Studies Related to Antitumor Antibiotics. Part VI. Correlation of Covalent Cross-linking of DNA by Bifunctional Aziridinoquinones with their Antineoplastic Activity

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Certain bisaziridinopyrrolidinoquinone analogs, which contain the structural moieties essential for physiological activity in the parent antitumor agent mitomycin C, have been synthesized. These compounds efficiently induce covalent cross-links in DNA as shown by the ethidium fluorescence assay which was confirmed by an independent S_1-endonuclease assay. The interaction of clinically active and structurally related antitumor aziridinoquinones with DNA have been examined similarly. The aziridinoquinones cross-link DNA efficiently with a marked p*H* dependence. Parallel dependence is observed on p*H* and concentration of alkylating species in the concomitant alkylation which does not result in cross-linking as measured by the suppression of the before heat fluorescence. The latter phenomenon was shown by the application of radiolabelled polynucleates not to be accompanied by depurination. A direct correlation exists between the extent of covalent cross-linking and (G + C) content of various DNA's of comparable molecular weight as in the case of mitomycin C. Estimates of the average number of cross-links per DNA molecule range from 0.61 to 1.71 depending on (G + C) content. The rate of acid assisted opening of a model aziridinoquinone measured spectrophotometrically at different p*H* values parallels the observed rate of covalent cross-linking and alkylation. It was shown independently that the intermediate 2,5 bis(2-acetoxyethylamino)-3,6-dimethoxy-1,4-benzoquinone does not cross-link DNA. A correlation is made of antineoplastic activity against a variety of tumors with covalent cross-linking ability using λ-DNA.

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On a synthétisé certains analogues de la bis-aziridinopyrrolidinoquinone qui contiennent la fraction structurale essentielle pour l'activité physiologique de la mitomycine C, agent actif contre les tumeurs. Ces composés induisent d'une façon efficace des liaisons transversales covalentes dans l'ADN; on a pu démontrer cette propriété par les tests de fluorescence à l'éthidium et la confirmer par un test indépendant avec la S_1-endonuclease. On a examiné, de la même manière, l'interaction entre l'ADN et les aziridinoquinones de structures semblables, actives du point de vue clinique contre les tumeurs. Les aziridinoquinones provoquent d'une façon efficace des liaisons transversales dans l'ADN; ce processus varie d'une façon importante avec le p*H* et la concentration des espèces alkylantes dans l'alkylation concomitante qui ne conduit pas à une liaison transversale telle que mesurée par la suppression de la fluorescence d'avant chauffage. On a démontré ce dernier phénomène par l'application de polynucléates marqués qui n'est pas accompagnée d'une dépurination. Une corrélation directe existe entre la quantité de liaisons transversales covalentes et le contenu (G + C) de divers ADN de poids moléculaires comparables à ceux utilisés dans le cas de la mitomycine C. On évalue que le nombre moyen de liaisons transversales par molécule d'ADN varie de 0.61 à 1.71 suivant le contenu (G + C). Le taux d'ouverture, aidée par les acides, de l'aziridinoquinone modèle, tel que mesuré spectrophotométriquement à divers p*H*, est parallèle au taux observé pour la formation de liaisons transversales covalentes et l'alkylation. On a démontré d'une façon indépendante que l'intermédiaire bis(acétoxy-2 éthylamino)-2,5 diméthoxy-3,6 benzoquinone-1,4 ne cause pas de liaisons transversales dans l'ADN. On présente une corrélation entre l'activité antinéoplastique contre une variété de tumeurs avec l'habilité de causer des liaisons transversales covalentes dans la λ-ADN.

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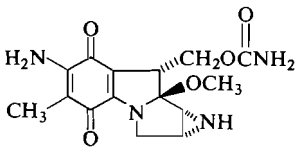
Introduction

In the alkylating class of antitumor antibiotics the most effective are bifunctional including many chemically important agents such as mitomycin C and synthetic agents *e.g.* Trenimon (1*a*). There is much evidence to indicate that DNA is the cell component most sensitive to the attack of such agents (2). As we described in Part V of this series the method of fluorescence enhancement employing the intercalative trypanocidal dye ethidium bromide which specifically detects double stranded DNA is a convenient procedure for investigating many aspects of the interaction of alkylating agents with the cell's genetic material (3). We describe the application of this technique to a study of the rate, extent, geometrical, substituent, and purine base specificity requirements for the covalent cross-linking of polynucleates, and attempt to correlate such factors with their antineoplastic properties.

Results and Discussion

Synthesis of Analogs of Mitomycin C which Covalently Cross-link DNA

In their studies on the mode of action of mitomycin C, **1**, Szybalski and Iyer (4) showed that the rate of cell death correlated well with the degree of DNA cross-linking and have calculated that one cross-link per genome is sufficient to cause cell death. An examination of the literature of cancer chemotherapy reveals that many effective bifunctional agents have quite widely different geometries (1*a*). To examine further the characteristics of cross-linking of DNA with a view ultimately of establishing a rapid and convenient screen for potential antitumor agents, we sought initially a group of analogs of mitomycin C. Despite intensive and ongoing efforts by several groups of workers, mitomycin C has not as yet yielded to a total synthesis (5). There is also interest in chemically modified forms of mitomycin C



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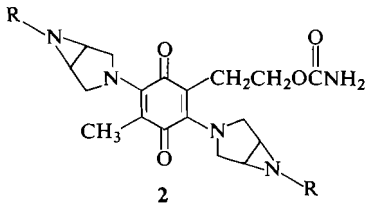
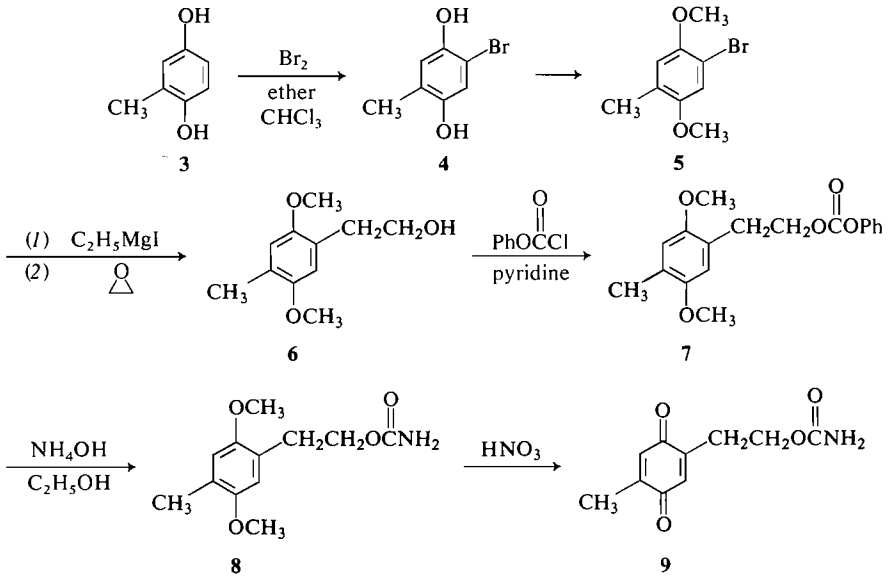


2

(5*h*). We have prepared a group of analogs which possess what are regarded as the essential structural moieties for physiological activity, *i.e.* the bisaziridinopyrrolidinoquinones, **2**.

The rationale is that compounds **2** retain the reactive aziridine and carbamate functions which separately have been shown to alkylate DNA (1*b*, 6). The distance between the two potential aziridine alkylating centers in **2** is comparable with that of the clinically useful 2,5-diaziridinoquinones (1). In addition, the greater conformational flexibility between the alkylating centers of **2** with the concomitant increase in the number of possible alkylating sites on DNA may compensate for the reduction in reactivity resulting from the loss of conjugative enhancement afforded by the indole nucleus in **1**. It has not been demonstrated that the rate of alkylation is significant in determining antitumor activity. The quinone function is retained for two reasons (*i*) so that the structure of analogs **2** should resemble **1** for possible intercalative properties (7) and (*ii*) we have established that mitomycin (3) in common with the structurally related antibiotic streptonigrin (8) degrades DNA employing its oxidative capacity which contributes significantly to their cytotoxic properties. In this connection there is much accumulated evidence that quinone containing substances display marked antibacterial and antitumor activity (5*h*, 9). In the event, analogs of type **2** proved to be quite efficient cross-linking agents which are useful in delineating the geometrical and conformational constraints in cross-linking of DNA.

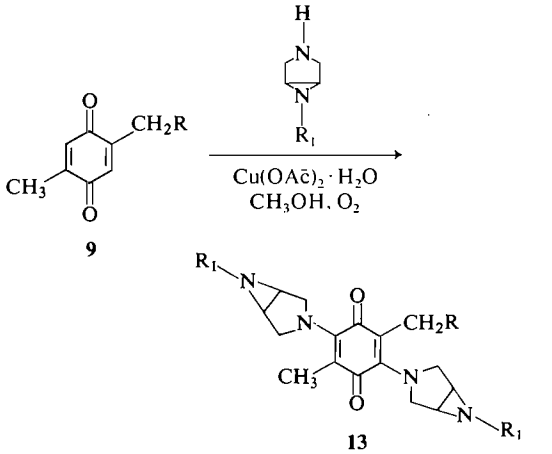
Functionalized quinones bearing the required carbamate side chain were prepared by the route illustrated in Scheme 1. The required bicyclic aziridines were prepared via 1,3-dipolar azide addition to 3-pyrroline and subsequent photolysis of the triazolines (10) (Scheme 2). The bicyclic aziridines were then coupled to the functionalized quinones using copper acetate as catalyst (11) (Scheme 3).



SCHEME 1

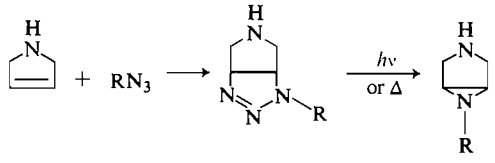
Detection of Covalent Cross-linking of DNA by Bicycloaziridinoquinones and Confirmation by S_1-Endonuclease

When the synthetic analogs **13** were incubated in 20% aqueous pyridine with λ-phage DNA (mol. wt. = 31 × 10^6) in an acetate buffer the induction of covalent cross-links was detected as summarized in Table 1. It is evident from the results that covalent cross-linking to the DNA is occurring quite efficiently across the two aziridine positions (compare **13***d* with **13***b*). The span between C_1 and C_{10} of the activated form of mitomycin is *ca.* 4.3 Å compared with the span between the alkylating centers of **13***d* of 10.1–10.8 Å indicating bifunctional agents of quite different dimensions may be accommodated by cross-linked DNA. Secondly, an electron-withdrawing group in R_1 enhances the efficiency of covalent cross-linking.



a R = CH_2OCONH_2; R_1 = C_6H_5
b R = CH_2OCONH_2; R_1 = $pBrC_6H_5$
c R = CH_2OCONH_2; R_1 = $pCH_3OC_6H_4$
d R = H; R_1 = $pBrC_6H_4$

SCHEME 3



a R = $pBrC_6H_4$
b R = C_6H_5
c R = $pCH_3OC_6H_4$

SCHEME 2

The induction of CLC sequences with compounds **13***d* received independent confirmation by the use of the S_1-endonuclease assay described previously employing *Escherichia coli* DNA (3) (see Table 2).

The substantially lower values for the induction of covalent cross-linking reflects the use of *E. coli* DNA of much lower molecular weight (14.8 × 10^6) than that of λ-DNA (31 × 10^6). Since only one cross-link is re-

TABLE 1. Induction of covalent cross-links in λ-DNA by mitomycin C analogs

Compound	Concentration (μg/μl)	Max in cross-linking (%)	Time to max in cross-linking (min)
13*d*	2.5	78	240
13*b*	2.5	61	180
13*a*	2.5	38	90
13*c*	2.5	34	240

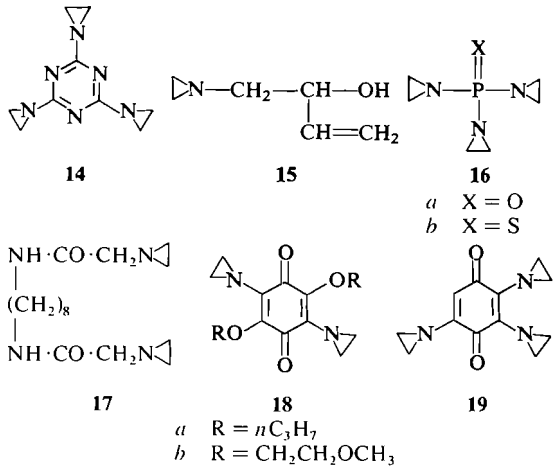
TABLE 2. Confirmation of covalent cross linking of DNA by mitomycin analogs with S_1-endonuclease assay

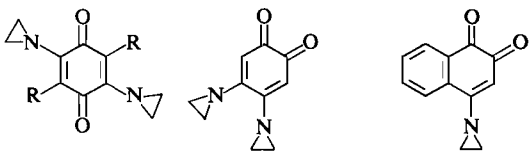
	Time (min)			
	10	90	195	270
% cross-linking by CLC assay	16	20	27	25
% cross-linking by S_1-endonuclease assay	9	15	19	16

quired per molecule to be detected by the CLC assay, a given concentration of alkylating agent will induce proportionately fewer cross-links in the *E. coli* DNA. The S_1-endonuclease assay gives lower values because the time required for digestion permits a small amount of spontaneous renaturation of the *E. coli* DNA especially at the neutral p*H* used to be compatible with the enzyme. In addition the ambient temperature used in the enzyme digestion probably permits some slow degradation of double stranded DNA presumably via the natural 'breathing' mechanism which produces transient single strand regions (12). There was no evidence of radical induced single strand cleavage of PM2 circular DNA by **13***d* as has been found for mitomycin C and streptonigrin (3).

Mode of Cytotoxic Action of Structurally Related Aziridinoquinones

Many aziridine containing compounds of quite different structures have useful antitumor properties (1*b*). Thus TEM (**14**, 2,4,6-tris(1-aziridinyl)-*s*-triazine) was the first alkylating agent found suitable for oral administration and is still in clinical use (13). Also included are tetramin (**15**) active against leukemia L1210 (14), TEPA (**16***a*), phosphoramides like tris(1-aziridinyl)phosphine oxide triethylene phosphoramide and thio-TEPA the sulfur analog **16***b* which effect complete regression of Flexner-Jobling rat carcinoma (15), and *N,N'*-octamethylenebis-1-aziridineacetamide (**17**) which is active against Ehrlich ascites carcinoma in mammals (16). Interest in the antitumor properties of the above compounds led to the synthesis and evaluation of many aziridinoquinones from which many effective agents were found including the clinically active benzoquinones 2,5-bis(1-aziridinyl)-3,6-dipropoxy-*p*-benzoquinone (**18***a*), 2,5-bis(1-aziridinyl)-3,6-bis(2-methoxyethoxy)-*p*-benzoquinone (**18***b*) (17), and tris(1-aziridinyl)-*p*-benzoquinone, Trenimon (**19**) (18). In common with other alkylating agents, they exhibit greatest effectiveness against leukemias and other lymphomas such as Hodgkin's disease. The mechanisms by which aziridine containing agents exert their antitumor activity cannot as yet be stated with confidence. Among the suggestions that have been made include (*i*) their alkylating ability (2*a*), (*ii*) their covalent cross-linking of DNA (1, 2), and (*iii*) that hydrogen peroxide or other oxidizing species formed by intracellular redox reactions of quinones are the real cytostatic agent (19). Efforts have been made, without notable success, to correlate antitumor activity with magnetic susceptibility and electron delocalization





20 21 22

(20), and with partition coefficients between benzene and aqueous phosphate buffer solutions (21).

The convenience of the ethidium fluorescence assay described above offers a means for investigating many of the suggestions that have been made with respect to the modes of action. Three general types of aziridinoquinones were synthesized **20**, **21**, and **22**. The induction of CLC sequences in λ-DNA was established for compounds of the type **20** and **21** in the concentration range 0.05–1.00 μg/μl using the ethidium assay (3, 22) as illustrated in Fig. 2 and Tables 6 and 7 where agents are listed in order of cross-linking ability. Independent confirmation that this assay detects CLC sequenced DNA for this group of bifunctional alkylating agents was obtained by the S_1-endonuclease assay (3) as summarized in Table 3.

A correspondence is again noted for these independent assays bearing in mind the remarks about the S_1 assay made earlier. It may be seen that for 1,4-benzoquinones alkoxy substituents enhance both antineoplastic activity and cross-linking efficiency whereas chloro substituents suppress both these phenomena. A carbamate side chain, unless activated as in the mitomycin C structure does not contribute to increased DNA cross-linking. *Ortho* substituted bifunctional agents are particularly effective in cross-linking and *in vivo* antitumor data are awaited with interest. The monofunctional aziridine *ortho* and *para* substituted naphthoquinones are included as controls and, as expected, do not exhibit cross-linking.

pH Dependence of Covalent Cross-Linking and Alkylation without Depurination of DNA by Aziridinoquinones

As has been observed with the antitumor antibiotic mitomycin C (3), the detection of CLC sequenced DNA is accompanied by a suppression of the before heat denaturation fluorescence reading. 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 decrease in before-heat fluorescence value was observed. The alkylated DNA's were washed with trichloroacetic acid and counted. The results summarized in Table 4 show that no depurination accompanies alkylation or covalent cross-linking as

TABLE 3. Confirmation of covalent cross-linking of DNA by aziridinoquinone (**20**) with S_1-endonuclease assay

	Time (min)			**20** R = CH_3
	0	45	135	270
% cross-linking by CLC assay	3.4	42.5	65.3	36.8
% cross-linking by S_1-endonuclease*	9.7	26.7	42.0	30.6

*Corrected for double strand digestion and the fact that FU/A260 for single stranded DNA ≈ 1/2 FU/A260 for double stranded DNA.

TABLE 4. Radioactivity assay for monoalkylation of polynucleates with aziridinoquinones with no depurination

Time (h)	^{3}H(FP/TCA channel) counts × 10^{-3}	^{14}C(FP/TCA channel) counts × 10^{-3}	^{3}H/^{14}C
0	11.4	16.0	0.713
2	11.3	17.5	0.646
18	12.6	20.4	0.618
46	14.3	22.4	0.638
65	16.7	23.6	0.708

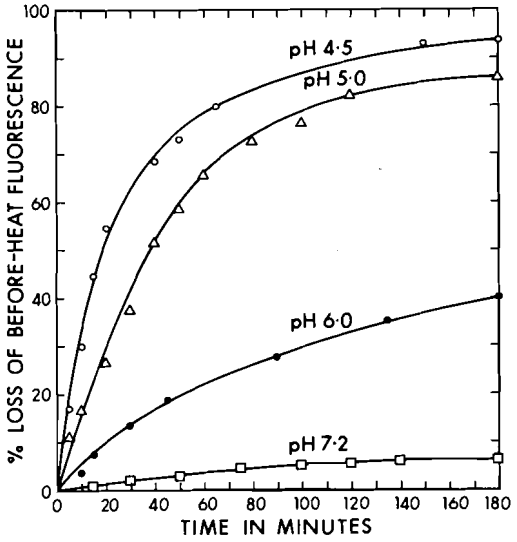


FIG. 1. The p*H* dependence of alkylation of λ-DNA by 2,5-bis(aziridinyl)-3,6-dimethoxy-1,4-benzoquinone at a final concentration of 0.8 μg/μl. Reactions were performed in 1.0 *M* buffered aqueous solutions at 37 °C with a final DNA concentration of 1.40 O.D.$_{260}$ units.

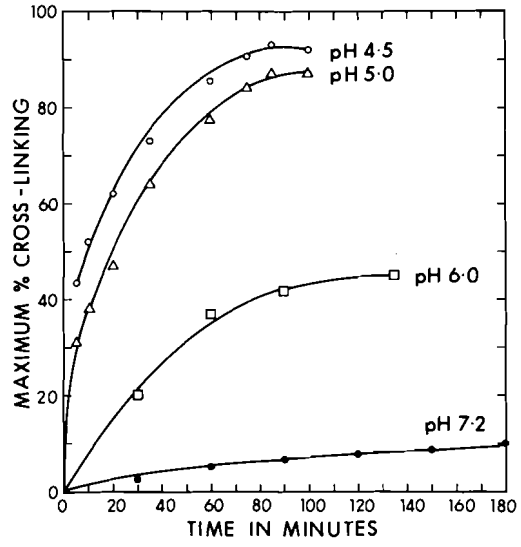
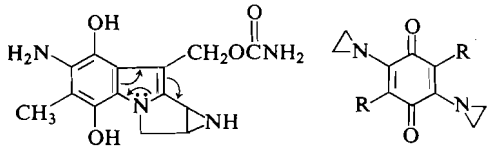


FIG. 2. The p*H* dependence of covalent cross-linking of λ-DNA by 2,5-bis(aziridinyl)-3,6-dimethoxy-1,4-benzoquinone at a final concentration of 0.05 μg/μl. Reactions were carried out in *ca.* 1.0 *M* buffered aqueous solutions at 37 °C with a final DNA concentration of 1.40 O.D.$_{260}$ units.

was found for the antibiotic mitomycin C. This evidence together with the close parallel between cross-linking and suppression of before-heat fluorescence (see Figs. 1 and 2) strongly suggest that the latter phenomenon is due to alkylation of DNA which does not lead to cross-links and is manifest by a destruction of potential ethidium intercalation sites. Several physical explanations are possible for the latter phenomenon, for example steric hindrance to approach of the ethidium or a referee has suggested quenching of the ethidium fluorescence by nearby bound drug or by drug modified bases. We are currently examining the physical cause by employing a range of ethidium analogs but meanwhile the decrease in before-heat fluorescence is used as a measure of single covalent attachment of drugs to DNA.

Freese and Cashel (23) have reported a small amount of induced cross-links in DNA by low p*H* alone. For example at p*H* 4.2 and at 25 °C their sample was cross-linked to the extent of 11% over a 2 h period. In our studies of p*H* dependence (Figs. 1 and 2) strict controls were run with λ-DNA at the corresponding p*H* but in the absence of the substrate. Over the time scale of our experiments no acid induced cross-linking was detected by the CLC assay.

The extent of alkylation as measured by the decrease in before-heat fluorescence showed a much more pronounced p*H* dependence than was found with mitomycin C (see Fig. 1). Similarly the concomitant covalent cross-linking of DNA by the aziridinoquinones shows a much more marked p*H* dependence than reduced mitomycin C (see Fig. 2). This reflects the structural differences; while the aziridine moiety of the activated mitosene **23** receives assistance to opening by conjugative interaction of the indole nitrogen lone pair at any p*H* value (4), the aziridine groups in **24** require protonation to assist alkylation.



23 **24**

Dependence of Extent of Covalent Cross-linking of DNA's by Aziridinoquinones on (G + C) Content of DNA

No evidence has as yet been presented for any base preference in the alkylation of DNA by aziridinoquinones. Therefore we examined

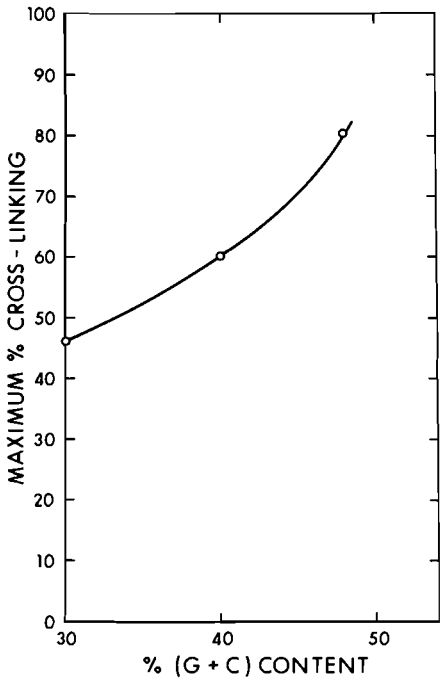


FIG. 3. The dependence of maximum percentage covalent cross-linking of DNA's by 2,5-bis(aziridinyl)-3,6-dimethoxy-1,4-benzoquinone (0.05 μg/μl) on the percentage (G + C) content of the DNA. The DNA's used were, *Clostridium perfringens* (30% G + C), mol. wt. 11.4×10^6; calf thymus (40% G + C), mol. wt. 10.8×10^6; *E. coli* (50% G + C), mol. wt. 14.8×10^6, with final concentrations respectively of 1.40, 1.145, and 1.03 $O.D._{260}$ units.

the interaction of **20** ($R = OCH_3$) with three different natural DNA's of different (G + C) content: *Clostridium perfringens*, 30%; calf thymus, 40%; and *E. coli* 50%. These three DNA's had comparable molecular weights as determined by sedimentation velocity studies and therefore their reactivity towards the aziridinoquinone could be compared directly. It may be seen from Fig. 3 that a direct correlation obtains between maximum extent of covalent cross-linking with higher (G + C) content for a given concentration of the cross-linking agents. Assuming a Poisson's distribution of the links and further that one link is sufficient to permit the spontaneous renaturation of the molecule an estimate of the average number of cross-links per molecule was made, $m = \ln(1/P_0)$ where P_0 is the proportion of the molecules unlinked as 0.62, 0.93, and 1.71 for the three DNA's. These are closely comparable with similar estimates made by Iyer and Szybalski (24) for mitomycin cross-linking. Recent work by Tomasz (25) rules out direct alkylation of the N-7 position of guanine by mitomycin C but does not exclude reaction at O-6 as originally suggested by Szybalski and Iyer (4). This may then apply to the aziridinoquinones.

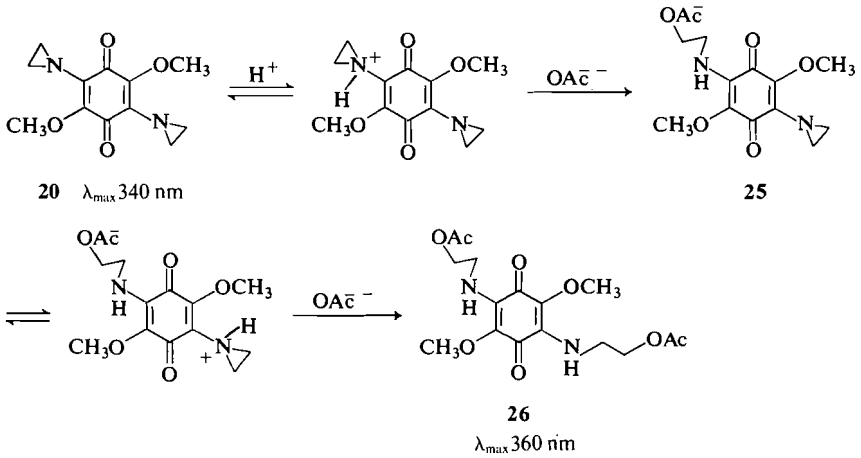
Kinetics of Acid Assisted Ring Opening of Aziridinoquinones

The rate of acid assisted opening of **20** in 1 *M* sodium acetate buffers in the range p*H* 4.0 to 6.0 in 25% tetrahydrofuran – 75% water was determined spectrophotometrically employing concentrations comparable with those used for DNA cross linking by measuring the rate of appearance of the ring opened species **26** at 380 nm. Since attempts at product isolation at intermediate stages afforded only **20** or **26** it was concluded that the lifetime of **25** was very short under the solvolysis conditions and therefore its equilibrium contribution to the absorbance at 380 nm could be neglected to a first approximation (Scheme 4).

The pseudo unimolecular rate constants at different p*H* values are listed in Table 5. It may be seen that the p*H* dependence of the aziridine opening parallels the rate of covalent cross-linking (Table 5 and Fig. 2) and of alkylation of DNA (Fig. 1). It was shown in an independent experiment that the diacetate **26** in which the aziridine rings were opened did not cross-link DNA. Therefore the strong p*H* dependence of the cross-linking suggests, as expected, that the active species involved directly in covalent bonding to DNA is the intermediate aziridinium ion.

Correlation of Covalent Cross-Linking with Antineoplastic Activity

Having established a convenient procedure for detecting alkylation and covalent cross-linking of various DNA's by aziridinoquinones and other alkylating agents it is encouraging to observe that 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 (see Table 6). This parameter may prove useful for prescreening antitumor agents for clinical trials. It is recognized that other biological and pharmacological parameters, in addition to DNA covalent cross-linking (*i.e.* drug uptake,



SCHEME 4

TABLE 5. Pseudo unimolecular rate constants for the acid catalyzed ring opening of 2,5-diaziridino-3,6-benzoquinone

p*H*	Rate constant $k \times 10^{-5}$ (s^{-1})
4.5	9.04
5.0	8.35
6.0	2.14
7.2	Too slow to measure
8.7	Too slow to measure

partition, metabolism, and toxicity) contributes to the ultimate effectiveness of cancer inhibitory properties. Table 7 lists covalent cross-linking results for additional aziridinoquinones for which, as yet, no *in vivo* antitumor data is available.

Experimental

Melting points were determined on a Fisher-Johns apparatus and are uncorrected. The i.r. spectra were recorded on a Perkin-Elmer model 421 spectrophotometer and only the principal, sharply defined peaks are reported. The n.m.r. spectra were recorded on Varian A-60 and A-100 analytical spectrometers. The spectra were measured on approximately 10–15% (w/v) solutions usually in $CDCl_3$, with tetramethylsilane as a standard. Line positions are reported in p.p.m. from the reference. Mass spectra were determined on an Associated Electrical Industries MS-9 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 15000. Kieselgel DF-5 (Camag, Switzerland) and Eastman Kodak precoated sheets were used for thin-layer chromatography. Microanalyses were carried out by Mrs. D. Mahlow of this department. 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.

Materials

Ethidium bromide, calf thymus DNA, and α-amylose powder were purchased from Sigma Chemical Co., Sephadex G-100 superfine was from Pharmacia and DEAE cellulose was obtained from Whatman and washed before use.

4-Phenyl-2,3,4,7-tetraazobicyclo[3.3.0]oct-1-ene

A mixture of 6.14 g (60 mmol) of phenylazide and 5.60 g (60 mmol) 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 **11***b* 6.75 g (57% yield) as a tan solid m.p. 111–112 °C.

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

The i.r. spectrum $\nu_{max}(CHCl_3)$: 3323 (NH); 1590 cm^{-1} (N=N). The n.m.r. 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, $J_{1'8'} = 4$ Hz), 5.15 (dd, 1H, H_5, $J_{56'} = 6$ Hz); 6.80–7.60 (m, 5H, ArH).

*4-(p-Methoxyphenyl)-2,3,4,7-tetraazabicyclo[3.3.0]-oct-1-ene (**11***c*)

This compound was prepared in a similar fashion from 2.2 g (15 mmol) of *p*-methoxyphenylazide and 1.4 g (15 mmol) of 3-pyrroline (75% pure) in 30% yield as a white crystalline solid m.p. 108.5–109 °C.

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

The i.r. spectrum $\nu_{max}(CHCl_3)$: 3305 (NH), 1580 cm^{-1} (N=N). The n.m.r. spectrum $\delta_{TMS}(CDCl_3)$: 1.46 (broad 1H, NH); 2.69–3.62 (m, 4H, CH_2); 3.79 (s, 3H,

TABLE 6. Correlation of covalent cross-linking of λ-DNA by bifunctional alkylating agents with antitumor activity against various tumors*

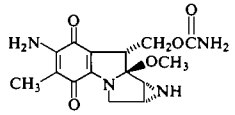
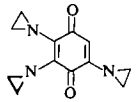
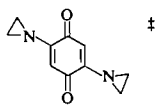
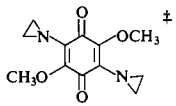
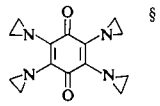
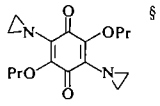
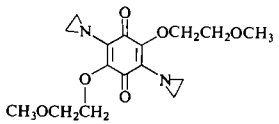
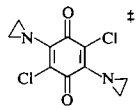
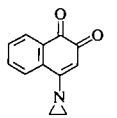
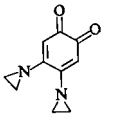
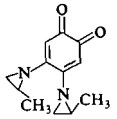
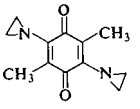
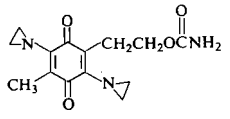
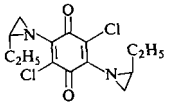
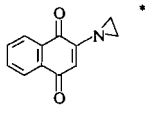
Structure	L1210†		W-256		CA-755		S-180		KB	λ-DNA cross-linking		
	OD	ILS	OD	ILS	OD	TWI	OD	TWI	ED_{50}	Concentration (μg/μl)	Maximum (%)	Time to reach maximum (min)
 1	1.5	60	1.0	95	1.0	90	3.0	75	0.04	0.04 0.02	100 93	5 5
	0.1	47	0.1	96	0.04	70	0.13	49	<0.01	0.4 0.04	100 100	2 10
 ‡	0.5	26	—	—	0.25	60	1.25	39	<1.0	0.01	92	10
 ‡	2.0	36	2.0	95	—	—	—	—	—	0.4 0.04	92 82	60 120
 §	0.01	39	—	—	0.05	80	0.13	65	4.9	0.4 0.04	90 85	25 100

TABLE 6 (*Concluded*)

Structure	L1210† OD	L1210† ILS	W-256 OD	W-256 ILS	CA-755 OD	CA-755 TWI	S-180 OD	S-180 TWI	KB ED_{50}	λ-DNA cross-linking Concentration (μg/μl)	λ-DNA cross-linking Maximum (%)	λ-DNA cross-linking Time to reach maximum (min)
 §	4	50	1.0	95	3.0	78	5.0	5.0	0.07	0.4 0.04	85.5 79	30 60
	2.0	7.0	0.98	84	2.0	80	3.0	63	—	0.4	55	255
 ‡	11, 22	0	—	—	11	50	25	35	—	0.4	18	120
	100	Toxic	—	—	—	—	—	—	—	0.4	0	—

*Abbreviations used: OD optimal dose, ILS increased life span of test animals; TWI percentage tumor weight inhibition; ED_{50} effective dose for 50% survival.
†L1210, leukemia 1210; W-256, Walker carcinoma 256; CA-755, carcinoma 755; S-180, Erlich ascites (9).
‡Shows antibacterial activity against *Staphylococcus aureus*, *E. coli*, and *Streptococcus faecalis*.
§Shows marked carcinostatic activity against Jensen sarcoma.

TABLE 7. Covalent cross-linking of λ-DNA by aziridinoquinones

Structure	Concentration	λ-DNA cross-linking	
		Maximum (%)	Time to reach maximum
	0.4	85.5	30
	0.04	80	105
	0.4	78.4	120
	0.04	32.3	45
	0.4	59	60
	0.4	38	30
	0.24	24	270
	0.4	0	—

*Shows substantial monoalkylation.

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 (m, 4H, ArH).

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

A solution of 2.60 g (14 mmol) of 4-phenyl-2,3,4,7-tetraazabicyclo[3.3.0]oct-1-ene 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 h. The solvent was removed *in vacuo* and the residue extracted with hot ether (6 × 100 ml). The ether extracts were concentrated giving **12***b* as a red brown oil, 1.95 g (87% yield).

Mol. Wt. Calcd. for $C_{10}H_{12}N_2$: 160.1001. Found (mass spectrum): 160.1007.

The n.m.r. spectrum $\delta_{TMS}(CDCl_3)$: 1.65–2.07 (broad 1H, NH), 2.66 (s, 2H, methine); 2.75 (AB quartet, 4H, CH_2, $J = 12.5$ Hz); 6.65–7.34 (m, 5H, ArH). The absorption spectrum: 239 (log ε 4.75), 277 nm (log ε 3.90).

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

A solution of 0.906 g (4 mmol) of 4-(*p*-methoxyphenyl)-2,3,4,7-tetraazabicyclo[3.3.0]oct-1-ene in 120 ml of tetrahydrofuran under nitrogen was irradiated as described in the above procedure. The solvent was evaporated and the residue treated with 100 ml of ether. The resulting precipitate was filtered. The filtrate was evaporated and the residue crystallized from benzene – light petroleum affording **12***c* 0.742 g (98% yield) as an off-white solid, m.p. 36–38 °C.

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

The n.m.r. spectrum $\delta_{TMS}((CD_3)_2CO)$: 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 (m, 4H, ArH). The absorption spectrum (CH_3CN): 238 (log ε 4.21), 295 nm (log ε 3.30).

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

A solution of 0.400 g (2.0 mmol) of freshly crushed cupric acetate monohydrate and 1.800 g (7.5 mmol) of 6-(*p*-bromophenyl)-3,6-diazabicyclo[3.1.0]hexane (23)

in 40 ml of methanol was purged with oxygen. While bubbling oxygen through the stirred solution, a solution of 0.313 g (1.5 mmol) of 2-(β-carbamoyloxyethyl)-5-methyl-1,4-benzoquinone (24) in 75 ml of methanol was added. The reaction mixture was stirred at room temperature for 2 days, then was concentrated to *ca.* 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 **13***b* as a purple solid, m.p. 111–113 °C, 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 (683, ebullioscopic): N, 9.89; Br, 23.05.

The i.r. spectrum ν_{max}($CHCl_3$): 3544, 3434 (NH_2), 1720 (carbamate C=O), 1585 cm^{-1} (quinone C=O). The absorption spectrum (CH_3CN): 248 (log ε 4.26), 282 (log ε 3.67), 396 (log ε 3.09), 503 nm (log ε 2.57). The e.p.r. spectrum (generated by treating a 1.2×10^{-2} *M* methanolic solution of **13***b* with sodium methoxide in air), developed a signal of maximum intensity in 30 min which persisted for 2½ h consisting of 10 lines h.f.s. 1.4–1.8 Oe of spectrum width 12.3 Oe.

2,5-Bis-3′-[6′-p-bromophenyl-3′,6′-diazabicyclo[3.1.0]-hexane]-3,6′dimethyl-1,4-benzoquinone

A similar reaction between 0.300 g (1.5 mmol) of freshly crushed cupric acetate monohydrate and 1.800 g (7.5 mmol) of 6-(*p*-bromophenyl)-3,6-diazabicyclo[3.1.0]-hexane (26) with 0.204 g (1.5 mmol) of 2,5-dimethyl-1,4-benzoquinone in methanol afforded **13***d* 0.511 g (56% yield) as a brownish purple solid, m.p. 107–110 °C, 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 i.r. spectrum ν_{max}($CHCl_3$): 1588 cm^{-1} (C=O). The absorption spectrum (CH_3CN): 250 (log ε 4.70), 285 (log ε 3.88), 328 (log ε 3.63), 395 (log ε 3.33), 501 nm (log ε 2.76). The e.p.r. spectrum (generated by treating a 1.2×10^{-2} *M* methanolic solution of **13***d* with sodium methoxide in air), developed a signal of maximum intensity within 30 min which persisted for 2½ h and consisted of 11 lines, h.f.s. 1.4–1.7 Oe of total spectrum width 14.2 Oe.

2,5-Bis-3′-[6′-phenyl-3′,6′-diazabicyclo[3.2.0]hexane]-3-(β-carbamoyloxyethyl)-6-methyl-1,4-benzoquinone

A similar reaction between 0.400 g (2.0 mmol) of cupric acetate and 1.60 g (10.0 mmol) of 6-phenyl-3,6-diazabicyclo[3.1.0]hexane with 0.418 g (2.0 mmol) of 2-(β-carbamoyloxyethyl)-5-methyl-1,4-benzoquinone(24) in methanol afforded **13***a* as a red-brown solid from ethyl acetate – light petroleum, 0.892 g (85% yield), m.p. 80–83 °C, which slowly turned brown on exposure to air and owing to its instability was characterized spectroscopically.

The i.r. spectrum ν_{max}($CHCl_3$): 3546, 3426 (NH_2); 1725 (carbamate C=O); 1595 cm^{-1} (quinone C=O). The absorption spectrum (CH_3CN): 241 (log ε 4.58), 282 (log ε 3.92), 394 (log ε 3.22), 503 nm (log ε 2.72). The e.p.r. spectrum (generated from **13***a* as described above) developed a signal due to the semiquinone of maximum intensity in 30 min which persisted for 2½ h and consisted of 10 lines h.f.s. 1.5–1.8 Oe of total spectrum width 12.5 Oe.

2,5-Bis-3′-[6′-p-methoxyphenyl-3′,6′-diazabicyclo[3.1.0]-hexane]-3-(β-carbamoyloxyethyl)-6-methyl-1,4-benzoquinone

A similar reaction of 0.400 (2.0 mmol) of cupric acetate and 1.900 g (10 mmol) of 6-*p*-methoxyphenyl-3,6-diazabicyclo[3.1.0]hexane in 50 ml of methanol with 0.418 g (2.0 mmol) of 2-(β-carbamoyloxyethyl)-5-methyl-1,4-benzoquinone (27) afforded **13***c* 0.199 g (17% yield) as a purple solid from ethyl acetate, m.p. 98–103 °C which slowly turned brown on exposure to light and air.

Anal. Calcd. for $C_{32}H_{35}N_5O_6$: C, 65.63; H, 6.02; N, 11.96. Found: C, 65.11; H, 6.41; N, 9.99.

The i.r. spectrum ν_{max}($CHCl_3$): 3520 and 3418 (NH_2); 2826 (OCH_3), 1723 (carbamate C=O), 1568 cm^{-1} (quinone C=O). The absorption spectrum (CH_3CN): 242 (log ε 4.52), 290 (log ε 3.97), 397 (log ε 3.47), 506 nm (log ε 2.94). The e.p.r. spectrum (generated from **13***c* as described above) developed a signal of the semiquinone within 30 min which persisted for over 2 h and consisted of an unresolved singlet of spectrum width 5.8 Oe.

Synthesis of Aziridinoquinones

Most of the aziridinoquinones studied were prepared by published procedures (17). The general method is illustrated by the following example.

Preparation of Tetramethoxy-1,4-benzoquinone

A slurry of 24.5 g (0.1 mol) of chloranil in 75 ml of methanol was added to a solution of 9.8 g (0.42 mol) of sodium in 250 ml of methanol. During addition the temperature of the reaction mixture was kept at 15–25 °C by means of external cooling. The reaction mixture was then heated on a steam bath for 6 h. The cooled reaction mixture deposited bright orange crystals which were collected, washed with cold water, and taken up in dichloromethane. 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 °C (lit. (28) m.p. 135–136 °C).

Reaction of Tetramethoxy-1,4-benzoquinone with Aziridine

To a suspension of 1.05 g (5 mmol) of tetramethoxy-1,4-benzoquinone in 30 ml of methanol was added a solution of 0.280 g (65 mmol) 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-dimethoxy-1,4-benzoquinone was collected by filtration, 0.875 g (79% yield), m.p. 189–190 °C (lit. (17*a*) m.p. 193–194.5 °C). The n.m.r. spectrum: δ_{TMS}($CDCl_3$): 2.28 (s, 8H, aziridinyl protons) and 3.92 (s, 6H, OCH_3).

New compounds prepared using these procedures include:—

(i) 2,5-Bis(2-methylaziridinyl)-3,6-dimethyl-1,4-benzoquinone in 63% yield, m.p. 147–150 °C.

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) 2,5-Bis(2-ethylaziridinyl)-3,6-dimethyl-1,4-benzoquinone in 50% yield, m.p. 125–126 °C.

Anal. Calcd. for $C_{16}H_{22}N_2O_2$: C, 70.44; H, 8.03; N, 10.08. Found: C, 70.04; H, 8.54; N, 10.38.

(iii) 4,5-Bis(2-methylaziridinyl)-1,2-benzoquinone in 47% yield, m.p. 135–138 °C.

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.

Fluorescence Assay for Detecting CLC Sequences and Monoalkylation

The general procedure employing a G. K. Turner and associates model 430 spectrofluorometer has been described previously. The assay mixture was prepared by mixing 2 ml of a 1 *M* K_2HPO_4–KOH buffer (at p*H* 12.0) with 0.2 ml of a 0.2 *M* solution of EDTA disodium salt (buffered at p*H* 8 with 2 *M* K_3PO_4) and with 0.05 ml of a 1 mg/ml solution of ethidium bromide in water. The resulting mixture was made up to 100 ml with distilled water and was covered with aluminum foil to protect it from the light. The instrument was blanked using 2 ml of the above solution. The cross-linking assays were carried out as follows.

Bicycloaziridinobenzoquinones

The cross-linking agents were added as 5 μg/μl solutions in 40% pyridine – 60% water. DNA was added as an aqueous solution. Reactions were buffered to p*H* 4.5 with 1 *M* sodium acetate – acetic acid buffer. Cross-linking reactions were carried out on a 60 μl scale. The reaction solutions were incubated at 37 °C and had concentrations of 1.06 O.D.$_{260}$ units of λ-DNA, 0.05 *M* of buffer, 2.5 μg/μl of cross-linking agent and 20% pyridine; 10 μl aliquots were removed at timed intervals and analyzed for the extent of cross-linking by CLC assay described previously (3). 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.

Bisaziridinoquinones

A 10 μl aliquot of the cross-linking reaction mixture (*ca.* 5 μg/μl of alkylating agent in 25% tetrahydrofuran – 75% water, 1 *M* sodium acetate buffer and DNA) was diluted in 2 ml of the solution. Similarly a 10 μl aliquot was withdrawn from a reaction mixture containing no alkylating agent (control) and was diluted in 2 ml of the assay solution. The solutions were analyzed by the CLC assay described previously (3). A typical run is given in complete form for 2,5-bis(aziridino)-3,6-dimethoxy-1,4-benzoquinone at p*H* 5.0 with a concentration of 0.8 μg/μl.

Time (min)	Before-heat fluorescence*	After-heat fluorescence	% Cross-linking
0	70	3.5	0
5	64	12.5	19.5
10	60	17	28.3
20	52	23	44.2
30	45	24	53
40	35	20	57
50	30	18	63
60	25	17	67
80	20	15	75
100	15	12	80
120	13	11	82
150	10	8	80
180	9	6	67

*Control fluorescence = 70 constant throughout experiment.

Covalent Cross-Linking of DNA's with Different (G + C) Content with 2,5-Bis(aziridino)-3,6-dimethoxy-1,4-benzoquinone

The CLC assays were performed at p*H* 4.5 and 37 °C as described above using the following solutions of natural DNA's.

Clostridium perfringens DNA (30% G + C)

The assay mixture was prepared using 7 μl of the DNA stock solution (O.D.$_{260}$ = 20), 5 μl of 1 *M* sodium acetate buffer p*H* 4.5, 8 μl of water, and 80 μl of a solution of **20** (R = OCH_3) in 25% aqueous tetrahydrofuran (final concentration of agent 0.05 μg/μl and final concentration of DNA was 1.40 O.D.$_{260}$). The control was similar but lacked **20** (R = OCH_3).

Calf Thymus DNA (40% G + C)

The assay mixture was prepared using 30 μl of the DNA stock solution (O.D.$_{260}$ = 4.58), 5 μl of 1 *M* sodium acetate buffer p*H* 4.5, 5 μl of water, and 80 μl of the solution of agent **20** (R = OCH_3) (final concentration of agent was 0.05 μg/μl and of DNA 1.145 O.D.$_{260}$). Control as above.

E. coli DNA (50% G + C)

The assay mixture was prepared using 25 μl of the DNA stock solution (O.D.$_{260}$, 1.03), 5 μl of 1 *M* sodium acetate p*H* 4.5, 5 μl of water, and 40 μl of a 25% aqueous solution of **20** (R = OCH_3) (final concentration of agent 0.05 μg/μl, and final concentration of DNA was 1.03 O.D.$_{260}$). Control as above.

Assay for Covalent Cross-Linking of E. coli DNA using S_1-Endonuclease

E. coli DNA which had been covalently cross-linked with the bifunctional alkylating agent was dialyzed overnight at 40 °C *vs.* 10 m*M* potassium phosphate at p*H* 11.5, 0.1 m*M* EDTA, neutralized with 0.5 *M* tris hydrochloride p*H* 7.5 (~25 m*M*), and heat denatured (5 min at 95 °C then ice treatment). To 80 μl were added 20 μl 5 × S_1 buffer p*H* 4.5 (final p*H* about 4.6). After removal of the first aliquot, 2 μl of purified S_1-endonuclease (610 U/mg, 1.28 mg/ml) was added and the mixture incubated at 45 °C. Aliquots were removed and examined with ethidium bromide solution p*H* 8.0 and read as described previously. Heat denatured and native *E. coli* DNA's were incubated as controls.

Assay for Depurination of Radioactively Labelled Polynucleates Treated with 2,5-Bis(aziridino)-3,6-dimethoxy-1,4-benzoquinone

Poly dG.dC(^{14}CG).d(^{3}HC) 0.339 A_{260}/ml was incubated at 37 °C in 50 m*M* sodium buffer p*H* 5.0 with 360 μg/ml of 2,5-bis(aziridino)-3,6-dimethoxy-1,4-benzoquinone in 18% aqueous tetrahydrofuran. At intervals duplicate samples were removed, placed on Whatman filter discs, washed with 5% trichloroacetic acid, then twice with ethanol, dried, and counted. The results summarized in Table 4 show no loss of purine or pyrimidine bases accompany treatment of the DNA by the aziridinoquinone.

Kinetic Studies of the Rate of Acid Catalyzed Ring Opening of Aziridinoquinones

A 1.04 × 10^{-3} *M* solution of the 2,5-bis(aziridinyl)-3,6-dimethoxy-1,4-benzoquinone was prepared in 25% THF – 75% H_2O at room temperature and transferred in 160 μl aliquots to reaction tubes and then 10 μl of water, 20 μl of λ-DNA, and 10 μl of 1 *M* sodium acetate

TABLE 8. Absorption spectra

Wavelength (nm)	Absorbance **20**	Absorbance **26**
330	0.29	0.20
340	0.31	0.29
350	0.275	0.375
360	0.20	0.43
370	0.135	0.39
380	0.08	0.270
400	0.03	0.055

TABLE 9. Rate of acid assisted ring opening of 2,5-bis(1-aziridinyl)-3,6-dimethoxy-1,4-benzoquinone

Time (min)	Optical density	$k \times 10^5$ (s^{-1})
0	0.08	—
30	0.11	9.15
60	0.135	9.04
90	0.154	8.97
120	0.169	9.08
150	0.185	9.03
210	0.211	8.75
270	0.225	9.04
330	0.240	9.14
420	0.251	9.13

buffer (of the appropriate p*H*) were added. The tubes were sealed with Parafilm and placed in a constant temperature bath. Several reaction tubes were withdrawn at convenient intervals, diluted with 25% THF – 75% H_2O to 5 ml and the concentration of 2,5-bis(2-acetoxyethylamino)-3,6-dimethoxy-1,4-benzoquinone was established by its absorbance at 380 nm. It was demonstrated that both the aziridinoquinone and the 2-acetoxyethylaminoquinone obeyed the Beer–Lambert Law in the optical density range of 0.02–0.4. Allowance was made for residual absorption at 380 nm due to the aziridine (see Table 8).

The reaction followed good first-order (pseudo unimolecular) kinetics for the formation of the diacetate the time dependence of which is shown in full for the run at p*H* 4.5 (see Table 9).

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