0	53%
		7.2	36%
		8.7	17%
		10.3	6%

duced by depurination or depyrimidation and small DNA fragments produced by degradation, while leaving behind intact polynucleotide molecules for radioactive counting. The results in Table 12 show that there was no loss of soluble radioactivity and that the ratio of $^{3}H/^{14}H$ counts is essentially constant under conditions where increasing concentrations of mitomycin C produce 16.1 to 79.6% reduction in ethidium fluorescence. This confirms that there was no detectable loss of either purine or pyrimidine bases or large scale degradation of DNA to small fragments.

Table 12

Radioactivity Assay for Alkylation of Polynucleotides by Mitomycin C*

Mitomycin C x 10^{-4} M	^{3}H c.p.m.	^{14}C c.p.m.	$^{3}H/^{14}C$ Ratio	% Decrease Fluore[illegible]
0.6	1292	1785.	0.724	16.1
1.2	1225	1682	0.728	43.5
1.8	1265	1733	0.730	61.7
2.4	1149	1418	0.810	72.2
3.0	1187	1528.	0.776	79.6

* The ratio of mitomycin C to sodium borohydride was 1:10 in all experiments.

In order to confirm that the drop in fluorescence was not due to some product of the reaction mixture inter-

fering with the fluorescence assay, *E. coli* DNA was treated with different concentrations of mitomycin C such that the fluorescence drop varied from 13 to 72%. The DNA was then dialyzed and fluorescence per A_{260} (Fu/A_{260}) was determined. The results presented in Table 13 show that a good correlation exists between the % decrease in fluorescence and the Fu/A_{260} ratio of the dialyzed DNA, thus indicating that there is no interference by reaction components with the fluorescence assay.

Table 13

Binding of Ethidium to Cross-Linked *E. coli* DNA After Dialysis

% Decrease in Fluorescence	Fu/A_{260}	Ratio Fu/A_{260} to Fu/A_{260} (Control)
Control (no MMC)	9,915	1
13	7,152	0.721
45	5,743	0.579
50	5,584	0.563
72	1,789	0.180

To confirm that the loss of fluorescence was due to alkylation of the DNA, an experiment was carried out to correlate the decrease in fluorescence with the extent of binding of mitomycin C to the DNA. The binding ratios of

mitomycin C to DNA for progressively increasing amounts of the antibiotic were determined by a modification of a procedure due to Tomasz.[24] Varying concentrations of mitomycin C were incubated with ^{3}H-labelled λ DNA. The decrease in fluorescence in each case was determined by the fluorescence assay. The reaction solutions were dialyzed to remove unbound mitomycin. The DNA concentration was determined by radioactive counting and the concentration of bound antibiotic was obtained from the UV. absorbance of the drug DNA complex at 314 nm. Table 14 and Figure 8 reveal that a good correlation exists between the loss of fluorescence and mitomycin C binding ratio over an extensive range.

Thus it is evident that the loss in fluorescence in reaction mixtures is not due to fragmentation of the DNA but rather to loss of ethidium intercalation sites by the mechanism described in (a) above. Therefore, using the fluorescence assay, the decrease in the fluorescence value may now be employed as a measure of alkylation of the DNA.

pH Dependence of Cross-Linking and Alkylation of DNA by Mitomycin C and the Sequence of Covalent Cross-Linking.

During studies on the interaction of mitomycin C with DNA, it was observed that the decrease in fluorescence due to the antitumor agent is strongly pH dependent with lower pH favoring loss of fluorescence *i.e.* favoring

Table 14

Correlation of % Loss of Fluorescence of DNA with Mitomycin C Binding Ratio

Initial MMC Conc $X10^{-6}$ M	% Loss of Fluorescence	DNA Conc After Dialysis $X10^{-5}$ M	Absorbance of Complex at 314 mμ	Bound MMC Conc $X10^{-6}$ M	Binding Ratio
0	0	7.33	0.017	0	-
1.2	19	7.07	0.032	1.42	50
1.8	37	6.63	0.037	1.97	34
2.4	58	7.03	0.048	2.88	24
3.0	67	6.23	0.048	3.05	20
4.2	85	5.66	0.087	6.72	8

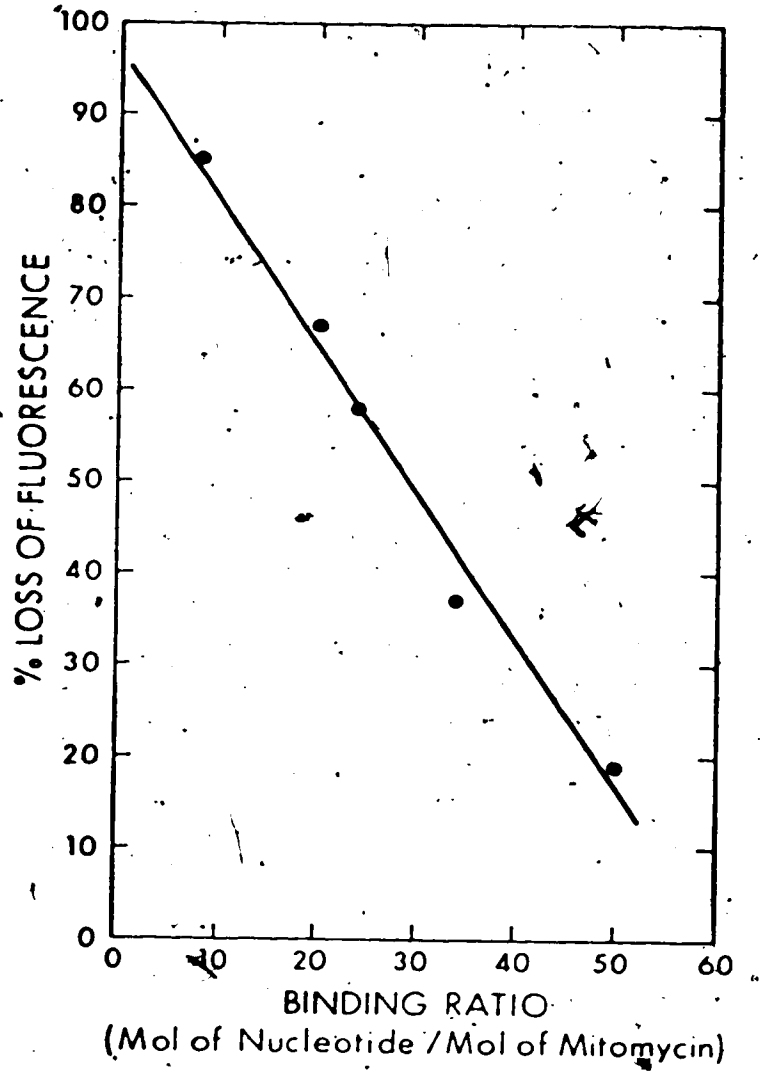


Figure 8. Dependence of % loss of fluorescence on the binding ratio of mitomycin C-DNA.

greater alkylation by one of the two alkylating groups on the mitomycin ring (Table 11 and Figure 9). This strongly suggests that under these conditions the aziridine function, which is known to be acid sensitive,[20a,59] is responsible for the alkylation of the DNA bases. The pH dependence of covalent cross-linking by mitomycin C was also examined and the results are presented in Table 9 and Figure 10. The trend towards more efficient covalent cross-linking with lower pH is clear. The results, which parallel those obtained for alkylation, again suggest that the first step in cross-linking is due to attack at the acid sensitive aziridine moiety under these conditions. Since tumor cells tend to have a lower pH as well as a more reducing environment as compared to normal cells, these factors could lead to selectivity of action.[60] It has been demonstrated that exposing DNA to low pH conditions can itself induce covalent cross-linking,[61] however, careful controls showed that there was no significant acid induced cross-linking under the conditions of our experiments.

Cross-Linking by Mitomycin C Without Reduction

As was described in Chapter I, Iyer and Szybalski[18b] have proposed that reductive activation of mitomycin C leads to elimination of methanol and activation of the two alkylating sites, C-1 and C-10, by conjugative interaction with the N-4 nitrogen (Scheme 8). However, we have found

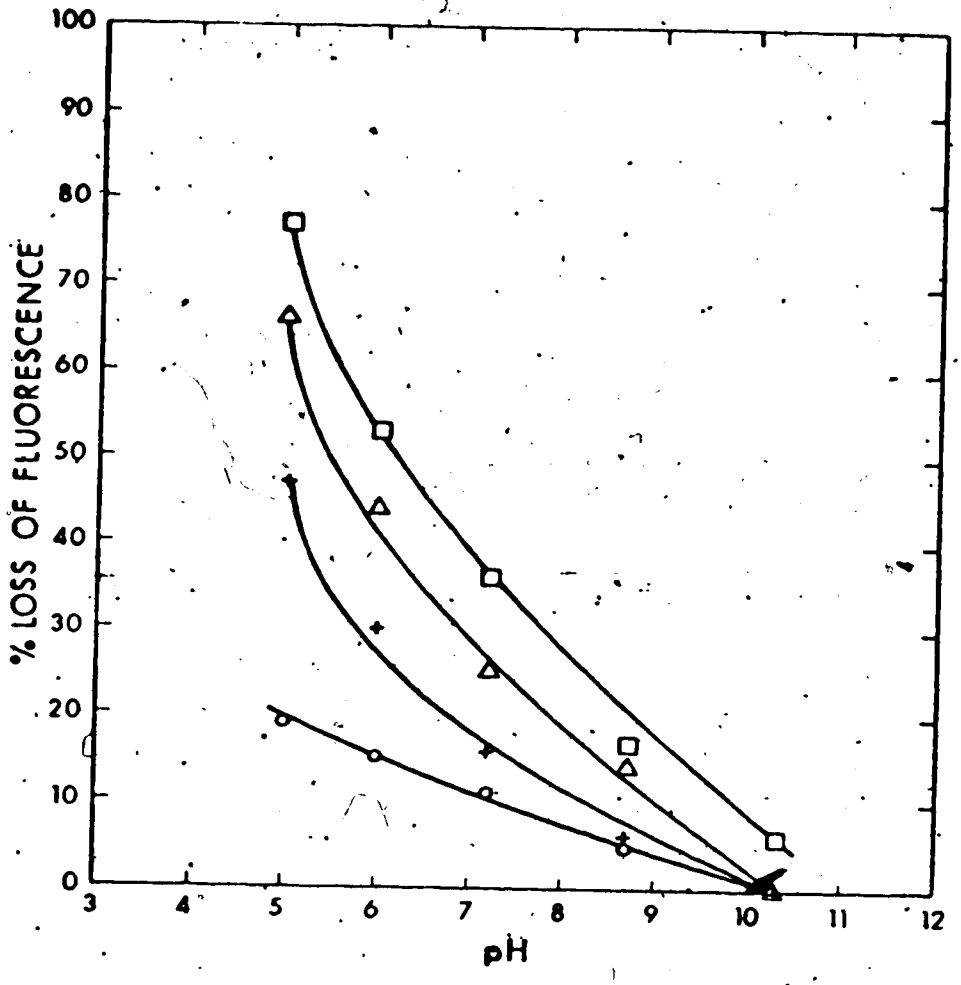


Figure 9. The pH dependence of alkylation of DNA by reduced mitomycin C. The reactions were at 22° in 50 mM phosphate at the appropriate pH and λ-DNA at 1.2 A_{260}. The mitomycin C concentrations were 0.6, 1.2, 2.0, and 4.0 x 10^{-4} M and the sodium borohydride 1.3, 2.2, 3.1 and 5.3 x 10^{-3} M for the following symbols respectively: o-o, +-+, Δ-Δ, and □-□.

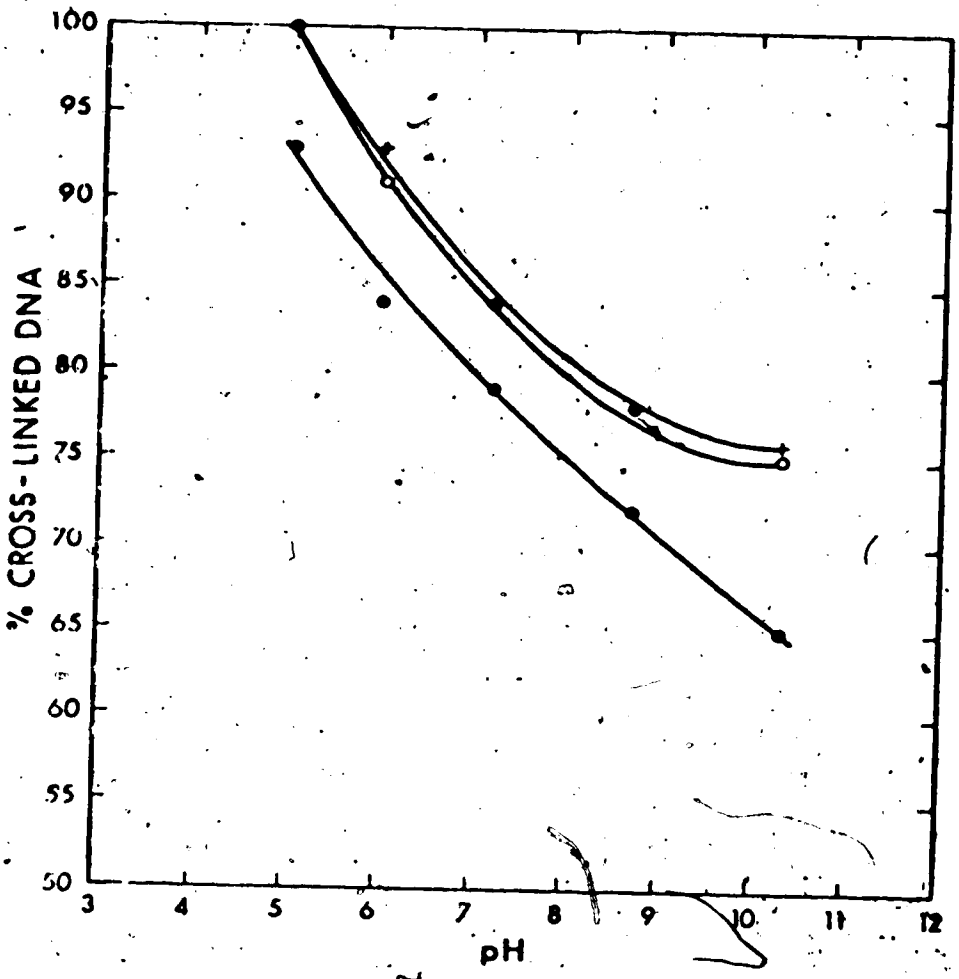


Figure 10. The pH dependence of cross-linking DNA by reduced mitomycin C. The conditions were as for Figure 9 but the fluorescence values were obtained after heating the DNA/ethidium mixture. The mitomycin C concentrations were 0.6, 1.2, 2.0×10^{-4} M, sodium borohydride 1.3, 2.2 and 3.1×10^{-3} M for the following symbols respectively: ●-●, o-o, and +-+.

that lowering the pH alone is sufficient to induce alkylation by the aziridine ring followed by covalent cross-linking *via* the carbamate. A control experiment showed that there was no significant acid induced cross-linking under these conditions. The kinetics of the cross-linking with unreduced mitomycin C shown in Figure 11, indicate that cross-linking is considerably slower with unactivated mitomycin than under reducing conditions. However, this result does raise the possibility that the antibiotic may induce covalent cross-linking *in vivo* without reduction.

Step-Wise Covalent Cross-Linking of DNA by Mitomycin C

The finding that unreduced mitomycin C can induce covalent cross-links in DNA under acid conditions, led us to carry out an experiment to show the step-wise nature of the cross-linking event. The covalent cross-linking by mitomycin C at low pH presumably involves an initial alkylation of the DNA at the acid sensitive aziridine group followed by a second bond formation at the carbamate. However, since the C-10 position is not activated by reduction, cross-linking is less efficient, presumably leaving mitomycin molecules attached to the DNA only by alkylation at the aziridine.

Mitomycin C was covalently attached to λ DNA without reduction by exposure at pH 4.0. The fluorescence assay showed that approximately 50% cross-linking of the

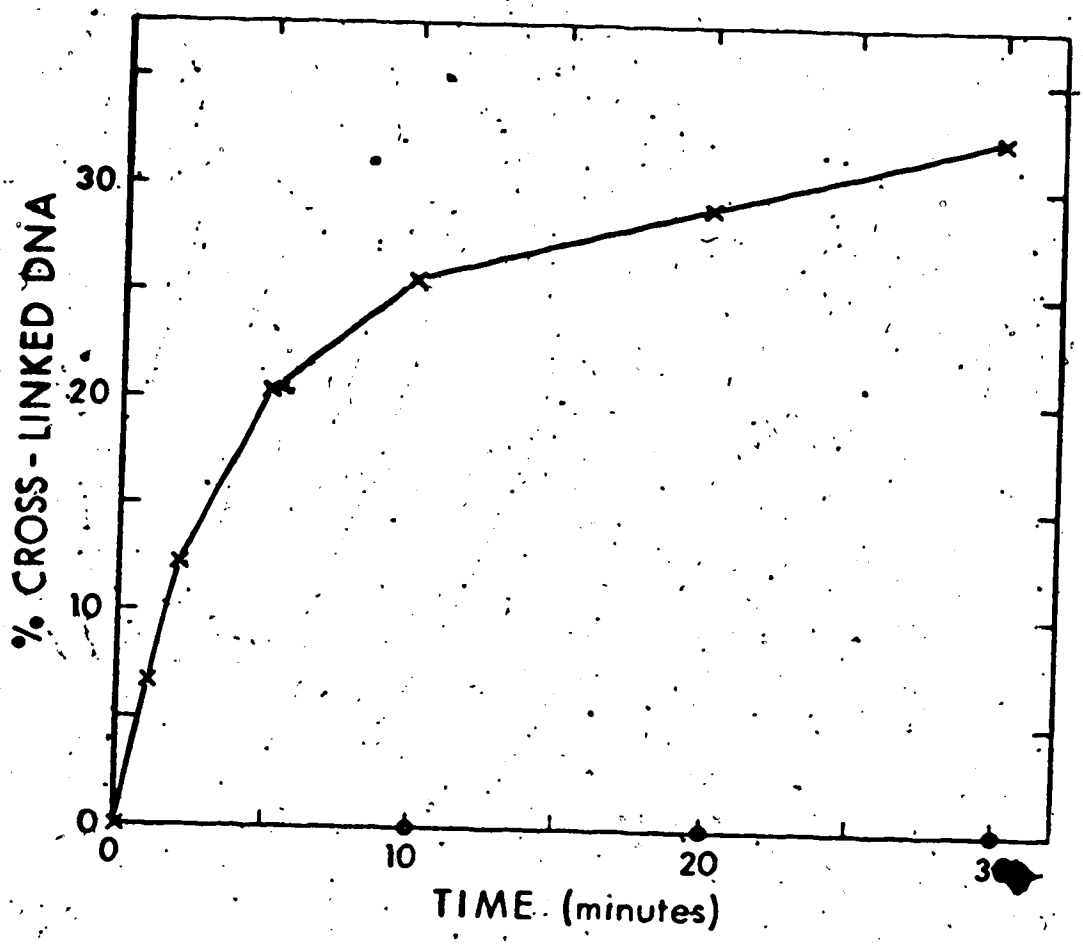


Figure 11. The cross-linking of λ DNA by mitomycin C without reduction. The reaction mixture contained λ DNA at 0.7 A_{260}, 50 mM sodium acetate pH 4 and mitomycin C 3.0×10^{-4} M, at 22°C. 15 µl samples were added to the alkaline ethidium assay mixture and the % cross-linked DNA was equated with the % of fluorescence remaining after the heat step. Under these conditions the fluorescence remained constant before heating. • Control experiment at pH 4 with no mitomycin C added.

DNA had occurred. Unreacted and unattached mitomycin molecules were removed by dialysis against a phosphate buffer, pH 7.0. The extent of cross-linking was unchanged after dialysis. The dialyzed DNA-drug complex was then treated with sodium borohydride resulting in a rapid increase in the extent of covalent cross-linking to 65%. This signified successful step-wise cross-linking of DNA by mitomycin C. Control experiments showed that the increased cross-linking was not due to free mitomycin, all of which had been removed by dialysis, or to sodium borohydride.

Dependence of the Efficiency of Covalent Cross-Linking of DNA by Mitomycin C on the (G+C) Content of the DNA

Iyer and Szybalski have shown that the degree of covalent cross-linking of DNA by mitomycin C increases with increasing (G+C) content of the DNA[18b] and have proposed the O-6 position of the guanines as the most likely site of attachment on the DNA.[18c] Attachment at other positions of the guanines has been ruled out by Tomasz.[26] We have applied the ethidium fluorescence assay to a similar study of the effect of (G+C) content of the DNA on the efficiency of covalent cross-linking. Three natural DNAs of different (G+C) content were used: *C. perfringens* (30%), calf thymus (40%), and *E. coli* (50%). The cross-linking efficiencies as determined by the ethidium fluorescence assay are not strictly comparable because of slight differences in the average molecular weights of the DNAs; as

determined by sedimentation velocities. Since one cross-link per DNA molecule is sufficient to produce rapid renaturation after cooling regardless of the length of the polynucleotide, DNAs of lower molecular weight require more cross-linking events on a nucleotide residue basis to obtain the same % cross-linking. Assuming a Poisson's distribution of the cross-links and also that one cross-link is sufficient to permit the renaturation of the molecule, an estimate of the average number of cross-links per molecule (m) was made from $m = \ln(1/P_o)$ (where P_o is the proportion of the molecules that are not cross-linked) for the three DNAs. Assuming an average molecular weight of 300 - 330 for each nucleotide, the average number of cross-links per nucleotide was calculated to make the results comparable for the three DNAs. From Table 15 and Figure 12, it is clear that a direct correlation exists between more efficient covalent cross-linking and higher (G+C) content of the DNA. The values obtained are closely comparable with similar estimates made by Iyer and Szybalski.[18]

Table 15

Dependence of Efficiency of Covalent Cross-Linking by Mitomycin C on the (G+C) Content of the DNA

MMC Conc $\times 10^{-6}$ M	$NaBH_4$ Conc $\times 10^{-3}$ M	% (G+C)	Max. % X-Linking/Time	Average X-Links per Molecule	Average X-Links per Nucleotide $\times 10^{-5}$
3.0	6.6	50	73%/5min	1.30	2.64
3.0	6.6	40	57%/5min	0.84	2.52
3.0	6.6	30	42%/5min	0.54	1.62
0.6	1.3	50	37%/5min	0.46	0.93
0.6	1.3	40	25%/5min	0.29	0.87
0.6	1.3	30	8%/5min	0.08	0.21

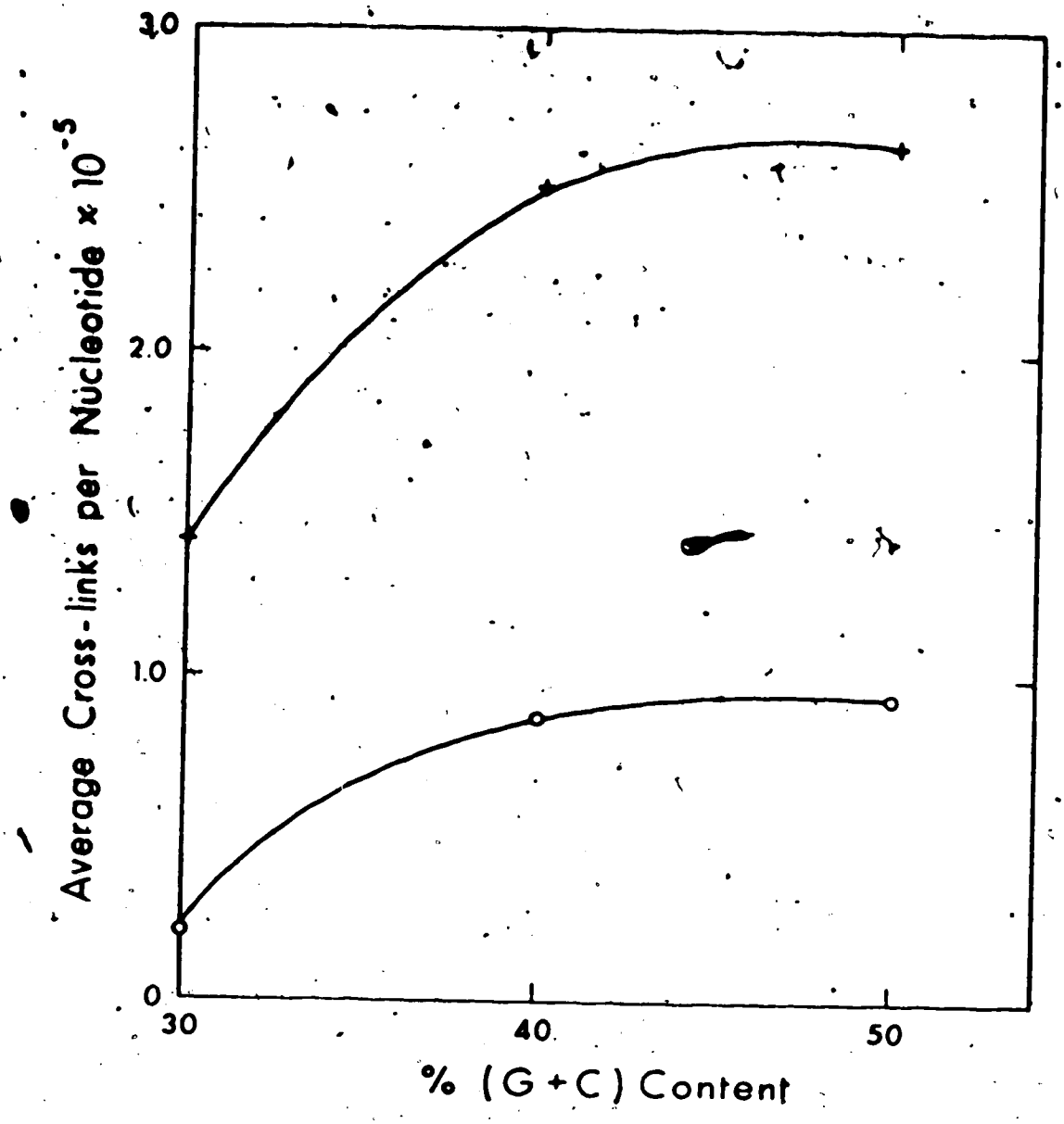


Figure 12. Dependence of efficiency of covalent cross-linking of DNAs by reduced mitomycin C on the (G+C) content of the DNA. Reactions contained DNA at 1.2 A_{260}, phosphate buffer pH 7.2 at 0.05 M, and mitomycin C and sodium borohydride at o .0.6 x 10^{-4} M, 1.3 x 10^{-3} M and + 3.0 x 10^{-4} M, 6.6 x 10^{-3} M respectively.

The Mechanism of DNA Degradation by Mitomycin C

Detection of Mitomycin C Induced Single Strand Scission of CCC-DNA by the Fluorescence Assay

It has been observed that mitomycin C produces extensive breakdown of DNA in many cases.[29] Although this process is relatively slow compared to covalent cross-linking, it has been suggested as a possible mode of action for the drug. It has been considered that the DNA degradation was due mainly to the activation of intracellular deoxyribonucleases.[31]

We examined the interaction of mitomycin C with CCC-DNA, by the ethidium fluorescence assay, in order to gain information about the DNA degradation. Since mitomycin C cross-links DNA efficiently, we expected to observe either case (c) or (d) illustrated in Figure 6. On exposing CCC-DNA derived from PM2 bacteriophage to activated mitomycin C, a rapid increase in fluorescence was observed. The loss of fluorescence normally observed on heat denaturation of nicked DNA was not seen and there was a 100% return of fluorescence after heating due to efficient covalent cross-linking of the DNA. A control experiment showed that the reducing agent, sodium borohydride, has no effect on the PM2 CCC-DNA. The results shown in Figure 13 confirm that activated mitomycin C induces single strand scission of DNA.

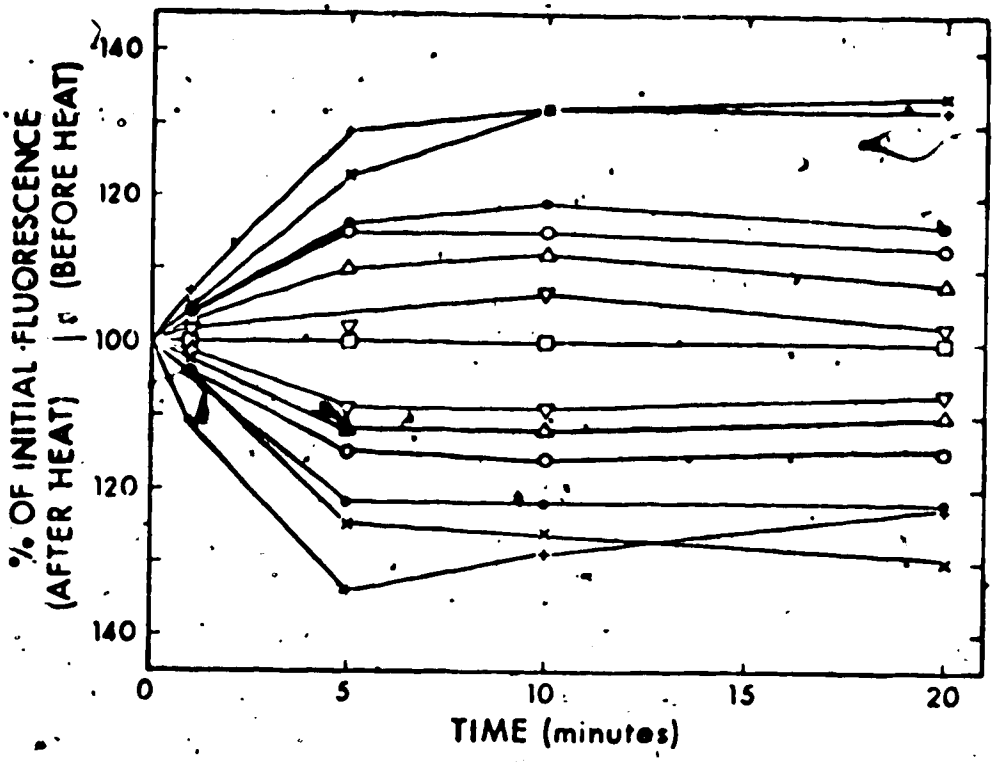
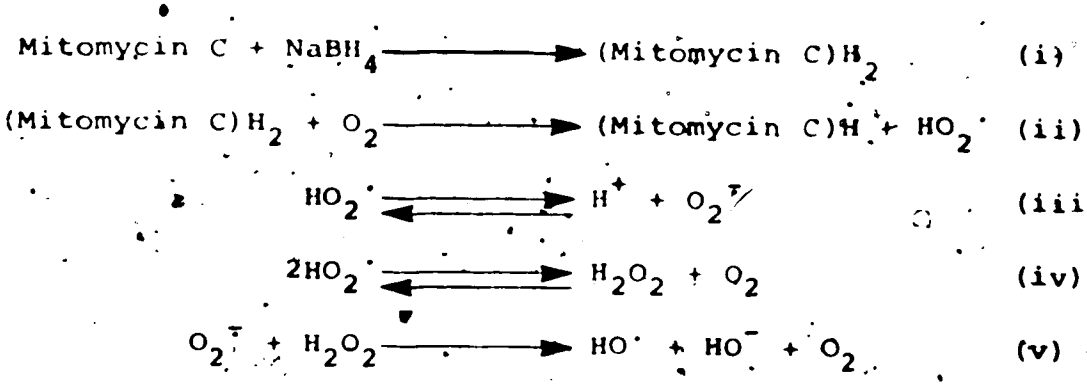


Figure 13. Single strand scission of PM2 CCC-DNA by mitomycin C. Reactions were performed at ambient temperature in phosphate buffer, pH 7.2 containing 1.13 A_{260} of PM2 DNA. Additional components: ▽ mitomycin C, 3.0×10^{-4} M, sodium borohydride 5.3×10^{-3} M, isopropyl alcohol 2.5×10^{-1} M; △ mitomycin C 3.0×10^{-4} M, sodium borohydride 5.3×10^{-3} M, sodium benzoate 5.0×10^{-2} M; ○ mitomycin C 3.0×10^{-4} M, sodium borohydride 5.3×10^{-3} M, catalase 4.1×10^{-6} M; ● mitomycin C 3.0×10^{-4} M, sodium borohydride 5.3×10^{-3} M; catalase 4.1×10^{-6} M, S.D. 6.1×10^{-5} M; + mitomycin C, 3.0×10^{-4} M, sodium borohydride 5.3×10^{-3} M, S.D. 3.0×10^{-5} M; x mitomycin C 3.0×10^{-4} M, sodium borohydride 5.3×10^{-3} M, □ control

In independent experiments it was shown that single strand cleavage can be retarded by catalase, a protective enzyme which removes hydrogen peroxide from the cell, and by a combination of catalase and superoxide dismutase. The latter is another protective enzyme found in the cell which catalyzes the dismutation of the superoxide radical ($O_2^{\cdot-}$). Free radical scavengers such as isopropyl alcohol and sodium benzoate were also efficient inhibitors of the single strand cleavage (Figure 13). This suggests a cleavage mechanism similar to that operating with reduced streptonigrin in which cleavage of the CCC-DNA is induced by hydroxyl radicals (Scheme 10).[30]

Scheme 10



This scheme requires the intermediacy of the semiquinone of mitomycin C which in contrast to that of streptonigrin is reported to have a very short lifetime of the order of several seconds.[62] This point is discussed further with the results of the electroanalytical data pre-

sented below. Recent work by Handa and Sato[63] confirming that mitomycin C facilitates the production of superoxide radical on reduction supports the above scheme.

Proximity Effect in the Mitomycin C Induced Single Strand Scission of PM2 CCC-DNA

It was of interest to determine if the radical induced cleavage of CCC-DNA is more efficient when the generating compound is covalently attached to the DNA. As a result, mitomycin C was covalently attached to DNA without reduction by exposure at pH 4.0. Unattached antibiotic was removed by gel filtration on an agarose column. The purified, alkylated DNA was then treated with sodium borohydride resulting in a characteristic rise in the ethidium fluorescence before denaturation, due to radical induced cleavage (Figure 14). Careful controls showed that all the unattached mitomycin C had been removed and that sodium borohydride did not cleave the DNA. In contrast to the previous experiment, catalase did not inhibit the cleavage. However, the radical scavenger, isopropyl alcohol protected the DNA to the same extent as with free mitomycin C. The progressive rise in the fluorescence after heat denaturation is due to the induction of further cross-links after reduction.

It is tempting to interpret these results as evidence for a proximity effect whereby superoxide and hy-

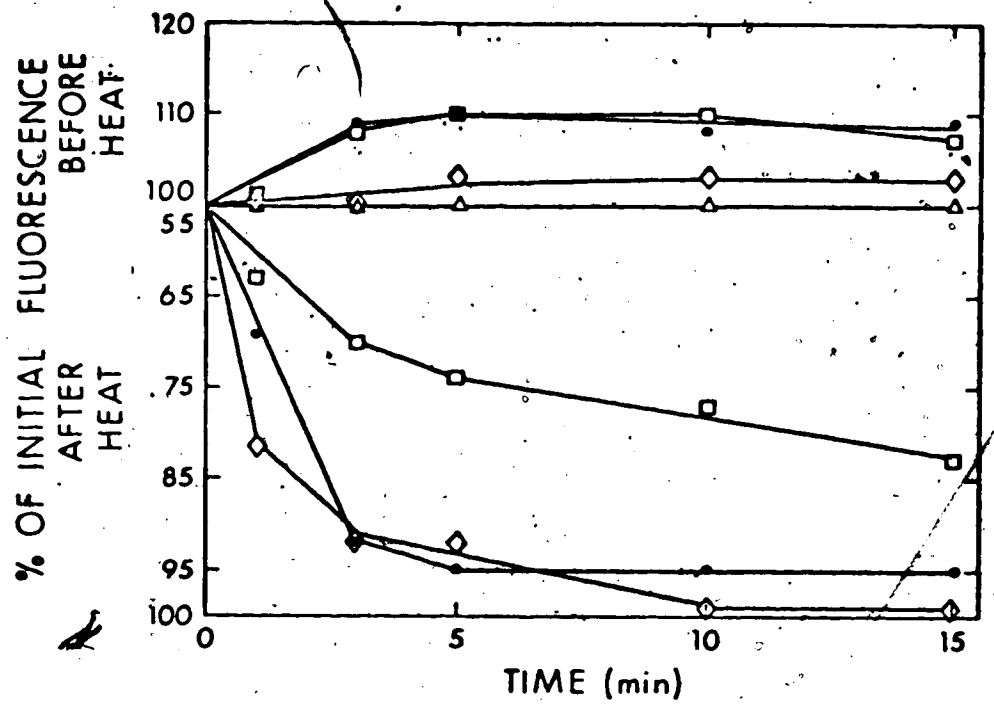


Figure 14. Single strand scission of mitomycin C alkylated PM2 CCC DNA. PM2 DNA was alkylated at ambient temperatures in 5×10^{-2} M sodium acetate buffer pH 4.0 containing 2.47 A_{260} of PM2 DNA and 5.1×10^{-4} M mitomycin C. Chromatography on 10 ml of agarose (Bio-Gel A-15 M, 5-100 mesh) afforded alkylated DNA. Scission reactions were performed at ambient temperature in phosphate buffer pH 7.0 containing 1.04 A_{260} of alkylated DNA. Additional components were ● sodium borohydride 5.3×10^{-3} M, ○ sodium borohydride 5.3×10^{-3} M, catalase 4.1×10^{-6} M; ◇ sodium borohydride 5.3×10^{-3} M, isopropyl alcohol 2.5×10^{-1} M; △ control.

droxyl radicals are generated close to the surface of the DNA. Catalase, being a very large molecule, could not adequately scavenge the associated hydrogen peroxide. On the other hand, the small isopropyl alcohol molecule is presumably able to penetrate within the double helix and scavenge the radicals more efficiently.

Investigation of the Possible Inactivation of the Protective Enzymes Superoxide Dismutase and Catalase by Mitomycin C

It has been shown that streptonigrin, in addition to producing extensive DNA degradation, also inactivates those enzymes in the cell (*i.e.* superoxide dismutase and catalase) which protect the DNA from such cleavage.[45] It was of interest to determine if mitomycin C had a similar effect on these enzymes. The results of experiments to detect such an effect are presented in Figures 15 and 16 and reveal that mitomycin C does not inactivate the protective enzymes superoxide dismutase or catalase.

Studies on Possible Metabolites of Mitomycin C

It has been proposed by Iyer and Szybalski[5] that the biochemical role of the NADPH mediated reductive activation of mitomycin C is to unmask the potential alkylating sites at C-1 and C-10 by causing rapid elimination of methanol (Scheme 8). However, the discovery of the radical induced cleavage of DNA by reduced mitomycin C requires

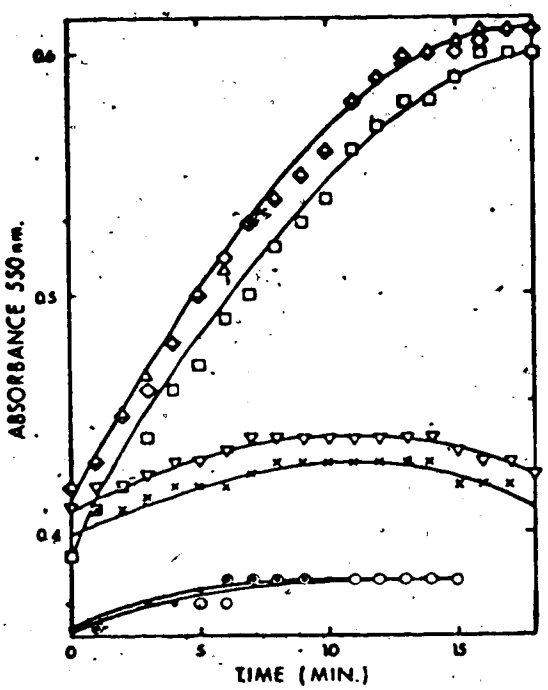


Figure 15. Control experiments for inactivation of superoxide dismutase action on the reduction of cytochrome C. Reactions were performed at 25° in a total volume of 3.5 ml, buffered at pH 7.8 by 0.05 M potassium phosphate containing 10^{-4} M EDTA, 10^{-4} M xanthine, 2×10^{-5} M cytochrome C and initiated by addition of 100 λ of a 0.18 μg/ml solution of xanthine oxidase. Preincubated components added were o S.D. 1.5×10^{-8} M; O mitomycin C 3.0×10^{-6} M, S.D. 1.5×10^{-8} M; x mitomycin C 3.0×10^{-6} M, sodium borohydride 5.3×10^{-5} M, S.D. 1.5×10^{-8} M; ▽ sodium borohydride 5.3×10^{-5} M, S.D. 1.5×10^{-8} M; Δ mitomycin C 3.0×10^{-6} M; sodium borohydride 5.3×10^{-5} M; ◇ mitomycin C 3.0×10^{-6} M. □ Control

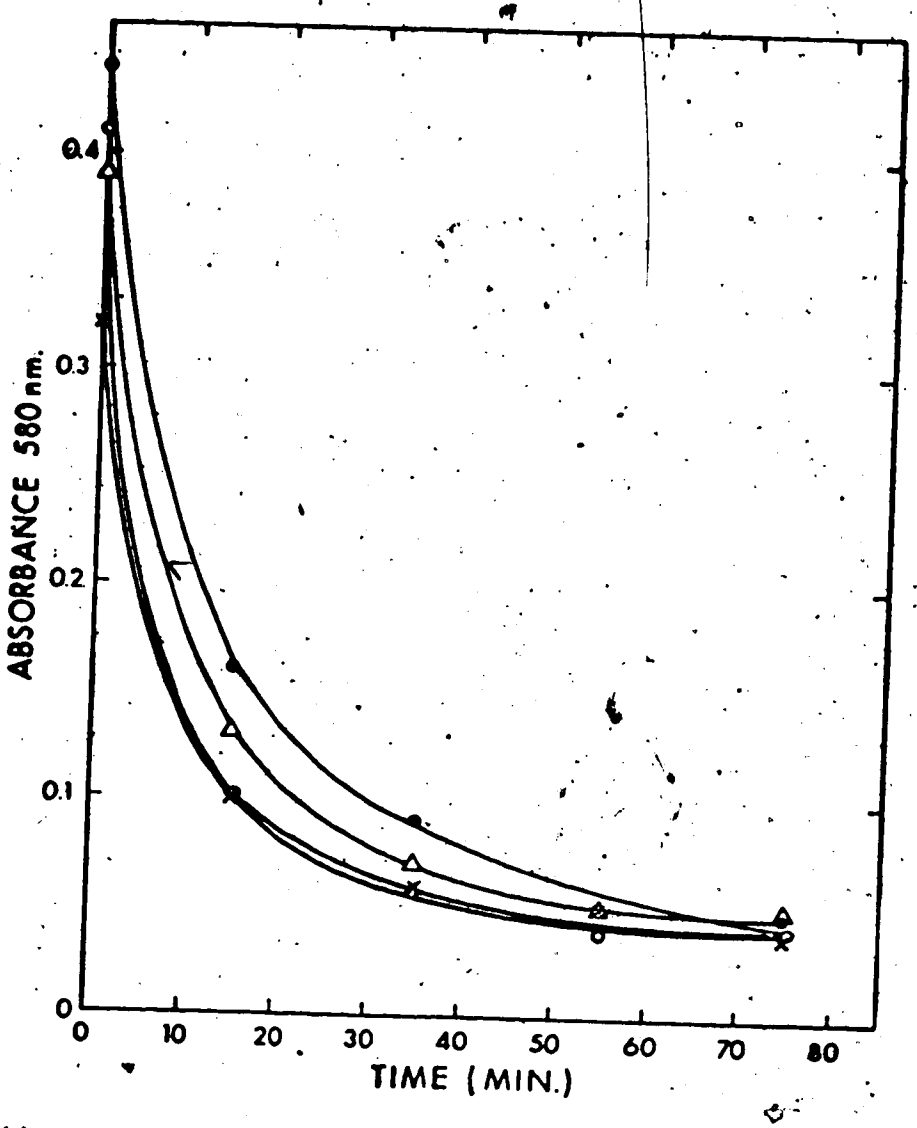
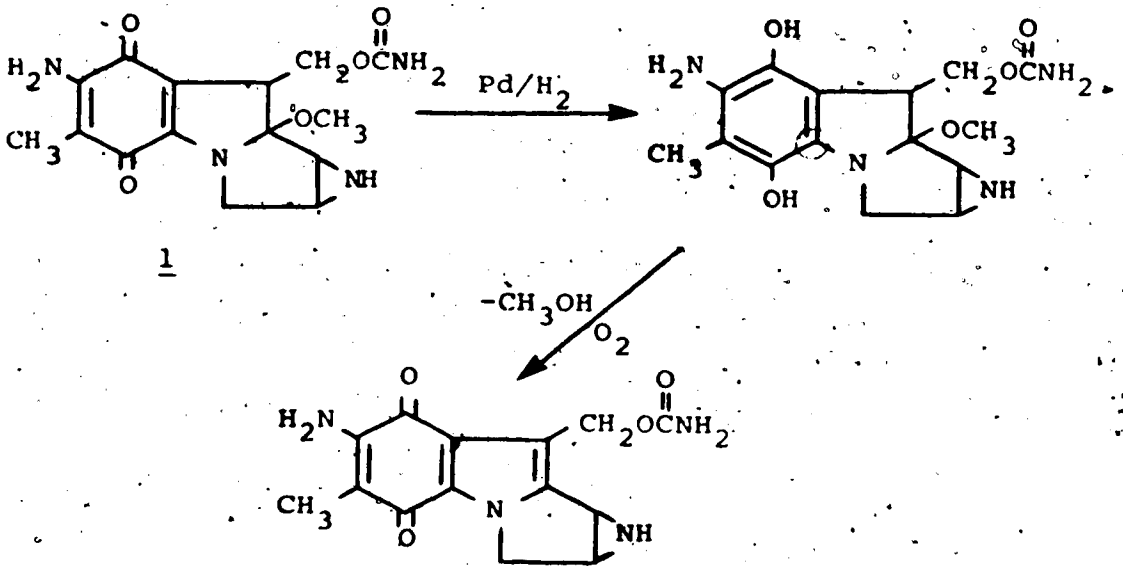


Figure 16. Control experiments for inactivation of catalase decomposition of hydrogen peroxide. Reactions were performed at 0° in a total volume of 10 ml buffered at pH 7.0 by 0.06 M potassium phosphate containing 0.02 M hydrogen peroxide and 8×10^{-9} M catalase. Catalase was preincubated with x mitomycin C 6.0×10^{-6} M; Δ sodium borohydride 1.1×10^{-4} M; • mitomycin C 6.0×10^{-6} M and sodium borohydride 1.1×10^{-4} M.

rapid reoxidation of a hydroquinone form to produce hydroperoxy and hydroxyl radicals. The reoxidation step might conceivably involve the hydroquinone of mitomycin C itself or one of its metabolites. As a result, several mitomycin C derivatives, which appeared to be plausible active intermediates in the breakdown of mitomycin C were prepared and investigated as possible active metabolites of the drug.

The demethoxylated derivative of mitomycin C 42 was prepared by catalytic hydrogenation at atmospheric pressure followed by rapid air oxidation. On reductive activation 42 should give rise to the presumed active form



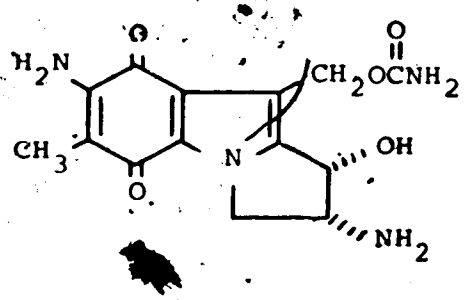
1

42

of mitomycin C. Therefore, the ability of this compound to cross-link λ-DNA was examined by the ethidium fluores-

cence assay. This mitomycin derivative showed very efficient covalent cross-linking. Concomitant alkylation of the DNA was shown by a loss of fluorescence. Incubation of reductively activated 42 with PM2 CCC-DNA resulted in a characteristic rise in the fluorescence which could be suppressed by the addition of the free radical scavenger, isopropyl alcohol (Figure 17), thus demonstrating the ability of this derivative to cause radical induced single strand scission of DNA.

No firm evidence has hitherto been presented for the direct covalent attachment of the C-10 carbamate of mitomycin C to DNA. In order to investigate this interaction, a derivative in which the aziridine ring had been opened was prepared. It has been reported that mild acid hydrolysis of mitomycin C affords a mixture of the two stereoisomeric ring opened compounds *cis* 1R, 2R 43 and *trans* 1S, 2R 44 in a ratio of 86:14.[59] Mitomycin C was



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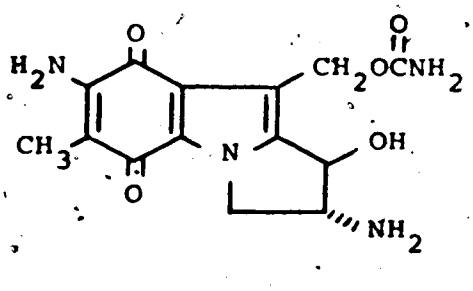

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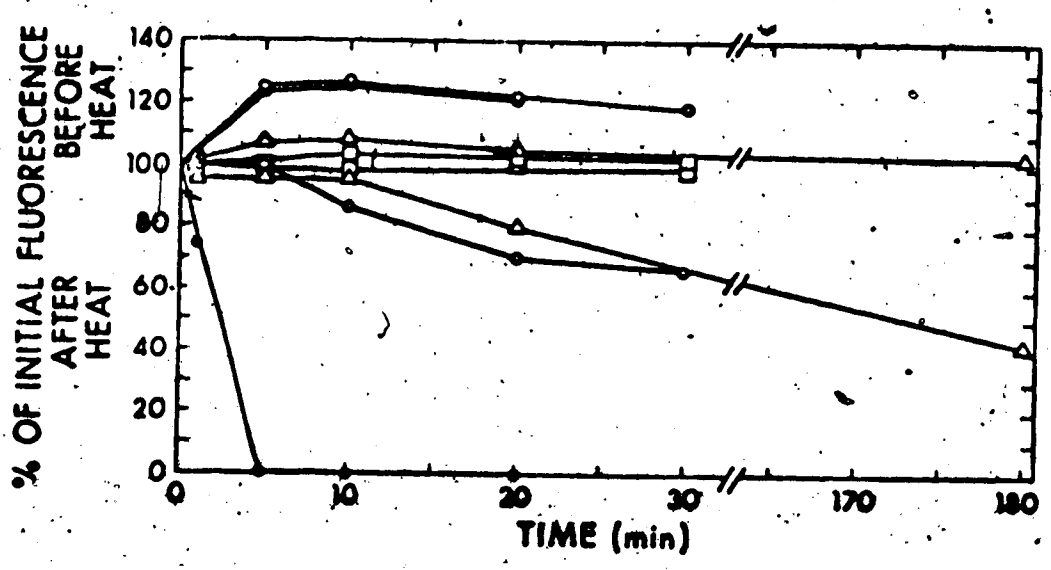


Figure 17. Single strand scission, covalent cross-linking and monoalkylation of PM2 CCC-DNA by mitomycin C derivatives. Reactions were performed at ambient temperature at pH 7.0 in the presence of 5.3×10^{-3} M sodium borohydride. Aziridine ring opened compound 43; • 3.2×10^{-4} M of 43, Δ 3.2×10^{-4} M of 43 and 0.25 M isopropyl alcohol. Aziridinomitosene 42; o 3.2×10^{-4} M of 42, □ 3.2×10^{-4} M of 42 and 0.25 M isopropyl alcohol.

acid hydrolyzed and the resulting product was recrystallized affording a material which gave a single spot on TLC and a single peak on high pressure liquid chromatography, to which was assigned structure 43. This ring opened derivative was reductively activated and incubated with PM2 CCC-DNA. As was anticipated from our previous work, a rise in fluorescence was observed due to efficient single strand scission resulting from the generation of superoxide and hydroxyl radicals (Figure 17). The DNA cleavage could be suppressed by the addition of isopropyl alcohol and under these conditions alkylation of the DNA due to the carbamate was detected by a slow decrease in the fluorescence after heat denaturation (Figure 6(c)). No covalent cross-linking of DNA by this derivative was observed. This provides the first firm evidence for the involvement of the C-10 carbamate in the covalent attachment to DNA.

From these results it seems reasonable that a number of degradation products of mitomycin C are capable of interacting directly with DNA, in addition to the parent antibiotic, to induce covalent cross-linking, alkylation, and cleavage.

Electroanalytical Examination of Mitomycin C and its Derivatives

We have shown that reduced mitomycin C and its quinone-containing metabolites produce extensive degradation of DNA by inducing the formation of superoxide and hydroxyl radicals which cause single-strand scission of the DNA. The proposed mechanism requires the intermediacy of the semiquinone of mitomycin C. It has been reported that the semiquinones of mitomycin B and of mitomycin C[62] have been observed by e.p.r. and that the lifetime of the semiquinone of mitomycin C is very short, of the order of several seconds. Therefore, we attempted to obtain information about the reduction process in mitomycin C and to gain confirmatory evidence for the existence of the semiquinone, and an estimate of its lifetime, by electroanalytical studies.

Polarographic analysis of aqueous solutions of mitomycin C gave graphs containing the cathodic waves IC-IVC, of which IVC is observable only at pH > 6.5. The IC wave ($E_{1/2}$ = -0.368 ± 0.003 V at pH 7) log plot gave a reversible slope. The pH dependence of $E_{1/2}$ is shown in Figure 18. The wave height remains approximately constant over the range pH = 5.4-8.5. At pH < 5.4, the height of the wave decreased markedly. The compound responsible for this wave is irrecoverably destroyed at pH < 4; the wave does not reappear when the pH of this solution is again

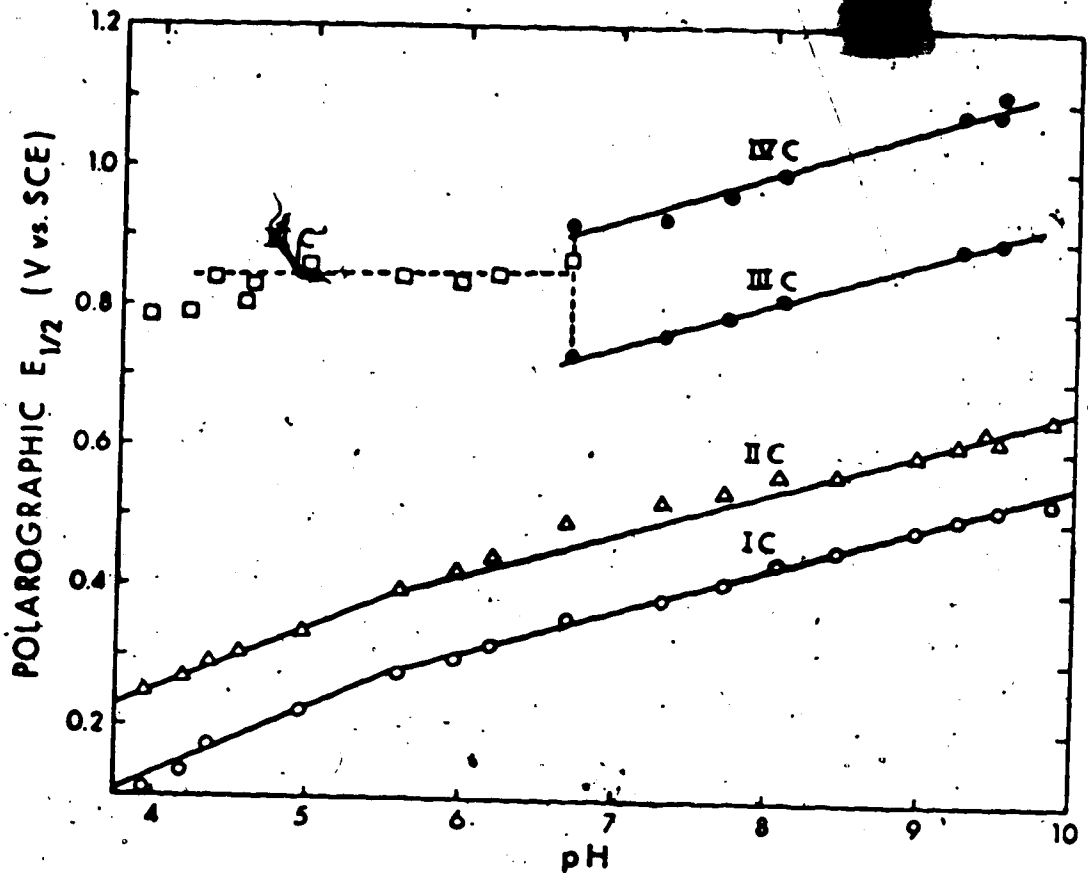


Figure 18. pH-dependence of half-wave potentials of aqueous mitomycin C 3.4×10^{-4} M solutions in solutions of buffer, acetate (pH < 5.9), phosphate (5.9 < pH < 8.1), trizma (pH > 8.1); o, first wave; Δ, second wave; □, third wave with no splitting, ●, third wave with splitting.

increased above 4, so that the decrease cannot be due to a reversible protonation equilibrium. The second wave IIC ($E_{1/2}$ = -0.486 ± 0.005 V at pH 7) is drawn out in shape and is shown by a log analysis not to be reversible. This wave is not affected by making the solution acidic (pH < 4) for a short period of time. The third wave, IIIC', is irreversible and appears to split into two smaller waves, IIIC and IVC, both irreversible, at pH > 6.5 (Figure 18).

Figure 19 shows a typical cyclic voltammogram of mitomycin C at 200 mV/s. It shows four cathodic peaks (IC - IVC) and one anodic peak (IIA). The appearance of IIIC and IVC and the absence of the corresponding anodic peaks IIIA and IVA, shows that these processes are not reversible, in agreement with the polarographic results. Most of the cyclic voltammetric studies were restricted to the potential range 0 to -0.85 V to avoid complications arising from these processes. With the range thus restricted, only IC and IIC are observed at slower scan rate (ν < 100 mV/s). At ν = 100 mV/s, IA and IIA become visible and at ν = 200 mV/s, IIC splits into two peaks. With increasing scan rate, the increase in the height of IC and IA is much greater than the corresponding increase in height of IIC and IIA. At very fast scan rates (2 V/s $\leq \nu \leq$ 10 V/s), IC and IA are very well defined and separated by 35 ± 5 mV (Figure 20).

Polarographic analysis of aqueous solutions of the

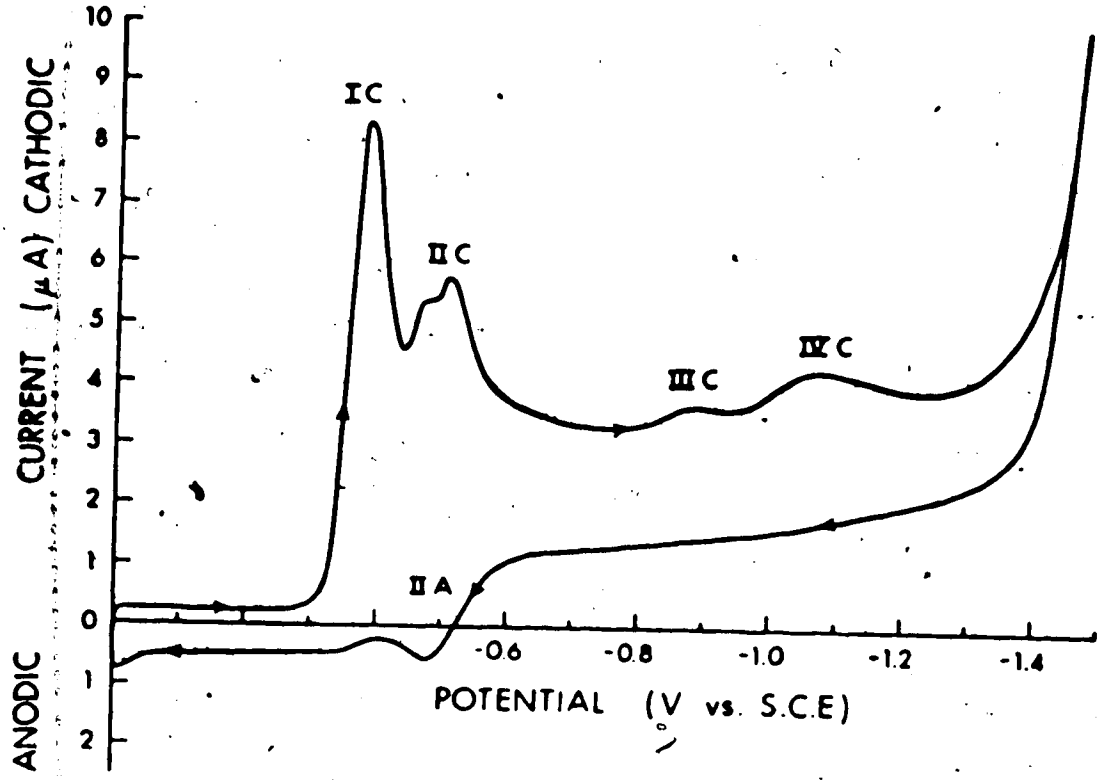


Figure 19. Cyclic voltammogram of mitomycin C (3.4×10^{-4} M), pH 6.98 ± 0.2 in phosphate buffer, 37.5°C, ν = 200 mV/s.

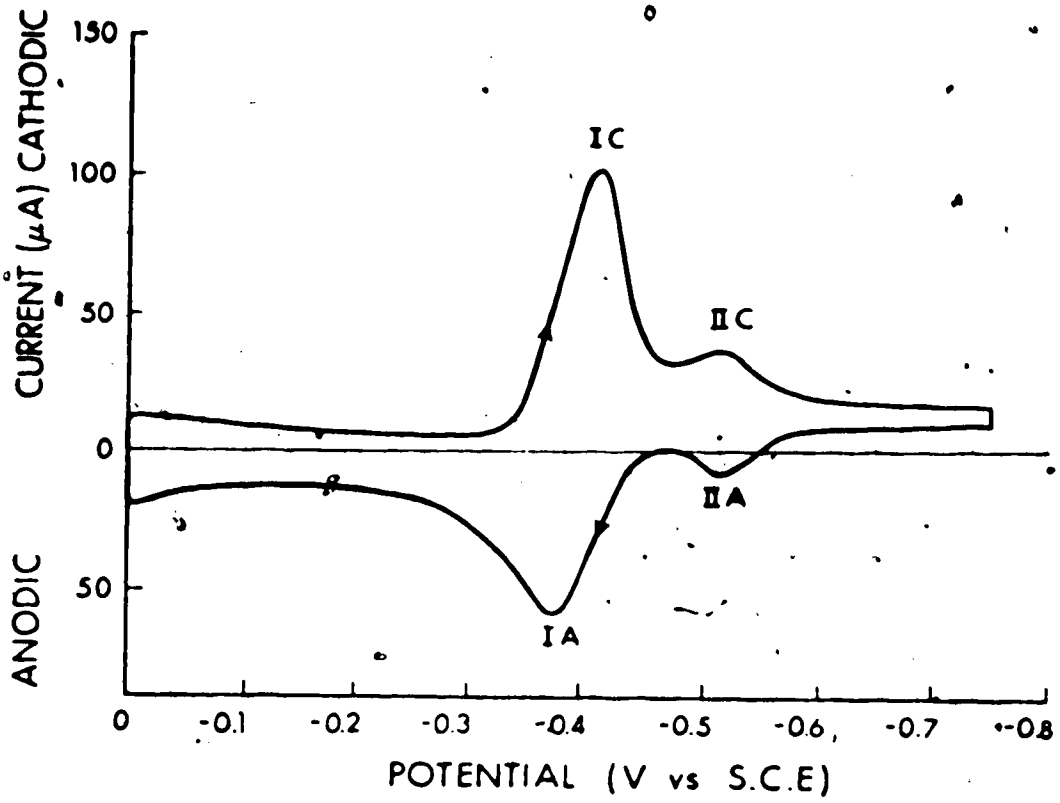


Figure 20 Cyclic voltammogram of mitomycin C (3.4×10^{-4} M), pH 6.98 ± 0.2 in phosphate buffer, 37.5°C, = 10 V/s.

ring opened mitomycin C derivative 43 gave curves in which IC is barely observable. Cyclic voltammetry of 43 shows very small peaks for IC and IA identical in potential and behavior with those of mitomycin C which may arise from < 5% of unreacted mitomycin C remaining in the preparation of the derivative. The irreversible waves IIIC and IVC of solutions of 43 were identical to those of the parent antibiotic. The polarographic wave for IIC is present in distorted form. The cyclic voltammetric peak IIC behaves similarly to peak IIC of mitomycin C for 200 mV/s $\leq \nu \leq$ 50 V/s. However, IIC splits into two peaks at scan rates of 200 mV/s and 100 mV/s. The height of the leading peak is slightly greater at 200 mV/s, while the height of the following peak becomes slightly greater at 100 mV/s. At $\nu <$ 100 mV/s, only one broad peak can be distinguished. The corresponding anodic peak IIA is visible at all scan rates and becomes sharper at faster scan rates.

Polarographic analysis of an aqueous solution of the demethoxylated mitomycin C derivative 42 gave waves identical with those of mitomycin C itself. The $E_{1/2}$ of IC for 42 differed from that of IC of mitomycin by less than 5 mV. The distortion of IIC, though of the same form as observed for 43, was barely noticeable. The cyclic voltammogram of the demethoxylated derivative was similar to that of the parent compound, however at $\nu >$ 1000 mV/s, both IC and IA of 42 became severely distorted. It was

also identical to that of 43 except for the IC and IA peaks.

A typical example of a multicycle sweep is shown in Figure 21 for 42; similar waves are observed for mitomycin C and for 43 except that for the ring opened derivative, the IC peak is extremely small. The compounds show a IC, a split IIC, and a IIA peak during the first cycle; mitomycin C also shows a small IA peak. In the second cycle, IC appears only as a shoulder. Only IIC and IIA appear after the second cycle. Although both derivatives reached steady state in five cycles, mitomycin reached steady state only after sixteen cycles.

Discussion of the Electroanalytical Studies

The mechanism of the reduction of mitomycin C at pH 7 appears to belong to the E.C.E. (electrochemical-chemical-electrochemical) class, where C represents one or more steps and the electrochemical steps are reversible.[65] Two pathways seem possible for this reduction and they are outlined below. As yet it has not been possible to distinguish between them.

If path A were operating, the polarographic wave IC would correspond to the two electron, two proton reduction to the hydroquinone; while for path B this wave would correspond to the one electron, one proton reduction to the semiquinone. In fact, the logarithmic $(i_d - i)/i$ plot yields a slope corresponding to two electrons, but this is not

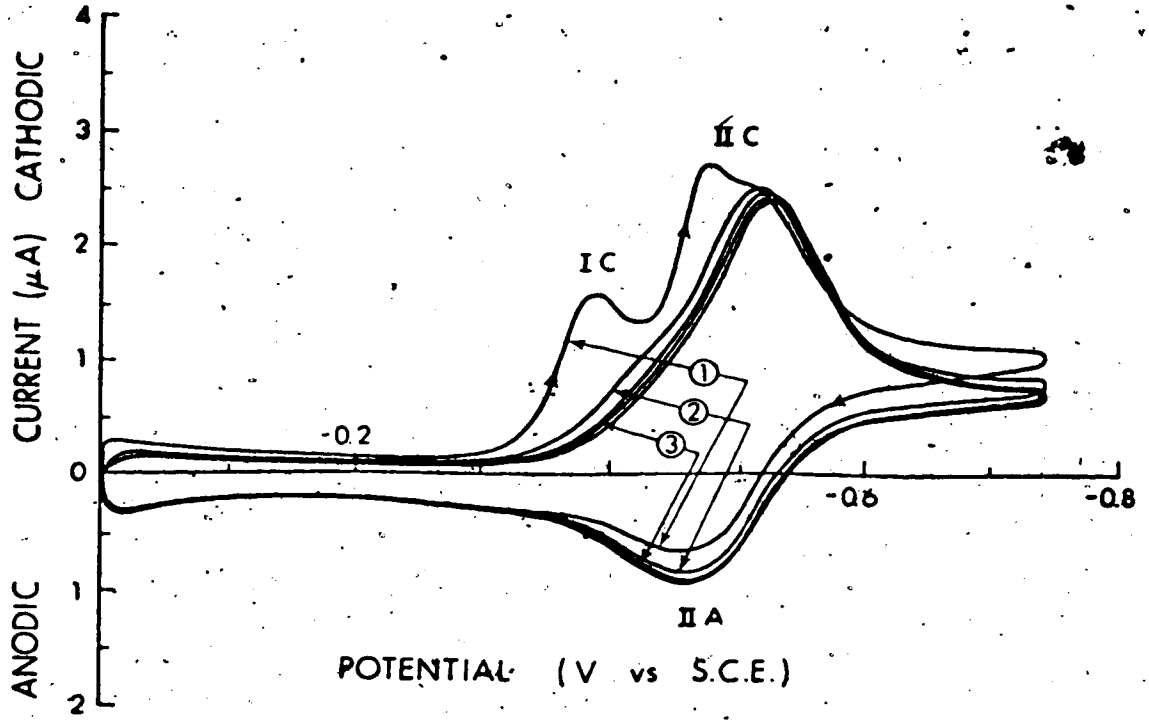
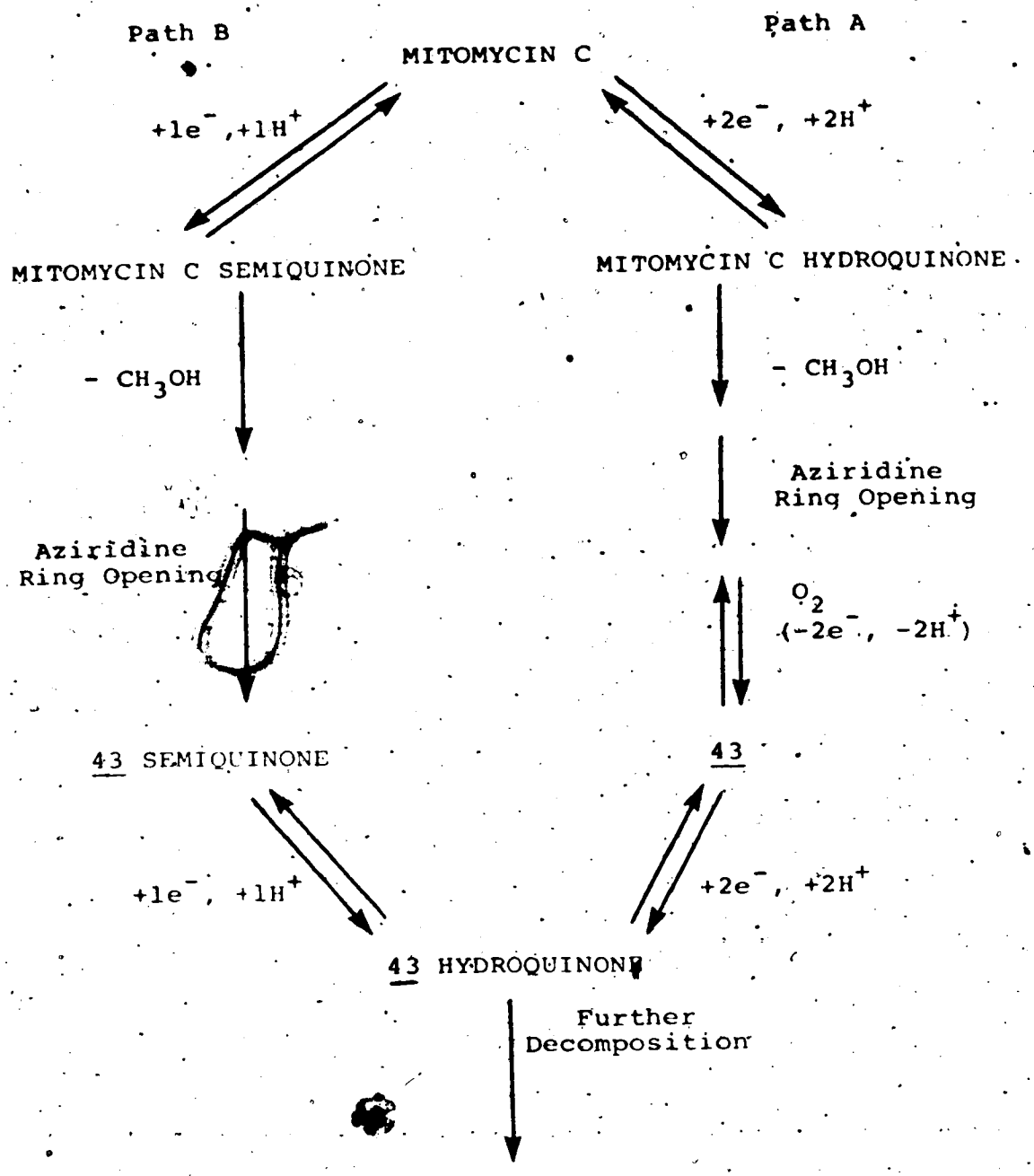


Figure 21. Multicycle voltammogram of 42 (3.4×10^{-4} M), pH 6.98 ± 0.2 in phosphate buffer, 37.5°C, ν = 200 mV/s.


Path B
MITOMYCIN C
Path A
+1e⁻, +1H⁺
+2e⁻, +2H⁺
MITOMYCIN C SEMIQUINONE
MITOMYCIN C HYDROQUINONE
- CH3OH
- CH3OH
Aziridine Ring Opening
Aziridine Ring Opening
O2 (-2e⁻, -2H⁺)
43 SEMIQUINONE
43
+1e⁻, +1H⁺
+2e⁻, +2H⁺
43 HYDROQUINONE
Further Decomposition

definitive in the present case because the rate of disappearance of the initial intermediate, presumably by reaction with itself is known from the voltammetric data to be fast on the five to seven second polarographic time scale. At lower pH values, the wave height, for either path, is due to the irreversible acid induced opening of the aziridine ring. Although it is not possible to obtain information about the chemical steps which follow the initial reduction from polarography, these steps may be characterized using the cyclic voltammetric data. The reactions must be both rapid and irreversible to explain the lack of a IA peak in the single scan voltammetric curves (except at very high scan speeds) and the absence of IC and IA from the later scans of the multicycle voltammetry. It seems likely that the reactions involved are the demethoxylation and aziridine ring openin[illegible]

The half-life of the semiquinone of mitomycin C may be estimated from the cyclic voltammetric data.[65] The time elapsed between IC and the subsequent IA is twice the difference between $E_{1/2}$ and the reversal potential of -0.75 V divided by the scan rate, ν, or $0.76/\nu$. For the first intermediate (either hydroquinone for path A or semiquinone for path B) to react to the point where it would not be detectable on the reverse scan would require five or six half-lives. This situation arises at ν = 100 mV/s. Thus the time required for these six half-lives is 7.6

seconds and the half-life is of the order of one second. If path A is operating this is the half-life of the hydroquinone and therefore also the maximum half-life of any semiquinone intermediate, while if path B is followed this value of approximately one second represents the half-life of the semiquinone itself.

The differences in the behavior of wave I of mitomycin C and the demethoxylated derivative 42, though slight, are real at high scan rates, which would be the case if the demethoxylation following the initial reduction proceeds at an extremely fast but finite rate.

The waves and peaks designated as II, if path A is operating, are due to the two electron, two proton reduction of the aziridine ring-opened derivative 43; for path B they correspond to the one electron, one proton reduction of the semiquinone to the hydroquinone of 43. This process is almost certainly electrochemically reversible but does not appear so because the chemical steps preceding it are not infinitely fast. Since wave I is absent for 43, path B predicts that the semiquinone of the ring-opened derivative is extremely short-lived to the point of nonexistence; thus wave II for the ring-opened derivative 43 corresponds to a two electron, two proton reduction directly to the hydroquinone. On the other hand, if path A is operating, the splitting of peak II into two voltammetric peaks at higher scan rates may indicate the

step-wise reduction of 43 *via* its semiquinone. Waves III and IV, present for all three compounds, appear to be due to further decomposition of the mitosane skeleton. Experiments designed to distinguish between the two pathways are presently underway.

Mitomycin C and compound 42 have very similar reduction potentials, whereas the ring-opened derivative is more difficult to reduce. Since the rates of cleavage of PM2 CCC-DNA by all three compounds in the presence of a limited amount of reducing agent are very similar, the controlling factor in the scission process may be the rate of reoxidation of the reduced forms from closely similar oxidation levels.

Summary and Conclusions

Making use of the rapid and convenient ethidium fluorescence assay, the interaction of mitomycin C and its derivatives with DNA has been studied. Studies with the ring-opened derivative 43 and studies of the pH dependence of covalent cross-linking and alkylation of DNA by mitomycin C, have confirmed the involvement of both the aziridine and carbamate groups in the covalent attachment to DNA. The step-wise nature of covalent cross-linking by mitomycin C has been demonstrated. The observed ability of the mitomycin derivatives 42 and 43 to form covalent bonds to DNA efficiently, raises the possibility that these

and other metabolites of mitomycin C may be involved in the mechanism of action of mitomycin C.

It has been shown that mitomycin C induces single strand scission in DNA by the generation of superoxide and hydroxyl radicals. This contrasts with the previously proposed mechanism of DNA degradation.[31] Furthermore, it is possible that the scission process may be enhanced by a proximity effect arising from the covalent interaction of the mitomycin with the DNA. The efficient strand scission produced by the mitomycin C derivatives 42 and 43 again raises the possibility that these and other metabolites may contribute to the biological activity of mitomycin C.

The electroanalytical studies have provided information about the steps involved in the reduction of mitomycin C. The short-lived nature of the semiquinone of mitomycin C described by Nagata and Matouyama[62] was confirmed. Continuing electroanalytical studies should provide more detailed information about the pathway by which mitomycin C is reduced.

A summary of the chemical transformations that may be involved in the biological activity of mitomycin C is presented in Figure 22.

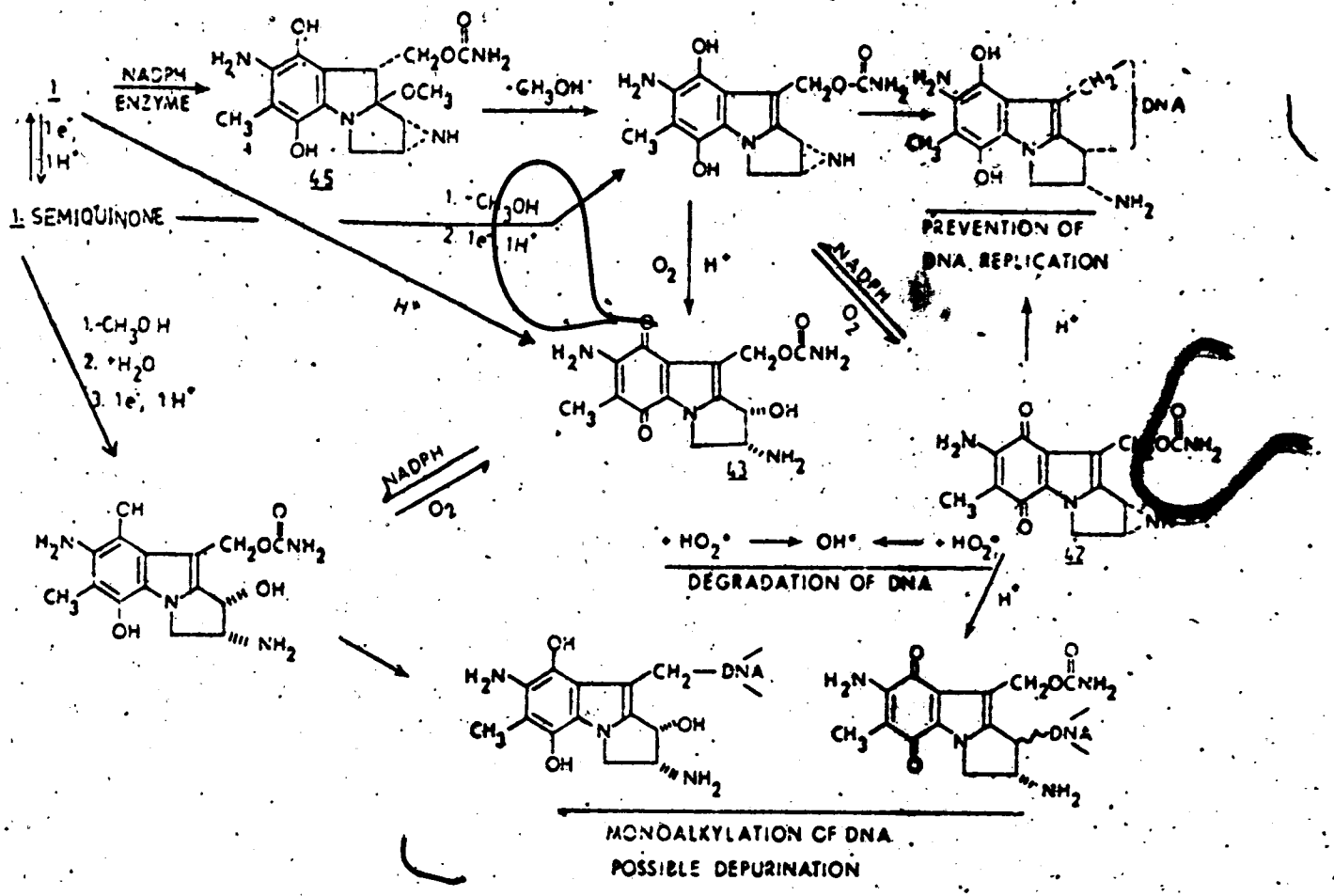


Figure 22. Possible chemical transformations involved in the antitumor activity of mitomycin C.

Experimental

All fluorescence measurements were performed on a G.K. Turner and Associates model 430 spectrofluorometer. Absorbance measurements were made on a Gilford 2400 spectrophotometer. Polarographic glass and teflon cells of conventional design were employed in a three-electrode assembly. The temperature of the cell was maintained at 37.5° ± 0.2°C by circulation of thermostated water. The dc polarographic studies were made with a Model 174A Polarographic Analyzer (Princeton Applied Research) equipped with Model 174/70 Drop Timer. Cyclic voltammetric measurements were made using the Model 173-176-175-9323 configuration of the same manufacturer. Curves were recorded on an X-Y recorder or photographed on an oscilloscope as required by scan rate, ν, which ranged from 10 mV/s to 50 V/s. Measurements of pH were carried out before and just after each run with a Accumet Model 520 pH Meter and combination glass-SCE electrode. Radioactivity was counted in Aquasol on a Beckman LS-250 Liquid Scintillation Counter.

Materials

Ethidium bromide, and catalase were purchased from Sigma Chemical Co. Mitomycin C was from Calbiochem and Kyowa Hakko Kogyo Chemical Co. Sephadex G-100 superfine was from Pharmacia and DEAE cellulose was obtained from

Whatman. λ, PM2, *C. perfringens*, *E. coli*, and calf thymus DNA were a gift of Dr. A.R. Morgan. Superoxide dismutase was the gift of Dr. Alan Davison.

Fluorescence Assay for Detecting CLC-Sequences

All measurements were performed on a G.K. Turner and Associates model 430 spectrofluorometer equipped with a cooling fan to reduce fluctuations in the xenon lamp source. Wavelength calibration was performed as described in the manual for the instrument. One centimeter round cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength 600 nm. Medium sensitivity (X100 scale) was generally used and water was circulated between the cell compartment and a thermally regulated bath at 22°. Small samples (5 - 20 µl) from reaction mixtures were added to 2 ml of the assay mixture which was 20 mM potassium phosphate pH 11.8, 0.4 mM EDTA and 0.5 µg/ml in ethidium bromide. The instrument was blanked with the assay mixture.

The cross-linking assay was carried out as follows: a 10 µl aliquot of the cross-linking reaction mixture was diluted in 2 ml of the assay solution. The fluorescence of the diluted solution was measured. The solution was then heat denatured at 96° for 2 minutes, cooled in ice, and equilibrated in a water bath at 22° for 5 minutes. The fluorescence of the solution was again measured. The

ratio of the fluorescence after heating to the fluorescence before heating gave the extent of covalent cross-linking.

General Procedure for Determination of Cross-linking and Alkylation of DNA with Reduced Mitomycin C

Mitomycin C was added as a 200 µg/ml solution in water, and DNA as aqueous solutions. Reaction mixtures were buffered to the appropriate pH with acetate at pH 5.0 or with potassium phosphate at pH 6.0, 7.2, 8.7 or 10.3. Mitomycin C was reduced in the cross-linking solutions by an aqueous solution of sodium borohydride. Cross-linking reactions were carried out on a scale of 40-100 µl. Reaction solutions had concentrations of approximately 1.2 A_{260} of λ DNA, 0.05 M of buffer, 0.6, 1.2, 2.0, and 4.0×10^{-4} M of mitomycin C and 1.3, 2.2, 3.1, and 5.3×10^{-3} M of sodium borohydride respectively. 10 µl samples were removed at timed intervals and analyzed for extent of cross-linking by the fluorometric assay described above. A control mixture prepared as above but containing no mitomycin C was run with each experiment. Assay of the control reactions showed no cross-linking in each case, and that none of the components of the reaction mixture interfered with the ethidium fluorescence.

Correlation of Loss of Fluorescence with Binding Ratio

Reactions were carried out on a 600 µl scale. Reaction solutions had concentrations of 0.520 A_{260} of

^{3}H-labelled λ DNA, 0.05 M of phosphate buffer pH 5.0, 0, 1.2, 1.8, 2.4, 3.0, and 4.2 x 10^{-4} M of mitomycin C, and 2.6, 2.6, 4.0, 5.3, 6.6, and 9.2 x 10^{-3} M of sodium borohydride. After 30 minutes the solutions were assayed for loss of fluorescence by the fluorometric assay described above. Two 100 µl aliquots were removed from each solution, added to Aquasol and counted in a liquid scintillation counter in order to correlate the radioactivity of the DNA with its absorbance at 260 nm. The counts were corrected for mitomycin C quenching.

Unbound mitomycin C was removed from the reaction solutions by dialysis *vs* 10 mM potassium phosphate, pH 11.8, 0.1 mM EDTA. Binding ratios were determined by the procedure of Tomasz[24] except that nucleotide concentrations were determined from radioactive counting. The nucleotide concentration was calculated using an extinction coefficient of 7,000 for DNA at 260 nm. The bound mitomycin concentration was calculated from the absorbance of the dialyzed reaction solutions at 314 nm using an extinction coefficient of 11,000 for bound mitomycin. The absorbance at 314 nm was corrected for DNA absorbance at this wavelength using an extinction coefficient of 232 for DNA at 314 nm, calculated from the control reaction (mitomycin C conc. = 0).

Cross-Linking by Mitomycin C Without Reduction

The reaction mixture contained λ DNA at 0.7 A_{260}, 0.05 M acetate buffer, pH 4.0, and mitomycin C at 3.0×10^{-4} M. 15 µl aliquots were analyzed for extent of cross-linking as described above. A control experiment at pH 4.0 with no mitomycin C added showed no cross-linking.

Procedure for the Study of Step-Wise Covalent Cross-Linking of DNA by Mitomycin C

The following three reaction solutions were prepared on a 1 ml scale: Solution A contained: λ DNA at 1.34 A_{260}, 0.05 M acetate buffer, pH 4.0, and 3.6×10^{-4} M mitomycin C; Solution B contained: λ DNA at I.34 A_{260} and 0.05 M acetate buffer, pH 4.0; Solution C contained: 0.05 M acetate buffer, pH 4.0 and 3.6×10^{-4} M mitomycin C. The three solutions were incubated for 1.5 hours at ambient temperature. 10 µl samples of solutions A and B were analyzed at time intervals by the ethidium assay described above.

Solution	Time	% Cross-Linking
A	1'	9%
A	40'	50%
A	90'	52%
B	1'	0%
B	40'	0%
B	90'	0%

The three solutions were individually dialyzed for 25 hours at 5° *vs.* 20 mM phosphate, pH 7.0, 0.4 mM EDTA.

The following reaction mixtures were prepared from dialyzed solutions A and B: 90 µl of the dialyzed solution was made up to a 100 µl solution containing 0.05 M phosphate buffer, pH 7.0 and 5.3×10^{-3} M sodium borohydride. The solutions were approximately 0.65 A_{260} in DNA. The solutions were incubated at ambient temperature and 15 µl aliquots were analyzed at timed intervals for extent of cross-linking.

Solution	Time	% Cross-Linking
Dialyzed A	0'	47%
	2'	49%
	5'	58%
	10'	65%
	15'	62%
Dialyzed B	0'	0%
	2'	0%
	5'	0%
	10'	0%
	15'	0%

A control experiment was carried out to show that all the free mitomycin C was removed by dialysis. A 100 µl solution containing 75 µl of dialyzed solution C, 0.05 M phosphate buffer, pH 7.0, native λ DNA at 1.01 A_{260}, and 5.3×10^{-3} M sodium borohydride was incubated at ambient

temperature. Analysis of 15 µl aliquots at timed intervals by the fluorescence assay showed no cross-linking.

Procedure for Determining Covalent Cross-Linking of DNAs of Different (G+C) Content by Activated Mitomycin C

The DNAs used were *E. coli* [M.W. = 14.8×10^6, (G+C) content = 50%]; calf thymus [M.W. = 10×10^6, (G+C) content = 40%]; *C. perfringens* [M.W. = 11.4×10^6, (G+C) content = 30%]. The molecular weights were determined by sedimentation velocity studies. Reaction solutions were prepared as described above. Concentrations in the final reaction mixtures were: DNA at 1.20 A_{260}, 0.05 M phosphate buffer, pH 7.2, mitomycin C at 0.6 or 3.0×10^{-4} M and sodium borohydride at 1.3 or 6.6×10^{-3} M respectively. Reactions were run at ambient temperature and aliquots were removed at timed intervals and analyzed for the extent of covalent cross-linking by the ethidium assay. The average number of cross-links per molecule, m, was calculated using the formula $m = \ln (1/P_o)$ where P_o is the proportion of molecules unlinked.

General Procedure for the Determination of Cleavage of PM2 CCC-DNA with Mitomycin C

Experiments were carried out on a 100 µl scale. Reaction mixtures contained: 3.0×10^{-4} M mitomycin C, 5.3×10^{-3} M sodium borohydride, PM2 CCC-DNA at 1.12 A_{260}, and

0.05 M phosphate buffer, pH 7.2. 15 µl samples were analyzed by the ethidium fluorescence assay as described above. A control experiment containing no mitomycin C was run with each experiment. Analysis of the control reaction showed a 75% return of fluorescence after heating (identical for DNA alone) indicating the PM2 was nicked to the extent of about 20%[53] (CCC-DNA gives a 30% increase in fluorescence on nicking).

Inhibition experiments were performed as above with the addition of the inhibitors listed in Table 16 and Figure 13.

Procedure for Studying the Proximity Effect in the Mitomycin C Induced Single Strand Scission of PM2 CCC-DNA

The following three solutions were prepared on a 1 ml scale: Solution A containing: PM2 CCC-DNA at 2.47 A_{260}, 0.05 M acetate buffer, pH 4.0, and 5.1×10^{-4} M mitomycin C; Solution B containing: PM2 CCC-DNA at 2.47 A_{260} and 0.05 M acetate buffer, pH 4.0; Solution C containing: 0.05 M acetate buffer, pH 4.0 and 5.1×10^{-4} M mitomycin C.

The three solutions were incubated for 1.5 hours at ambient temperature and then applied to a 10 ml column of agarose (Bio Gel A-15 M, 50-100 mesh) eluting with 1 mM potassium phosphate at pH 8.0, 10^{-5} M EDTA. The fraction between 3.5 and 5 ml of eluent was collected in each case.

Table 16

Inhibition of Mitomycin C Induced Cleavage of PM2 CCC-DNA

Time (Min.)	Inhibitor											
	None		Cat.[a] (4.1×10^{-6} M)		S.D.[b] (3.0×10^{-5} M)		Cat. (4.1×10^{-6} M) S.D. (6.1×10^{-5} M)		Isopropyl Alcohol (0.25 M)		Sodium Benzoate (0.5 M)	
	B.H.[c]	A.H.[d]	B.H.	A.H.	B.H.	A.H.	B.H.	A.H.	B.H.	A.H.	B.H.	A.H.
1	56	54	57	57	60	63	57	57	58	56	57	56
5	65	66	61	63	72	75	64	67	58	61	61	62
10	70	67	63	64	[illegible]	72	65	67	61	58	62	61
20	71	69	62	63	74	69	64	67	58	62	60	61

[a] Cat. Catalase

[b] S.D. Superoxide Dismutase

[c] B.H. Before heat denaturation ethidium fluorescence

[d] A.H. After heat denaturation ethidium fluorescence

Fluorescence analysis of this fraction from solutions A and B indicated that it contained most of the DNA.

Scission reactions with chromatographed solutions A and B were performed at ambient temperature in 0.05 M phosphate buffer, pH 7.0 containing approximately 8.1 A_{260} of chromatographed DNA and 5.3×10^{-3} M sodium borohydride. Additional experiments also contained 4.1×10^{-6} M catalase or 0.25 M isopropyl alcohol.

A control experiment was carried out to show that all the free mitomycin was removed by the gel filtration. A 100 µl solution containing 85 µl of chromatographed solution C, 0.05 M phosphate buffer, pH 7.0, native PM2 CCC-DNA at 0.99 A_{260}, and 5.3×10^{-3} M sodium borohydride was incubated at ambient temperature. Analysis of 15 µl aliquots at timed intervals by the ethidium assay showed no single strand scission of the DNA.

Control Experiments for Possible Inactivation of Superoxide Dismutase by Mitomycin C

In a typical experiment a solution of 1.5×10^{-7} M superoxide dismutase, 3.0×10^{-5} M mitomycin C, and 5.0×10^{-4} M sodium borohydride in phosphate buffer at pH 7.8 was incubated at ambient temperature for 1 hour. In a 5 ml flask was mixed a 10^{-3} M xanthine solution (0.5 ml), a 10^{-3} M EDTA solution (0.5 ml), a 5×10^{-5} M cytochrome C solution (2 ml) and 0.5 ml of the superoxide dismutase-

mitomycin C-borohydride solution. After diluting to 5 ml with phosphate buffer at pH 7.8, 3.5 ml of the solution was transferred to a cuvette. After the absorbance at 550 nm was recorded, 100 µl of xanthine oxidase solution (0.18 µg/ml) was added. The solution was mixed and the absorbance at 550 nm was followed. The results for this and additional experiments are shown in Figure 15 where no inhibition of superoxide dismutase by either mitomycin C or its reduced form is evident.

Control Experiments for Possible Inactivation of Catalase by Mitomycin C

In a typical experiment a solution containing 3.0×10^{-5} M mitomycin C and 5.3×10^{-4} M sodium borohydride in pH 7.0 phosphate buffer was incubated with catalase at 4×10^{-8} M at 0° for 1 hour. 2 ml of this solution was added to hydrogen peroxide such as to obtain a final volume of 10 ml, 0.02 M in H_2O_2. The decomposition of H_2O_2 at 0° was determined by the following method. At timed intervals, 1 ml of the reaction mixtures was withdrawn and transferred to a 50 ml volumetric flask containing 5 ml of 10% sulfuric acid and the whole made up to the mark with water. To 0.5 ml of this solution in a 10 ml volumetric flask was then added successively 1 M potassium iodide (0.5 ml), 1 mM acidified ammonium molybdate solution (0.5 ml) and 2% starch solution (0.25 ml). The re-

sulting blue solution was then diluted to 10 ml and the absorbance at 580 nm recorded. The results of this and additional experiments are shown in Figure 16 where no interference with the action of catalase by either mitomycin C or its reduced form is apparent.

Preparation of Demethoxylated Mitomycin C 42

Mitomycin C (33 mg, 0.1 mmole) was hydrogenated over 5% palladium on charcoal in 10 ml of ethyl acetate. After one equivalent of hydrogen was absorbed, the solution was filtered and the filtrate exposed to air oxidation. Removal of the solvent *in vacuo* gave 8 mg of 42 as a purple solid which was purified by recrystallization from ethyl acetate: petroleum ether.

The absorption spectrum $\lambda_{max}(H_2O)$: 248 nm (log ε 3.90); 318 nm (log ε 3.60); 364 nm (log ε 3.79); 547 nm (log ε 2.52).

The infrared spectrum ν_{max}(KBr disc): 3320 (br); 1713 (C=O of $OCONH_2$); 1650 (C=O of quinone); 1600; 1548; 1325; 1210 cm^{-1}. This preparation follows the general procedure of reference 20d.

Acid Catalyzed Opening of the Aziridine Ring of Mitomycin C. Preparation of 43

Hydrolysis of mitomycin C was carried out according to the procedure of Stevens and coworkers[20a] using dilute hydrochloric acid. The crude hydrolysate was recrystal-

lized from dimethylformamide : water to give 17% yield of 42 as dark purple crystals.

The absorption spectrum λ_{max} (CH_3OH): 248 nm (log ε 3.95); 309 nm (log ε 3.78); 343 nm (sh) (log ε 3.32); 562 nm (log ε 2.93).

The infrared spectrum ν_{max} (KBr disc): 3390 (br); 1695 (C=O of $OCONH_2$); 1650 (C=O of quinone); 1594; 1376 cm^{-1}.

General Procedure for Determination of Cross-Linking of DNA with Reduced Mitomycin C Derivatives

Experiments were performed as described above for mitomycin C. Reaction solutions had concentrations of approximately 1.01 A_{260} of λ DNA, 0.05 M phosphate buffer, pH 7.0, 4.3×10^{-4} M of mitomycin derivative, and 5.3×10^{-3} M sodium borohydride. 15 µl aliquots were analyzed for extent of cross-linking by the fluorometric assay. Control reaction mixtures run with each experiment and containing no mitomycin derivative showed no cross-linking.

Table 17

Cross-Linking of λ DNA by Reduced Mitomycin C Derivatives

Compound	Time (Min.)	Fluorescence Before Heat Denaturation	Fluorescence After Heat Denaturation	% Cross-Linking
42	5	40	34	85%
	10	40	34	85%
43	5	57	0	0%
	10	63	0	0%

General Procedure for the Determination of Interaction of Reduced Mitomycin C Derivatives with PM2 CCC-DNA

Experiments were performed as described above for mitomycin C. Reaction solutions had concentrations of approximately 1.00 A_{260} of PM2 CCC-DNA, 0.05 M phosphate buffer, pH 7.0, 3.2×10^{-4} M mitomycin C derivative, and 5.3×10^{-3} M sodium borohydride. Additional experiments also contained 0.25 M isopropyl alcohol. 15 µl samples were analyzed by the ethidium assay. Control experiments lacking mitomycin derivative showed a 55-60% return of fluorescence after heating (identical to DNA alone) indicating this sample of PM2 DNA was nicked to the extent of about 40%.

The following experiments were carried out by Dr. A.R. Morgan and D. Johnson in the Department of Biochemistry at the University of Alberta..

Purification and Fluorometric Assay of S_1-Endonuclease

The S_1-endonuclease was purified by the method of Vogt[66] with the omission of the SP-Sephadex C-50 chromatography, the final step being Sephadex G-100 superfine chromatography in a buffer containing 30 mM sodium acetate pH 4.5, 10 mM sodium chloride, 30 mM zinc sulfate, and 10% aqueous glycerol. The G-100 fraction on SDS polyacrylamide gel electrophoresis[67] gave one major protein band and one minor band and was essentially inactive on

duplex DNA. The standard S_1-endonuclease reaction contained 30 mM sodium acetate pH 4.5, 50 mM NaCl, 1 mM $ZnSO_4$, $2A_{260}$ of heat denatured calf thymus DNA with incubation at 45°C. 20 µl samples of the reaction were added to 2 ml of ethidium bromide assay solution at pH 8, (5 mM Tris HCl pH 8, 0.5 mM EDTA and 0.5 µg/ml ethidium bromide). Under these conditions denatured DNA exists with about 50% of its structure being in short intramolecular duplex regions.[53] On a nucleotide residue basis, the fluorescence enhancement is 50% that of native DNA, and this is lost on degradation with S_1-endonuclease. Therefore in calculations on the extent of cross-linked DNA using the S_1 assay, the DNA resistant to S_1 is taken to have twice the fluorescence enhancement per nucleotide residue of that which is degraded. Due to the slight activity of the S_I-endonuclease preparation on duplex DNA the kinetics of degradation were always followed. After the initial very rapid degradation of denatured DNA, the cross-linked DNA was very slowly degraded.

E. coli DNA which had been covalently cross-linked with reduced mitomycin C was dialyzed overnight at 4° in 10 mM potassium phosphate pH 11.5, 0.1 mM EDTA, neutralized with 1.0 M Tris HCl pH 8 (final concentration 25 mM) and heat denatured (5 minutes at 95°). To 80 µl were added 20 µl 5X S_1 assay pH 4.3 (final pH about 4.6). After removal of the first 10 µl sample, 1.5 units of puri-

fied S_1-[illegible]nuclease were added and the mixture incubated at 45°. 10 µl samples were added to 2 ml of the ethidium bromide pH 8 solution and read as described previously. Heat denatured and native *E. coli* DNA were incubated as controls.

Assay for Depurination or Depyrimidation of Polynucleotides Treated with Reduced Mitomycin C

Poly dG·dC·(^{14}C G)·(^{3}H C) was incubated at 22° in 0.05 M sodium acetate buffer pH 4.5 with increasing concentrations of mitomycin C ($0.6 - 3.0 \times 10^{-4}$ M) and with a constant molar ratio of sodium borohydride ($NaBH_4$/mitomycin C = 10:1). After 10 minutes, duplicate samples were removed, placed on Whatman filter discs, washed 3 times with 5% trichloroacetic acid, then two times with ethanol, dried and counted.

Under these conditions *E. coli* DNA showed a direct relationship between the magnitude of the loss of fluorescence and the concentration of mitomycin C.

Binding of Ethidium Bromide to DNA Covalently Cross-Linked and Monoalkylated with Mitomycin C

DNA samples which had been treated with reduced mitomycin C were dialyzed against 10 mM potassium phosphate, pH 11.7, 0.1 mM EDTA. The % of cross-linking was determined by ethidium fluorescence before and after dialysis. The absorbance of the dialyzed DNA was measured at 260 nm

on a Gilford 2400 spectrophotometer. Increasing amounts of this DNA were added to an ethidium bromide solution, pH 8.0 and the fluorescence per A_{260} added was calculated *versus* a known amount of DNA as standard.

The following work was carried out by Dr. J.A. Plambeck and Dr. G.M. Rao in the Department of Chemistry at the University of Alberta.

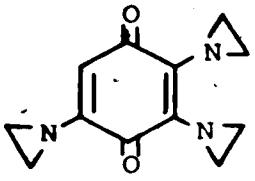
Electroanalytical Procedures

A stock solution of 1.4×10^{-3} M mitomycin C was prepared. Acetate (pH < 5.9), phosphate (5.9 < pH < 8.1) and tris (pH > 8.1) buffers of 0.133 M were prepared in 0.133 M potassium chloride solution. The samples were prepared in the cell to give 3.4×10^{-4} M of mitomycin C in 0.1 M buffer and also 0.1 M/KCl. The mitomycin C derivatives were treated in a similar manner. The solutions used for cyclic voltammetry were buffered at pH 6.98 ± 0.02 with phosphate. All solutions were deaerated with purified nitrogen gas for 10 minutes and blanketed with it during each run. The polarographic drop time was controlled at 1.0 second and the mercury flow rate was 0.93 mg/sec. All potentials are quoted relative to the saturated calomel electrode. The reversibility of the polarographic electrode reaction was determined from conventional log $[(i_d - i)/i]$ plots.

CHAPTER IV

STUDIES OF BENZOQUINONES RELATED TO MITOMYCIN C

A great many benzoquinone derivatives have been shown to be good antibiotics and antineoplastic agents.[37] For the most part, their mode of action has been proposed to involve covalent alkylation and cross-linking of DNA and the most effective compounds are usually bifunctional. In addition to the quinone ring system many of these compounds, including the clinically important antitumor agents, trenimon 22,[68] 2,5-bis(1-aziridinyl)-3,6-dipropoxy-1,4-benzoquinone 46a, and 2,5-bis(1-aziridinyl)-3,6-bis(2-methoxyethoxy)-1,4-benzoquinone 46b,[69,70] have other features in common with the mitomycins, such as aziridine or carbamate groups. Consequently, we decided to prepare a



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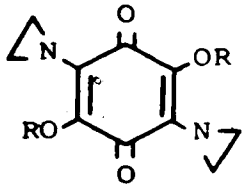
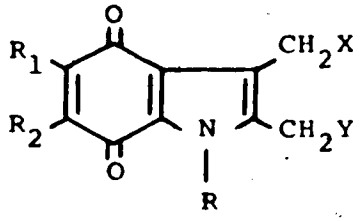


46 a R=nC_3H_7
b R=$CH_2CH_2OCH_3$

series of benzoquinones containing the aziridine group and to investigate their interaction with DNA by the convenient ethidium fluorescence assay in order to investigate any

possible correlation between cross-linking ability and antitumor activity. In addition, we hoped to obtain information about the reactivity of the aziridine group in relation to the reactivity of this group in mitomycin C.

It has been proposed that the essential portions of the mitomycin structure can be represented by 23.[20d] Charge delocalization of the corresponding hydroquinone



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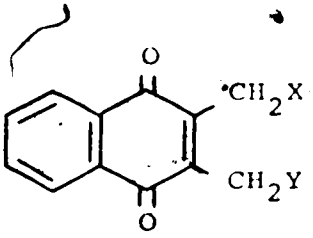
can then result in *o*-quinone methide-like intermediates as illustrated in Scheme 9. Sartorelli and coworkers prepared a series of substituted naphthoquinones 24 and *p*-benzoquinones 25 as potential bioreductive alkylating



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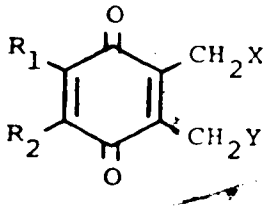

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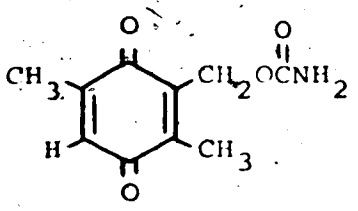
agents since on reduction these compounds are capable of forming quinone methides.[28,35] These compounds were tested for antitumor activity and many were found to show significant activity.

We undertook to prepare a series of *p*-benzoquinones having a carbamate as the leaving group (47-50) in order



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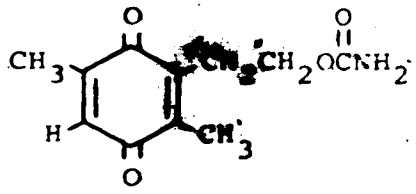

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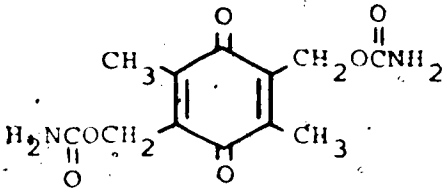

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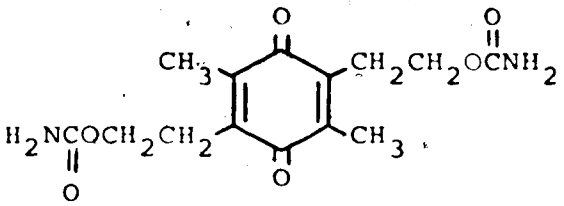
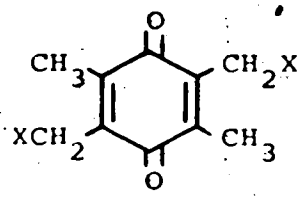


50

to obtain information about the alkylating ability of the carbamate group in mitomycin C. The effectiveness of these compounds in alkylating and cross-linking DNA was examined by the ethidium fluorescence assay.[36] By comparison of compounds 47 and 48 and compounds 49 and 50, it was hoped to

determine the influence of charge delocalization of the corresponding hydroquinones on the alkylating ability of such compounds. Arrangements were made to have these compounds tested for antitumor activity so that possible correlations between cross-linking and activity could be examined.

A series of 2,5-disubstituted *p*-benzoquinones 51 was also prepared. Their ability to alkylate and cross-link DNA was examined and compared with the series of cor-



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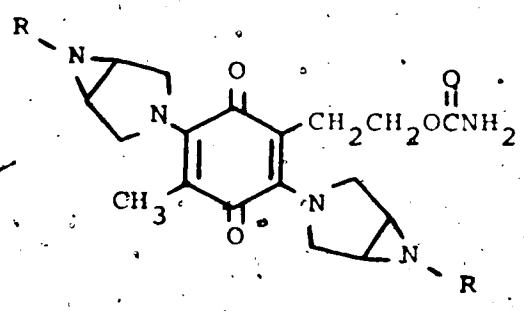
responding 2,3-disubstituted *p*-benzoquinones prepared by Sartorelli.[28a,35d]

Studies of Mitomycin C Analogs

Synthesis of Analogs of Mitomycin C

Because of the relatively short span (4.3 Å) between the alkylating sites of mitomycin C, the number of potential alkylating sites on DNA is limited. In order to provide greater conformational flexibility between the alkylating centres, we prepared a series of analogs in which the C-9, C-9a bond has been broken. This group of analogs 52 possesses what are regarded as the essential



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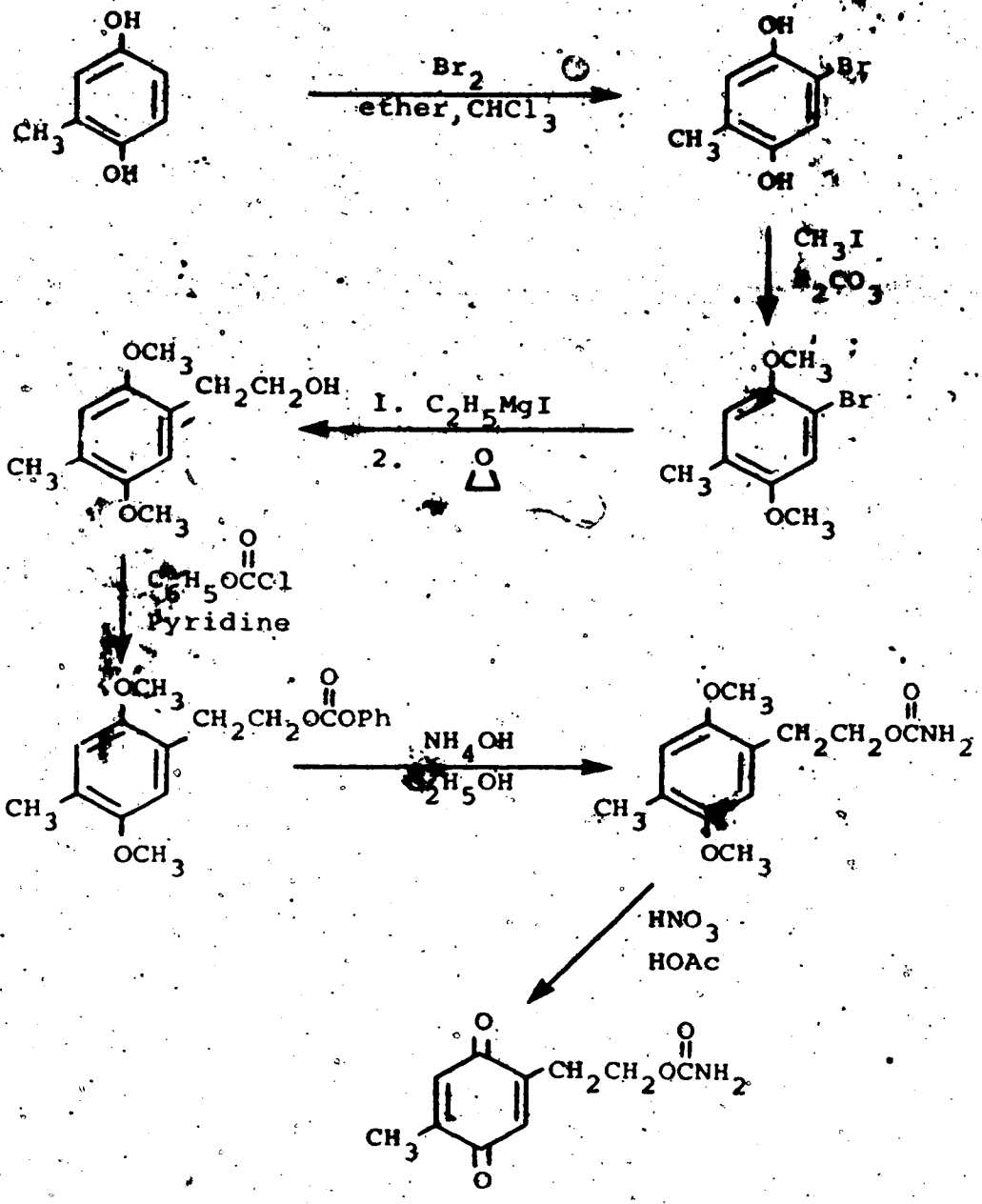
structural features necessary for physiological activity. Compounds 52 retain the reactive aziridine and carbamate groups which have been shown to alkylate DNA. The distance between the two potential aziridine alkylation centres is comparable to that of the clinically useful 2,5-diaziridinoquinones. In addition, it was hoped that the

greater conformational flexibility between the alkylating centres of 52 would compensate for the loss of conjugative interaction provided by the indole nucleus of mitomycin C. The quinone function was retained for two reasons: (i) so that the structure of the analogs would resemble mitomycin for possible intercalative properties and (ii) to determine if analogs of this type can degrade DNA as has been established for mitomycin C and streptonigrin.[30]

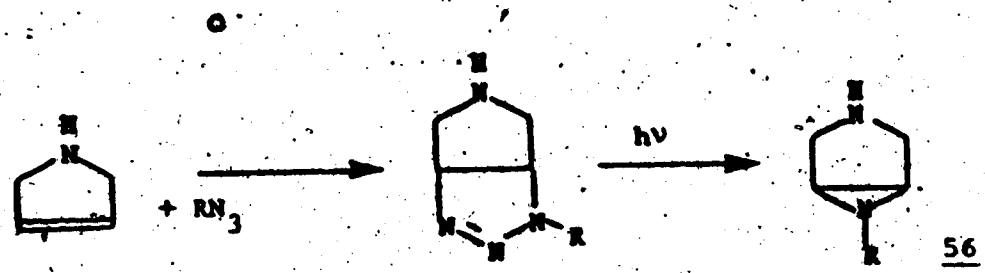
The mitomycin analogs were prepared by a converging scheme. Functionalized quinone having the required carbamate side-chain 53 was prepared by the following route due to Nakao and Arakawa[34a] (Scheme 11). The required bicyclic aziridines were prepared by 1,3-dipolar azide addition to 3-pyrroline 54 to give a triazoline 55 which was subsequently photolyzed (Scheme 12).[49] The mitomycin analogs were obtained by coupling the bicyclic aziridine to functionalized quinones using copper acetate as a catalyst (Scheme 13).[39]

Scheme 11


OH
CH3
OH
Br2
ether, CHCl3
Br
CH3I
K2CO3
OCH3
CH2CH2OH
I. C2H5MgI
2.
Br
C6H5OCCl
Pyridine
CH2CH2OCOPh
NH4OH
C2H5OH
CH2CH2OCNH2
HNO3
HOAc
O

53

Scheme 12



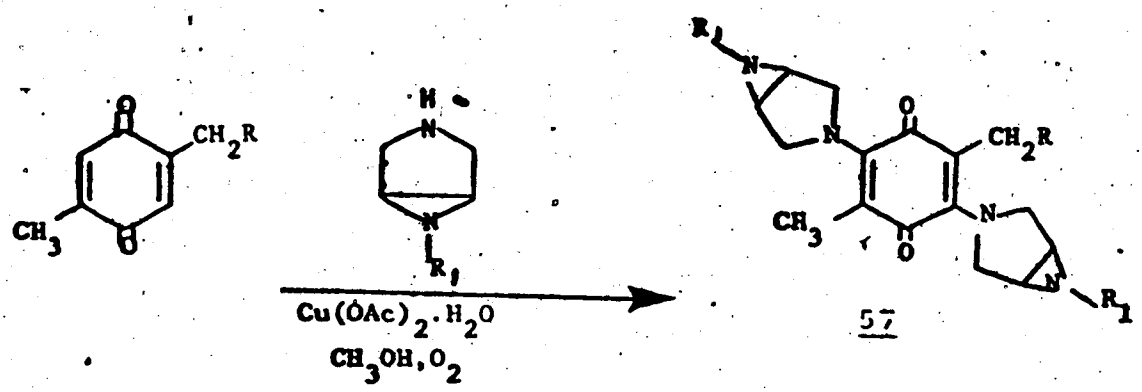
54 55 56

a R = $pBrC_6H_4$

b R = C_6H_5

c R = $pCH_3OC_6H_4$

Scheme 13



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a R = CH_2OCONH_2, R_1 = $pBrC_6H_4$

b R = CH_2OCONH_2, R_1 = C_6H_5

c R = CH_2OCONH_2, R_1 = $pCH_3OC_6H_4$

d R = H R_1 = $pBrC_6H_4$

Detection of Covalent Cross-Linking of DNA by Bicyclo-aziridinoquinones and Confirmation by S_1-Endonuclease

The synthetic analogs 57 were incubated in 20% aqueous pyridine with λ DNA in an acetate buffer at pH 4.5. The induction of covalent cross-links was detected by the ethidium fluorescence assay as summarized in Table 18. From the results it is evident that cross-linking is

Table 18

Induction of Covalent Cross-Links in λ DNA by Mitomycin Analogs

Compound	Concentration μg/μl	Maximum % Cross-Linking	Time to Reach Maximum
57d	2.5	78%	240 min.
57a	2.5	61%	180 min.
57b	2.5	38%	90 min.
57c	2.5	34%	240 min.

occurring at the two aziridine groups. The carbamate does not appear to be involved in the covalent binding to DNA. The span between the alkylating centres of 57d is *ca.* 10.1-10.8 Å as compared to *ca.* 4.3 Å in mitomycin C indicating that bifunctional alkylating agents of quite different dimensions may be accommodated by cross-linked DNA. An

electron-withdrawing group in R_1 seems to enhance the efficiency of covalent cross-linking.

The induction of CLC sequences with compound 57d was confirmed independently by the S_1-endonuclease assay described in Chapter III (Table 19). The lower values for

Table 19

Confirmation of Covalent Cross-Linking of DNA by Mitomycin Analogs by S_1-Endonuclease Assay

	Time (min.)			
	10	90	195	270
% Cross-Linking by Ethidium Fluorescence Assay	16%	20%	27%	25%
% Cross-Linking by S_1-Endonuclease Assay	9%	15%	19%	16%

covalent cross-linking are due to the use of *E. coli* DNA of much lower molecular weight (14.8×10^6) than that of the λ DNA (31×10^6). The two assays show a satisfactory correlation.

No loss of fluorescence due to alkylation of the DNA was observed, reflecting the lower alkylating ability of the analogs as compared to mitomycin C. On incubating 57d under reducing conditions with PM2 CCC-DNA, no single strand cleavage was observed.

The four mitomycin analogs 57a-d were tested for antitumor activity against Sarcoma 180 ascites cells.

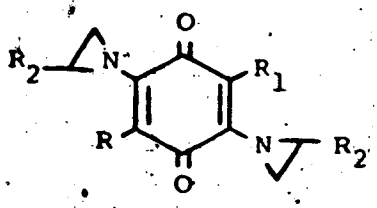
in mice by Professor A.C. Sartorelli at the School of Medicine of Yale University. All of them showed little or no activity against this neoplasm.

Studies on Aziridinoquinones

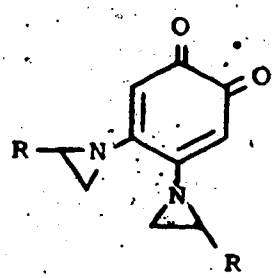
Mode of Cytotoxic Action of Aziridinoquinones

Many aziridinoquinones are active antitumor agents. Although the mechanism by which these compounds exert their activity is not known with any certainty, suggestions that have been made include: (i) alkylation and cross-linking of DNA[71] (ii) production of hydrogen peroxide and other oxidizing species by intracellular redox reactions of the quinones.[72] Efforts have been made, without much success, to correlate antitumor activity with magnetic susceptibility and electron delocalization[73] and with partition coefficients between benzene and aqueous phosphate buffer solutions.[74]

We have prepared aziridinylbenzoquinones of the general types 58 and 59 and investigated their interaction



58



59

with DNA by the ethidium fluorescence assay. Most of the compounds were prepared by published procedures.[34,69,70] Covalent cross-linking and alkylation of λ DNA by compounds of this type in the concentration range 0.01-0.4 μg/μl was established by the fluorescence assay as is illustrated in Figure 23 and Table 22 where agents are listed in order of decreasing cross-linking ability. Independent confirmation that this assay detects CLC sequenced DNA for this group of bifunctional alkylating agents was again obtained by the S_1-endonuclease assay as summarized in Table 20.

Table 20

Confirmation of Covalent Cross-Linking of DNA by Aziridinoquinone 58* with the S_1-Endonuclease Assay

	Time (min.)		
	0	45	135
% Cross-Linking by Ethidium Fluorescence Assay	3.4%	42.5%	63.3%
% Cross-Linking by S_1-Endonuclease Assay	9.7%	26.7%	42.0%

* $R = R_1 = CH_3$; $R_2 = H$

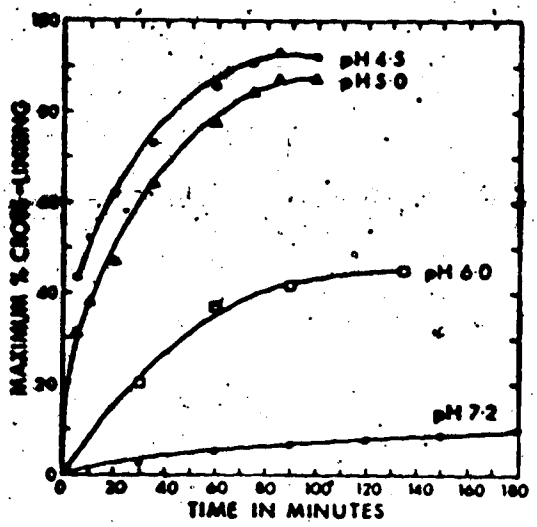


Figure 23. pH dependence of covalent cross-linking of λ DNA by 2,5-bis(aziridinyl)-3,6-dimethoxy-1,4-benzoquinone at a final concentration of 0.05 µg/µl. Reactions were carried out in approximately 0.05 M buffered aqueous solutions at 37° with a final DNA concentration of 1.40 A_{260}.

pH Dependence of Covalent Cross-Linking and Alkylation of DNA by Aziridinoquinones

As was observed with mitomycin C, the detection of aziridinoquinone induced covalent cross-linking of DNA was often accompanied by a suppression of the ethidium fluorescence. By analogy with the results obtained with mitomycin C, it seemed likely that this phenomenon could be attributed to alkylation of the DNA with a resulting loss of potential intercalation sites for ethidium. An experiment similar to that described for mitomycin C was carried out to show that the loss of fluorescence was not due to depurination or depyrimidation or to large scale degradation of the DNA.

A synthetic polynucleotide containing selective radioactive labels in the purine and pyrimidine bases was treated with an aziridinoquinone to the stage where in a control experiment an appreciable loss of fluorescence was observed. The alkylated DNA was washed with trichloroacetic acid and counted. The results in Table 21 confirm that the loss of fluorescence is not due to loss of bases or large scale degradation of the DNA. Experiments designed to correlate loss of fluorescence with binding ratios, as was done for mitomycin C, are now underway in our laboratories.

The pH dependence of covalent alkylation and cross-

Table 21

Radioactivity Assay for Alkylation of Polynucleotides with Aziridinoquinone 58*

Time (hr.)	^{3}H c.p.m.	^{14}C c.p.m.	$^{3}H/^{14}C$ Ratio
0	2285	3195	0.713
2	2252	3500	0.646
18	2520	4075	0.618
46	2850	4476	0.638
65	3342	4713	0.708

* $R = R_1 = CH_3$; $R_2 = H$

linking by aziridinoquinones was studied. Figures 23 and 24 reveal that these quinones show a much more pronounced pH dependence than reduced mitomycin C. This difference reflects the structural differences of the two alkylating agents. The aziridine moiety of activated mitomycin C receives assistance to opening by conjugative interaction of the indole nitrogen lone pair at any pH. On the other hand, aziridinoquinones receive no such activation and require protonation to assist the aziridine ring opening. Careful controls were run with λ DNA at the corresponding pH but in the absence of the quinone. Over the time scale of these experiments no acid induced cross-linking was detected.

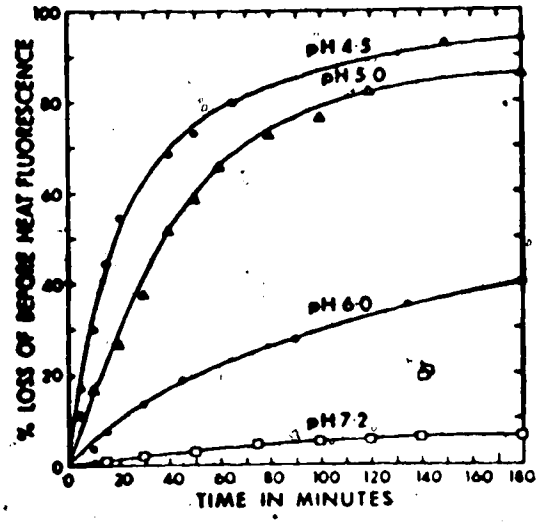


Figure 24. pH dependence of alkylation of λ DNA by 2,5-bis(aziridinyl)-3,6-dimethoxy-1,4-benzoquinone at a final concentration of 0.8 μg/μl. Reactions were performed in 0.05 M buffered aqueous solutions at 37° with a final DNA concentration of 1.40 A_{260}.

Dependence of Extent of Covalent Cross-Linking of DNAs by Aziridinoquinones on (G+C) Content of the DNA

No evidence has yet been presented for any base preference in the covalent cross-linking of DNA by aziridinoquinones. Therefore the interaction of 2,5-bis(aziridinyl)-3,6-dimethoxy-1,4-benzoquinone (58, R = R_1 = OCH_3, R_2 = H) with three different natural DNAs of different (G+C) content, *C. perfringens* (30%), calf thymus (40%), and *E. coli* (50%) was examined. Due to slight differences in the average molecular weights of the DNAs, as determined by sedimentation velocities, the cross-linking efficiencies as determined by the fluorescence assay are not strictly comparable. The average number of cross-links per molecule (m) was estimated from $m = \ln (1/P_o)$ as was done for mitomycin C. The values obtained, 0.62, 0.93, and 1.71 for the three DNAs are comparable to similar estimates for mitomycin C.[18] Assuming an average molecular weight of 300 - 330 for each nucleotide, the average number of cross-links per nucleotide was calculated to make the results comparable for the three DNAs. It is clear from Figure 25 that the extent of covalent cross-linking increases with increasing (G+C) content and as in the case of mitomycin C this probably indicates a preference for attack on guanine.

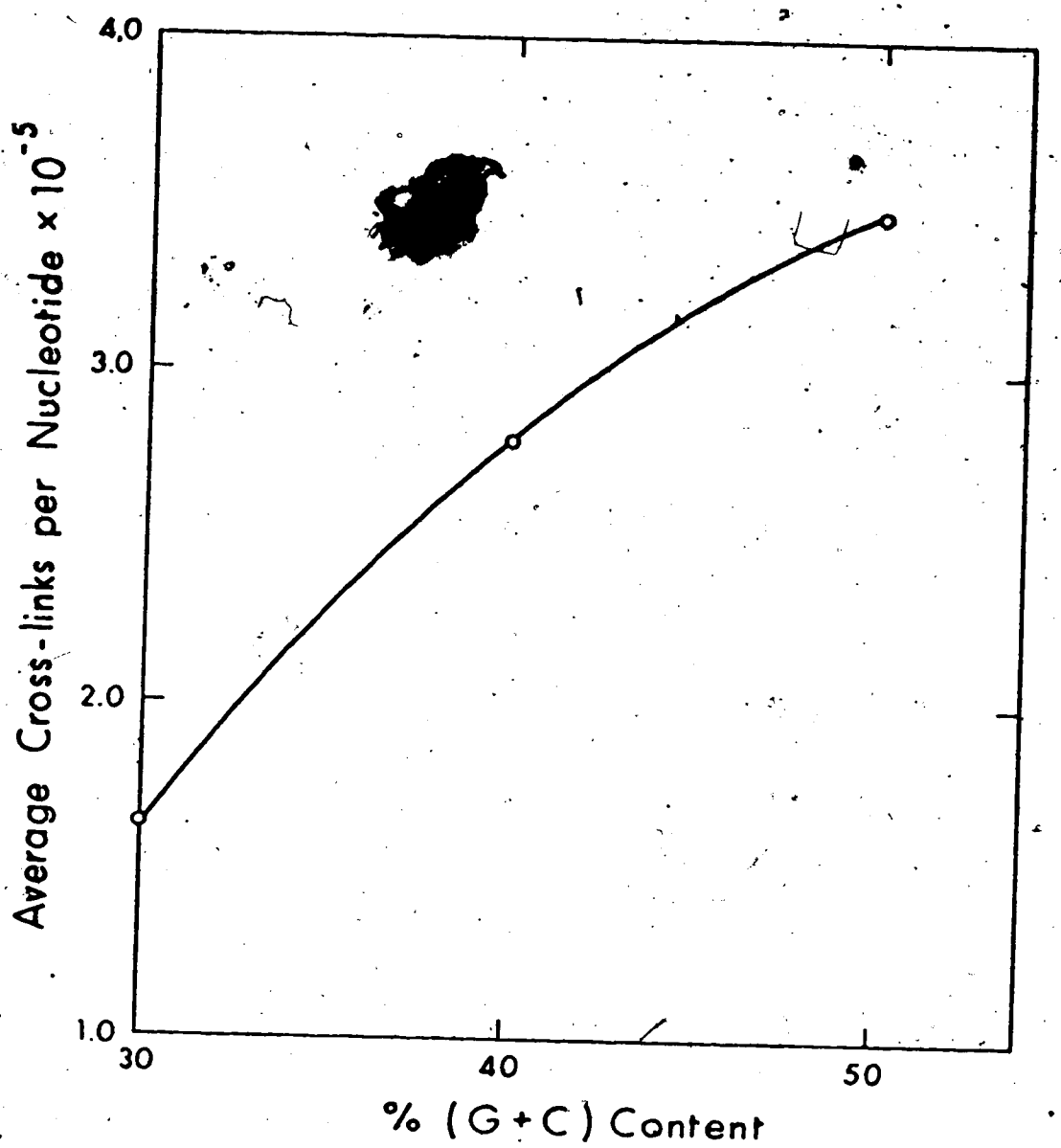


Figure 25. Dependence of efficiency of covalent cross-linking by 2,5-bis(aziridinyl)-3,6-dimethoxy-1,4-benzoquinone on the (G+C) content of the DNA. Reactions were carried out at 37° and contained DNA at approximately 1.2 A_{260}, 0.05 M acetate buffer pH 4.5, and 0.05 µg/µl quinone in 20% aqueous tetrahydrofuran.

Correlation of Structure, Covalent Cross-Linking and Antitumor Activity

It may be seen from Table 22 that for 1,4-benzoquinones, alkoxy substituents appear to enhance both antineoplastic activity and cross-linking efficiency while chloro substituents suppress both these phenomena. A carbamate group, unless activated as in reduced mitomycin C, does not contribute to increased cross-linking. Alkyl substituents on the aziridines decrease the cross-linking efficiency. 1,2-Benzoquinones show both efficient cross-linking of DNA and good antitumor activity.

In general, a fairly good correlation exists between the extent and rate of covalent cross-linking of DNA and antineoplastic activity against Leukemia L1210 and several solid tumors. It is possible that this parameter may prove useful for the pre-screening of antitumor agents, recognizing of course that other biological and pharmacological parameters, in addition to covalent cross-linking, (*i.e.* drug uptake, partition, metabolism, and toxicity) contribute to the ultimate effectiveness of the cancer inhibitory properties.

TABLE 22

Correlation of Covalent Cross-Linking of λ-DNA by Bifunctional Alkylating Agents with Antitumor Activity Against Various Tumors[a]

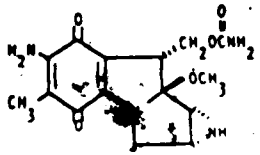
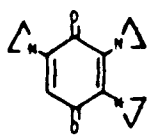
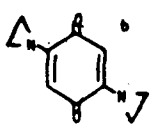
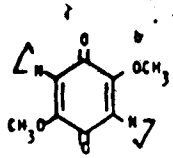
Structure	L1210[d,e]		M-366[e]		CA-755[e]		S-180[f]		KB[e]	Cross-Linking of λ-DNA		
	OD	ILS	OD	ILS	OD	TWI	OD	ILS	ED50	Conc μg/μl	Maximum %	Time to Reach Maximum (Min)
	2.0	85[g]	1.0	95	1.0	90	0.8	259	0.04	0.04 0.02	100 [illegible]	5 5
	0.15	114[g]	0.1	96	0.04	70	-	-	<0.01	0.4 0.04	100 100	2 10
	0.5	26	-	-	0.25	60	-	-	<1.0	0.01	92	10
	1.7	90[g]	2.0	95	-	-	0.4	159	-	0.4 0.04	92 82	60 120

TABLE 22 (continued)

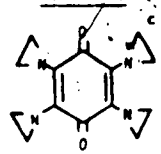
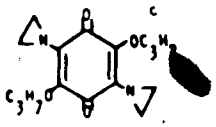
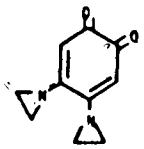
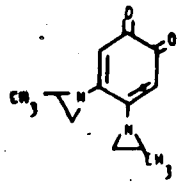
Structure	L1210		W-256		CA-755		S-180		KB	Cross-Linking of k-DNA		
	OD	ILS	OD	ILS	OD	TWI	OD	ILS	ED50	Conc µg/µl	Maximum %	Time to Reach Maximum (Min)
	0.01	39	-	-	0.05	80	0.1	155	4.9	0.4 0.04	90 85	25 100
	4.0	50	1.0	95	3.0	78	2.5	202	0.07	0.4 0.04	85.5 79	30 60
	-	-	-	-	-	-	0.1	>202	-	0.4 0.04	85.5 80	30 105
	-	-	-	-	-	-	5.0	221	-	0.4 0.04	78.4 32.3	120 45

TABLE 22 (continued)

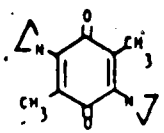
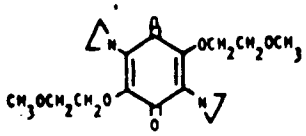
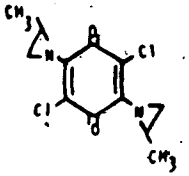
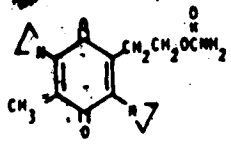
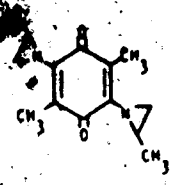
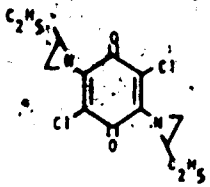
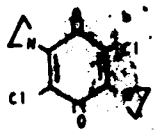
Structure	L1210 OD	L1210 ILS	M-256 OD	M-256 ILS	CA-755 OD	CA-755 TVI	S-180 OD	S-180 ILS	KB ED50	Cross-Linking of λ-DNA: Conc μg/ml	Maximum %	Time to Reach Maximum (Min)
	0.96	107	-	-	-	-	0.4	169	-	0.4	59	60
	2.0	70	0.98	84	2.0	80	-	-	-	0.4	55	255
	-	-	-	-	-	-	7.5	177	-	0.4	54	360
	0.5	140	-	-	-	-	-	-	-	0.4	38	30

TABLE 22 (continued)

Structure	L1210		W-256		CA-755		S-180		RS	Cross-Linking of DNA		
	OO	ILS	OO	ILS	OO	TVI	OO	ILS	ED50	Conc μg/ml	Maximum %	Time to Reach Maximum (min)
	10	40	-	-	-	-	5.0	206	-	0.4	26	60
	-	-	-	-	-	-	7.5	130	-	0.24	24	270
	11	0	-	-	11	90	-	-	-	0.4	18	120

a. OO = Optimal dose, mg/kg/day. ILS = Percentage increased life span of test animals. TVI = Percentage tumor weight inhibition. ED50 = Effective dose for 50% survival, μg/ml.

b. Shows antibacterial activity against *Staph. aureus*, *E. coli*, and *Strept. faecalis*

c. Shows marked carcinostatic activity against Ianocone Sarcoma

d. L1210 = Leukemia 1210. W-256 = Walker carcinoma 256. CA-755 = Carcinoma 755. S-180 = Sarcoma 180 ascites

e. Reference 75 except where noted.

f. Reference 76.

g. Reference 34.

Studies on Carbamate Quinones

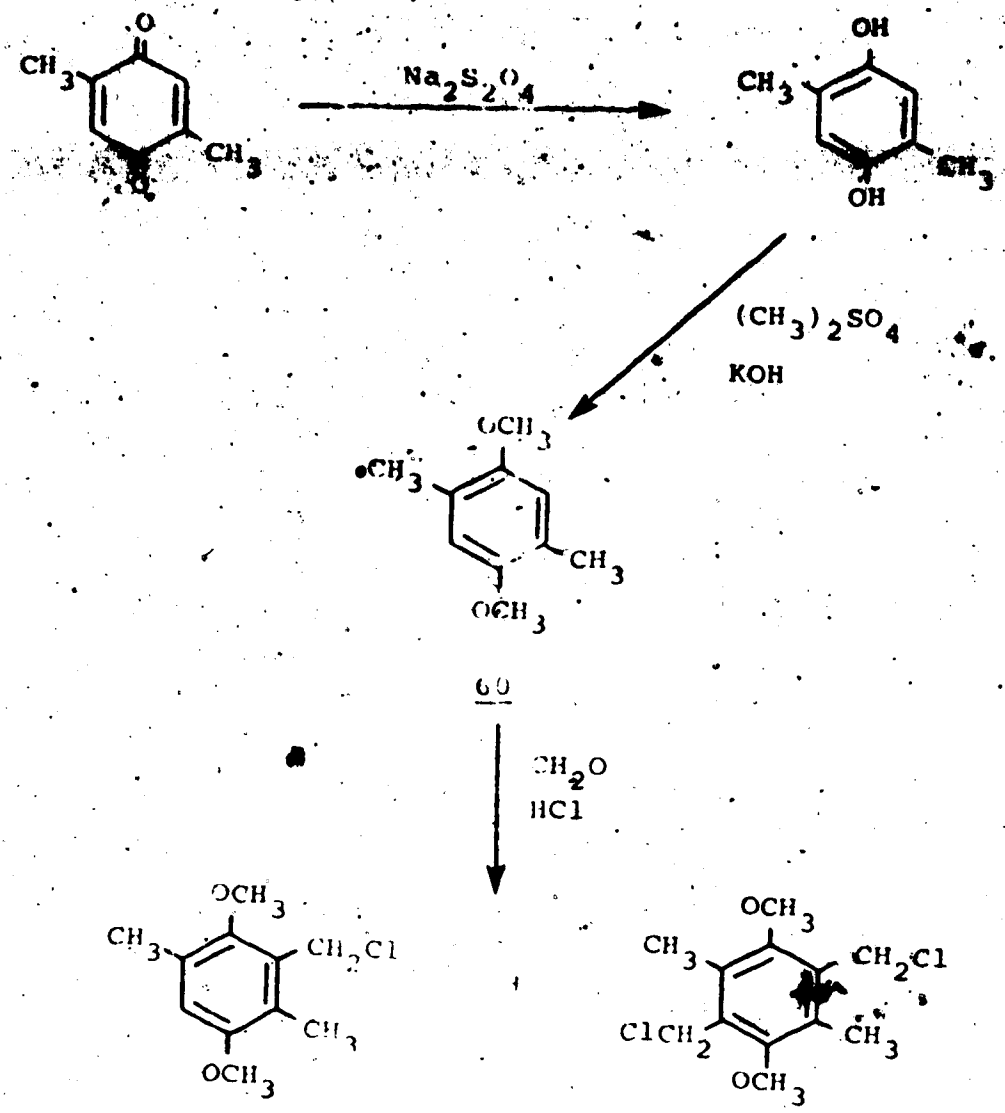
Preparation of Carbamate Quinones

A series of carbamate quinones (47, 48, and 50) was prepared in order to study the effectiveness of the carbamate group in alkylation reactions. These compounds, which are capable of forming methide-like intermediates, are similar to the bioreductive alkylating agents prepared by Sartorelli[28,35] and are also of interest as potentially active antitumor agents. All attempts to prepare the fourth compound in this series 49 were unsuccessful.

The synthetic routes for the preparation of all four carbamate quinones began with a common starting material, 1,4-dimethoxy-2,5-dimethylbenzene 60, which was prepared by a known route as shown in Scheme 14.[77] Chloromethylation of the dimethyl ether gave a mixture of monochloromethyl compound 61 and dichloromethyl compound 62. The relative yields of the two products could be varied by changing the reaction time and also by changing the amount and form of the formaldehyde used. The use of paraformaldehyde seemed to favor monosubstitution while the use of 40% formalin resulted in more dichloromethyl compound being formed. The two products were easily separable by fractional crystallization.

The reaction sequence for the preparation of the monosubstituted carbamate quinone 47 made use of the mono-

Scheme 14


O
CH3
CH3
Na2S2O4
OH
CH3
CH3
OH
(CH3)2SO4
KOH
OCH3
CH3
CH3
OCH3
60
CH2O
HCl
OCH3
CH3
CH2Cl
CH3
OCH3
61
OCH3
CH3
CH2Cl
ClCH2
CH3
OCH3
62

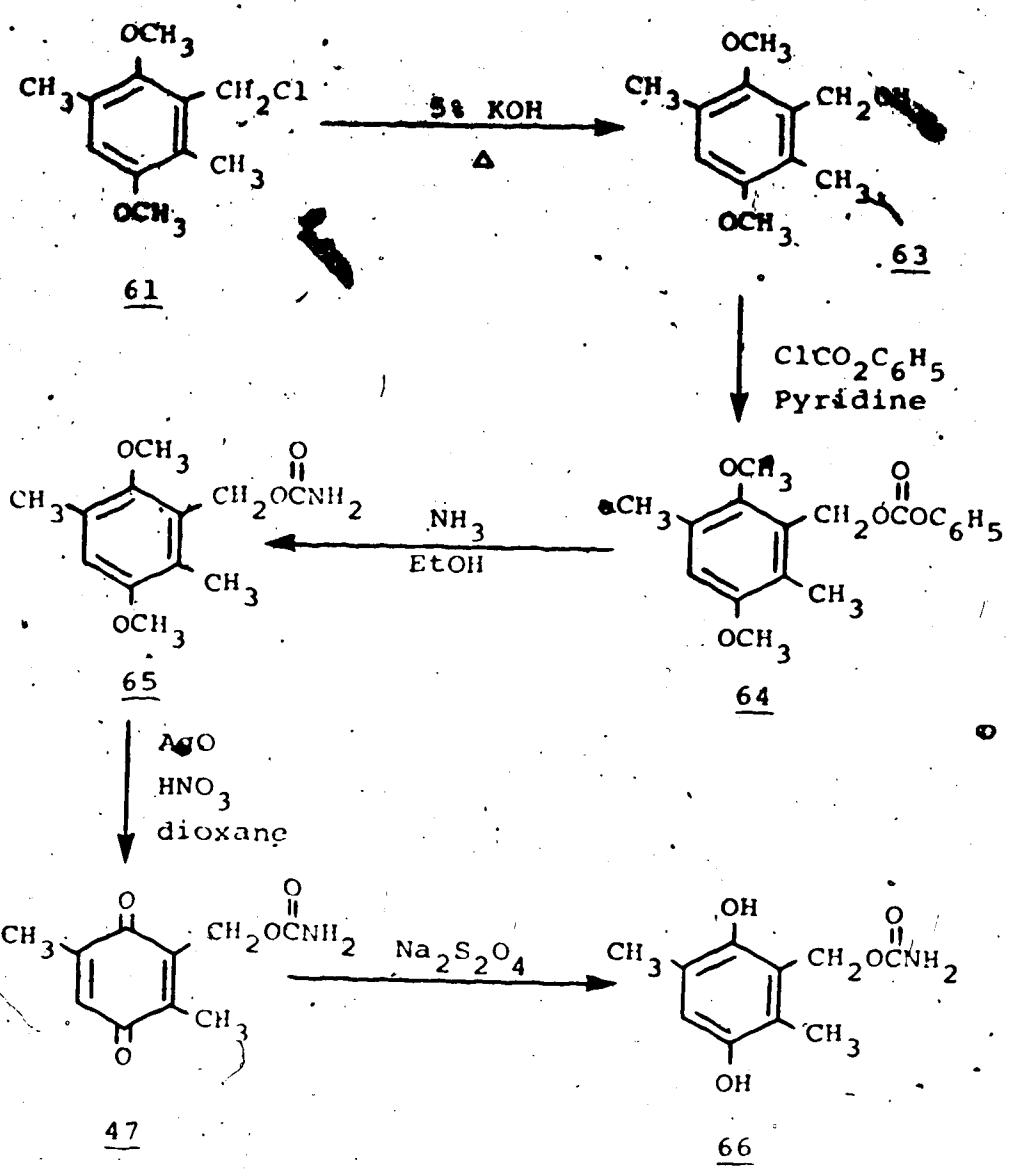
chloromethylbenzene derivative 61 (Scheme 15). Alkaline hydrolysis gave the alcohol 63. The carbamate side chain was introduced *via* the phenyl carbonate ester 64.[28a] Oxidation with silver oxide and nitric acid gave the quinone 47. The corresponding hydroquinone 66 was prepared by reduction with sodium dithionite.

The homologous monocarbamate quinone 48 was prepared as illustrated in Scheme 16. The side chain was extended by reaction with potassium cyanide followed by acid hydrolysis and reduction to the alcohol 70. The yield of the hydrolysis step was reduced by the formation of the lactone 69, probably by acid catalyzed displacement of the methoxy group. Introduction of the carbamate and oxidation of the dimethyl ether gave the quinone 48 in poor yield.

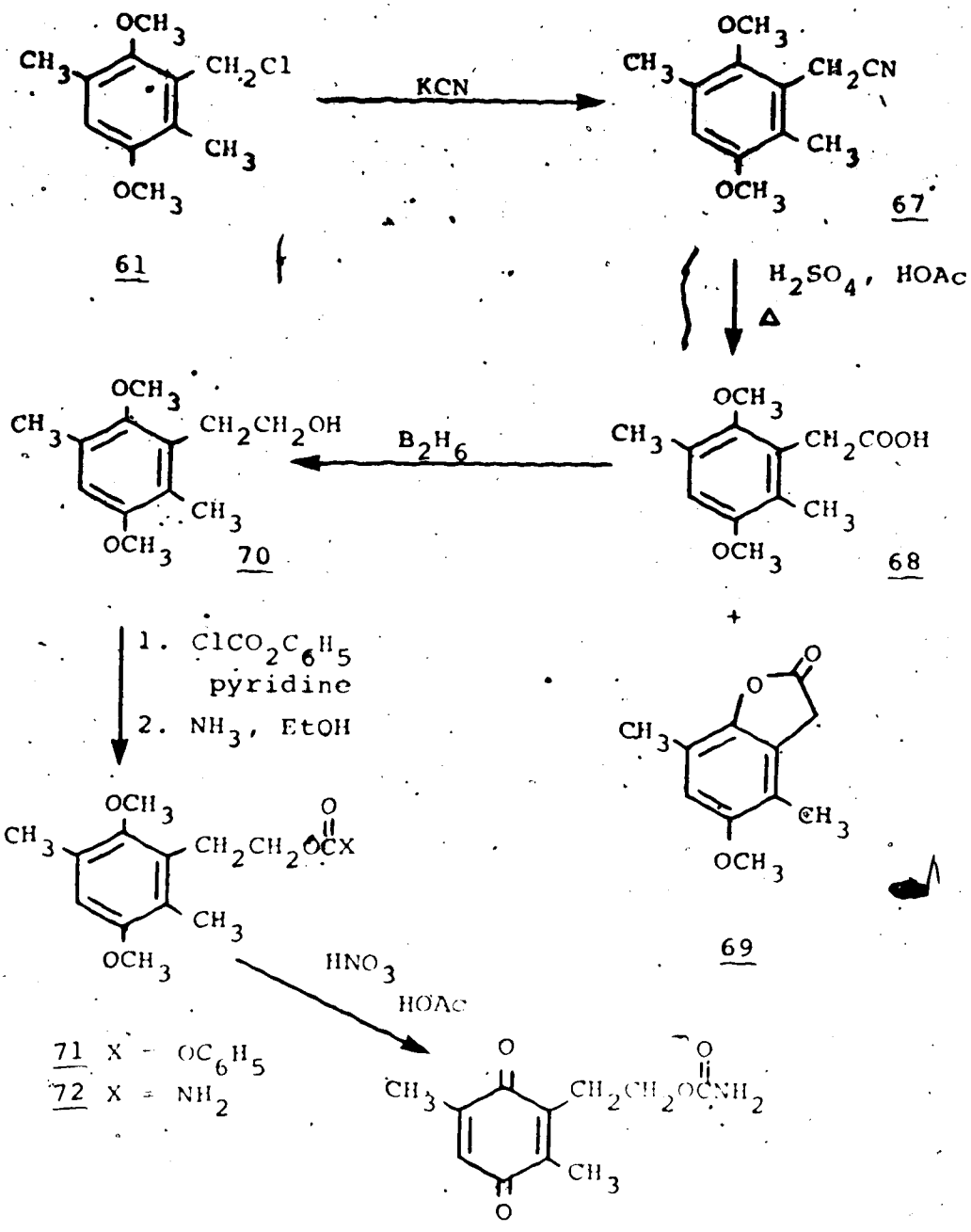
The synthetic scheme for the preparation of the dicarbamate quinone 50 is shown in Scheme 17. Stepwise reduction of the diacid 74 *via* the ester 75 was investigated but was found to have no advantage over the direct reduction with diborane. The dicarbamate quinone 50 was reduced to the corresponding hydroquinone 79 with sodium dithionite.

The synthetic scheme for preparing the dicarbamate quinone 49 (Scheme 18) proceeded smoothly to give the dimethyl ether 82. However, oxidation by silver oxide and nitric acid or nitric acid and acetic acid under various conditions was unsuccessful.

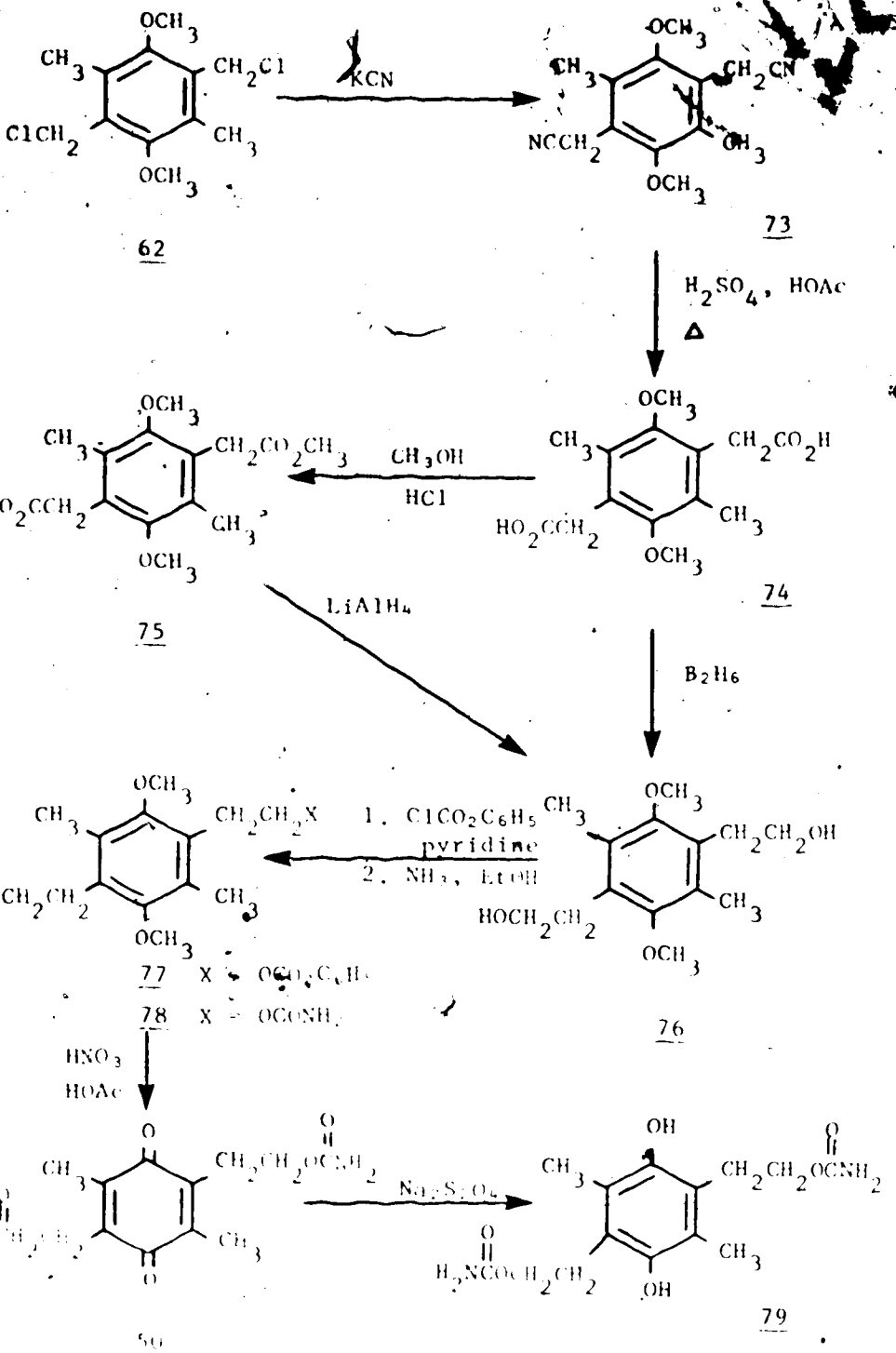
Scheme 15


OCH3
CH3
CH2Cl
CH3
OCH3
5% KOH
Δ
CH2OH
61
63
ClCO2C6H5
Pyridine
CH2OCOC6H5
64
NH3
EtOH
CH2OCNH2
65
AgO
HNO3
dioxane
47
Na2S2O4
OH
66

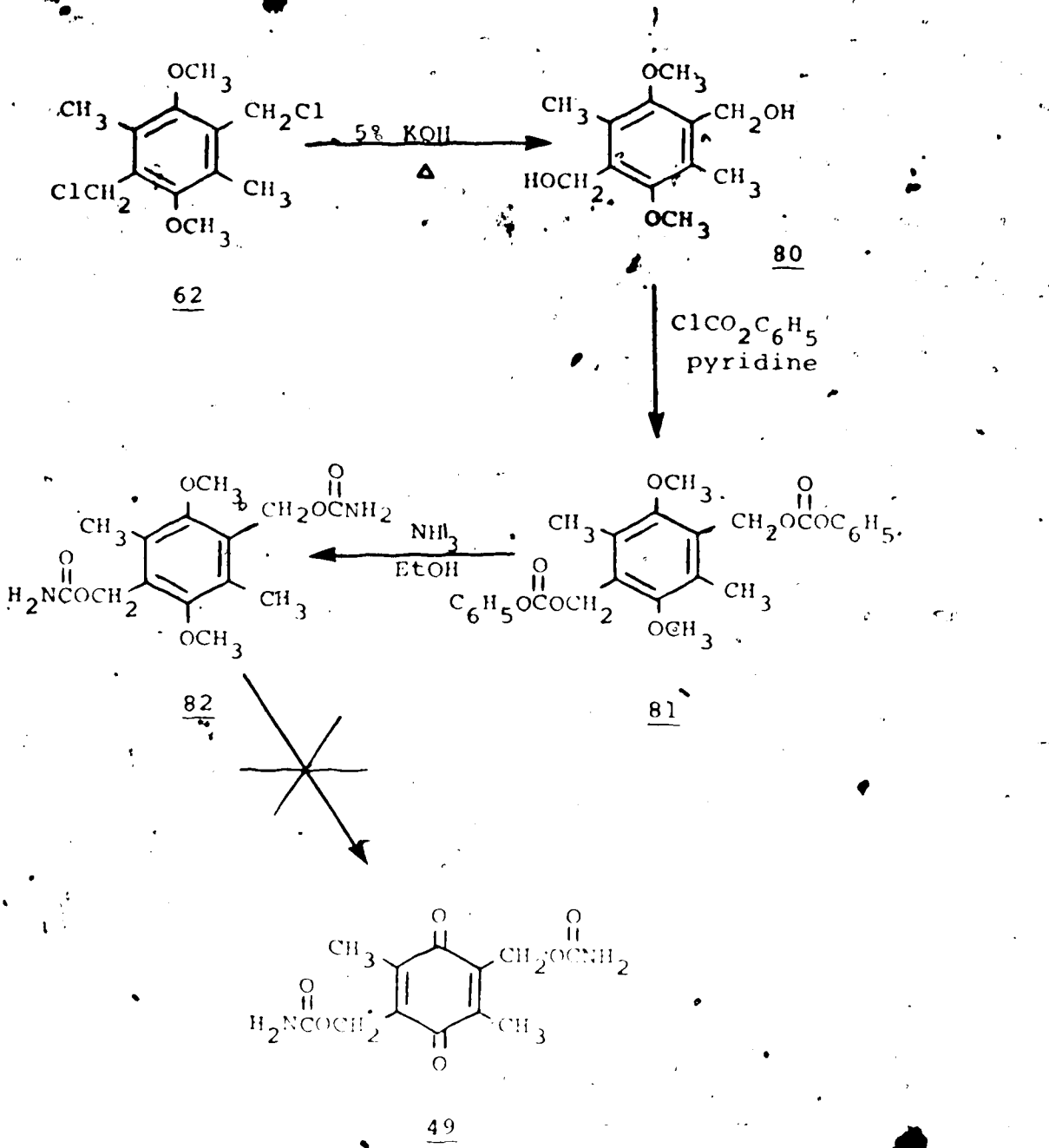
Scheme 16


OCH3
CH3
CH2Cl
CH3
OCH3
61
KCN
CH2CN
67
H2SO4, HOAc
Δ
CH2COOH
68
B2H6
CH2CH2OH
70
1. ClCO2C6H5
pyridine
2. NH3, EtOH
O
CH2CH2OCX
71 X = OC6H5
72 X = NH2
69
HNO3
HOAc
CH2CH2OCNH2
48

Scheme 17


62
KCN
73
H_2SO_4, HOAc
Δ
74
CH_3OH
HCl
75
$LiAlH_4$
B_2H_6
1. $ClCO_2C_6H_5$
pyridine
2. NH_3, EtOH
77 X = $OCO_2C_6H_5$
78 X = $OCONH_2$
76
HNO_3
HOAc
$Na_2S_2O_4$
79

Scheme 18



62

80

81

82

49

Study of the Interaction of Carbamate Quinones with DNA

The interaction of the carbamate quinones 47, 48, and 50 with DNA was examined by means of the ethidium fluorescence assays. Incubation of 25% aqueous dimethyl sulfoxide solutions of the quinones, both unreduced and reduced *in situ* with sodium borohydride, with λ DNA at 37°, pH 7.0, produced no detectable alkylation or cross-linking. The hydroquinones 66 and 79 gave the same result. The interaction of quinones 47 and 50, reduced *in situ* with sodium borohydride, and hydroquinones 66 and 79 with λ DNA at pH 5.0 and 8.7 was also examined. Again no alkylation or cross-linking could be detected. The interaction of carbamate quinones with CCC-DNA was examined. Quinone 47, reduced *in situ* with sodium borohydride, and hydroquinone 66 were incubated with PM2 CCC-DNA at 37°, pH 7.0. An initial small increase in fluorescence of approximately 10% was observed with both compounds in the first 15 minutes of reaction. A corresponding decrease in the fluorescence after heat denaturation was also observed. These results indicate that a small amount of quinone-hydroquinone induced single strand scission of the CCC-DNA was taking place. However, after this initial observation no additional decrease in the fluorescence after heat denaturation was observed as would be expected if alkylation of the DNA were taking place. Thus there is no evidence for

covalent alkylation or cross-linking of DNA by any of the carbamate quinones or hydroquinones.

These results support the results obtained with mitomycin C and its derivatives, that the carbamate group is much less reactive than the aziridine group. Even the possible conjugative interaction between the hydroquinone oxygen and the leaving group in 66 was not able to activate the carbamate.

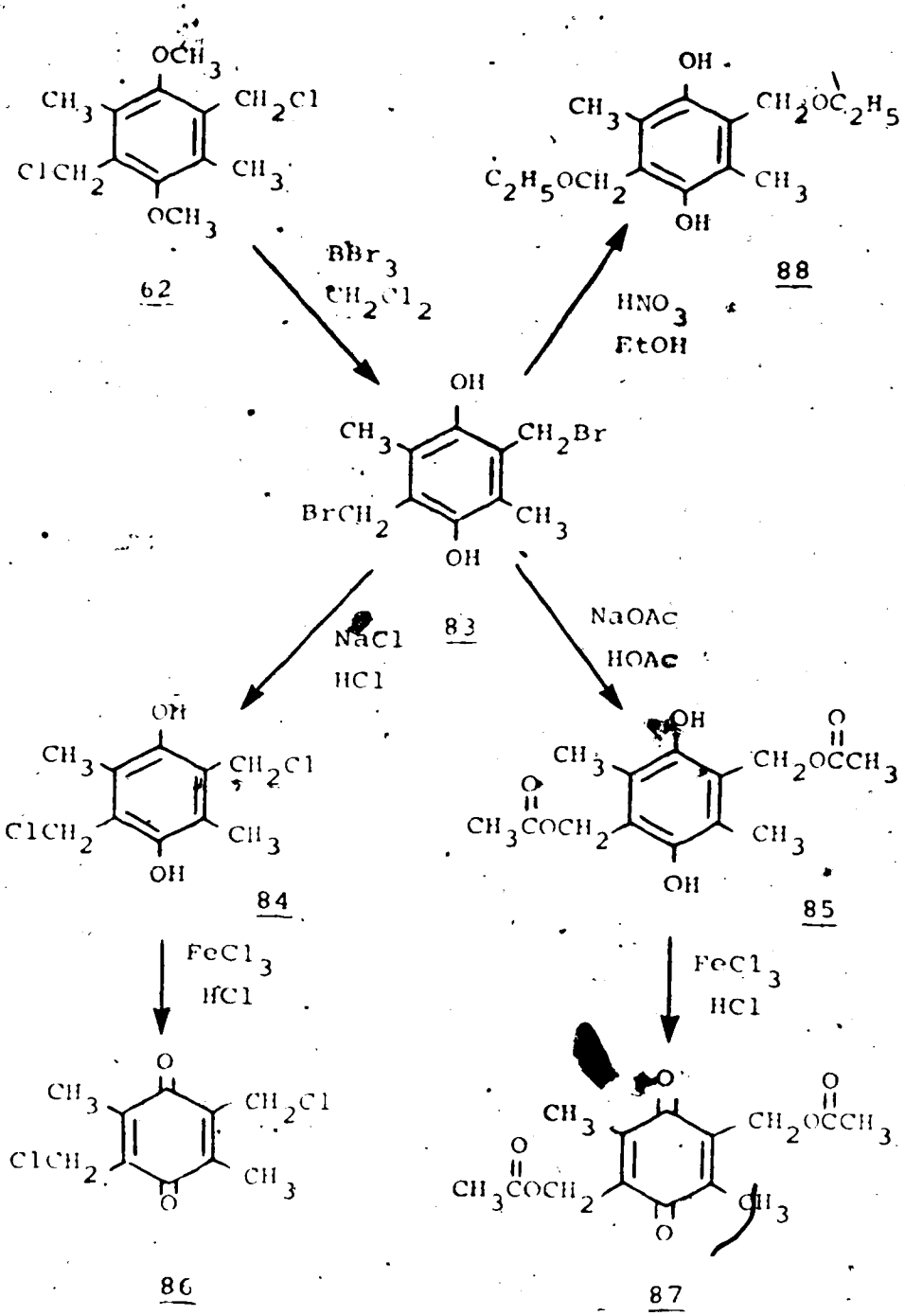
These carbamate quinones and hydroquinones are being tested for antitumor activity.

Studies on Potential Bioreductive Alkylating Agents

Synthesis of Potential Bioreductive Alkylating Agents

A series of 2,5-disubstituted benzoquinones of the type 51 was prepared by the reaction sequence shown in Scheme 19. The removal of the ether groups from 62 with boron tribromide led unexpectedly to complete replacement of chlorine with bromine. A similar ether cleavage with boron trichloride to give the dichloromethylhydroquinone 84 was unsuccessful. However, the bromine atoms of the dibromomethylhydroquinone 83 could be replaced by chlorine by stirring with concentrated hydrochloric acid and sodium chloride. Displacement of bromine by acetate proceeded readily to give 85. The hydroquinones were oxidized to the corresponding benzoquinones, 86 and 87, with ferric chloride and hydrochloric acid. Oxidation of the dibromo-

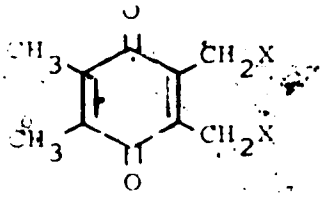
Scheme 19


OCH3
CH3
CH2Cl
ClCH2
CH3
OCH3
62
BBr3
CH2Cl2
OH
CH3
CH2OC2H5
C2H5OCH2
CH3
OH
88
HNO3
EtOH
OH
CH3
CH2Br
BrCH2
CH3
OH
83
NaCl
HCl
NaOAc
HOAc
OH
CH3
CH2Cl
ClCH2
CH3
OH
84
OH
CH3
CH2OCCH3
O
CH3COCH2
CH3
OH
85
FeCl3
HCl
FeCl3
HCl
O
CH3
CH2Cl
ClCH2
CH3
O
86
O
CH3
CH2OCCH3
O
CH3COCH2
CH3
O
87

hydroquinone 83 with this reagent resulted in displacement of bromine as well as oxidation. Oxidation with ferric bromide and hydrobromic acid however proved unsuccessful. Oxidation with other oxidizing agents resulted in displacement of the labile bromine atoms. Warming 83 in ethanol containing nitric acid gave the diether 88.

Interaction of Bioreductive Alkylating Agents with DNA

Sartorelli and coworkers have prepared a series of 2,3-disubstituted benzoquinones of the type 89.[28a,35d]



89 a X = Cl
b X = $OCOCH_3$

These have proven to be relatively active antitumor agents. It was of interest to compare the cross-linking and antitumor activity of these compounds with the corresponding 2,5-isomers, as all these compounds are potential bioreductive alkylating agents. Since both the quinones and hydroquinones of some of the 2,5-isomers were available, the efficiency of in vivo reduction of the quinones could be examined. Again we wished to look for a possible

correlation between cross-linking and alkylating ability and antitumor activity.

The quinones and hydroquinones were incubated with λ DNA at 37°, pH 7.0. The results of cross-linking measurements are shown in Table 23 and Figure 26. None of the compounds produced a loss of fluorescence due to alkylation of the DNA. The results indicate that all these compounds are relatively efficient cross-linking agents. The 2,3 and 2,5-isomers show similar activities, however a number of anomolies are apparent. For example, the unreduced dichloroquinones show more efficient cross-linking than those reduced *in situ*. In addition, the maximum per cent cross-linking with the 2,5-dichloromethylhydroquinone lower than with the quinone reduced *in situ*, while the reverse is true for the 2,5-diacetates. The diether 88, as expected, shows no cross-linking or alkylation.

The efficient cross-linking induced by the dichloromethylquinones may be due to the fact that the chlorines in these compounds are allylic and thus are relatively easily displaced. On reduction, however, the halogens, which are now benzylic, become even more labile to displacement owing to conjugative interaction with the hydroquinone oxygen. They may very well be so activated in this form that they are rapidly hydrolyzed in solution before they can interact chemically with the DNA. This would result in lowered cross-linking efficiency. The results

TABLE 23

Cross-Linking of λ-DNA by Bifunctional Benzoquinones

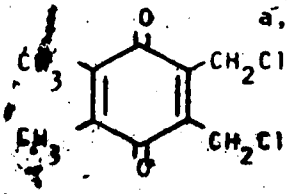
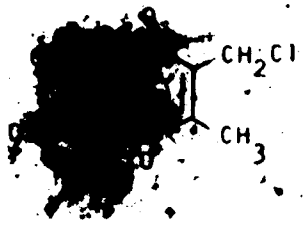
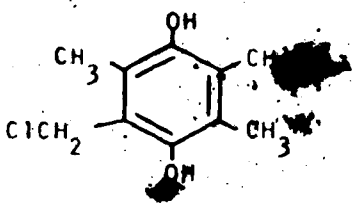
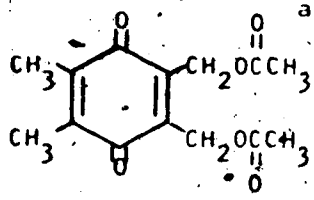
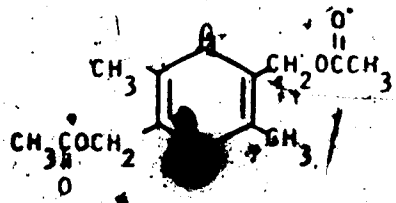
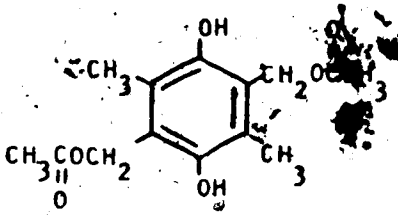
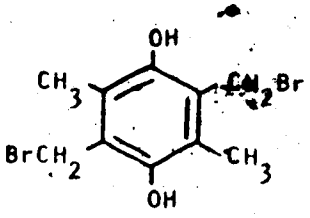
Compound (Conc = 1.0 μg/μl)	Maximum % Cross-Linking		Time to Reach Maximum (Min)
	Reduced	Not Reduced	
 [a,c]	64[b]	71	180
	37[b]	43	60
	18	-	60
 [a,d]	12[b]	0	120

TABLE 23 (continued)

Compound (Conc = 1.0 mg/ml)	Maximum % Cross-Linking		Time to Reach Maximum (Min)
	Reduced	Not Reduced	
	18[b]	0	
	81	0	30
	37	-	45

a. Supplied by Dr. A. C. Sartorelli. Conc = 0.8 mg/ml.

b. Reduced for 1 hr with 1 mg/ml $NaBH_4$.

c. Shows activity against Sarcoma 180 in mice.

d. Shows activity against Adenocarcinoma 755 in mice.

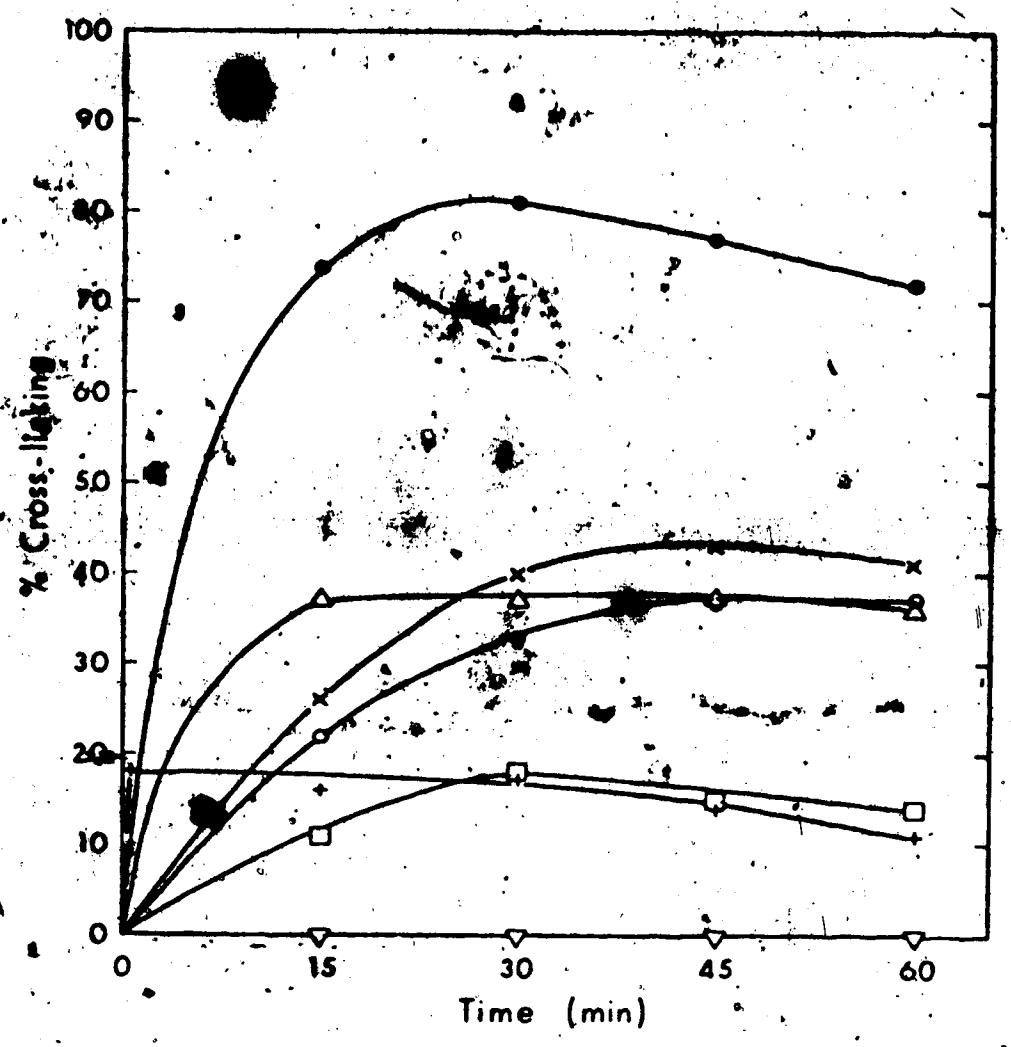


Figure 26: Covalent cross-linking of substituted quinones and hydroquinones with λ DNA. Reactions contained λ DNA at 1.0 A_{260}, phosphate buffer pH 7.0 at 0.05 M. Additional components were: ▽ 87 at 1.0 μg/μl; □ 87 at 1.0 μg/μl and sodium borohydride at 1.0 μg/μl; + 84 at 1.0 μg/μl; ○ 83 at 1.0 μg/μl; △ 86 at 1.0 μg/μl and sodium borohydride at 1.0 μg/μl; × 86 at 1.0 μg/μl; ● 85 at 1.0 μg/μl.

obtained with the 2,5-dichlorohydroquinones are consistent with this explanation since in this case the hydrolysis could be immediate since no prior reduction is necessary. The very rapid initial cross-linking observed for this compound (Figure 26) also appears to support this explanation.

The results obtained with the diacetate quinones may be complicated by reaction at the acetate groups during the reduction process. Preparative scale reduction of the 2,5-diacetate quinone 87 with sodium borohydride indicated some attack at the acetate function, however, no products could be identified. Therefore, the relatively low cross-linking values obtained for the two diacetate quinones reduced in vitro (i.e. 12% and 18% as compared to 81% for the hydroquinone) may be due to loss of the acetate groups during the reduction process. Studies on alkylating agents of this type are continuing.

The 2,5-disubstituted benzo- and hydroquinones are being tested for antitumor activity with the view of correlating cross-linking ability and antitumor activity.

Summary and Conclusions

A series of benzoquinone derivatives having structural features related to mitomycin C were prepared and their interaction with DNA was examined. The results of cross-linking interactions with DNA support the conclusions obtained with mitomycin C, that the aziridine group interacts with DNA much more efficiently than does the carbamate group and thus that the initial step in the cross-linking of DNA by mitomycin C is alkylation at the aziridine. In fact, it was found that the carbamate group was unable to alkylate DNA at all, except when highly activated as in mitomycin C.

It was demonstrated that aziridinoquinones are able to efficiently cross-link DNA as has been suggested for their mode of action.[71] The results of cross-linking experiments with DNAs of different (G+C) content suggest that alkylation by aziridinoquinones may occur preferentially at the guanine bases. In addition, there was fairly good correlation of cross-linking ability with antitumor activity. Thus the rapid and efficient ethidium fluorescence assays for alkylation and cross-linking of DNA may prove to be convenient pre-screening procedures for compounds having potential antitumor activity.

Experimental

First derivative e.p.r. spectra were measured on a Varian V-2503 spectrometer fitted with a V-4532 dual cavity operating at a nominal frequency of 9.5 GHz. The microwave power incident on the cavity was attenuated to 10 db below maximum. Hyperfine couplings were measured by comparison with a peroxylamine disulfonate solution in the audio cavity. The triplet spacing of the standard was taken to be 13.0 Oe.

6-(*p*-Bromophenyl)-3,6-diazabicyclo[3.1.0]hexane 56a

This compound was prepared in 48% yield by an adaption of a procedure due to Oida[49] using a Pyrex filtered 200 W Hanovia high pressure mercury lamp for 6 hours, m.p. 114-115.5° (lit. m.p. 11[redacted].[49b]

4-Phenyl-2,3,4,7-tetraazabicyclo[3.3.0]oct-2-ene 55b

A mixture of 6.14 g (60 mmole) of phenylazide and 5.60 g (60 mmole) of 3-pyrroline (75% pure) was set aside in the dark at ambient temperature for 3 weeks. The resulting precipitate was collected, washed with light petroleum and recrystallized from ethyl acetate: petroleum ether to give 55b, 6.75 g (57% yield) as a tan solid m.p. 111-112°.

Anal. Calcd. for $C_{10}H_{12}N_4$ [mol. wt. 188.1062]: C, 63.81; H, 6.43; N, 29.76. Found [(mass spectrum) 188.1072]: C, 63.80; H, 6.19; N, 29.45.

The infrared spectrum $\nu_{max}(CHCl_3)$: 3323 (NH); 1590 cm^{-1} (N=N).

The ^1H nmr spectrum $\delta_{TMS}(CDCl_3)$: 1.43 (s, 1H, NH); 2.68-3.60 (m, 4H, CH_2); 4.32 (dd, 1H, H_1, J_{15} = 10 Hz, J_{18}, = 4 Hz); 5.15 (dd, 1H, H_5, J_{56}, = 6 Hz); 6.80-7.60. (m, 5H, aryl protons).

6-Phenyl-3,6-diazabicyclo[3.1.0]hexane 56b

A solution of 2.60 g (14 mmole) of 4-phenyl-2,3,4,7-tetraazabicyclo[3.3.0]oct-2-ene 55b in 280 ml of tetrahydrofuran under nitrogen was irradiated with a Hanovia high pressure mercury lamp (200 W, fitted with a Pyrex filter) with stirring and cooling for 6 hours. The solvent was removed and the residue extracted with hot ether (6 x 100 ml). The ether extracts were concentrated giving 56b as a red brown oil 1.95 g (87% yield).

Anal. Calcd. for $C_{10}H_{12}N_2$ [mol. wt. 160.1001]. Found [(mass spectrum) 160.1007].

The ^1H nmr spectrum $\delta_{TMS}(CDCl_3)$: 1.65-2.07 (broad, 1H, NH); 2.66 (s, 2H, methine); 2.75 (AB quartet, 4H, methylenes, J = 12.5 Hz); 6.65-7.34 (multiplet, 5H, aryl protons).

The absorption spectrum $\lambda_{max}(CH_3CN)$: 239 nm (log ε 4.75); 2.77 nm (log ε 3.90).

2,5-Bis[3'-(6'-p-bromophenyl)-3',6'-diazabicyclo[3.1.0]-hexane]-3-(β-carbamoyloxyethyl)-6-methyl-1,4-benzoquinone 57a

A solution of 0.400 g (2.0 mmole) of freshly crushed cupric acetate monohydrate and 1.800 g (7.5 mmole) of 56a in 40 ml of methanol was purged with oxygen. While bubbling oxygen through the stirred solution, a solution of 0.313 g (1.5 mmole) of 2-(β-carbamoyloxyethyl)-6-methyl-1,4-benzoquinone 53[34a] in 75 ml of methanol was added. The reaction mixture was stirred at room temperature for 2 days, then was concentrated to approximately 5 ml and subjected to chromatography on a neutral alumina (Woelm) column eluting with methanol. The dark red band was collected and concentrated and the residue was crystallized from ethyl acetate: light petroleum to afford 57a as a purple solid m.p. 111-113° which slowly turned brown on exposure to light and air.

Anal. Calcd. for $C_{30}H_{29}Br_2N_5O_4$ [mol. wt. 683]: N, 10.25; Br, 23.39. Found [(ebullioscopic) 683]: N, 9.89; Br, 23.05.

The infrared spectrum ν_{max} ($CHCl_3$): 3544, 3434 (NH_2); 1720 (carbamate C=O); 1585 cm^{-1} (quinone C=O).

The absorption spectrum λ_{max} (CH_3CN): 248 nm (log ε 4.26); 282 nm (log ε 3.67); 396 nm (log ε 3.09); 503 nm (log ε 2.67).

The e.p.r. spectrum (generated by treating a 1.2×10^{-2} M methanolic solution of 57a with sodium methoxide in air), developed a signal of maximum intensity in 30 minutes which persisted for 2.5 hours consisting of 10 lines h.f.s. 1.4-1.8 Oe of spectrum width 12.3 Oe.

2,5-Bis[3'-(6'-phenyl)-1',6'-diazabicyclo[3.1.0]hexane]-3-(β-carbamoyloxyethyl)-6-methyl-1,4-benzoquinone 57b

A similar reaction between 0.40 g (2.0 mmole) of cupric acetate and 1.60 g (10.0 mmole) of 56b with 0.418 g (2.0 mmole) of 53[34a] in methanol afforded 57b as a red-brown solid from ethyl acetate: light petroleum 0.89 g (85% yield) m.p. 80-83° which slowly turned brown on exposure to air and owing to its instability was characterized spectroscopically.

The infrared spectrum ν_{max}($CHCl_3$): 3546, 3426 (NH_2); 1725 (carbamate C=O); 1595 cm^{-1} (quinone C=O).

The absorption spectrum λ_{max}(CH_3CN): 241 nm (log ε 4.58); 282 nm (log ε 3.92); 394 nm (log ε 3.22); 503 nm (log ε 2.72).

The e.p.r. spectrum (generated from 57b as described above) developed a signal due to the semiquinone of maximum intensity in 30 minutes which persisted for 2.5 hours and consisted of 10 lines h.f.s. 1.5-1.8 Oe of total spectrum width 12.5 Oe.

2,5-bis[6'-(6''-methoxyhexyl)-3',6'-diazabicyclo[3.1.0]-hexane]-3-(6''-methoxyethyl)-6-methyl-1,4-benzoquinone 57c

A similar reaction of 0.400 g (2.0 mmole) of cupric acetate and 1.06 g (10 mmole) of 6-methoxyhexyl-3,6-diazabicyclo[3.1.0]hexane 56c (prepared as described in Chapter II) in 250 ml of methanol with 0.418 g (2.0 mmole) of [illegible] 57c 0.199 g (17 yield) as a purple solid from ethyl acetate m.p. 98-103° which slowly turned brown on exposure to light and air.

Anal. Calcd. for $C_{32}H_{52}N_6O_6$: C, 65.63; H, 6.02; N, 11.96. Found: C, 65.11; H, 6.41; N, 9.99.

The infrared spectrum $\nu_{max}^{(CHCl_3)}$: 3520, 3418 (CH_2); 2826 (OCH_3); 1724 (urethane C=O); 1568 cm^{-1} (quinone C=O).

The absorption spectrum $\lambda_{max}^{(CH_3CN)}$: 242 nm (log ε 4.52); 299 nm (log ε 3.97); 397 nm (log ε 3.47); 506 nm (log ε 2.94).

The e.s.r. spectrum (generated from 57c as described above) developed a signal of the semiquinone within 30 minutes which persisted for over 2 hours and consisted of an [illegible] of spectra width 5.8 G.

2,5-bis[6'-(6''-hydroxyhexyl)-3',6'-diazabicyclo[3.1.0]-hexane]-3-(6''-hydroxyethyl)-6-methyl-1,4-benzoquinone 57d

A similar reaction between 0.800 g (1.5 mmole) of freshly prepared cupric acetate monohydrate and 1.800 g

(7.5 mmole) of 56a with 0.204 g (1.5 mmole) of 2,5-dimethyl-1,4-benzoquinone in methanol afforded 57d 0.511 g (56% yield) as a brownish purple solid m.p. 107-110° which slowly changed to a brown solid on exposure to light and air.

Anal. Calcd. for $C_{28}H_{24}Br_2N_4O_2$: N, 9.18; Br, 26.18. Found: N, 9.06; Br, 27.89.

The infrared spectrum ν_{max} ($CHCl_3$): 1588 cm^{-1} (quinone C=O).

The absorption spectrum λ_{max} (CH_3CN): 250 nm (log ε 4.70); 285 nm (log ε 3.88); 328 nm (log ε 3.63); 395 nm (log ε 3.33); 501 nm (log ε 2.76).

The e.p.r. spectrum (generated from 57d as described above) developed a signal of maximum intensity within 30 minutes which persisted for 2.5 hours and consisted of 11 lines h.f.s. 1.4-1.7 Oe of total spectrum width 14.2 Oe.

General Procedure for Determination of Covalent Cross-Linking and Alkylation of DNA with Bicycloaziridinobenzoquinones 57

The cross-linking agents were added as 5 μg/ml solutions in 40% pyridine-60% water. DNA was added as an aqueous solution. Reactions were buffered to pH 4.5 with 1 M acetate buffer. Cross-linking reactions were carried out on a 60 ml scale. The reaction solutions were incubated at 37° and had concentrations of approximately

1.06 A_{260} of [illegible] DNA, 0.05 M of buffer, 2.5 µg/ml of quinone and 20 [illegible] pyridine. 10 µl aliquots were removed at timed intervals and analyzed for the extent of cross-linking and alkylation by the ethidium fluorescence assay described previously. A control reaction mixture prepared exactly as above but containing no cross-linking agent was run with each experiment. In no case was there evidence for acid induced covalent cross-linking.

General Procedure for Determination of Cross-Linking and Alkylation of DNA with Aziridinoquinones

The cross-linking agents were added as 0.5 µg/µl solutions in approximately 30% tetrahydrofuran: 70% water. All DNA was added as an aqueous solution. Reactions were buffered with 1 M acetate buffers at pH 4.5 or 5.0 or with 1 M phosphate buffers at pH 6.0 or 7.2. Reactions were carried out on an approximately 100 µl scale. The reaction solutions were incubated at 37° and had concentrations of approximately 1.2 A_{260} of [illegible] DNA, 0.05 M of buffer, 0.01-0.4 µg/µl of quinone and 15% tetrahydrofuran. 10 µl aliquots were removed at timed intervals and analyzed for extent of cross-linking and alkylation by the ethidium fluorescence assay described previously. A control mixture [illegible]pared exactly as above but containing no cross-linking agent was run with each experiment and showed no evidence for acid induced covalent cross-linking.

1-Chloromethyl-2,5-dimethoxy-3,6-dimethylbenzene 61 and 1,4-Dichloromethyl-2,5-dimethoxy-3,6-dimethylbenzene 62

These compounds were prepared by the procedure outlined by Smith and Nichols[77] with the following modifications. 40% formalin could be used instead of paraformaldehyde. Reaction time and quantities of paraformaldehyde used could be decreased to increase the yield of 61. After the reaction was complete, the reaction mixture was extracted three times with methylene chloride. The combined organic extracts were washed with water until the water was neutral, dried over sodium sulfate and evaporated. The resulting white solid was recrystallized from 95% ethanol using 40 ml per gram of solid giving white needle-shaped crystals of 62 in 32% yield m.p. 162-162.5° (lit. m.p. 165-165.5 °).[77]

The infrared spectrum $\nu_{max}(CHCl_3)$: 2824 cm^{-1} (OCH_3).

The ^1H nmr spectrum δ_{TMS} ($CDCl_3$): 2.32 (s, 6H, $Ar-CH_3$); 3.80 (s, 6H, OCH_3); 4.69 (s, 4H, CH_2).

Water was added to the mother liquor giving fluffy white crystals of 61 in 22% yield m.p. 65-66°.

Anal. Calcd. for $C_{11}H_{15}O_2{}^{35}Cl$ [mol. wt. 214.0761]: C, 61.54; H, 7.04; Cl, 16.51. Found [(mass spectrum) 214.0766]: C, 61.44; H, 7.25; Cl, 16.55.

The infrared spectrum $\nu_{max}(CHCl_3)$: 2824 cm^{-1} (OCH_3).

The ^1H nmr spectrum $\delta_{TMS}(CDCl_3)$: 2.28 (s, 6H, $Ar-CH_3$); 3.80 (s, 6H, OCH_3); 4.73 (s, 2H, CH_2); 6.68 (s, 1H aryl proton).

1,4-Dimethoxy-2,5-dimethyl-3-(hydroxymethyl)benzene 63

A solution of 1.50 g (7.0 mmole) of the chloride 61 in 50 ml of 1,4-dioxane and 35 ml of 5% aqueous potassium hydroxide was refluxed for 24 hours. Water was added and the dioxane was removed under reduced pressure. The residue was extracted three times with chloroform and the organic solution was evaporated. The resulting solid was recrystallized from benzene: petroleum ether to give 0.93 g (68% yield) of the alcohol 63 m.p. 60-61°.

Anal. Calcd. for $C_{11}H_{16}O_3$ [mol. wt. 196.1100]: C, 67.32; H, 8.22. Found [(mass spectrum) 196.1094]: C, 66.85; H, 8.26.

The infrared spectrum $\nu_{max}(CHCl_3)$: 3595 (OH); 2823 cm^{-1} (OCH_3).

The ^1H nmr spectrum $\tau_{TMS}(CDCl_3)$: 2.22 and 2.25 (2s, 6H, Ar-CH_3s); 2.51 (broad, 1H, D_2O exchangeable, OH); 3.71 and 3.77 (2s, 6H, OCH_3s); 4.70 (broad d, 2H, collapses to singlet on D_2O exchange, CH_2); 6.60 (s, 1H, aryl proton).

2,5-Dimethoxy-3,6-dimethylphenmethyl Phenyl Carbonate 64

Phenyl chloroformate (1.2 ml) was added dropwise to an ice cooled, stirred solution of the alcohol 63 (0.686 g, 3.5 mmole) in 18 ml of pyridine. The solution was stirred in the cold for 1 hour and then at room temperature for 24 hours. Water was added slowly (35 ml) and

the reaction mixture was extracted 3 times with 35 ml of chloroform. The chloroform extracts were washed twice with 50 ml of cold dilute hydrochloric acid, dried over sodium sulfate and evaporated. The resulting yellow oil was crystallized from ether: petroleum ether giving 0.723 g (66% yield) of a white solid 64 m.p. 77.5-78.5°. This preparation essentially follows a literature procedure.[28a]

Anal. Calcd. for $C_{18}H_{20}O_5$ [mol. wt. 316.1311]: C, 68.34; H, 6.37. Found [(mass spectrum) 316.1320]: C, 68.30; H, 6.44.

The infrared spectrum $\nu_{max}(CHCl_3)$: 2824 (OCH_3); 1755 cm^{-1} (C=O).

The ^1H nmr spectrum $\tau_{TMS}(CDCl_3)$: 2.26 and 2.29 (2s, 6H, Ar-CH_3s); 3.73 and 3.78 (2s, 6H, OCH_3s); 5.41 (s, 2H, CH_2); 6.71 (s, 1H, aryl proton); 7.00-7.58 (m, 5H, phenyl protons).

1-Carbamoylmethyl-2,5-dimethoxy-3,6-dimethylbenzene 65

Anhydrous ammonia was bubbled through a solution of 0.562 g (1.8 mmole) of the phenyl carbonate 64 in 25 ml of 95% ethanol for 15 minutes. The reaction mixture was stirred for 18 hours in a closed pressure bottle. The solution was concentrated to approximately 1/3 its volume and 20 ml of 5% aqueous sodium hydroxide was added. The resulting precipitate was filtered and washed with water

giving 0.373 g (87% yield) of the carbamate 65 m.p. 118.5-119.5°.

Anal. Calcd. for $C_{12}H_{17}NO_4$ [mol. wt. 239.1158]: C, 60.24; H, 7.16; N, 5.85. Found [(mass spectrum) 239.1167]: C, 60.06; H, 7.16; N, 5.78.

The infrared spectrum ν_{max} ($CHCl_3$): 3534 and 3421 (NH_2); 2834 (OCH_3); 1717 cm^{-1} (C=O).

The ^1H nmr spectrum δ_{TMS} ($CDCl_3$): 2.18 and 2.27 (2s, 3H each, Ar-CH_3s); 3.69 and 3.78 (2s, 3H each, OCH_3s); 4.91 (broad, 2H, NH_2); 5.18 (s, 2H, CH_2); 6.68 (s, 1H, aryl proton).

2-Carbamoylmethyl-3,6-dimethyl-1,4-benzoquinone 47

This compound was prepared by a modification of a literature procedure.[28a] To 0.956 g (4.0 mmole) of the dimethyl ether 65 in 15 ml of 1,4-dioxane was added 2.020 g (16.0 mmole) of AgO and 4 ml of 6 N nitric acid. The mixture was stirred for 2.5 hours., 60 ml of water was added and the solution was extracted three times with 75 ml of ether. The combined ether layers were washed with water, dried over sodium sulfate, and evaporated. The residue was crystallized from ether: Skelly B giving 0.680 g (81% yield) of the quinone as yellow crystals m.p. 110.5-112°.

Anal. Calcd. for $C_{10}H_{11}NO_4$ [mol. wt. 223.0844]: C, 57.41; H, 5.30; N, 6.70. Found [(mass spectrum) 223.0841]: C, 57.39; H, 5.34; N, 6.12.

The infrared spectrum $\nu_{max}(CHCl_3)$: 3535 and 3421 (NH_2); 1730 (carbamate C=O); 1650 cm^{-1} (quinone C=O).

The ^1H nmr spectrum $\delta_{TMS}(CDCl_3)$: 2.08 (d, 3H, (C-6)-CH_3, J_{H,CH_3} 1.5 Hz); 2.16 (s, 3H, (C-3)-CH_3); 4.50-5.34 (broad, 2H, NH_2); 5.05 (s, 2H, CH_2); 6.63 (q, aryl proton).

2-Carbamoylmethyl-3,6-dimethyl-1,4-benzoquinone 66

A solution of the quinone 4[illegible]05 g, 0.5 [illegible] in 40 ml of ether was shaken with 3 portions of 40 ml freshly prepared saturated aqueous sodium dithionite. The ether solution was washed with water, dried over sodium sulfate, and evaporated. On recrystallizing the resulting solid from benzene: petroleum ether the hydroquinone 66 was obtained as white crystals (0.055 g, 52% yield) m.p. 130-132° which slowly turned brown due to decomposition.

Anal. Calcd. for $C_{10}H_{13}NO_4$ [mol. wt. 211.0845]. Found [(mass spectrum) 211.0837].

The infrared spectrum ν_{max} (nujol): 3210 (broad, OH and NH_2); 1715 cm^{-1} (C=O).

The ^1H nmr spectrum δ_{TMS} (acetone-d_6): 2.11 and 2.20 (2s, 3H each, Ar-CH_3); 5.09 (s, 2H, CH_2); 6.68 (s, 1H, aryl proton).

1-Cyanomethyl-2,5-dimethoxy-3,6-dimethylbenzene 67

A solution of the chloride 61 (3.21 g, 15 mmole) in 100 ml of ethanol was added dropwise and with stirring to a warm (steam bath) solution of potassium cyanide (1.625 g, 25 mmole) in 7 ml of water. The mixture was refluxed for 8 hours, concentrated to one-half its volume and added to 800 ml of water. The resulting precipitate was filtered and recrystallized from ether: petroleum ether to give 2.639 g (86% yield) of the nitrile 67 m.p. 84.5-85.5°. This procedure is a modification of a literature preparation.[77]

Anal. Calcd. for $C_{12}H_{15}NO_2$ [mol. wt. 205.1103]: C, 70.22; H, 7.37; N, 6.82. Found [(mass spectrum) 205.1107]: C, 70.41; H, 7.44; N, 7.04.

The infrared spectrum $\nu_{max}(CHCl_3)$: 2824 (OCH_3); 2240 cm^{-1} (CN).

The ^1H nmr spectrum $\delta_{TMS}(CDCl_3)$: 2.18 and 2.22 (2s, 6H, Ar-CH_3); 3.72 and 3.76 (2s, 6H, OCH_3); 3.74 (s, 2H, CH_2); 6.64 (s, 1H, aryl proton).

1-Carboxymethyl-2,5-dimethoxy-3,6-dimethylbenzene 68

A solution of the nitrile 67 (2.050 g, 10 mmole) in 50 ml of glacial acetic acid, 25 ml of concentrated sulfuric acid, and 25 ml of water was refluxed for 3 hours. 400 ml of water was added and the reaction mixture was extracted twice with 200 ml of ether. The ether extracts

were washed with water and extracted twice with 300 ml of 10% aqueous sodium carbonate. The aqueous layer was acidified with hydrochloric acid and cooled. The resulting precipitate was filtered, decolorized and recrystallized from benzene: petroleum ether to give 0.575 g (26% yield) of the carboxylic acid 68 m.p. 136-137° (lit. m.p. 137°).[78]

Anal. Calcd. for $C_{12}H_{16}O_4$ [mol. wt. 224.1049]. Found [(mass spectrum) 224.1056].

The infrared spectrum ν_{max} (nujol): 3400-2300 (broad, COOH); 1690 cm^{-1} (acid C=O).

The ^1H nmr spectrum δ_{TMS} (acetone-d_6): 2.06 and 2.23 (2s, 3H each, Ar-CH_3s); 3.62 and 3.74 (2s, 3H each, OCH_3s); 3.59 (s, 2H, CH_2); 6.67 (s, 1H, aryl proton).

The ether layer was dried over sodium sulfate and evaporated. The resulting solid was decolorized and recrystallized from aqueous methanol giving 0.366 g (19% yield) of the lactone 69 m.p. 94-95°.

Anal. Calcd. for $C_{11}H_{12}O_3$ [mol. wt. 192.0787]: C, 68.74; H, 6.29. Found [(mass spectrum) 192.0783]: C, 69.71; H, 7.66.

The infrared spectrum ν_{max}($CHCl_3$): 2824 (OCH_3); 1810 and 1765 cm^{-1} (C=O).

The ^1H nmr spectrum δ_{TMS}($CDCl_3$): 2.16 (s, 6H, CH_3s); 3.73 (s, 5H, OCH_3 and CH_2); 6.66 (s, 1H, aryl proton).

The yield of the acid 68 was increased to 44% by reducing the reaction time to 2 hours. This procedure is a modification of a preparation due to Smith and Nichols.[77]

1-(β-Hydroxyethyl)-2,5-dimethoxy-3,6-dimethylbenzene 70

A 1 M solution of diborane in tetrahydrofuran (50 ml) was added to a stirred mixture of the carboxylic acid 68 (0.448 g, 2 mmole) in 25 ml of dry tetrahydrofuran under nitrogen. The reaction was stirred for 3 hours. Just enough water was added to destroy the excess diborane and to decompose borate esters. The tetrahydrofuran was removed and ether and water were added till all the solid dissolved. The ether layer was separated, washed with water, dried over sodium sulfate and evaporated. The resulting oil was purified by chromatography on a neutral alumina (Woelm) column eluting with chloroform. Evaporation of the solvent gave the alcohol 70 as an oil (0.262 g, 62% yield).

Anal. Calcd. for $C_{12}H_{18}O_3$: [mol. wt. 210.1256]. Found [(mass spectrum) 210.1263].

The infrared spectrum $\nu_{max}(CHCl_3)$: 3600 cm^{-1} (OH).

The ^1H nmr spectrum $\tau_{TMS}(CDCl_3)$: 2.15 and 2.25 (2s, 3H each, Ar-CH_3s); 2.73 (s, 1H, OH); 2.93 (t, 2H, ArCH_2, J = 7.0 Hz); 3.66 and 3.74 (2s, 3H each, OCH_3s); 3.72 (t, 2H, CH_2-O); 6.67 (s, 1H, aryl proton).

2,6-Dimethoxy-3,5-dimethylphenethyl Phenyl Carbonate 71

This compound was prepared by the procedure described above for the preparation of 64 using 0.262 g (1.25 mmole) of alcohol 70, 0.6 ml of phenyl chloroformate, and 10 ml of pyridine. An oil was obtained which was a mixture of the desired phenyl carbonate ester 71 and phenol. Attempts to purify the product were unsuccessful so the oil was used without further purification for the following reaction.

The ^{1}H nmr spectrum ($CDCl_3$): 2.15 and 2.22 (2s, 6H, Ar-CH_3); 3.05 (t, 2H, Ar-CH_2, J = 7.0 Hz); 3.67 and 3.72 (2s, 6H, OCH_3); 4.32 (t, 2H, CH_2-O); 6.58 (s, 1H, Ar-H); 6.93-7.60 (m, phenyl protons).

1-(2-Chloroethyl)-2,6-dimethoxy-3,5-dimethylbenzene 72

This compound was prepared by the procedure described above for the preparation of the carbamate 65 using the mixture of phenyl carbamate 71 and phenol in 20 ml of ethanol. The carbamate 72 was obtained as a white solid wt. 0.165 g (52% yield from the alcohol 70) m.p. 122.5-123°.

Anal. Calcd. for $C_{12}H_{17}ClO_2$ [m.w. 228.1341]: C, 63.03; H, 7.50; Cl, 15.53. Found [mass spectrum 228.1308]: C, 63.02; H, 7.62; Cl, 15.31.

The infrared spectrum ν_{max} ($CHCl_3$): 3544 and 3434 (OH); 2833 (OCH_3); 1721 cm^{-1} (C=O).

The ^{1}H nmr spectrum $\delta_{TMS}^{CDCl_3}$: 2.18 and 2.26 (2s, 3H each, CH_3); 2.60 (t, 2H, CH_2, J = 7.0 Hz); 3.70 and 3.76 (2s, 3H each, OCH_3); 4.18 (t, 2H, CH_2-O); 4.56-5.08 (broad, 2H, NH_2); 6.64 (s, 1H, aryl proton).

2-(2-Carbomethoxyethyl)-3,6-dihydroxy-1,4-benzoquinone 48

To a solution of ether 25 (0.127 g, 0.5 mmole) in 5 ml of glacial acetic acid was added 0.5 ml of concentrated nitric acid. The reaction was stirred for 2.5 hours. 30 ml of water was added and the solution was concentrated to remove acetic acid. The concentrate was extracted four times with 50 ml of ether. The combined ether extracts were washed twice with 50 ml of saturated aqueous sodium bicarbonate then twice with water. The organic layer was dried over sodium sulfate and evaporated. The residue was crystallized from ether/ petroleum ether to give 0.018 g (16% yield) of the quinone 48 as a yellow solid m.p. 148.5-150°.

Anal. Calcd. for $C_{11}H_{13}NO_4$ [mol. wt. 223.0844]. Found [(mass spectrum) 223.0841].

The infrared spectrum $\nu_{max}^{CHCl_3}$: 3550 and 3436 (NH_2); 1722 (carbonate C=O); 1644 cm^{-1} (quinone C=O).

The ^{1}H nmr spectrum $\delta_{TMS}^{CDCl_3}$: 2.03 (d, 3H, (C=C)CH_3, J_{H,CH_3} = 1.5 Hz); 2.06 (s, 3H, (C-3)CH_3); 2.86 (t, 2H, CH_2, J = 7.0 Hz); 4.17 (t, 2H, CH_2-O); 6.59 (q, 1H, aryl); 6.6 (s).

1,4-Di(cyanomethyl)-2,5-dimethoxy-3,6-dimethylbenzene 73

This compound was prepared in 80% yield by the procedure of Smith and Nichols,[77] m.p. 204-205° (lit. m.p. 205-206°).[77]

The infrared spectrum $\nu_{max}(CHCl_3)$: 2828 (OCH_3); 2242 cm^{-1} (CN).

The ^1H spectrum $\delta_{TMS}(CDCl_3)$: 2.37 (s, 6H, $ArCH_3$); 3.75 (s, 4H, CH_2); 3.80 (s, 6H, OCH_3).

1,4-Dicarboxymethyl-2,5-dimethoxy-3,6-dimethylbenzene 74

This compound was prepared in 93% yield by the procedure of Smith and Nichols,[77] m.p. 267-269° (lit. m.p. 270-271°).[77]

The infrared spectrum $\nu_{max.}$ (KBr disc): 3200-2400 (broad, COOH); 1695 cm^{-1} (C=O).

The ^1H nmr spectrum δ_{TMS} (dimethyl sulfoxide-d_6): 2.12 (s, 6H, $ArCH_3$); 3.58 (s, 6H, OCH_3); 3.61 (s, 4H, CH_2).

1,4-Di(carbomethoxymethyl)-2,5-dimethoxy-3,6-dimethylbenzene 75

To the diacid 74 (0.795 g, 2.5 mmole) in 25 ml of methanol was added 5 ml of concentrated hydrochloric acid and the reaction mixture was refluxed for 3 hours. The methanol was removed under reduced pressure, the solution was cooled, and the precipitate was filtered giving 0.534 g (69% yield) of the diester 75 m.p. 108-109°.

Anal. Calcd. for $C_{16}H_{22}O_6$ [mol. wt. 310.1416]: C, 61.92; H, 7.15. Found [(mass spectrum) 310.1422]: C, 61.73; H, 7.07.

The infrared spectrum $\nu_{max}(CHCl_3)$: 2828 (OCH_3); 1734 cm^{-1} (C=O).

The ^1H nmr spectrum $\tau_{TMS}(CDCl_3)$: 2.16 (s, 6H, $ArCH_3$); 3.65 and 3.70 (2s, 12H, OCH_3s); 3.73 (s, 4H, CH_2).

1,4-Di(α-hydroxyethyl)-2,5-diethoxy-3,6-dimethylbenzene (76)

This compound was prepared by the procedure described above for the preparation of the monoalcohol 70 using 2.60 g (9.2 mmole) of the diacid 74 in 100 ml of dry tetrahydrofuran and 200 ml of 1 M diborane in tetrahydrofuran. Evaporation of the ether solution resulted in an oil which was crystallized from ether: Skelly B giving 1.04 g (45% yield) of the dialcohol 76 m.p. 146-147°.

Anal. Calcd. for $C_{14}H_{22}O_4$ [mol. wt. 254.1518]: C, 66.12; H, 8.72. Found [(mass spectrum) 254.1523]: C, 65.68; H, 8.66.

The infrared spectrum ν_{max} (nujol): 3326 and 3256 cm^{-1} (OH).

The ^1H nmr spectrum τ_{TMS} (acetone-d_6): 1.82 (s, 6H, Ar-CH_3); 2.45 (s, 2H, OH); 2.50 (t, 4H, Ar-CH_2, J = 7.0 Hz); 3.27 (s, 6H, OCH_3); 3.26 (t, 4H, CH_2O).

Alternatively, 0.403 g (1.3 mmole) of the diester

75 in 50 ml of ether was added to a stirred mixture of 0.395 g (10.4 mmole) of lithium aluminum hydride in 150 ml of ether. The reaction mixture was stirred for 4.5 hours. 10% aqueous sodium and potassium tartrate was added dropwise to decompose unreacted hydride. The mixture was filtered and the solid was washed several times with ether. The ether solution and washings were combined, washed with water, dried over sodium sulfate, and evaporated. The residue was crystallized from ether: Skelly B giving 0.126 g (38% yield) of the dialcohol 76 m.p. 146-147°. The overall yield of alcohol from the acid 74 by this route was 26%.

2,5-Dimethoxy-3,6-dimethylphendiethyl Diphenyl Dicarbonate 77

This compound was prepared by the procedure outlined for the preparation of compound 64 using 0.178 g (0.7 mmole) of the dialcohol 76, 0.6 ml of phenyl chloroformate in 6 ml of pyridine. Recrystallization from ether: petroleum ether gave 0.280 g (81% yield) of the carbonate m.p. 82.5-83.5°.

Anal: Calcd. for $C_{28}H_{30}O_8$ [mol. wt. 494.1941]: C, 68.09; H, 6.11. Found [(mass spectrum) 494.1936]: C, 67.91; H, 6.17.

The infrared spectrum ν_{max} ($CHCl_3$): 2812 (OCH_3); 1758 cm^{-1} (C=O).

The ^{1}H nmr spectrum δ_{TMS} ($CDCl_3$): 2.29 (s, 6H, $Ar-CH_3$); 3.10 (t, 4H, $Ar-CH_2$, J = 7.5 Hz); 3.69 (s, 6H, OCH_3); 4.37 (t, 4H, OCH_2); 7.00-7.62 (m, 10H, phenyl protons).

1,4-Bis(β-carbamoylethyl)-2,5-dimethoxy-3,6-dimethylbenzene 78

This compound was prepared by the procedure outlined above for the preparation of the carbamate 65 using 1.218 g (2.5 mmole) of the phenyl carbonate 77 in 50 ml of ethanol. The carbamate 78 was obtained as a white solid (0.702 g, 83% yield) which was recrystallized from aqueous ethanol m.p. 210-210.5°.

Anal. Calcd. for $C_{16}H_{24}N_2O_6$ [mol. wt. 340.1634]: C, 56.46; H, 7.11; N, 8.23. Found [(mass spectrum) 340.1639]: C, 56.02; H, 7.17; N, 8.08.

The infrared spectrum ν_{max} (nujol): 3441, 3339, 3305, and 3205 (NH_2); 1702 cm^{-1} (C=O).

The ^{1}H nmr spectrum δ_{TMS} (dimethyl sulfoxide-d_6): 2.19 (s, 6H, $Ar-CH_3$); 2.85 (t, 4H, $Ar-CH_2$, J = 7.0 Hz); 3.62 (s, 6H, OCH_3); 3.98 (t, 4H, OCH_2); 6.43 (s, 4H, NH_2).

2,5-Bis(β-carbamoylethyl)-3,6-dimethyl-1,4-benzoquinone 50

To a stirred mixture of 0.510 g (1.5 mmole) of the carbamate 78 in 15 ml of glacial acetic acid was added 1.5 ml of concentrated nitric acid. The reaction mixture was stirred for 5 hours. 100 ml of water was added and

the mixture was cooled. The resulting precipitate was filtered and recrystallized from aqueous ethanol to give 0.274 g (59% yield) of the quinone 50 m.p. 234-235°.

Anal. Calcd. for $C_{14}H_{18}N_2O_6$ [mol. wt. 310.1165]: C, 54.19; H, 5.85; N, 9.03. Found [(mass spectrum) 310.1158]: C, 54.05; H, 5.75; N, 7.76.

The infrared spectrum ν_{max} (nujol): 3430, 3318, 3245, and 3194 (NH_2s); 1722 (carbamate C=O); 1633 cm^{-1} (quinone C=O).

The ^1H nmr spectrum δ_{TMS} (dimethyl sulfoxide-d_6): 2.01 (s, 6H, Ar-CH_3); 2.73 (t, 4H, Ar-CH_2, J = 6.0 Hz); 3.48 (broad, 2H, NH), 4.00 (t, 4H, CH_2O), 6.45 (broad, 2H, NH).

2,5-Bis(β-carbamoylethyl)-3,6-dimethyl-1,4-hydroquinone 79

A solution of 0.090 g (0.29 mmole) of the quinone 50 in 50 ml of ether was shaken with 5 portions of 50 ml of freshly prepared saturated aqueous sodium dithionite. The ether layer was washed twice with water, dried over sodium sulfate, and evaporated. The resulting oil was crystallized from ether: petroleum ether giving 0.011 g (12% yield) of the hydroquinone as pale yellow crystals m.p. 95-97°.

Anal. Calcd. for $C_{14}H_{20}N_2O_6$ [mol. wt. 312.1321]. Found [(mass spectrum) 312.1309].

The infrared spectrum ν_{max} (nujol): 3440 and 3324

(NH_2 and OH); 1685 cm^{-1} (C O).

The ^1H nmr spectrum δ_{TMS} (acetone-d_6): 2.22 (s, 6H, Ar-CH_3); 2.98 (t, 4H, Ar-CH_2, J = 7.0 Hz); 4.06 (t, 4H, CH_2O).

1,4-Di(hydroxymethyl)-2,5-dimethoxy-3,6-dimethylbenzene 80

This compound was prepared by the procedure described for the preparation of the alcohol 63 using 4.08 g (15.5 mmole) of the dichloride 62 in 150 ml of 1,4-dioxane and 80 ml of 5% aqueous potassium hydroxide. Recrystallization from benzene: petroleum ether gave 3.41 g (97% yield) of the dialcohol 80 m.p. 188-189°.

Anal. Calcd. for $C_{12}H_{18}O_4$ [mol. wt. 226.1205]: C, 63.70; H, 8.02. Found [(mass spectrum) 226.1210]: C, 63.57; H, 7.92.

The infrared spectrum ν_{max} ($CHCl_3$): 3592 (OH); 2828 cm^{-1} (OCH_3).

The ^1H nmr spectrum δ_{TMS} (dimethyl sulfoxide-d_6): 2.28 (s, 6H, Ar-CH_3); 3.68 (s, 6H, OCH_3); 4.53 (s, 4H, CH_2); 4.43-4.96 (broad, 2H, OH).

2,5-Dimethoxy-3,6-dimethylphendimethyl Diphenyl Dicarbonate 81

This compound was prepared by the procedure described previously for the preparation of the carbonate 64, using 0.60 g (2.6 mmole) of dialcohol 80, 1.5 ml of phenyl chloroformate, and 1.2 ml of pyridine. Recrystallization

from ethyl acetate: petroleum ether gave 1.02 g (84% yield) of the carbonate ester 81 m.p. 180-181°.

Anal. Calcd. for $C_{26}H_{26}O_8$ [mol. wt. 466.1628]: C, 66.94; H, 5.62. Found [(mass spectrum) 466.1644]: C, 66.90; H, 5.82.

The infrared spectrum ν_{max} ($CHCl_3$): 2824 (OCH_3); 1750 cm^{-1} (C=O).

The ^1H nmr spectrum δ_{TMS} ($CDCl_3$): 2.37 (s, 6H, Ar-CH_3); 3.78 (s, 6H, OCH_3); 5.43 (s, 4H, CH_2); 3.10-3.63 (m, 10H, phenyl protons).

1,4-Bis(carbamoylmethyl)-2,5-dimethoxy-3,6-dimethylbenzene 82

This compound was prepared by the procedure described above for the preparation of compound 65 using 0.466 g (1.0 mmole) of the phenyl carbonate 81 in 25 ml of ethanol. The carbamate 82 was obtained as a white solid (0.259 g, 83% yield) m.p. 248-249°.

Anal. Calcd. for $C_{14}H_{20}N_2O_6$ [mol. wt. 312.1321]: C, 53.84; H, 6.45; N, 8.97. Found [(mass spectrum) 312.1314]: C, 54.11; H, 6.51; N, 8.93.

The infrared spectrum ν_{max} (nujol): 3420, 3302, 3240, 3168 (NH); 1692 cm^{-1} (carbamate C=O).

The ^1H nmr spectrum δ_{TMS} (dimethyl sulfoxide-d_6): 2.23 (s, 6H, Ar-CH_3); 3.64 (s, 6H, OCH_3); 5.02 (s, 4H, CH_2); 6.47 (broad s, 4H, NH_2).

General Procedure for Determination of Covalent Cross-Linking and Alkylation of DNA with Carbamate Quinones and Hydroquinones

The alkylating agents were added as 2 µg/µl solutions in 50% dimethyl sulfoxide: 50% water. Reactions were buffered with 1 M acetate pH 5.0 or 1 M phosphate pH 7.0 or 8.7. Cross-linking reactions were carried out on a 100 µl scale. The reaction solutions were incubated at 37° and had concentrations of approximately 1.04 A_{260} of λ DNA, 0.05 M of buffer, 1.0 µg/µl of cross-linking agent and 25% dimethyl sulfoxide. Quinones were run both unreduced and reduced *in situ* with sodium borohydride, 1.0 µg/µl. 10 µl aliquots were removed at timed intervals and analyzed for the extent of cross-linking and alkylation by the ethidium fluorescence assay. Control reaction mixtures prepared exactly as above but containing no alkylating agent were run with each experiment and showed no covalent cross-linking or alkylation.

The experiment with PM2 CCC-DNA was performed as above at pH 7.0 and a DNA concentration of 1.24 A_{260}.

2,5-Di(bromomethyl)-3,6-dimethyl-1,4-hydroquinone 83

A solution of the dimethyl ether 62 (1.315 g, 5.0 mmole) in 30 ml of methylene chloride was added to a solution of boron tribromide (3.765 g, 1.43 ml, 15.0 mmole) in 15 ml of methylene chloride. The solution was stirred

overnight under a drying tube. 75 ml of water was added and the mixture was extracted twice with 100 ml of ether. The organic layers were dried over sodium sulfate and evaporated. The resulting solid was decolorized and recrystallized from benzene to give 1.096 g (68% yield) of the dibromohydroquinone 83 m.p. 174-175° (dec.). This preparation is a modification of a literature procedure.[79]

Anal. Calcd. for $C_{10}H_{12}O_2{}^{79}Br{}^{81}Br$ [mol. wt. 323.9173]: C, 37.07; H, 3.73; Br, 49.32. Found [(mass spectrum) 323.9174]: C, 37.39; H, 3.74; Br, 49.51.

The infrared spectrum ν_{max} (nujol): 3180 cm^{-1} (broad, OH).

The ^1H nmr spectrum δ_{TMS} (acetone-d_6): 2.28 (s, 6H, Ar-CH_3); 4.69 (s, 4H, CH_2); 4.78 (s, 2H, OH).

2,5-Di(chloromethyl)-3,6-dimethyl-1,4-hydroquinone 84

A mixture of the dibromohydroquinone 83 (0.972 g, 3.0 mmole) and 3.482 g (6.0 mmole) of sodium chloride in 75 ml of ether and 75 ml of concentrated hydrochloric acid was stirred for 3 days. 100 ml of water was added and the solution was extracted twice with 100 ml of ether. The ether solution was dried over sodium sulfate and evaporated giving a solid which was recrystallized from ether: Skelly B (0.555 g, 79% yield) m.p. 148-149.5°.

Anal. Calcd. for $C_{10}H_{12}O_2{}^{35}Cl_2$ [mol. wt. 234.0215]: C, 51.09; H, 5.14; Cl, 30.16. Found [(mass spectrum)

234.0206]: C, 50.89; H, 5.18; Cl, 30.19.

The infrared spectrum ν_{max} (nujol): 3200 cm^{-1} (broad, OH).

The ^1H nmr spectrum δ_{TMS} (acetone-d_6): 2.30 (s, 6H, Ar-CH_3); 4.85 (s, 4H, CH_2).

2,5-Di(acetoxymethyl)-3,6-dimethyl-1,4-hydroquinone 85

The dibromohydroquinone 83 (1.072 g, 3.0 mmole) and 1.500 g (18.0 mmole) of sodium acetate in 30 ml of glacial acetic acid were stirred for 4 hours. 50 ml of water was added and the resulting precipitate was filtered and recrystallized from ether giving 0.676 g (80% yield) of the diacetate 85 m.p. 133° (dec.).

Anal. Calcd. for $C_{14}H_{18}O_6$ [mol. wt. 282.1103]: C, 59.57; H, 6.43. Found [(mass spectrum) 282.1111]: C, 59.53; H, 6.31.

The infrared spectrum ν_{max} ($CHCl_3$): 3308 (broad, OH); 1698 cm^{-1} (C=O).

The ^1H nmr spectrum δ_{TMS} ($CDCl_3$): 2.08 (s, 6H, CH_3CO); 2.31 (s, 6H, Ar-CH_3); 5.19 (s, 4H, CH_2); 7.68 (s, 2H, OH).

2,5-Di(chloromethyl)-3,6-dimethyl-1,4-benzoquinone 86

A solution of 2.300 g (8.5 mmole) of ferric chloride in 35 ml of water to which had been added 0.43 ml of concentrated hydrochloric acid was added to the dichlorohydroquinone 84 (0.399 g, 1.7 mmole) in 35 ml of chloro-

form. The mixture was stirred vigorously for 2.5 days. The chloroform layer was separated and the aqueous layer was extracted twice with 35 ml of chloroform. The combined chloroform layers were washed twice with water, dried over sodium sulfate, and evaporated. The residue was crystallized from benzene: petroleum ether giving 0.288 g (73 yield) of the dichloroquinone 86 m.p. 109-110°.

Anal. Calcd. for $C_{10}H_{10}O_2{}^{35}Cl_2$ [mol. wt. 232.0059]: C, 51.53; H, 4.32; Cl, 30.42. Found [(mass spectrum) 232.0002]: C, 51.38; H, 4.34; Cl, 30.25.

The infrared spectrum $\nu_{max}(CHCl_3)$: 1655 cm^{-1} (C=O).

The ^1H nmr spectrum $\delta_{TMS}(CDCl_3)$: 2.19 (s, 6H, Ar-CH_3); 4.48 (s, 4H, CH_2).

2,5-Di(acetoxymethyl)-3,6-dimethyl-1,4-benzoquinone 87

The procedure outlined above for the preparation of the dichlorobenzoquinone 86 was followed using 0.564 g (2.0 mmole) of the diacetoxyhydroquinone 85 in 50 ml of chloroform and 2.700 g (10 mmole) of ferric chloride, 0.50 ml of concentrated hydrochloric acid in 50 ml of water. Crystallization from ether: Skelly B gave 0.399 g (71% yield) of the quinone 87 as yellow crystals m.p. 115-116°.

Anal. Calcd. for $C_{14}H_{16}O_6$ [mol. wt. 280.0947]: C, 60.00; H, 5.75. Found [(mass spectrum) 280.0940]: C, 60.01; H, 5.80.

The infrared spectrum $\nu_{max}(CHCl_3)$: 1735 (acetate C=O); 1650 cm^{-1} (quinone C=O).

The ^{1}H nmr spectrum $\delta_{TMS}(CDCl_3)$: 2.07 (s, 6H, CH_3CO); 2.16 (s, 6H, Ar-CH_3); 5.04 (s, 4H, $^*CH_2$).

2,5-Di(ethoxyethoxy)-3,6-dimethyl-1,4-hydroquinone 88

To a solution of 0.108 g (0.3 mmole) of the dibromohydroquinone 83 in 10 ml of ethanol was added 0.1 ml of concentrated nitric acid. The mixture was heated on the steam bath for 1 hour. Water was added and the solution was cooled. The resulting precipitate was filtered giving 0.042 g (55% yield) of the diether 88 m.p. 73-74°.

Anal. Calcd. for $C_{14}H_{22}O_4$ [mol. wt. 254.1518]: C, 66.12; H, 8,72. Found [(mass spectrum) 254.1518]: C, 65.81, H, 8,10.

The infrared spectrum $\nu_{max}(CHCl_3)$: 3310 cm^{-1} (OH).

The ^{1}H nmr spectrum $\delta_{TMS}(CDCl_3)$: 1.26 (t, 6H, ethyl CH_3, J = 7.0 Hz); 2.12 (s, 6H, Ar-CH_3); 3.59 (q, 4H, ethyl CH_2); 4.72 (s, 4H, Ar-CH_2); 7.83 (s, 2H, OH).

General Procedure for Determination of Covalent Cross-Linking and Alkylation of DNA with Bioreductive Alkylating Agents

The alkylating agents were added as solutions in 50% dimethyl sulfoxide: 50% water. Reactions were buffered to pH 7.0 with 1 M phosphate. Cross-linking solutions of 100 µl were incubated at 37° and had concentrations of approximately 1.0 A_{260} of * DNA, 0.05 M of buffer, 1.0 µg/µl of the 2,5-disubstituted quinones and hydroquinones,

and 25% dimethyl sulfoxide. Quinones were run both unreduced and reduced [illegible] with sodium borohydride, 1.0 mg/ml. 10 µl aliquots were removed at timed intervals and analyzed for the extent of cross-linking and alkylation by the ethidium fluorescence assay. Control reactions run with each experiment showed no cross-linking or alkylation.

Experiments with the 2,3-disubstituted quinones were performed by L.W. McLaughlin in our laboratories as described above except that the quinone concentration was 0.8 mg/ml.

Synthesis of Aziridinoquinones

The aziridinoquinones were prepared in our laboratories by Dr. M.H. Akhtar, L.W. McLaughlin, and T. Ali by published procedures.[34,69,70] The general method is illustrated by the following example.

(i) Preparation of Tetramethoxy-1,4-benzoquinone

A slurry of 24.5 g (0.1 mole) of chloranil in 75 ml of methanol was added to a solution of 9.8 g (0.42 mole) of sodium in 250 ml of methanol. During addition the temperature of the reaction mixture was kept at 15-25° by means of external cooling. The reaction mixture was then heated on a steam bath for 6 hours. The cooled reaction mixture deposited bright orange crystals which were collected, washed with cold water, and taken up in dichloro-

methanol. The solution was decolorized with charcoal, filtered and the solvent removed in vacuo to give bright orange crystals of the product 16.7 g (73.2% yield) m.p. 133-134° (lit. m.p. 135-136°).[80]

(ii) Reaction of tetramethyl-2,5-bromomethyl-1,4-benzoquinone with aziridine

To a suspension of 1.05 g (3 mmole) of tetramethyl-2,5-bromomethyl-1,4-benzoquinone in 30 ml of methanol was added a solution of 0.28 g (6.5 mmole) of aziridine in 10 ml of methanol. The reaction mixture was stirred at room temperature for 2 days. The resulting reddish-brown 2,5-bis(aziridinyl)-3,6-dimethyl-1,4-benzoquinone was collected by filtration: 0.87 g (74% yield) m.p. 189-190° (lit. m.p. 193-194.5°).[69a]

New compounds prepared using the above procedures included:

(i) 2,5-bis(2-methylaziridinyl)-3,6-bromomethyl-1,4-benzoquinone in 63% yield m.p. 147-150°.

Anal. Calcd. for $C_{14}H_{18}N_2O_2$: C, 68.29; H, 7.31; N, 11.38. Found: C, 68.21; H, 7.61; N, 11.72.

(ii) 4,5-bis(2-methylaziridinyl)-1,2-benzoquinone in 47% yield m.p. 135-138°.

Anal. Calcd. for $C_{12}H_{14}N_2O_2$: C, 66.05; H, 6.42; N, 12.85. Found: C, 65.85; H, 6.51; N, 13.01.

Experiments on the interaction of the aziridinoquinones with DNA were carried out jointly by the author,

Dr. M.H. Akhtar, and L.W. McLaughlin in our laboratories.

The following experiment was carried out by Dr. M.H. Akhtar in our laboratories.

Covalent Cross-linking of DNAs of Different (G+C) Content with 2,5-Bis(aziridinyl)-3,6-dimethoxy-1,4-benzoquinone

Reactions were carried out at pH 4.5 at 37° using the following solutions of natural DNAs. The assays were carried out as described previously.

(a) [illegible] DNA (30% G+C). The reaction solution was approximately 1.40 A_{260} in DNA, 0.05 M in acetate buffer pH 4.5, 0.05 µg/µl in quinone and 20% in tetrahydrofuran. An identical control reaction containing no quinone was also prepared and assayed.

(b) Calf Thymus DNA (40% G+C). The reaction solution was approximately 1.15 A_{260} in DNA, 0.05 M in buffer, 0.05 µg/µl in quinone and 20% in tetrahydrofuran. An identical control reaction containing no quinone was also prepared and assayed.

(c) [illegible] DNA (50% G+C). The reaction solution was approximately 1.03 A_{260} in DNA, 0.05 M in buffer, 0.05 µg/µl in quinone and 20% in tetrahydrofuran. An identical control reaction containing no quinone was also prepared and assayed.

The following experiments were carried out by Dr. A.R. Morgan and D. Johnson in the Department of Biochemistry at the University of Alberta.

Assay for Covalent Cross-Linking of [illegible] DNA using S_1-Endonuclease

These assays were carried out as described in Chapter III for mitomycin C using 57d and 58 ($R=R_1=CH_3$; $R_2=H$). Results are presented in Tables 19 and 20.

Assay for Depurination or Depyrimidation of Polynucleotides Treated with 2,5-Bis(aziridinyl)-3,6-[illegible]thyl-1,4-benzoquinone

Poly dG·dC·(^{14}C G)·(^{3}H C) 0.339 A_{260} was incubated at 37° in 0.05 M sodium acetate buffer pH 5.0 with 0.36 mg/ml of the quinone in 18% aqueous tetrahydrofuran. At intervals duplicate samples were removed, placed on Whatman filter discs, washed twice with 5% trichloroacetic acid, then twice with ethanol, dried, and counted.

SUMMARY

In this work various aspects of the physical and chemical properties of mitomycin C have been studied in order to gain information about the mode of action of this clinically important antibiotic and antitumor agent. The ^{13}C nmr spectra of mitomycin C and the structurally related streptonigrin were analyzed and the peaks assigned. The strong conjugative interaction between the N-4 nitrogen and the quinone group of mitomycin C, which has been proposed as the reason for the stability of the unreduced form of the antibiotic, was confirmed. The fully assigned ^{13}C nmr spectra may prove useful for possible biosynthetic studies involving ^{13}C incorporation studies.

The interaction of mitomycin C with DNA was examined using rapid and convenient ethidium fluorescence assays. This led to an assay for measuring the ability of various alkylating agents to covalently alkylate DNA. Work is continuing in this area to quantify this assay and to extend it to other systems. Studies of the pH dependence of covalent cross-linking and alkylation of DNA by mitomycin C, as well as work with mitomycin derivatives have confirmed, for the first time, the involvement of both the aziridine and carbamate groups in the attachment to DNA. The step-wise nature of covalent cross-linking by mitomycin C and the sequence involved has been

demonstrated. These results were supported by studies of the relative reactivities of the two alkylating groups in mitomycin analogs. The observed ability of possible mitomycin C metabolites to cross-link and alkylate DNA raised the possibility of the involvement of such metabolites in the action of mitomycin C.

It was demonstrated that mitomycin C induces single strand scission in DNA by the generation of superoxide and hydroxyl radicals. This result contrasts with the previously proposed mechanism for mitomycin C induced DNA degradation[31] and is supported by the recent confirmation that mitomycin C facilitates the production of superoxide radical on reduction.[63] Evidence was presented for the operation of a proximity effect in the scission process, arising from the covalent interaction with the DNA. Experiments to elucidate the role of the degradation mechanism in the antitumor action of mitomycin C can be envisioned.

Electroanalytical experiments confirmed the short life-time of the semiquinone of mitomycin C and continuing experiments should provide a detailed description of the entire reduction process.

Work with mitomycin analogs supported the contention that the aziridine group of mitomycin C is preferentially involved in the interaction with DNA. The proposed

covalent cross-linking of DNA by aziridinoquinones was confirmed and evidence was presented for preferential alkylation on guanine. It was demonstrated that cross-linking ability shows a fairly good correlation with anti-tumor activity raising the possibility of using the ethidium fluorescence assay as a convenient pre-screening procedure for potentially active compounds. The recent finding that the clinically useful aziridinoquinone, Streptonigrin[63], facilitates the formation of superoxide radicals along with the detection of quinone induced single strand scission of DNA suggests the utility of an investigation into the role of DNA degradation in the mechanism of action of quinone antitumor agents.

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