DNA−DNA Interstrand Cross-Linking by FR66979: Intermediates in the Activation Cascade

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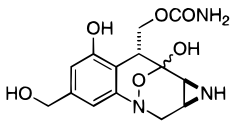
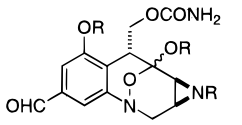
Abstract: The antitumor antibiotics FR66979 (**1**), FR900482 (**2**), and FK973 (**3**) are similar in structure and biological activity to the DNA cross-linking antitumor antibiotic mitomycin C (**4**). The cytotoxic effects of **1**−**3** have been proposed to result from sequential bioreductive cleavage of the N−O bond and condensation of the thus-exposed amine and ketone functions to yield an indole (e.g., **9**) which is structurally analogous to the mitosene nucleus of reductively activated mitomycins. We report herein evidence substantiating this proposal based upon study of the reductive activation chemistry of **1** and **2** using thiols and iron(II) in the absence and presence of DNA. Prolonged exposure of reductively activated **1** to sodium borohydride afforded the dihydroindole **11**, presumably through trapping of the iminium ion precursor (**16**). Kinetics measurements strongly implicate a relatively long-lived precursor to the iminium ion, which accumulates following iron(II)-catalyzed thiol-promoted reduction of **1**, proposed herein to be one or both of the isomeric aminals **12**. Under appropriate conditions, some step or steps between this intermediate and the iminium ion are shown to be rate limiting in DNA cross-linking, in production of the dihydroindole by borohydride trapping, and in the decay of the intermediate(s) competent to produce those same products. These studies clearly demonstrate the strong similarities in the cascade of reactions which follow reductive activation of FR66979 (**1**) [and presumably by extension FR900482 (**2**) and FK973 (**3**)] and the mitomycins.

Introduction

FR66979 (**1**),[1] FR900482 (**2**),[2] and FK973 (**3**)[3] are members of a relatively newly discovered family of antitumor antibiotics possessing a unique dihydrobenzoxazine structure. These



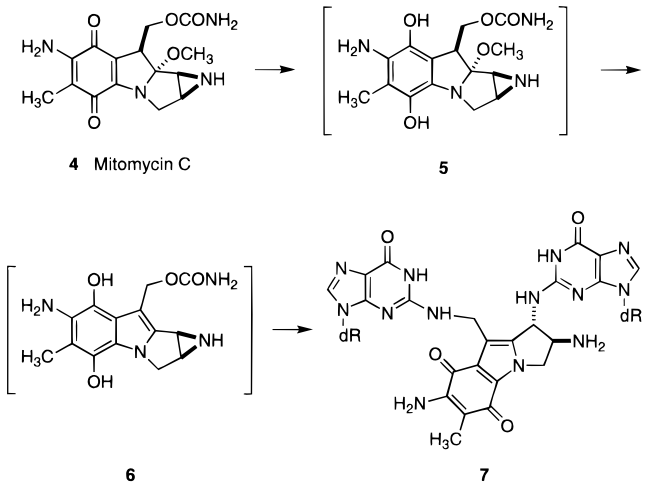
1 FR66979



2 R = H FR900482
3 R = Ac FK973

substances are similar in both structure and biological activity to the well-known antitumor antibiotics of the mitomycin family (e.g., **4**), and it is plausible that the two families share a similar mechanism of biological action. It is generally accepted that the mitomycins exert their cytotoxic effects by a mechanism involving covalent adducts with DNA.[4] Mitomycin C, for example, produces DNA monoadducts, intrastrand and interstrand cross-links in DNA, probably through the intermediacy of substances such as **5** and **6** or their close relatives, formed by sequential reduction of the quinone moiety and expulsion of the elements of methanol (Scheme 1).[4] This mechanism is

Scheme 1. Alkylation of DNA by Reductively Activated Mitomycin C



4 Mitomycin C **5** **6** **7**

supported by the characterization of DNA adducts such as **7** (as its peracetylated derivative),[5] the lesion responsible for interstrand cross-links, and by the identification of several intermediates formed upon in vitro reductive activation.[6]

It has been reported that both FR900482 and FK973 produce DNA interstrand cross-links in cultured murine L1210 tumor cells.[7] The observation that FK973 did not cross-link DNA in isolated nuclei was taken to indicate a requirement for cytoplasmic activation. To explain the DNA cross-linking activity of FR900482, Fukuyama and Goto proposed a reductive

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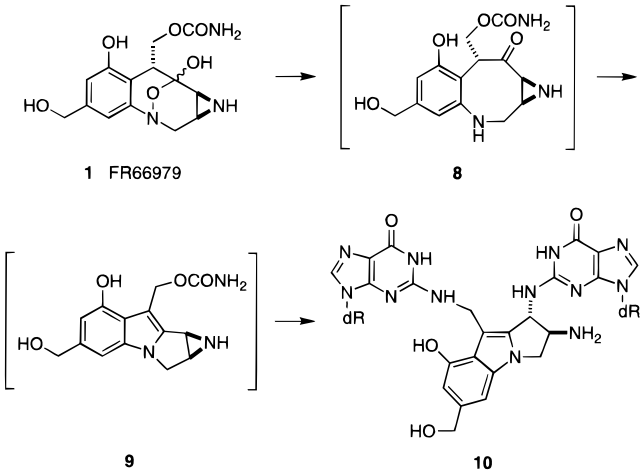
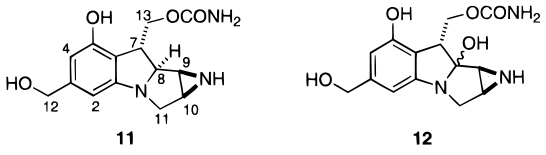
Scheme 2. Alkylation of DNA by Reductively Activated FR66979



activation mechanism analogous to that seen in the mitomycins.[8] Specifically, they proposed that reductive cleavage of the N−O linkage (shown in Scheme 2 for FR66979) affords an amino ketone (**8**). Intramolecular cyclization could then in principle provide a mitosene-like indole (**9**) as a reactive, bifunctional electrophile. In vitro studies of the DNA interstrand cross-linking activity of FR66979 and FR900482 have demonstrated the requirement for reductive activation, both sodium dithionite[9,10] and dithiothreitol[11−13] having been shown to be effective. Studies on the sequence specificity of **1** and **2** showed a strong preference for cross-linking at 5′-CG sites, as is well-known for mitomycin C.[9,12] That this cross-link bridges the N2 atoms of the diagonally adjacent deoxyguanosine residues (analogous to **7**) was shown by complete characterization of adduct **10** (as its peracetylated derivative) derived from **1**.[10]

The above findings all argue indirectly in favor of the analogy implicit in Schemes 1 and 2. This analogy posits the intermediacy of substances such as **8** and **9**; to date there has been no compelling characterization of the structures or reactivity of such intermediates. We report herein a study of the products of reductive activation of **1** and **2** in the absence and presence of DNA. We report the trapping of a putative iminium ion precursor to indole **9** by its reduction to the stable dihydroindole **11**. We also report the results of kinetics measurements which



strongly implicate a relatively long-lived precursor to the iminium ion, which accumulates following reduction of **1**. The available evidence suggests that this intermediate is one or both of the isomeric aminals **12**. Under appropriate conditions, some step or steps between this intermediate and the iminium ion are shown to be rate limiting in the *appearance* of the dihydroindole and DNA cross-links as well as in the *disappearance* of the intermediate(s) competent to produce those same products.

Results

Development of Conditions for Reductive Activation of 1 and 2. We attempted to reduce the N−O bond of **1** and **2** in the absence of DNA, but failed using thiols or sodium dithionite, reducing agents which had previously been reported to activate **1** and **2** for DNA interstrand cross-linking. This led us to reinvestigate the reductive activation of **1** and **2** in the presence of DNA, resulting in the discovery that the presence of iron(II) is critical for efficient DNA cross-linking with FR66979 or FR900482 with either of these agents.[14,15] The modest levels of DNA cross-linking which were observed when iron(II) was not explicitly added no doubt involved contaminating metal ions, as this cross-linking was virtually eliminated by the inclusion of EDTA. Likewise, iron(II) was critical for the reduction of FR66979 and FR900482 in the absence of DNA, as measured by their consumption and the appearance of reduction products. The requirement for metal ions explained the puzzling observation that in a mixture FR900482 and mercaptoethanol or DTT did not react with one another appreciably as monitored by NMR, despite the fact that thiols activate FR900482 for DNA cross-linking:[11] Sufficient levels of metal ion impurity must have been present to activate the traces of FR900482 required for a positive result in the highly sensitive cross-linking assay, but the level of iron(II) must have been insufficient to convert FR900482 to reduction products in quantities detectable by ^{1}H NMR.

Preliminary Structural Characterization of Products of Reductive Activation. A buffered aqueous solution of FR66979 was treated with DTT and catalytic iron(II) under anaerobic conditions at 25 °C. The progress of the reaction was monitored by HPLC. FR66979 was consumed completely in 8 h, producing two major compounds in a roughly 1:2 ratio that reached maximum intensity after about 5 h. After this time, the quantities of these major products decreased in favor of compounds with higher retention times, disappearing essentially completely after 48 h. Analogous reactions employing mercaptoethanol or dihydrolipoamide in place of DTT gave initially the same two compounds in the same ratio, but the HPLC retention times of the ultimate products differed from one thiol to the next. This suggested that the thiol was not incorporated in the initial products, but was incorporated in the final products.

The UV absorbance spectra of these two initially formed compounds, recorded from solutions freshly collected from the HPLC, were essentially identical with one another, with maximum absorbance at 232 nm, indicative of an indole substructure.[10] Electrospray LCMS performed on a mixture of FR66979 activated with DTT/iron(II) afforded essentially identical mass spectra for the two substances. We tentatively interpret the observed abundant ions at *m*/*e* 303, 287, and 247 (the latter being the largest peak above *m*/*e* 200) as the M + K^+, M + Na^+, and M + H^+ − H_2O ions originating from the diastereoisomeric pair **13**. Because it is possible that the mass spectrometric analysis is accompanied by hydrolysis reactions, other compositions are not excluded.[17] Unfortunately, all attempts to isolate those two compounds to permit further characterization were unsuccessful, owing to their instability.

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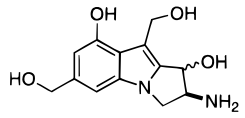

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A different picture of the contents of the reductive activation mixture emerged when it was prepared and analyzed differently. FR66979 could be activated with excess iron(II) (2.5 equiv) in the absence of any added thiol (pH 8.9 borate buffer), resulting in about 50% consumption of FR66979 and the appearance in the HPLC of the peaks previously tentatively assigned as diols **13**. Electrospray MS of the mixture *without* the liquid chromatography step afforded a much more complex mass spectrum than in the previous experiment. In addition to the $M + H^+$, $M + Na^+$, and $M + K^+$ ions for residual FR66979, this spectrum afforded ions of *m*/*e* 308 (consistent with $M + H^+$ for ketone **8** or the aminals **12**), 290 (consistent with $M + H^+$ for the mitosene analog **9**), and 229 (consistent with $M + H^+ - HOCONH_2$ for **9**). Remarkably, two of the ions characteristic of **13** (*m*/*e* 287, $M + Na^+$, and 303, $M + K^+$, from the LCMS) were *not* observed. The *m*/*e* 247 ion attributed above to $M + H^+ - H_2O$ for **13** was present in relatively reduced size, but this ion could also have been derived from hydrolysis of a less advanced intermediate. While these data did not reveal the details of the composition of the mixture, they did suggest that the mixture contained substances such as **8** or **12** which precede **13** in the reaction cascade (Scheme 3). We believe that the appearance of indoles such as the putative **13** in the HPLC analysis of the reduction mixture is an artifact of the analysis, and that some earlier, moderately stable intermediate(s) actually predominate in the activation mixture. This unusual circumstance was strongly supported by the series of experiments described below aimed at trapping this intermediate.

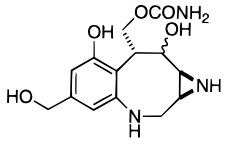
The activation of FR900482 under these conditions gave similar results. The HPLC analysis of a mixture of FR900482, DTT, and catalytic iron(II) revealed the formation initially of two major compounds in a 2:1 ratio and the LCMS showed the same mass spectrum for both compounds. The fragmentation pattern was equivalent to that obtained in the LCMS for the initial products from FR66979, except that each ion was lower in mass by 2 units.

Trapping of the Iminium Ion. To pursue the identity of the early products of reductive activation of FR66979, we attempted to immortalize some intermediate by trapping with sodium borohydride. HPLC analysis of a reaction mixture containing FR66979, DTT, iron(II), and sodium borohydride showed initially a product profile similar to the reaction without borohydride. Again, over a multi-hour period the putative diastereoisomers **13** disappeared, but in this case a new substance was produced.[18] This material could be isolated and manipulated without decomposition. Experiments employing other thiols (dihydrolipoic acid, mercaptoethanol) provided ultimately a peak with the same retention time, suggesting that thiol had not been incorporated. It was subsequently learned that this substance could be optimally produced (80−90% yield) by exposure of FR66979 to iron(II) and sodium borohydride, without the need for thiol.[19] The UV spectrum of this material showed a maximum at 218 nm, indicating that the aromatization to the indole substructure had not occurred.[10] The electrospray mass spectrum of this substance revealed proton, sodium, and potassium ion adducts consistent with a molecular weight of 291, and the only important fragments corresponded to the loss of carbamic acid ($-HOCOHN_2$). These data are consistent with the dihydroindole **11**, the stereochemistry of which was proven as described below.

Table 1. ^{1}H NMR Chemical Shifts for Aliphatic Carbon-Bound Hydrogens of **11**

	δ (ppm)		
hydrogen	pH 7.5	pH 5.8	$\Delta\delta$ (ppm)
H7	3.81	3.96	0.15
H8	3.96	4.36	0.40
H9	2.91	3.83	0.92
H10	2.81	3.72	0.91
H11a	3.44	3.84	0.40
H11b	3.27	3.62	0.35
H12	4.38	4.41	0.03
H13	4.26	4.28	0.02

The dihydroindole **11** was formed in a yield that allowed further characterization. Attempts to grow crystals of the putative **11** or acylated derivatives for X-ray analysis failed. Homonuclear ^{1}H NMR decoupling experiments clearly revealed the substructure $CH_2-CH-CH-CH-CH-CH_2$. Although consistent with structure **11**, this substructure is also present in the alcohols **14**, which could in principle arise from trapping



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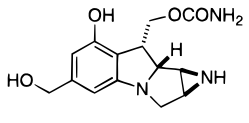
of **8** by borohydride. This ambiguity was resolved in favor of structure **11** by two observations. Firstly, the chemical shifts for the aliphatic, carbon-bound hydrogens were measured after acidification of the sample (Table 1). All protons separated by three or more bonds from a nitrogen atom (H7, H12, H13) shifted modestly to insignificantly (0.15 to 0.02 ppm), while those separated by two bonds (H8, H9, H10, H11) shifted more substantially downfield (0.92 to 0.35 ppm). Shifts of this magnitude, particularly that of H8 (0.40 ppm), are consistent with structure **11**, but not **14**. Secondly, peracetylation of this substance with acetic anhydride in pyridine afforded a triacetyl derivative (MS, ^{1}H NMR analyses), again consistent with **11** but not **14**.

The stereochemistry at the newly created chiral center C8 in **11** was assigned by NOE experiments (Table 2). Irradiation of H8 produced strong NOEs to protons H9 and H13, and a weaker NOE to H7. The reverse outcome would have been expected for **15**, the stereoisomer at H8.



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The isolation of dihydroindole **11** supports the conclusion that intermediates prior to indoles such as **13** in fact predominate in the reductive activation mixture, as it is implausible that any indole would revert to the dihydroindole **11** on exposure to sodium borohydride.[20] This again suggested that **13** was an

(17) For example, equally compatible with the UV and mass spectral data are the two diastereoisomers resulting from monohydrolysis of the scissile bond of the aziridine moiety of **9**.

(18) This situation is complicated by the chance occurrence that the new substance (**11**) and the same retention time on HPLC as the major diastereoisomer of the initial products (**13**).

(19) The presence of iron(II) was critical: FR66979 is essentially inert to treatment with sodium borohydride alone, as evidenced by the fact that this is one method of preparing FR66979 from FR900482.

Scheme 3. Reductive Activation Cascade for FR66979

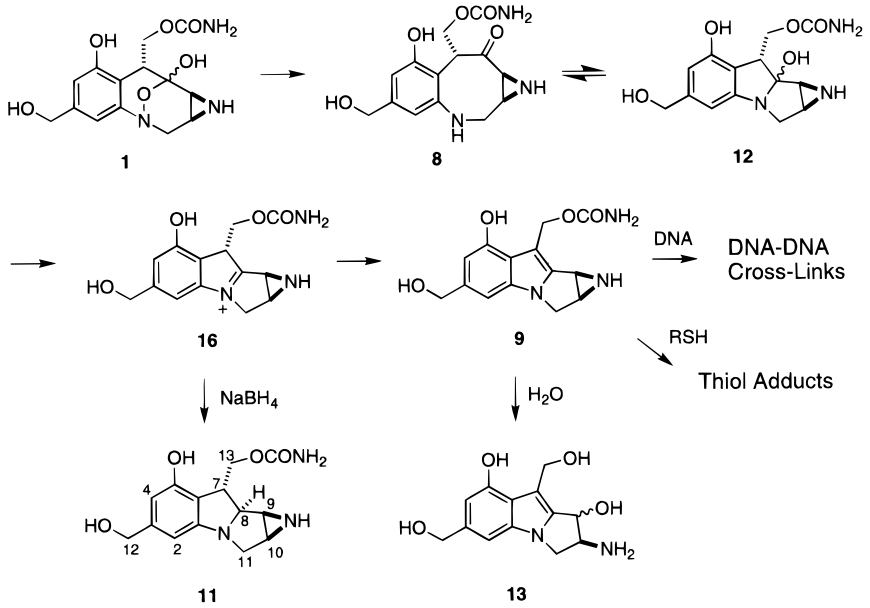


Table 2. NOE Data Distinguishing **11** from **15**

		interproton distance	
irradiated H	NOE (%)	**11**[a] (Å)	**15**[a] (Å)
H7	−1.7 (H8)	3.0	2.3
	2.2 (H9)	3.0	3.5
	5.1 (H13)	2.4	2.4
H8	0.9 (H7)	3.0	2.3
	3.9 (H9)	2.4	2.9
	2.3 (H13)	2.8	3.6
H9	1.7 (H7)	3.0	3.5
	4.5 (H8)	2.4	2.9
H13	7.3 (H7)	2.4	2.4
	2.8 (H8)	2.8	3.6

[a] From an energy-minimized computer model (AM1).

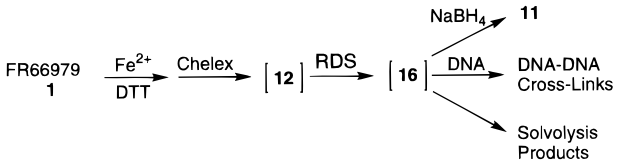
artifact of the HPLC analysis, and that some other relatively persistent intermediate or intermediates were rapidly hydrolyzed by HPLC to afford the putative **13**.

The findings described above are summarized in Scheme 3, which posits that reduction of FR66979 rapidly affords one or both of the aminals **12**, which, in this pH range, is slowly converted to iminium ion **16**. In the presence of sodium borohydride, **16** is trapped to yield the dihydroindole **11**, which has been rigorously characterized. In the absence of borohydride, it escapes to form the mitosene analog **9**, which is captured by the nucleophiles DNA, thiol, or water to form adducts. The conversion of **12** to indolic substances is evidently accelerated by exposure to HPLC conditions.

A Common Rate Limiting Step for Dihydroindole Production and DNA Cross-Linking. We hypothesized that some step involved in the conversion of the stable intermediate (**12**?) to the iminium ion **16** might in fact be rate limiting for the variety of products formed in the presence of various nucleophiles, as summarized in Scheme 4. The outcome of the following experiments supports this hypothesis. A single rate limiting step accounts for the rates of (a) appearance of dihydroindole **11** in the presence of sodium borohydride, (b) appearance of DNA−DNA interstrand cross-links in the presence of DNA, and (c) decay of substances competent to form the dihydroindole or DNA−DNA interstrand cross-links. The simplest possibility is that this rate determining step is expulsion of hydroxide from one isomer of **12**, but other possibilities are not excluded.

(20) This was confirmed by exposing freshly collected "**13**" from the HPLC to excess sodium borohydride, with the result that no **11** was produced.

Scheme 4. A Unifying Hypothesis That the Production of Dihydroindole **11**, DNA Interstrand Cross-Linking, and Decay of the Active Intermediate by Solvolysis Share a Common Rate Determining Step (RDS)



The kinetics of these reactions were probed by reductively activating FR66979 for a period of time, then stopping the activation by removal of iron(II) and measurement of (a) the rate of *appearance* of the dihydroindole in the presence of excess sodium borohydride, (b) the rate of *appearance* of DNA interstrand cross-links in the presence of DNA, and (c) the rate of *disappearance* of intermediates capable of producing dihydroindole or DNA interstrand cross-links (Scheme 4). FR66979 was treated with DTT/iron(II) in Tris buffer at pH 8.9 containing deoxythymidine as an inert, internal standard. When HPLC analysis indicated that the consumption of FR66979 was higher than 90% (typically 5−6 h) the reaction mixture was filtered through Chelex resin to remove iron, which halted the consumption of residual FR66979. The resulting sample of "activated FR66979" was treated as described in A−C below. In all cases, the appearance or disappearance rate monitored could be satisfactorily fit to a first-order rate law. Data and a summary of the observed rate constants (k_{obs}) are shown in Figure 1 and Table 3, respectively.

(A) The activated mixture at pH 8.9 was treated with a large excess of sodium borohydride, and the rate of *appearance* of dihydroindole **11** was determined by HPLC. This provided k_{obs} of 3.1×10^{-3} min^{-1}. This rate was unchanged on doubling of the sodium borohydride concentration, indicating that the reduction reaction involving sodium borohydride was not rate limiting. The long-lived intermediate and the rate limiting step for dihydroindole formation must therefore *precede* the iminium ion **16** (Scheme 3).[21]

(B) The activated mixture at pH 8.9 was treated with a large excess of an oligodeoxynucleotide duplex containing an FR66979-

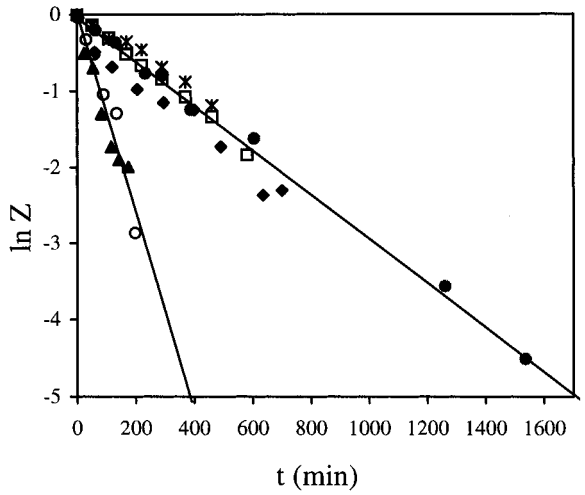


Figure 1. Kinetics measurements: (◆) Appearance of dihydrindole **11** (pH 8.9), $Z = 1 - [\mathbf{11}]_t/[\mathbf{11}]_{t_\infty}$; (●) appearance of DNA interstrand cross-links (pH 8.9), $Z = 1 - [X]_t/[X]_{t_\infty}$, where X is the yield of interstrand cross-links at time *t*; (□) decay of yield of **11** by aging a reductively activated mixture (pH 8.9) prior to exhaustive incubation with sodium borohydride, $Z = [\mathbf{11}]_t/[\mathbf{11}]_{t_0}$, where *t* is the length of aging prior to adding sodium borohydride; (▲) same as previous, except at pH 7.5; (*) decay in yield of interstrand cross-links by aging reductively activated mixture (pH 8.9) prior to exhaustive incubation with DNA, $Z = [X]_t/[X]_{t_0}$, where X is the yield of interstrand cross-links and *t* is the length of aging prior to adding DNA; (○) same as previous except at pH 7.5. Indicated lines are the least-squares best fit through *all* data points at a given pH.

Table 3. Observed Rate Constants

expt[a]	pH	k_{obs} 10^{-3} (min^{-1})	R^2
A	9.5	3.1[b]	0.97
B	8.9	2.9[c]	0.99
C	8.9	2.9[b]	0.99
C	8.9	2.5[c]	0.98
C	7.5	12.0[b]	0.96
C	7.5	13.8[c]	0.94

[a] See text. [b] Values determined from integration of HPLC peaks corresponding to **11** and dT (internal standard). [c] Values determined from phosphorimager quantitation of DPAGE.

cross-linkable [5′-d(CG)]$_2$ site, and the rate of appearance of DNA interstrand cross-links was monitored by denaturing polyacrylamide gel electrophoresis. Cross-links appeared with a k_{obs} of 2.9×10^{-3} min^{-1}, in good agreement with the rate measured in (A). Thus, it appeared possible that the rate limiting step in DNA cross-linking, like dihydroindole production, was a step *subsequent* to the long-lived intermediate, and *preceding* the iminium ion (Scheme 3).

(C) We measured the rates at pH 8.9 and 7.5 of the *disappearance* of substances competent to yield dihydroindole **11** on trapping with sodium borohydride or competent to yield DNA cross-links on trapping with DNA. These were accomplished by aging for various lengths of time the unadulterated activated mixture, then quenching for an extended time with either sodium borohydride or DNA, and measuring the yield of dihydroindole **11** or cross-linked DNA, respectively. At pH 8.9, the rates of decay of the yield of dihydroindole and cross-linked DNA were 2.9×10^{-3} and 2.5×10^{-3} min^{-1}, respectively. The good agreement of these values with those measured for appearance of the dihydroindole and DNA cross-links is again consistent with a single rate limiting step for all of these processes. The decay of the competence of the solution to produce dihydroindole **11** indicates that in the absence of sodium borohydride, steps subsequent to the rate determining step *rapidly* and *irreversibly* progress beyond the iminium ion **16**. The decay of competence to produce interstrand cross-links indicates that in the absence of DNA, steps subsequent to the rate determining step *rapidly* and *irreversibly* progress beyond the mitosene analog **9**. At pH 7.5, the rate of decay of competence for dihydroindole production was 12.0×10^{-3} min^{-1}; at this pH, decay of cross-linking activity occurred at 13.8×10^{-3} min^{-1}. Decay of the active intermediate was thus accelerated with decreasing pH in this range.[22,23]

Discussion

The discovery of the catalytic effect of iron(II) on the reduction of the N−O bond of FR66979 was crucial to the structural and kinetics studies described herein. The isolation of **11** from the activation of FR66979 in the presence of sodium borohydride provided the first characterization of any intermediate, in this case the iminium ion **16**, enroute from FR66979 to solvolysis products or DNA adducts. As described in more detail below, from the kinetics measurements, it was clear that reduction of FR66979 with thiols and catalytic iron(II) generates one or more relatively long lived intermediates which are capable of generating the iminium ion **16**, and in turn trapped by sodium borohydride to form the dihydroindole **11**. This sequence of events is summarized in Scheme 3.

Based largely upon the kinetic evidence, we believe that the most reasonable candidate for the stable intermediate is one or both of the aminals **12**. Firstly, the observations that the rate of dihydroindole **11** production is independent of sodium borohydride concentration, has a relatively long half life of several hours at pH 8.9, and is pH dependent all argue that the stable intermediate *precedes* the iminium ion, making one or both of the aminal diastereoisomers **12** the most reasonable candidates for the long-lived intermediate. Secondly, ketone **8** is unlikely to be this abundant intermediate, because it should react more rapidly, with a rate that is dependent upon sodium borohydride concentration, and provide one or both of the alcohols **14**, none of which is observed to be the case. The remote possibility that ketone **8** is inert to sodium borohydride and that its conversion to iminium ion **16** is rate limiting cannot be rigorously excluded.

These kinetic observations likewise narrow considerably the range of possibilities for the rate determining step. The simplest explanation for the identity of all the rates described in (A) through (C) is that, at these borohydride and DNA concentrations, a single rate determining step is common to all of the measured processes. This step must precede the iminium ion **16**, as evidenced by the production of the dihydroindole **11** being controlled by this rate determining step at a rate independent of sodium borohydride concentration. Given this and the high probability that **12** is the abundant intermediate, only a modest

(21) This rate constant is probably the least reliable of those we have measured, because in the course of the incubation the pH rose from 8.9 to roughly 9.5.

(22) A variant of this experiment provided support for the hypothesis that the hydrolysis of the relatively long-lived intermediate was accelerated by exposure to the packing material of an HPLC column. The reductively activated sample was aged *in the presence of some C-18 silica gel* from a used HPLC column, resulting in roughly a doubling of the rate at which the intermediate was diverted to materials incapable of being converted to dihydroindole **11** during subsequent treatment with sodium borohydride.

(23) The proposal that the indoles (the putative diastereoisomers **13** or their indole predecessors) observed in the HPLC analysis of activated FR66979 are in reality the signature of an active, long-lived intermediate which precede the iminium ion in Scheme 3 suggests that their rate of disappearance should be identical with the rates measured in (A) through (C) above. This was found to be the case: the rate of disappearance of the putative diols **13** was monitored by HPLC, affording rate constants k_{obs} of 3.1×10^{-3} min^{-1} at pH 8.9 and 12.7×10^{-3} min^{-1} at pH 7.5.

number of possibilities remain for the rate determining step(s). The simplest among these is that a single diastereoisomer of **12** is the vast majority of the stable intermediate, and the rate controlling step is the acid-catalyzed[24] expulsion of the hydroxyl group of the aminal to yield the iminium ion **16**. In the more complex possibilities, the production of iminium ion **16** would require the interconversion of the two diastereoisomers of **12** through ketone **8**. (This interconversion cannot proceed through the iminium ion **16**, given that the rate of dihydroindole production is independent of sodium borohydride concentration.) This would arise, for example, if one isomer of **12** was greatly predominant, but its diastereoisomer more readily progressed to iminium ion **16**. In these cases, the observed rate would depend upon the rate of some combination of the steps **16** ← **12a** ↔ **8** ↔ **12b** → **16**. These schemes would need to account for the failure of the borohydride trapping experiments to provide the reduction product **14** from ketone **8**. None of the available data distinguish among these various possibilities.

Conclusions

These studies extend the body of information indicating strong similarities in the chemistry and biochemistry of FR66979 (**1**) [and presumably by extension FR900482 (**2**) and FK973 (**3**)] and the mitomycins, in particular the best studied member of that class, mitomycin C (**4**). For mitomycin C, it is amply demonstrated that reductive activation, in this case of the quinone function, exposes a masked indole substructure, affording a mitosene that acts as a bifunctional electrophile, alkylating and cross-linking DNA. A related pathway for FR66979 and FR900482, in this case initiated by reduction of the N−O bond, has previously been inferred from the fact of the identity of the nucleotide sequences which are interstrand cross-linked by **1**, **2**, and **4** [5′-(CG)].[9,12] The common structural elements present in the lesions responsible for these cross-links (cf., **7** from mitomycin C, **10** from FR66979) lend further support to the case. The proposal herein that the relatively stable intermediate formed upon reductive activation of FR66979 at pH 8.9 has the structure **12**, a structural analog of mitomycins, does not substantially extend the proof of analogy, because the structural characterization of **12** rests largely on the analogy itself. In contrast, the well-characterized dihydroindole **11** reported herein to result from trapping of the relatively stable product of reductive activation does extend the analogy. The isolation of **11** provides substantive proof that the masked indole substructure of FR66979 is revealed prior to its electrophilic (alkylation) reactions. The finding that the rate of production of the dihydroindole **11** is identical with the rate of decay of the stable intermediate as well as the rate of production of interstrand cross-links provides compelling evidence that the indole intermediate is on the mechanistic pathway leading to alkylation and cross-linking of DNA, rather than being some adventitious side process.

We note in closing that these studies raise the question of the mechanism of the in vivo reductive activation of these antitumor antibiotics. It is tempting to speculate that a metal ion containing enzyme may play a critical role. The finding herein that the product of reductive activation of FR66979 is an intermediate which at physiological pH possesses a half-life of roughly 1 h suggests that this activation need not occur in close proximity to the DNA target, consistent with the earlier conclusion for FK973 that the activation occurs in the cytoplasm rather than the nucleus.[7]

(24) (a) Koehler, K.; Sandstrom, W.; Cordes, E. H. *J. Am. Chem. Soc.* **1964**, *86*, 2413. (b) Benkovic, S. J.; Benkovic, P. A.; Comfort, D. R. *J. Am. Chem. Soc.* **1969**, *90*, 1860.

Experimental Section

Materials and Methods. Materials and their sources were as follows: DNA synthesis reagents were from Applied Biosystems. FR900482 was a gift from Fujisawa Pharmaceutical Co., Ltd. (Japan). FR66979 was prepared by reduction of FR900482 with $NaBH_4$ as reported.[12] Water was purified on a Millipore Milli-Q deionizer. All other reagents were commercial and used as received. Unless otherwise specified, solutions were aqueous. Samples were concentrated on a Savant Speed Vac concentrator. The Tris buffer for cross-linking reactions was 200 mM Tris/Tris·HCl (pH 7.5). The loading buffer was 90% aqueous deionized formamide containing 10 mM Tris/Tris·HCl (pH 7.5) and 1 mM Na_2EDTA. The 1 × TBE buffer was 90 mM Tris/90 mM boric acid (pH 8.9) and 1.8 mM Na_2EDTA. The oligonucleotide was synthesized on an Applied Biosystems Model 392 synthesizer and purified by preparative DPAGE. UV spectra were measured on a Hewlett-Packard 8452A or a Perkin-Elmer Lambda 3A spectrophotometer. Nuclear magnetic resonance spectra were measured on a Bruker AM300 (300 MHz) or Bruker AM500 (500 MHz) spectrometer and are reported in parts per million from internal H_2O (4.67 ppm). Electrospray ionization mass spectra (ESIMS) were measured on a FISONS VG Quattro II, triple quadrupole model with a megaflow electrospray source at a voltage of 25−35 V. LCMS was performed in the previously described mass spectrometer, connected to an HPLC system formed by a Shimadzu LC-10AD controller and a Shimadzu SPD-10AV UV/vis detector. Except where otherwise noted, selected ions, diagnostic for the substance of interest, are reported. High resolution mass spectra (HRMS) were obtained on a FISONS VG 70SEQ tandem hybrid mass spectrometer. Analytial and semipreparative HPLC was performed on a Keystone, 5 mm, C18, 250 mm × 10 mm Econosphere or an Alltech, 5 mm, C18, 250 mm × 4.6 mm column, using an SSI 200B/220B dual pump system with an SSI controller and sequential SSI 500 UV/vis (output to a Linear 255/M recorder and an HP 3390A electronic integrator) detector. The solvent was run at 2 mL/min. Gradient (solvent A, 100 mM ammonium acetate (pH 7.0); solvent B, 75% A, 25% CH_3CN): isocratic 97% A for 4 min, 11 min linear gradient to 75% A, 10 min linear gradient to 60% A, 5 min linear gradient to 100% B, 10 min 100% B, the 10 min linear gradient to initial conditions.

Activation of FR66979 and FR900482 with Thiols and Fe(II). The procedure presented here for the DTT/Fe(II) activation of **1** is representative of the analytical scale HPLC and LCMS experiments. Other experiments, involving different reducing agents or **2**, were performed similarly. FR66979 (40 μL of a 30 mM solution in H_2O, 1.2 μmol) was diluted with 40 μL of 200 mM Tris buffer (pH 8.9), and the mixture was deaerated by bubbling N_2 through the solution for 10 min. DTT (11 μL of a 225 mM solution in deaerated H_2O, 2.4 μmol) and $Fe(SO_4)_2(NH_4)_2$ (25 μL of a 2.5 mM solution in deaerated H_2O, 62 nmol) were then added. The mixture was stirred at 25 °C. Aliquots of the mixture were withdrawn *via* syringe and analyzed by HPLC using the gradient described above.

Dihydroindole 11. A solution of FR900482 (11 mg, 34 μmol) in H_2O (1.0 mL) was treated with $NaBH_4$ (10 mg, 300 μmol) and stirred for 1 h. After this period, TLC analysis showed complete conversion to FR66979. The resulting mixture was deaerated by bubbling Ar through the solution for 10 min, and a solution of $Fe(SO_4)_2(NH_4)_2$ (1.0 mg, 2.5 μmol) in deaerated H_2O (50 μL) was added. The mixture was stirred at 25 °C for 24 h. The excess of $NaBH_4$ was destroyed by addition of 0.2 mL of 100 mM HCl. A precipitate appeared, and was removed by centrifugation, washing the precipitate with H_2O (2 × 500 μL). The combined supernatants were concentrated to a volume of 200 μL and purified by HPLC using the gradient described above. The fractions containing the compound (retention time 30.5 min) were collected and concentrated to give **12**·AcOH (9.9 mg, 28 μmol, 82%) as a white solid, ^{1}H NMR (D_2O, pH 7.5) δ (ppm) 2.81 (dd, J = 2 Hz, 4.8 Hz, 1 H, H-10), 2.91 (dd, J = 2 Hz, 5 Hz, 1 H, H-9), 3.27 (dd, J = 2 Hz, 13 Hz, 1 H, H-11b), 3.44 (d, J = 13 Hz, 1 H, H-11a), 3.81 (dd, J = 4 Hz, 2 Hz, 1 H, H-7), 3.96 (t, J = 2 Hz, 1 H, H-8), 4.26 (m, 2 H, H-13), 4.38 (s, 2 H, H-12), 6.17 (s, 1H, H-2), 6.27 (s, 1H, H-4); ^{1}H NMR (D_2O, pH 5.8) δ (ppm) 3.67 (dd, J = 2 Hz, 14.2 Hz, 1 H, H-11b), 3.72 (dd, J = 2 Hz, 5 Hz, 1 H, H-10), 3.83 (dd, J = 2 Hz, 5 Hz, 1 H, H-9), 3.84 (d, J = 14 Hz, 1 H, H-11a), 3.96 (m, 1 H, H-7), 4.28 (m, 2 H, H-13), 4.36 (t, J = 2 Hz, 1 H, H-8), 4.41 (s, 2 H, H-12),

6.26 (s, 1H, H-2), 6.34 (s, 1H, H-4); ^{13}C NMR (D_2O, off-resonance ^{1}H decoupled) δ (ppm) 36.9 (CH), 39.4 (CH), 47.9 (CH), 53.9 (CH_2), 64.5 (CH_2), 66.8 (CH_2), 69.4 (CH), 103.3 (CH), 108.1 (CH), 115.9 (C), 144.6 (C), 153.8 (C), 158.4 (C), 160.7 (C); HRMS calcd for $C_{14}H_{18}N_3O_4$ (**11**·H^+) 292.1297, found 292.1290.

Triacetyl Derivative of 11. A solution of **11** (2 mg, 6.8 μmol) in pyridine (0.25 mL) was treated with excess Ac_2O (40 μL) and stirred for 3 h. After this period, HPLC analysis of an aliquot showed the formation of a single compound. The resulting mixture was concentrated to dryness, and the residue was redissolved in toluene (0.3 mL) and concentrated again to give a yellow solid. ESIMS: *m*/*e* 456 (M + K^+), 440 (M + Na^+), 418 (M + H^+); ^{1}H NMR (D_2O) δ (ppm) 1.88 (s, 3 H), 2.00 (s, 3 H), 2.25 (s, 3H), 3.26 (dd, J = 1 Hz, 13 Hz, 1 H, H-11b), 3.41 (dd, J = 1 Hz, 5 Hz, 1 H, H-10), 3.51 (dd, J = 2 Hz, 4 Hz, 1 H, H-9), 3.62 (d, J = 13 Hz, 1 H, H-11a), 3.84 (m, 1 H, H-7), 3.93 (t, J = 2 Hz, 1 H, H-8), 4.16 (m, 2 H, H-13), 4.91 (s, 2 H, H-12), 6.51 (s, 1H, H-2), 6.52 (s, 1H, H-4).

Activation of FR66979 and Kinetic Measurements. A solution of FR66979 (40 μL of a 30 mM solution in H_2O, 1.2 μmol) was admixed with dT (10 μL of a 5 mM solution in H_2O) and Tris buffer (pH 8.9, 40 μL of 200 mM). The mixture was deaerated by bubbling N_2 through the solution for 10 min. DTT (11 μL of a 225 mM solution in deaerated H_2O, 2.4 μmol) and $Fe(SO_4)_2(NH_4)_2$ (25 μL of a 2.5 mM solution in deaerated H_2O, 60 nmol) were then added. The mixture was stirred at 25 °C until HPLC showed a conversion greater than 90% (typically 5−6 h). The resulting mixture was then filtered through Chelex 100TM 200−300 mesh, sodium form (Biorad) using Alltech micro-spin centrifuge filters−Nylon 66. The resulting solution was used directly for the experiments at pH 8.9 or acidified to pH 7.5 with 100 mM HCl for the experiments at that pH.

(a) Experiment A: An aliquot of the mixture was treated with excess $NaBH_4$. Aliquots of this reaction mixture were withdrawn at time intervals, quenched with 100 mM aqueous HCl, and kept at −20 °C until the moment of being analyzed by HPLC. Retention times (Alltech column): dT, 21 min; **11**, 29 min. The k_{obs} was calculated assuming a t_∞ of 24 h.

(b) Experiment B: The mixture (30 μL) was incubated with partially 5′-^{32}P-phosphoryl-radiolabeled 5′-[d(TATAATACGTATTATA)]$_2$ (2 OD units) diluted to 100 μL with Tris buffer at pH 8.9. Aliquots of this mixture (10 μL) were removed and admixed with cold oligonucleotide (1 OD unit) and cooled to and stored at −20 °C. The DNA was precipitated by addition of 100 μL of 0.3 M NH_4OAc (pH 5.2) and 800 μL of absolute EtOH. The sample was centrifuged, the supernatants were separated, and the resulting pellet was dried. The pellets of DNA were dissolved in 20 μL of a 1:1 mixture of water and loading buffer and analyzed by DPAGE as described by Kirchner et al.[16] Quantitation was performed by phosphorimagery. The yield of cross-link at t_∞ was calculated by optimization of the fit of the data to a first-order decay.

(c) Experiments C: Aliquots of the mixture were removed at time intervals, treated with excess $NaBH_4$ for 24 h, then quenched with 100 mM aqueous HCl and analyzed by HPLC. Retention times (Alltech column): dT, 21 min; **11**, 29 min. Alternatively, aliquots of the reaction mixture (3 μL) were removed at time intervals and incubated with partially 5′-^{32}P-phosphoryl-radiolabeled 5′-[d(TATAATACGTATTATA)]$_2$ (0.2 OD units) diluted to 20 μL with Tris buffer at pH 7.6. The mixture was vortexed and incubated at 25 °C for 24 h. The DNA was precipitated by addition of 100 μL of 0.3 M NH_4OAc (pH 5.2) and 800 μL of absolute EtOH. The sample was centrifuged, the supernatants were separated, and the resulting pellet was dried. The pellets of DNA were dissolved in 20 μL of a 1:1 mixture of water and loading buffer and analyzed by DPAGE as described by Kirchner et al.[16] Quantitation was performed by phosphorimagery.

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