DNA Cross-Linking Agents as Antitumor Drugs

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Contents

1. Introduction

The double helical structure of deoxyribonucleic acid (DNA) represents the richest source of information within a living organism. Importantly, its sequence codes not only for protein/enzyme synthesis via the process of translation, but it also codes for RNA synthesis, which, in light of the discovery of ribozymes, is likely to play a much larger cellular role than previously believed.[1] Composed of the four nucleotides deoxyadenosine, deoxyguanosine, deoxycytidine, and deoxythymidine (dA, dG, dC, and dT respectively), DNA is a rather uniform structure when compared to proteins for which there are 20 amino acids to choose from upon translation. The vast majority of naturally occurring DNA is in the canonical B-form, consisting of the two polynucleotide strands wound about a common axis with a right-handed twist (Figure 1).[2] The duplex is roughly 20 Å in diameter with the two strands running antiparallel to each other.[2] The core contains the nitrogenous bases while the sugar−phosphate chains occupy the periphery so as to minimize electrostatic repulsions between the two strands.

Two hydrogen bonding motifs exist for DNA: the Watson and Crick model, which is by far the most relevant in considering B-DNA duplexes; and the Hoogsteen motif, which is favored under slightly acidic conditions (Figure 2).[3] Hoogsteen bonding also requires multivalent cations in order to reduce electrostatic repulsions between the phosphate backbone of the Watson−Crick duplex and the negatively charged backbone of the "triplex" forming polypyrimidine strand.[3]

Hydrogen bonding is crucial to the ability of the two strands to stay annealed to each other, which, in turn, is critical for a host of macromolecular interactions necessary for the survival of the organism. Some of these types of interactions involve the recognition of specific DNA duplex structures by proteins responsible for tumor suppression,[4] transcription activation,[5] and DNA repair,[6] to name just a few. Equally important is the ability of the two strands to separate from one another.

The necessity that the DNA molecule be capable of undergoing strand separation was first hinted at by Watson and Crick in the statement: "It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material."[7] Not long after their ground-breaking paper in which this comment appeared, they did indeed demonstrate that one DNA strand could act as the template for synthesis of the complementary strand.[8] Since then a great deal has been learned about the intricacies of DNA replication. A host of different enzymes are involved and numerous functions must be performed. At its most rudimentary level of understanding (and that most

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relevant to the issue of DNA interstrand cross-linking), DNA is replicated by DNA polymerases which utilize single-stranded DNA as a template upon which the complementary strand is synthesized.[9] This synthesis is dictated by Watson−Crick base pairing of an incoming nucleotide with the template strand thus affording a new strand capable of annealing with the original template. Absolutely crucial is the progressive separation of the two parental strands leading to the synthesis of their complements yielding two semiconservatively replicated "daughter" strands.[10] The point at which parental strand separation occurs is referred to as the replication fork. The replication fork has held the interest of biologists and chemists alike in that its viability is critical to DNA replication. To block it is to halt replication, thus inflicting a catastrophic event upon the cell.[11]

The vast majority of clinically employed alkylating agents behave as electrophilic traps for macromolecular nucleophiles. Such nucleophiles often include amino acids such as cysteine, lysine, tyrosine, and threonine. Additionally, the nucleobases of DNA and RNA represent likely targets. It is often the case that bifunctional alkylating agents may give rise to a number of different lesions, including those depicted in Figure 3.[12] Although knowledge of the biological consequences of such lesions is increasing, it is generally agreed that the formation of interstrand cross-links represents by far the most toxic of all alkylation events, since this results in seizure of the replication fork.[11]

That interstrand cross-links (ISCs) completely shut down replication has been demonstrated on several occasions. In 1969 Lawley et al. found that T7 phage were much less infective in an *Escherichia coli* host after reaction with the sulfur mustard di-2-chloroethyl sulfide than by its monohalogenated analogue.[13] Phage inactivation occurred with an average of seven monoalkylations per phage resulting in 1.3 equivalents of interstrand cross-link (note that not all monoalkylations can result in cross-links due to geometric constraints). However, utilizing the singly electrophilic mustard, a total of 280 alkylations per phage were required to achieve a similarly low level of infectivity. More recently, Patierno and Manning have demonstrated the DNA base-specific arrest of T7 DNA polymerase at sites of chromium-mediated interstrand cross-linking.[14] Utilizing ascorbate reduction of hexavalent chromium to the trivalent state (5−60 μM in Cr(III)) between 2 and 40 chromium adducted sites were formed per 1000 nucleotides (nts) of substrate DNA. Of these lesions 18.5% resulted in polymerase arrest.

Chromium binds not only the nucleobases, but also the phosphate backbone (giving rise to interhelical binding events). However, only the chromium-bound bases were capable of polymerase arrest and of these, only the interstrand cross-link sites terminated polymerase activity, thus accounting for the 18.5% "arrest ratio".

DNA interstrand cross-linking agents comprise an extremely important class of clinical agents not only in the treatment of cancers, but also for diseases such as psoriasis, and various anemias.[15] Their ability to induce DNA−protein cross-links has also been ex-

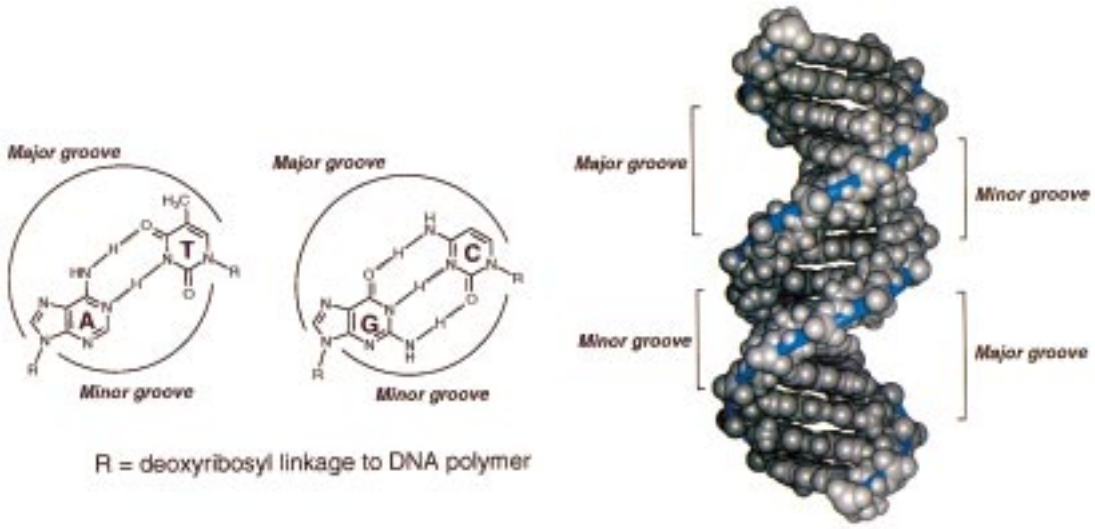


Figure 1. Depiction of major and minor grooves within B-DNA as viewed from top to bottom of the duplex. Grooves are defined with respect to the glycosyl linkage of each base to its respective deoxyribose. See Figure 4 for base numbering scheme.

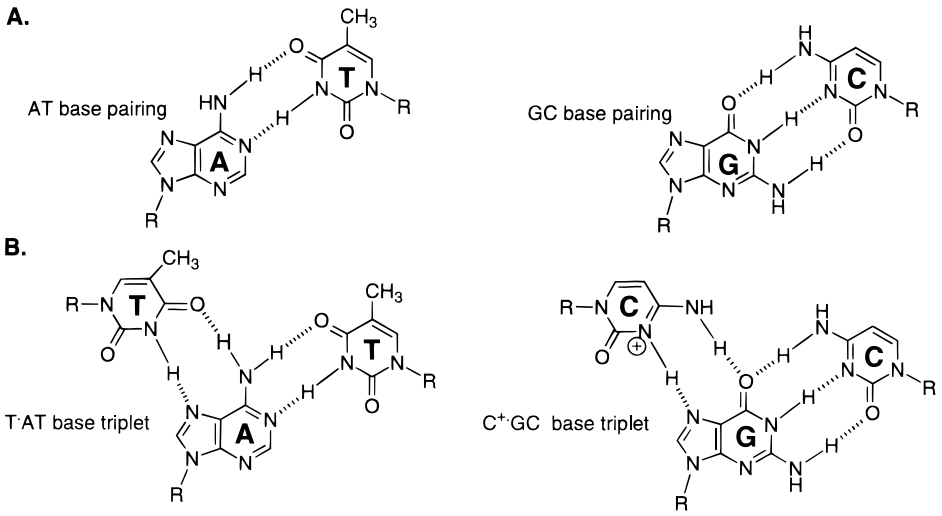


Figure 2. (A) Watson−Crick AT and GC base pairs and (B) Hoogsteen hydrogen-bonding motif for pyrimidines bound in the major groove to the Watson−Crick-bonded GC and AT pairs.

ploited in molecular recognition studies aimed at understanding DNA−protein interactions.[11c,16] The importance of these agents and their biological activities has, in many cases, been known for several decades. For example, DNA−DNA interstrand and DNA−protein cross-linking was achieved in the early 1960s.[17] However, the ability to elucidate the functionalities involved at the atomic level has been realized only recently, due largely to the advent of solid-phase oligodeoxyribonucleotide (ODN) synthesis, and the discovery of restriction endonucleases. Arguably, the isolation of drug−DNA complexes following enzymatic digestion of large DNAs (bacterial plasmids, calf thymus, etc.) represents an exception to this statement. However, the inherent instability and extremely low yields of many interstrand lesions has hampered characterization via this method. Additionally, the enzymatic digestion/isolation route has only provided information pertaining to the nucleotide−drug−nucleotide complex but not the influence of flanking sequences or tertiary structure of the target DNA. In this vein, solid-phase ODN synthesis and exploitation of restriction endonucleases has allowed studies aimed at understanding the interactions necessary for interstrand cross-linking by many agents about which very little was previously known. This knowledge has been used to design more efficient and/or selective agents and has also allowed the design of cross-linking agents from the simple dimerization of DNA monoalkylating agents. Triple helix formation has also been employed in the design of cross-linking agents vastly more discriminating in selectivity than the parent compound. Given this, it seems surprising that no reviews have appeared in the literature pertaining to cross-linking agents. This review, in addition to providing some background regarding the significance of DNA interstrand cross-linking, seeks to provide a brief overview of compounds (or classes thereof) capable of DNA cross-linking. Particular emphasis has been placed upon the molecular recognition studies of the last 10 years, a great deal of which has been possible for the reasons stated above. The four subsections cover (1) inherently labile

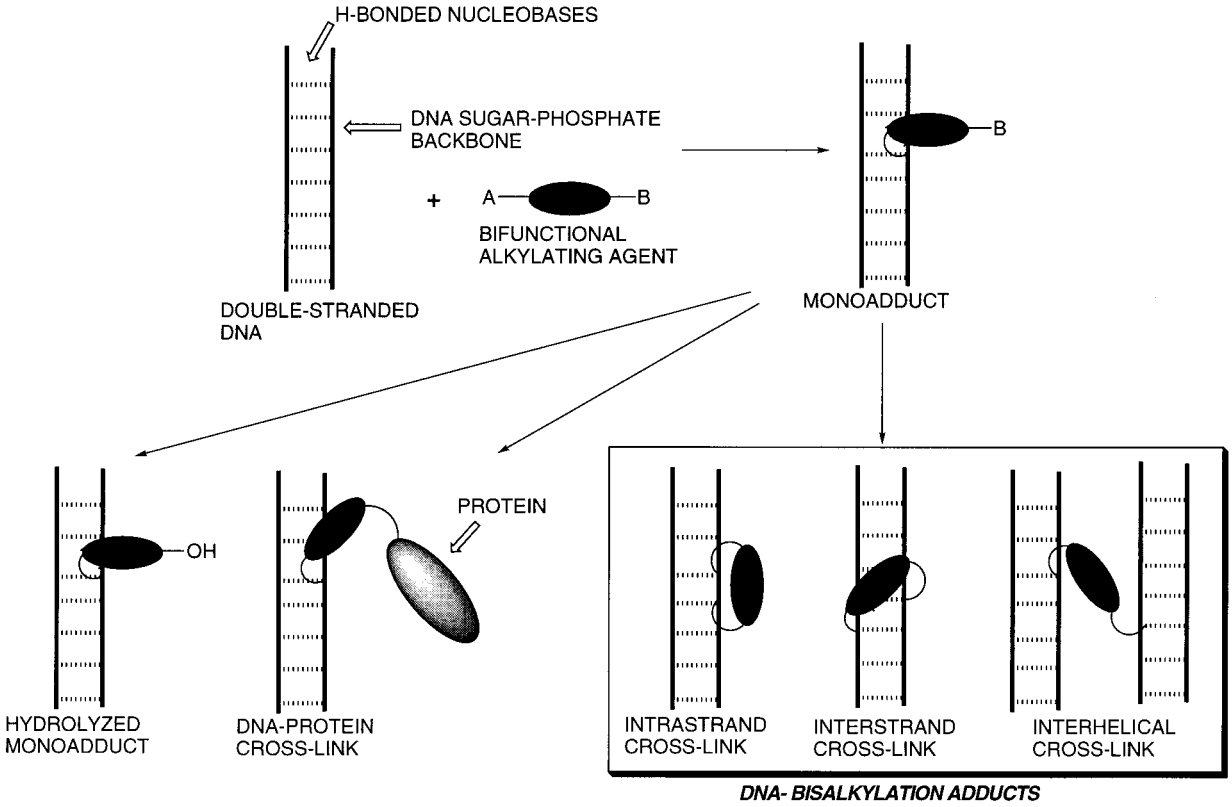


Figure 3. Mechanistic pathway for DNA functionalization by interstrand cross-linking agents. A and B represent electrophilic moieties within the cross-linking agent of interest.

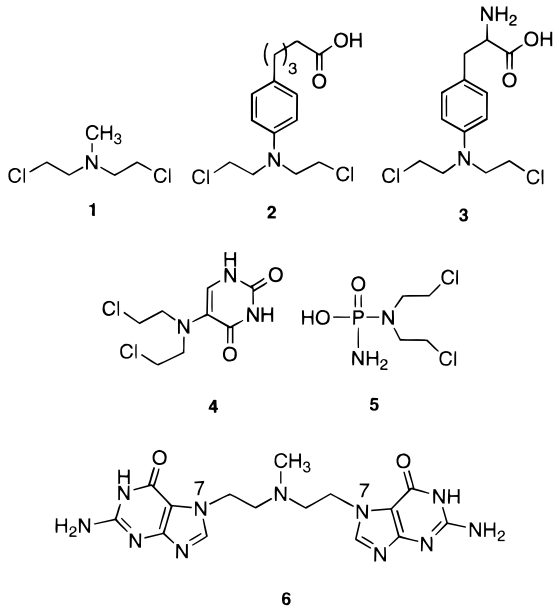
compounds, (2) photoactivated agents, (3) oxidatively activated cross-linkers, and (4) reductively activated agents. Numerous compounds reviewed are structurally similar to those in subsection 2 but have been modified to fall into one of the other three subsections. In many cases this was done to increase the clinical utility of the parent compound.

2. Inherently Reactive Agents

2.1. The Nitrogen Mustards

The nitrogen mustards represent the earliest and perhaps most extensively studied of the DNA interstrand cross-linking agents.[11b] Despite their long history, mechlorethamine **1** and chlorambucil **2** are two of the most heavily employed clinical anticancer agents in use today.[18] Along with a number of other mustard based structures (**3**−**5**), their high degree of cytotoxicity is attributed to their ability to induce DNA interstrand cross-links thereby inhibiting replication. In the case of mechlorethamine, this was originally inferred from the anomalously fast renaturation kinetics of mustard-treated DNA.[1,7a] Although these compounds have been studied and clinically exploited for over 30 years, they still provide an area of extremely intense and progressive investigation.

The site specificity of the mustards was originally assigned as being the $^{5'}$GpC$^{3'}$ sequence within B-form DNA. This was based on the isolation of the lesion **6** in which two guanine residues are bridged via their respecitve N^7 atoms by one mechlorethamine derived pentylene chain.[19] The original isolation involved



reaction of mechlorethamine **1** with the simple monomer GMP (guanosine monophosphate), and was soon followed by experiments involving isolation from mechlorethamine-treated yeast RNA.[20]

From the RNA experiments, **6** was postulated to result from interstrand bisalkylation at the sequence $^{5'}$GpC$^{3'}$. Assumed to be a unimolecular reaction with respect to the mustard, this assignment was later substantiated by the molecular modeling studies of Lewensohn et al. for a closely related compound.[21] These studies strongly suggested the ideal N^7 to N^7 distance match of $^{5'}$GpC$^{3'}$ for cross-linking by mechlorethamine within the interstrand motif. On these

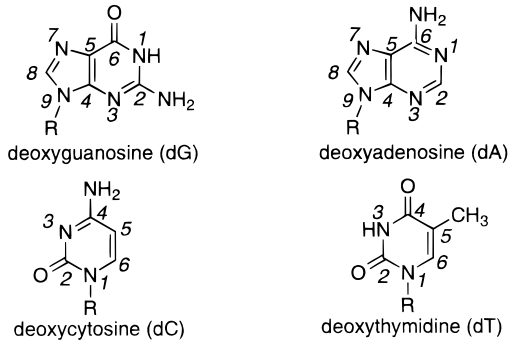


Figure 4. Deoxynucleotide numbering system where R = deoxyribose.

grounds alone the mechanistic origin of **6** was assigned as being interstrand, although in fact, the possibility of interhelical or intrastrand interactions could not be unambiguously dismissed.

The early 1990s represented a time of intense study in the area of molecular recognition by the nitrogen mustards. The Grueneberg and Hopkins groups independently reported that the distal deoxyguanosines of $^{5'}$GNC$^{3'}$ (N = dA, dG, dC, or dT) are cross-linked much more efficiently by **1** than is $^{5'}$GpC$^{3'}$.[22] This discovery revealed the true sequence selectivity of the mustards and in doing so, pointed out an important dilemma pertaining to the mechanism of ISC formation by the mustards.

The nitrogen mustards covalently cross-link DNA via a sequence of aziridinium (**7**) formation followed by alkylation (to afford **8**) and then presumed repetition of the cycle to ultimately afford bisalkylated material (**10**) as shown in Figure 5.[23a]

Molecular modeling of the unstrained pentylene tether resulting from such a mechanism suggests a "greatest" distance of 5.1 Å.[20] Given that the planes occupied by adjacent base pairs within canonical B-DNA are separated by roughly 3.4 Å, to span three bases (as in $^{5'}$GNC$^{3'}$) would require a minimum possible distance (assuming a single perpendicular line) of 6.8 Å.[20] This, in conjunction with the fact that **6** had never been unambiguously shown to arise from interstrand cross-linking, led to conjecture involving dimerization of **1** en route to the $^{5'}$GNC$^{3'}$ cross-link. Such a lesion would easily account for this specificity and would result in minimal perturbation of the B-DNA target structure.

In 1993, the Hopkins group presented results which refuted the possibility of such a dimerization manifold en route to cross-linking by **1**.[23] Utilizing ODN duplexes of differing sizes, it was found that **1** was incapable of interhelical cross-linking as judged

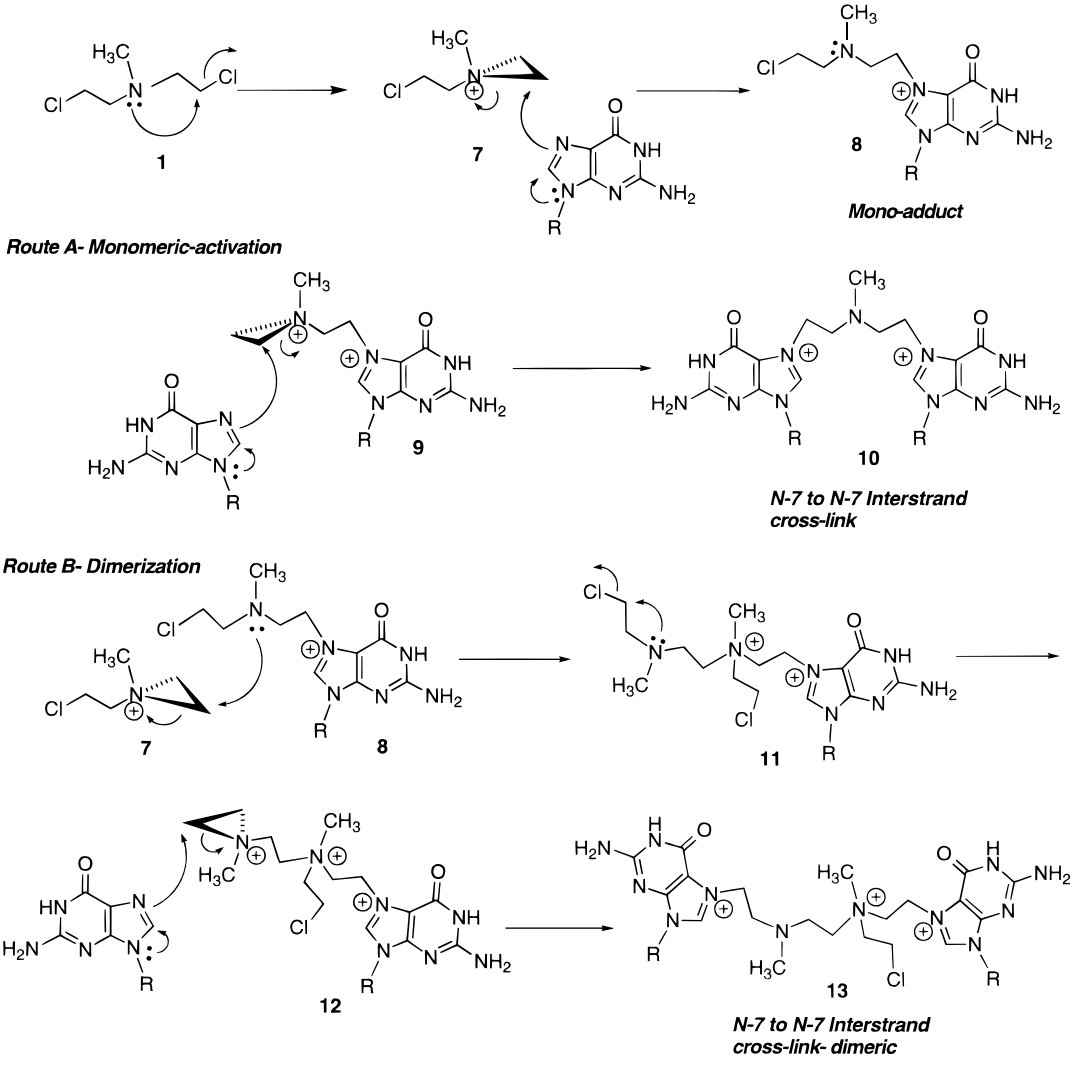


Figure 5. Proposed mechanisms of DNA−DNA interstrand cross-link formation by mechlorethamine **1**.

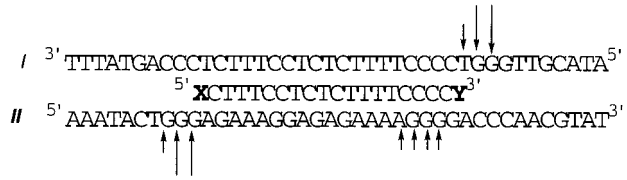


Figure 6. Histogram of observed base-labile lesion formation for ODN−**2** conjugate upon reaction with the strand I/II Watson−Crick duplex.

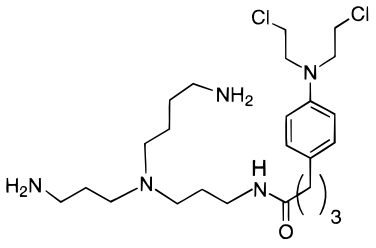
by the absence of "crossed" adducts in reactions involving multiple DNA substrates. More importantly, the isolation and characterization of **6** was repeated using ODNs in which predominant cross-linking was relegated to $^{5'}$GNC$^{3'}$ thus fortifying the monomeric cross-linking route depicted in Figure 5. That this was the sole lesion isolated strongly suggested the inoperability of mechlorethamine dimerization involved in ISC generation. Furthermore, the correlation of **6** with the $^{5'}$GNC$^{3'}$ cross-link implied that the nitrogen mustards cause a high degree of structural distortion in their DNA targets. Indeed, later studies by Hopkins and Rink showed that mechlorethamine cross-linking of 5′-d(GNC) does, in fact, induce a static bend into the B-form DNA substrate; the result of local deformations giving rise to the necessarily shortened N^7−N^7 distance within $^{5'}$GNC$^{3'}$.[23b] This was demonstrated by molecular modeling and ligation experiments involving gel retardation of mechlorethamine-modified duplex ligation products (as discussed in Figure 17).[23b] By inducing the disclosed distortional processes, the mustards represent a unique departure from most DNA-reactive agents whose base pair selectivities are dictated largely by minimization of target distortion. Importantly, it has been noted that agents capable of DNA distortion are often capable of altering recognition/affinity properties of DNA−protein interactions and that this alteration may often play a significant role in the expression of biological activity.[23b]

Despite their clinical importance, the usefulness of many DNA-modifying drugs is often limited by a number of pharmacological deficiencies resulting from the intrinsic chemical reactivity of the agent. Low affinity for the target molecule of interest (in this case, DNA) and rapid quenching via hydrolysis prior to DNA alkylation or hydrolysis of the monoadduct, place severe restrictions on the potential utility of DNA-reactive agents. Efforts to increase the target affinity of the mustards have produced a number of successes and continues to constitute an area of intense interest.

Utilizing the reactivity of chlorambucil **2**, triple helix approaches to genome-specific cross-linking have been examined. Kutyavin et al. have shown that the polypyrimidine ODN $^{5'}$CTTTCCTTCTCTTTTCCCC$^{3'}$ tethered at either the 3′ or 5′ end with **2**, was capable of covalently linking the synthetic ODN to one strand of the target duplex or the other.[24] Interstrand cross-linking of the target duplex was seen only in the case where both 5′ and 3′ ends of the ODN bore the mustard **2** (Figure 6).

That no interstrand cross-link was observed for a monofunctionalized ODN is directly related to the absence of 5′-GNC-3′ within the vicinity of either end of the functionalized polypyrimidine ODN. Strand II proximal to **Y** does bear $^{5'}$GNC$^{3'}$; however, the aminohexyl moiety employed to tether **2** places the mustard too far away for "normal" interstrand cross-linking to occur. The lesions derived from either mono- or bisalkylation were attributable to N^7 alkylation on one of several possible dG residues proximal to the 3′ or 5′ ODN terminus, as depicted by the histogram resulting from piperidine treatment of the alkylation adducts. Chlorambucil functionalization at the ODN 3′ end gives rise to base-labile alkylations on both sides of the Watson−Crick duplex. Major cleavage sites are seen in strand I while minor cleavage is detected on strand II. This is most likely due to a high degree of flexibility imparted by the aminohexyl linker upon the ODN−mustard conjugate, allowing **2** to inflict DNA damage by folding back over to the 5′ side of the ODN-**2** connection.

In a much simpler vein, Cohen et al. synthesized the chorambucil−spermidine conjugate **14** in hopes that under physiological pH, the polycationic spermidine chain would associate with the duplex DNA target thus increasing the target affinity of the chlorambucil moiety.[25] Additionally, the observation that many cancer cell lines possess polyamine uptake systems made the choice of spermine or spermidine conjugation an attractive means of enhancing in vivo drug delivery.[26] In fact, what was observed was a much greater efficiency of interstrand cross-linking (in vitro) by **14** versus the parent compound **2** although biological activity profiles (in vivo) revealed poor cellular uptake of the conjugate. In similar efforts, Stark and Meadows devised the slightly altered conjugate **15** which also demonstrated unexpectedly low cytotoxicity against BL6 melanoma cells and equally poor polyamine uptake.[27]



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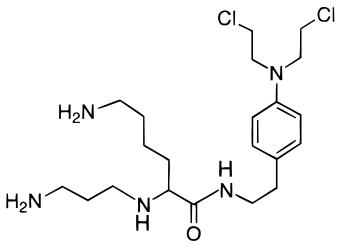

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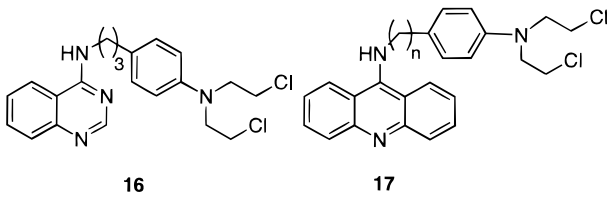
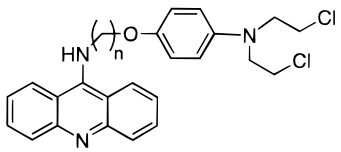
In addition to increasing drug-to-target affinity by attachment to potential hydrogen bonding groups or cationic matrixes, the appending of DNA intercalating groups has also been examined. By tethering an intercalating chromophore to the drug of interest, drug affinity for the DNA substrate is considerably increased via the π-stacking interactions of the

nucleobases with the attached chromophore. Prakash and co-workers have recently demonstrated this approach by conjugation of aniline mustards to a number of quinazoline and 9-aminoacridine chromophores (**16**−**18**).[28]



16 **17**



18

In all cases, the tethered mustards resulted in a much greater degree of monoalkylation than observed with the untethered analogues. On average, only one cross-link occurred per 20 monoalkylation events. This rate of cross-linking closely parallels that of the native mustards and represented a rather disappointing result. It was believed that by increasing the inductive electron density of the aniline, facilitation of both DNA alkylation events would ensue. This was not the case with interstrand bisalkylation, but to some extent this was in fact observed with the initial monoalkylation. Substitution para- to the mustard resulted in the following order of reactivity: **17** > **18** > thioether analogue of **18** > sulfoxide analogue of **18**. In fact, the sulfoxide analogue of **18** yielded no discernible DNA−drug adducts. Of these compounds (**17**, **18**, and the thioether analogue of **18**), the thioether was found considerably less efficient at in vitro interstrand cross-linking than were **17** and **18** and in vivo activity was much lower as well. It is noteworthy that these three tethered compounds showed much higher cytotoxicity values than the untargeted mustards. Drugs bearing the core structure of either **17** or **18** (*n* was variable from 2 to 5) were much more potent (optimal dose of 20−30 mg/kg) than was the aniline mustard chlorambucil **2** (optimal dose 225 mg/kg) or any other untethered mustards. On the other hand, the thioether and sulfoxide congeners of **18** showed weak in vivo activity.

The Prakash study also investigated the differential alkylation studies of structures **17** and **18** from the perspective of tether length (*n*) alteration and its influence upon DNA base selectivity.[28] These studies, in conjunction with evaluation of the quinazoline **16**, revealed that the aminoacridine−mustard conjugates were capable of adenine alkylation. This work represented the first demonstration of adenine alkylation by any of the nitrogen mustards. Experimental work in addition to evaluation of CPK models indicated that use of short tethers (3−4 atoms total) induced a degree of rigidity to the conjugate. This rigidity orients the mustard group at right angles to the acridine plane thereby placing the electrophilic mustard in line with the guanosine N^7 of the sequence $^{5'}GTA^{3'}$ (preferred monoalkylation site). Use of longer tethers, however removes this rigidity, thus giving rise to the observed adenine N^7 alkylation on the complementary strand ($^{3'}CAT^{5'}$). Later studies by Denny et al. in 1994 revealed that the lesion previously assigned as adenine N^7 by the Prakash group was in fact not N^7 but rather adenine N^1.[29] Utilizing a "singly-armed" (only one chloroethyl group on the aniline nitrogen) version of compound **18** (with $n = 5$) it was demonstrated that connectivity to adenine N^1 (demonstrating *the first example* of any agent that selectively alkylates this position), was formed in greater than 90% yield. This amazing selectivity is proposed to result from acridine intercalation with concomitant projection of the mustard−acridine tether into the major groove, thus giving rise to base pair distortions. Such distortions would likely result in increased access to the normally hydrogen-bonded/deactivated adenine N^1 adjacent to the site of intercalation. From these efforts it becomes clear that initiatives to increase drug−DNA affinity often provide frameworks upon which to design and construct new drugs of higher efficacy or altered selectivity. As such, the targeting of new DNA sequences by the aminoacridine−mustard conjugates offers fertile ground for research and will no doubt increase the utility of the nitrogen mustards.

2.2. Chloroethylating Agents

The majority of DNA interstrand cross-linking agents give rise to lesions which bear a distinct structural similarity to the DNA reactive starting material (i.e., the cross-linking agent). One such example is that of interstrand cross-linking by mitomycin C in which the cross-link bears the indoloquinone core of the original starting material (Figure 7).[30] However, some instances do exist in which the cross-linking agent undergoes dramatic decomposition or metabolism thereby releasing the active cross-linking agent (now representing only a fraction of the starting material's structure). The lesions generated are often very simple in structure and bear little similarity to the transient parent compound. Examples include the nitrosoureas, triazenes, and the alkylsulfonates.

2.3. Nitrosoureas

Chloroethylnitrosoureas (CENU's) are clinical antineoplastic agents employed specifically in the treat-

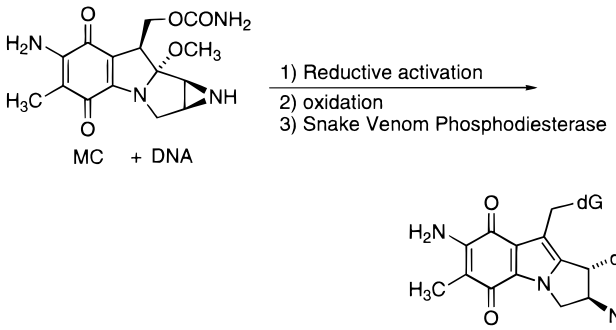


Figure 7. Generic scheme for DNA cross-linking by reductively activated Mitomycin C (MC). Note that ISC lesion retains the majority of the mitomycin core structure.

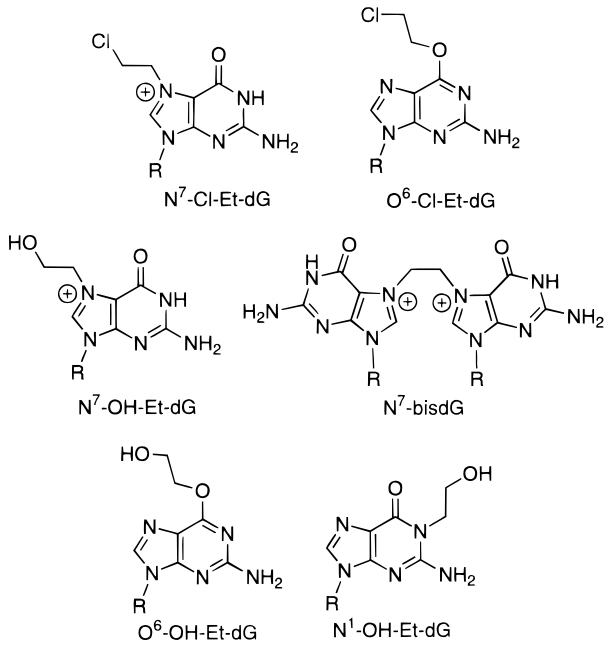
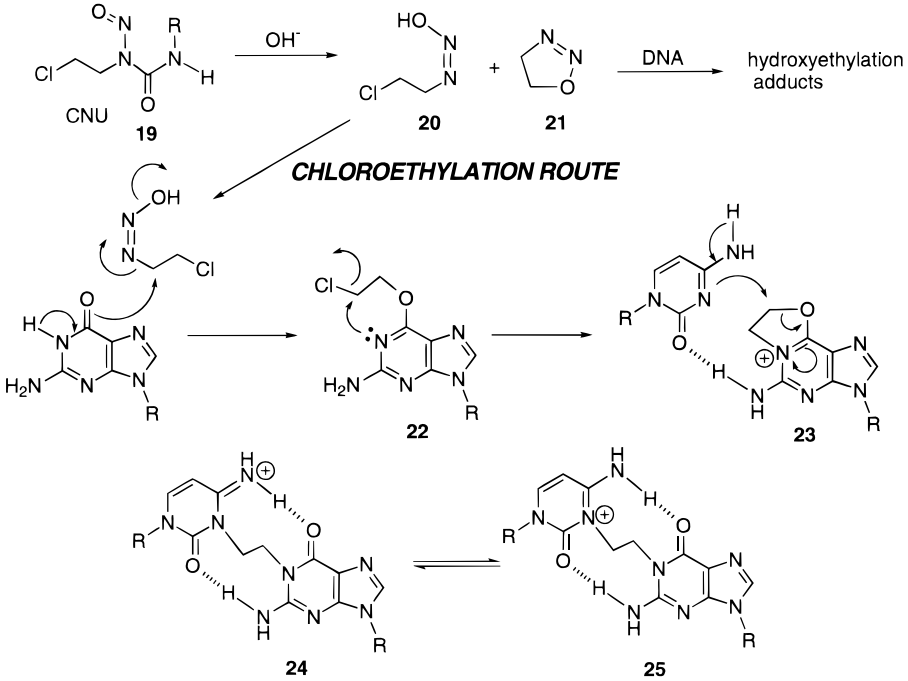


Figure 8. Commonly observed adducts resulting from CENU treatments of double-stranded DNA.[34]

ment of brain tumors.[31] Although these agents are capable of inducing numerous alkylation adducts (Figure 8), it is the formation of an ethane linkage between the N^1 of deoxyguanosine and the N^3 of its base pairing partner deoxycytosine which gives rise to the critical interstrand cross-link.[32] The generally accepted mechanism by which these agents decompose in aqueous solution to generate the chloroethyl and hydroxyethyl nucleotides shown below is depicted by Figure 9.[33]

Mechanistic uncertainties involve the formation of the active chloroethylating moiety and that of the O^6 to N^1 chloroethyl group shift (**22** to **24**) depicted in Figure 9.[32c] The importance of a chloronium ion in monoalkylation of deoxyguanosine O^6 has been implied although never demonstrated experimentally.[32b] Alternatively, one may envision the direct S_N2 attack shown in Figure 9 resulting in loss of N_2 and water. The interstrand lesion had the structure shown in Figure 9. However, the questionable reactivity of dG N^1 led Kohn et al. to propose that dG chloroethylation proceeds via initial O^6 chloroethylation followed by rearrangement to the N^1 alkylated material.[35] This was supported by studies involving the DNA repair enzyme O^6-methylguanine-DNA methyltransferase.[36] Known to remove substituted ethyl groups from dG O^6 and dT O,[4] this enzyme was incapable of CNU cross-link reversal. However, when included in the cross-link reaction, the enzyme completely shut down cross-link formation. In earlier support of this, Kohn et al. had demonstrated that in mammalian cell lines possessing reduced capacity to repair O^6 alkylguanosine, CNU-induced cross-linking was observed at much higher levels than in normal cells.[37] This phenomenon has also been demonstrated more recently by the Gold group.[38]

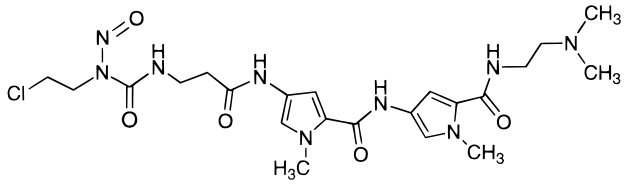
Utilizing conjugates of the general form **26**, they found that chloronitrosourea-induced cross-links were formed preferentially in 9L cell lines lacking O^6 methyltransferase activity versus those which possess normal levels of the DNA repair enzyme. Interestingly, tethering of CNU to the minor groove-binding peptide shown in **26** resulted in a decreased level of cross-link formation over that of the untethered CNU. Mismatching of the CNU (major groove alkylator) and the polypyrrole peptide (minor groove binding at dA -rich sites) results in the formation of a new minor groove alkylation (at adenine N^3) at the expense of major groove alkylation (required en route to dG-dC cross-linking). That adenine N^3 alkylation



Tautomeric forms of CNU induced dC-dG interstrand cross-link

Figure 9. Mechanism of chloroethylnitrosourea decomposition resulting in dC-dG interstrand cross-link.

does not give rise to any of the DNA cross-links observed with **26** has been inferred but not unambiguously demonstrated. As with the acridine-tethered aniline mustards, attempts to simply increase target−drug affinities resulted in the inadvertent design of a new alkylation specificity.



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2.4. Triazenes

The chemical decomposition of the 1,3-dialkyl-3-acyltriazenes has received a great deal of attention, owing to the biological activity of the degradation products involved. These compounds result in the transfer of the N^1 and N^3 alkyl groups to the reactive deoxyguanosine N^7 and O^6 positions.[39] Two degradative routes have been demonstrated (Figure 10).[40] Immediate deacylation followed by tautomerization resulting in transfer of the N^3 alkyl group represents the minor pathway while heterolytic scission of the N^2-to-N^3 bond results in generation of the alkanediazonium. Generation of the diazonium followed by DNA alkylation or hydrolysis (also giving rise to dG adducts) ensues and completes the major pathway of triazene decomposition. In the case where the N1 alkyl group is chloroethyl, quenching of the diazonium yields chloroethylation at either dG N^7 or O^6. As with the nitrosoureas, O^6 chloroethylation ultimately gives rise to the dG-dC interstrand cross-link while the N^7 event may result in the dG-dG intrastrand lesion should there be another deoxyguanosine proximal to the initial N^7 lesion.[41]

Kroeger Smith et al. have recently shown that a high degree of control over the deacylation route may be obtained by using an N3 attached acyl group bearing a terminal methyl ester.[42] Enzymatic conversion of the ester to the corresponding acid (Figure 11) allows cyclization followed by release of the N3 methyl triazene which may then result in guanosine alkylation by either the major and to a lesser extent, the minor pathway shown in Figure 10.

Using a similar strategy of acyl group functionalization on N3 of the triazene, Schmidt and co-workers selectively targeted gastrin receptor (GR) expressing tumor cells.[43] GR is expressed in a number of human cancers (one example being colon adenocarcinoma) and a critical factor in the recognition of gastrointestinal peptides by this receptor is the C-terminus sequence Trp-Met-Asp-Phe amide. Using the conju-

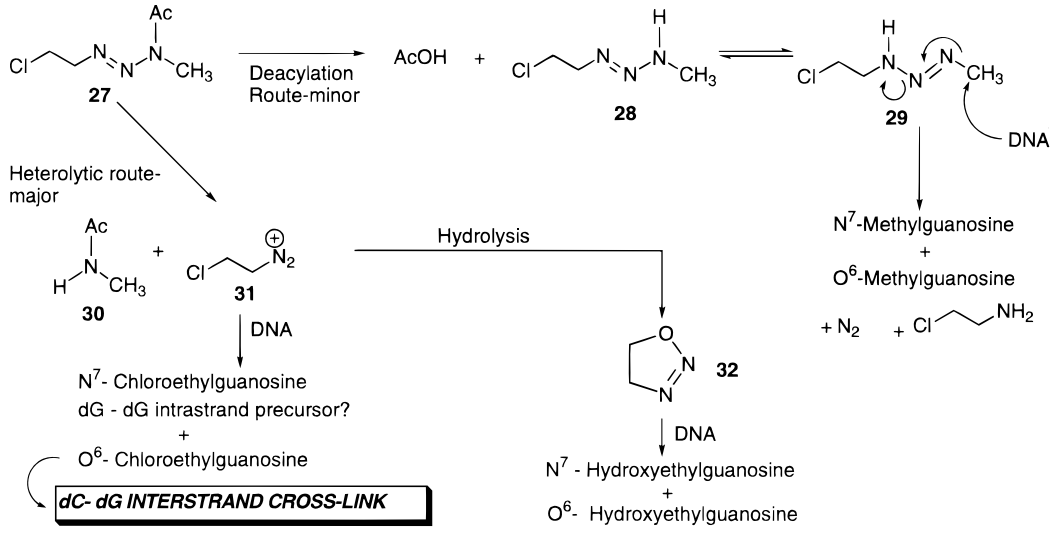


Figure 10. Degradation of 1,3-dialkyl-3-acyltriazenes to afford DNA alkylation adducts.

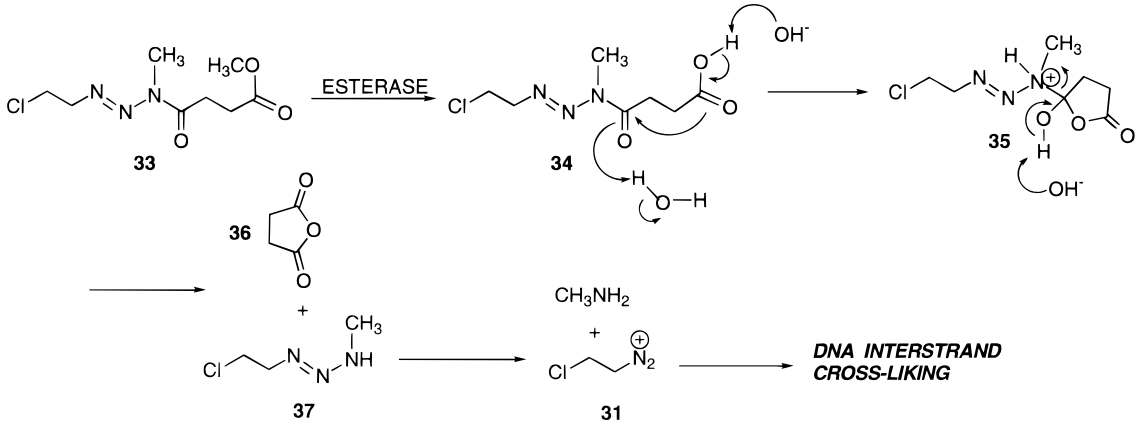


Figure 11. Enzymatic activation of triazene decomposition en route to DNA interstrand cross-linking.

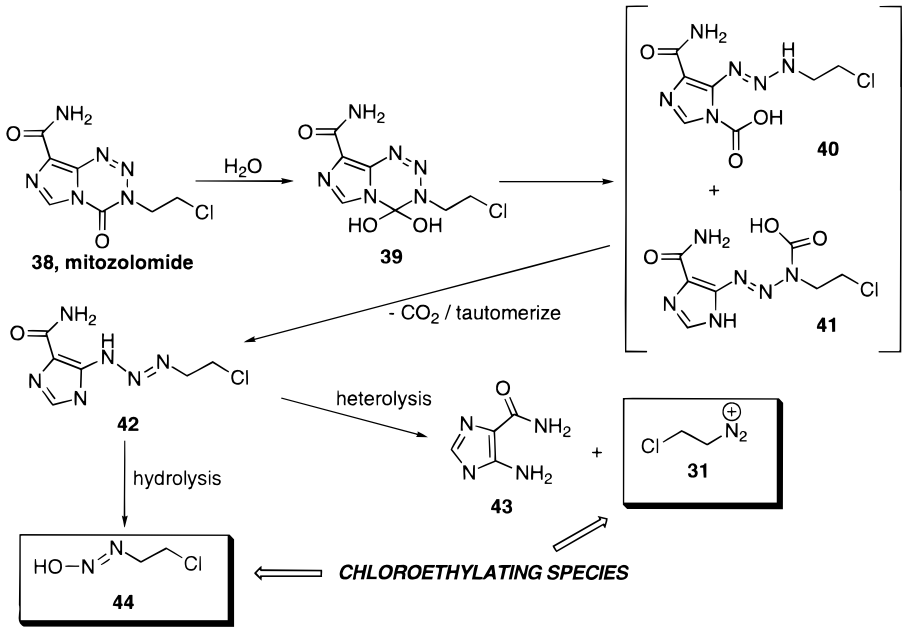


Figure 12. Mitozolomide decomposition pathway.

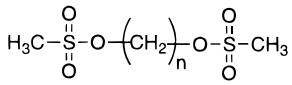
gate *N*-[3-benzyl-3-(carboxypropanoyl)-1-(2-chloroethyl)triazene]-*β*-Ala-Trp-Met-Asp-Phe amide, cytotoxicity was directed selectively at the gastric AR42J cancer cells. A549 human lung cancer cells (not capable of GR expression) were completely unaffected by the conjugate.[43]

On the basis of the proposed ring cleavage of the cyclic triazenes under physiological conditions, the cyclic triazenylimidazoles have prompted investigation and have been found to closely resemble acyclic triazenes in their DNA cross-linking ability. Representative of the cyclic triazenylimidazoles, mitozolomide **38** (Figure 12) is simply a prodrug of 5-[3-(2-chloroethyl)-1-triazenyl]imidazole-4-carboxamide **41** (MCTIC).[44a]

Despite enhanced stability relative to MCTIC (and thus less reactive toward cross-linking), mitozolomide has proven extremely effective against TLX5 lymphoma, B16 melanoma, colon 26, colon 38, and L1210 leukemia (resistant to cyclophosphoramide) cell lines.[44b] Indeed a number of very promising DNA cross-linking agents have been designed with the general structure of mitozolomide in mind, all of which operate upon the basic mechanism of chloroethylation to afford dC-dG cross-links.

2.5. Alkyl Sulfonates

As stated earlier, the correlation between biomolecule alkylation and therapeutically useful agents relies heavily on the electrophilicity of those agents. This realization was brought about no doubt by the nitrogen mustards and was the impetus for the study of alkyl sulfonates. Particular emphasis was originally placed on the potential DNA cross-linking ability of the "homo-bifunctional" sulfonates many of which consisted of sulfonylated cyclic or acyclic alkanes.[45] Many of these agents are active against numerous cancer cell lines, although the exact mechanisms by which they effect cytotoxic action has not been thoroughly elucidated.[46] Busulfan **45a** and its related congeners **45b** and **d**−**f** represent the only

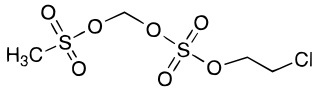


45

a: n = 4, **Busulfan**
b: n =1
c: n = 2
d: n = 9
e: n = 8
f: n = 6

di-sulfonyl compounds shown conclusively to effect ISC formation.[47] Characterization of the lesion obtained from busulfan treated DNA has revealed the generation of 1,4-bis(7-guanyl)butane as well as, 7-(4-hydroxybutyl)guanosine upon hydrolysis of the modified DNA.[47]

Concomitant with the development of the bis-(methanesulfonates), monosulfonic agents were examined as a means of chloroethyl introduction into DNA. The 2-chloroethyl methanesulfonates received considerable attention but were fraught with low activities in the tumor cell lines chosen. So too were the vast majority of 2-chloroethyl alkene/arenesulfonates examined. The next generation of sulfonates afforded vast improvements in activity against leukemia P388 cell lines over that seen with the 2-chloroethyl methanesulfonates. These agents, the 2-chloroethyl chloroethanesulfonates in turn, gave rise to the development of [(2-chloroethylsulfonyl)-methyl] methanesulfonate (clomesone, **46**). Studies



46, **clomesone**

by the Kohn, Pegg, and Smith groups have confirmed that **46** does, in fact chloroethylate dG N^1 (via O^6 transfer as per Figure 9) en route to formation of the

anticipated dC-dG interstrand cross-link.[32b,48] Some differences regarding general reactivity patterns exist between **46** (the most prevalent of the sulfonate chloroethylating agents) and the chloronitrosoureas and triazenes. First, **46** is much more selective in its DNA specificity. The vastly predominant product from the reaction of calf thymus DNA with **46** is N^7-chloroethylguanosine, whereas both the triazenes and nitrosoureas produce a wide array of adducts (many incapable of interstrand cross-linking) as shown in Figure 8.[49a] Additionally, **46** (and all the alkyl sulfonates in general) is devoid of carbamoylation activity (a common side reaction seen in chloronitrosourea treated proteins), this being a route leading to nonselective toxicities resulting in the therapeutically unproductive consumption of drug.[49b] The chloroethylsulfonates (specifically **46**-based structures) thus represent the most efficient of the chloroethylating agents owing largely to the specificity of alkylation.

2.6. Epoxide-Containing Agents

It has been realized for a long time that epoxides represent potent DNA-alkylating agents. This reactivity has been demonstrated most vividly by the benzo[*a*]pyrenes which, in mammals, undergo oxidative metabolism to the four stereoisomers **47**−**50** (Chart 1) of the carcinogenic 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene BPDE. Bearing the activated benzylic epoxide, these compounds are highly reactive toward DNA, forming numerous adducts; the predominant ones (both in vivo and in vitro) resulting from epoxide opening and subsequent connection of the bulky BPDE C-10 to DNA via the exocyclic amine N^2 of deoxyguanosine.[50] Surprisingly, only the (+)-anti-BPDE dG lesion results in carcinogenic/mutagenic cellular events while alkylation of the other stereoisomers has comparatively little biological significance.[51] The lesion **51** blocks RNA[52] and DNA[53] polymerases and very recently has been shown to inhibit the DNA helicase activity (necessary for replication) of the bacteriophage T7 gene 4 protein in addition to blocking dNTPase activities.[54]

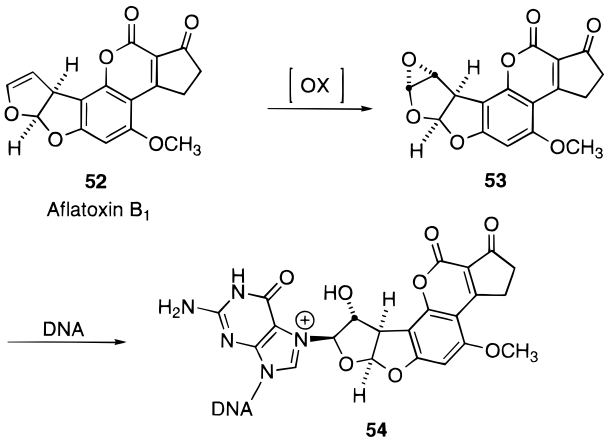
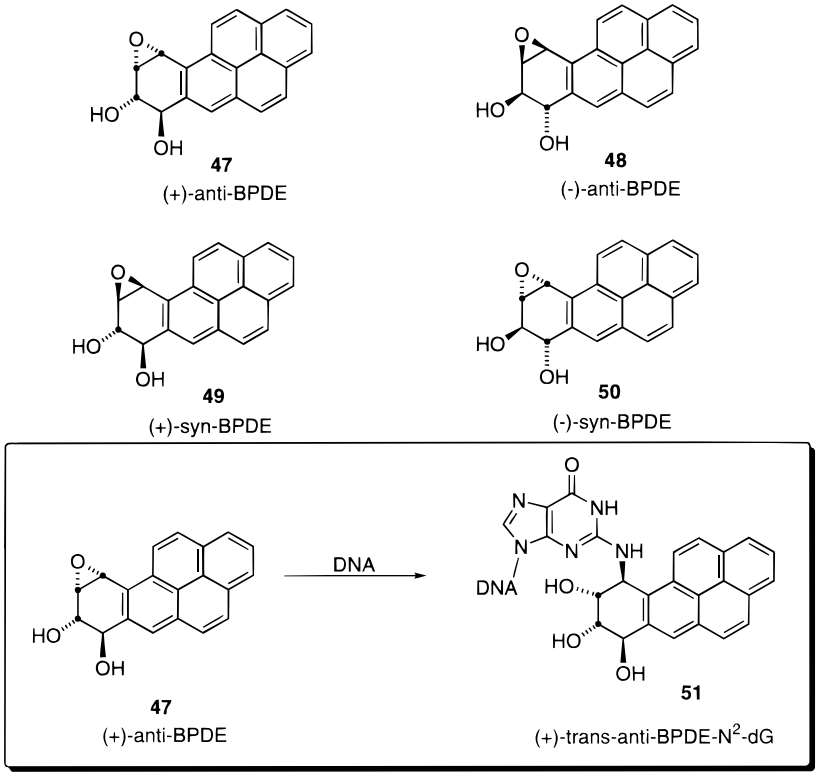


Figure 13. Oxidative activation of aflatoxin B_1 and ensuing reaction with deoxyguanosine.

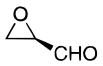
The in vivo generation of epoxides via cytochrome P-450 oxidation is a common theme in epoxide biochemistry; prime examples being the oxidative activation of aflatoxin B alkylation shown below in Figure 13,[55] and the conversion of glycidyl ethers to glycidaldehyde.[56] Much simpler epoxides (not resulting from metabolic activation) are also capable of DNA alkylation. Examples include propylene oxide,[57] styrene oxide, and trichloropropylene oxide.[58]

Chart 1



The DNA residues most susceptible to alkylation by these agents include dG N^7, dA N^6, and dC N^3. It is noteworthy that most examples of DNA-modifying epoxides result only in monoalkylation adducts.

It is interesting that review of the literature reveals little or no mechanistic detail pertaining to the mechanism of action of the few known epoxide cross-linking agents. One case in point is glycidaldehyde (**55**). Although it is often reported as a DNA cross-



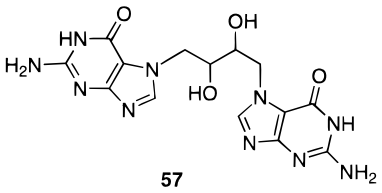
55

linking agent,[59c] structure elucidation (of the ISC lesion) has not been achieved nor has there been a DNA sequence assignment for its preferred ISC site. Its reactions with dA and dG have been thoroughly studied but data pertaining to any interstrand events of importance have been extremely elusive.[59] Given this, glycidaldehyde will not be discussed although the interested reader is referred to ref 59 for insight into its ring extension reactions (similar to chloroacetaldehyde) with dA and dG and the importance of **55** in probing DNA structure perturbations.

2.6.1. Diepoxybutane

Diepoxybutane (DEB, **56**) is the simplest of the epoxide cross-linking agents and the first epoxide to be reported as carcinogenic.[60] Lawley and Brookes proposed that DEB behaves similarly in its cross-linking behavior to the N-mustards.[61] This was based upon the 1967 isolation of the diol **57**, implicating two dG N^7 residues as those responsible for the interstrand event.[61] As with the mustards, the

56



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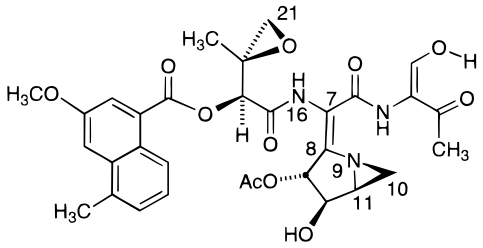
original supposition was that $^{5'}GpC^{3'}$ was the preferred DNA sequence targeted by DEB. More recently, Millard and White have shown using synthetic ODNs of varying sequences that, indeed, the preferred sequence for cross-linking by DEB is $^{5'}GNC^{3'}$ thus refuting the proposal put forth by Lawley and Brooks.[62]

Interesting to note is that diepoxybutane (DEB), unlike the nitrogen mustards, cross-links a number of different sequences, the order of reactivity being $^{5'}GGCC^{3'} \gg TGCA$, TCGA > CCGG > TATA with the $^{5'}GNC^{3'}$ (i.e., $^{5'}GGCC^{3'}$) predominating over the next reactive sequence by ~4-fold. Piperidine cleavage of cross-linked material confirmed N^7 base-labile alkylation at both dG residues involved in the cross-link. This finding refutes the original $^{5'}GpC^{3'}$ specificity assignment but, more surprisingly, points out a discrepancy pertaining to the molecular distances available within B-form DNA. As discussed previously with the nitrogen mustards, the N^7-to-N^7 distance within the targeted $^{5'}GNC^{3'}$ sequence of B-DNA greatly exceeds that which can be spanned by the cross-linking agent. A best case scenario dictates that 6.8 Å separates the N^7 moieties (assuming retention of base stacking interactions) while the minimized pentylene chain of mechlorethamine affords a distance of only 5.1 Å between the terminal electrophilic sites. The DEB case is even shorter in that it lacks the central nitrogen present with mechlorethamine and all the N-mustards. Thus, the target $^{5'}GNC^{3'}$ must substantially distort in order to accommodate the DEB-induced cross-link. How this occurs and the implications with respect to subsequent DNA−protein (repair?) interactions has yet to be addressed and most certainly represents an area of interest with respect to molecular recognition of small molecules and the different forms (B-DNA and otherwise) of DNA.

2.6.2. Carzinophilin/Azinomycin B

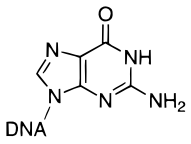
Considerably more complex than any other epoxide cross-linking agent, carzinophilin (**58**, also called azinomycin B) possesses not only the labile epoxide, but also bears the novel bicyclic aziridinopyrolidine in conjugation with the C6 amide. Isolated from broths of *Streptomyces sahachiroi* this potent antitumor antibiotic was recently shown to be identical in structure to azinomycin B which is isolated from *Streptomyces griseofuscus* S42227.[63]



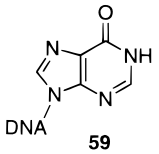
58, Carzinophilin / Azinomycin B

To date, the most detailed mechanism of action studies have been carried out by the Armstrong group. It has been determined that DNA cross-linking specificity is for $^{5'}GNC^{3'}$ and $^{5'}GNT^{3'}$ in which the N^7 moieties of two deoxyguanosines (in the case $^{5'}GNC^{3'}$) or the N^7 of one deoxyguanosine and the N^7 of the neighboring deoxyadenosine (the $^{5'}GNT^{3'}$ case) are alkylated.[64] These assignments were derived from examination of piperidine labile sites of cleavage, as well as the use of ODN substrates bearing the modified bases deoxyinosine and 7-deazaguanosine in probing the effects of functional group deletions on cross-linking efficiency. Additionally, treatment of cross-linked substrates with the chemical nuclease reagent bis(1,10-phenanthroline)copper was used to confirm connectivity assignments derived from the piperidine reactions.

The alkylation sites on DNA have been assigned, but no assignments regarding drug connectivities have been reported. Terashima et al. have recently reported the generation of ring-opened structures **62** and **63** upon treatment of synthetic intermediate **61** with thiophenol and triethylamine at room temperature.[65] This transformation is consistent with the



Deoxyguanosine



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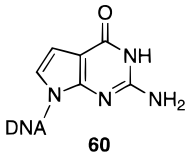
Deoxyinosine



60

7-Deazaguanosine

anticipated lability of the epoxide and aziridine rings of the natural product and has prompted the mechanism of action proposed by Terashima depicted in Figure 14. Particularly susceptible to ring opening is the aziridine, given the preponderance of **62** over **63** in cases where reaction times were shortened. From this it is anticipated that **63** is derived by way of **62** and thus aziridine cleavage gives rise to the initial monoadduct en route to interstrand cross-linking (Scheme 1).

This is further supported by the conversion of aziridine **64** to the ring-opened adducts **65a**−**c** under extremely mild conditions via nucleophilic attack of the aziridine C10 position. Further, in vitro cytotoxicity assays against P388 murine leukemia utilizing **61**−**63** demonstrated prominent values for **61** (IC_{50} = 0.0023 μg/mL), whereas **62** and **63** demonstrated much weaker activities (IC_{50} = 0.035 and 1.9 μg/mL, respectively).[65]

According to the mechanism proposed by Terashima,[65] conjugation of the aziridine nitrogen with the N5−C6 amide likely activates this position for initial DNA attack at C10, leading to the imine monoadduct **66** (Figure 14). Tautomerization restores the enamine **67** which *may* then undergo attack at epoxide C21 in an intramolecularly assisted fashion via deprotonation of amide N16. Tautomerization of the resulting imidate **68** back to the amide yields the proposed interstrand cross-link.

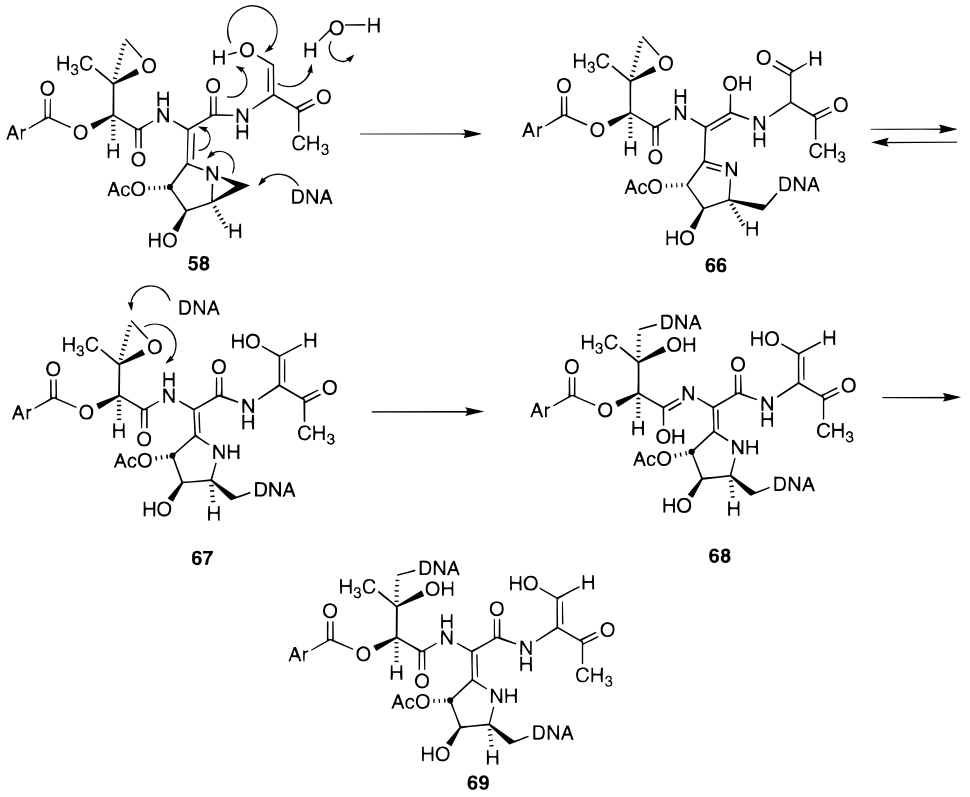
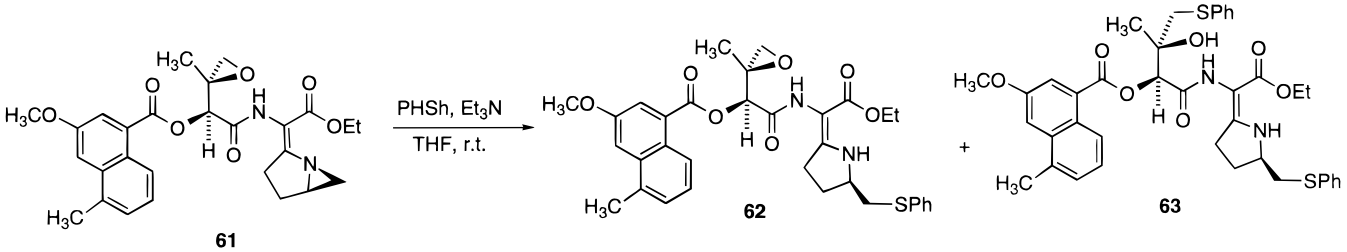
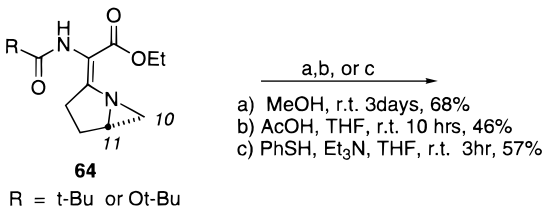
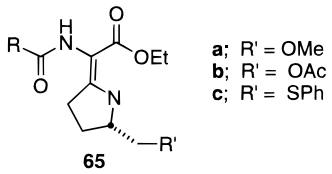


Figure 14. Terashima model for DNA interstrand cross-linking by carzinophilin. Regiospecificity and reaction ordering derived from synthetic reactivity studies.[65]

Scheme 1



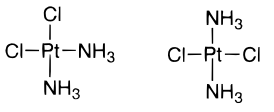


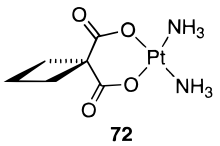


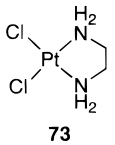
Despite the compelling nature of this mechanism and its resulting covalencies, the amount of supporting evidence has been limited. No DNA−drug lesion has been isolated nor have extensive DNA-binding studies been performed aside from the original Armstrong findings. As such, carzinophilin represents a novel natural product due not only to its complex structure but also its relatively obscure mechanism of DNA interaction.

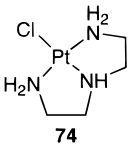
2.7. *cis*-Diamminedichloroplatinum(II)

cis-Diamminedichloroplatinum(II) (*cis*-platin or *cis*-DDP, **70**) was originally described 150 years ago but has only within the last 30 years been recognized as a potent antitumor agent.[66] The cytotoxic properties of this and numerous analogues have been attributed to its ability to form both inter- and intrastrand cross-links within deoxyguanosine-rich runs of DNA. Interestingly, its biological activity was initially observed in electrochemical studies involving the effects of electric fields on the growth rates of *E. coli*.[67] Cells exposed to electric currents (aerobic conditions) in the presence of NH_4Cl underwent abnormal filament elongation (as much as 300 times the normal length) at the expense of cell division. Further investigation of this phenomenon revealed that generation of *cis*-DDP (in addition to the *trans*-isomer **71**) from electrolysis at the platinum electrodes was responsible for the abnormal cellular behavior.[68] Despite extensive studies and clinical trials leading to the drug's FDA approval in 1979 the mechanism of action of *cis*-DDP has continued to provide fertile ground for interesting experimental investigations. Particular emphasis of late has involved the ability of **70** to induce DNA mutations[69] and to radically alter DNA−protein binding motifs.[70,254]

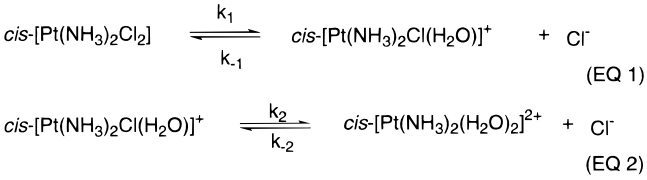








Evidence suggests that *cis*-DDP undergoes sequential chloride displacements via a two-step hydration process. Studies with [Pt(*en*)Cl_2] **73** have shown that chloride displacement does not occur in plasma (where chloride ion concentration is about 100 mM) but rather, it occurs upon membrane penetration.[71] Once inside the cell, the chloride concentration drops to around 5 mM and bisaquation of the *cis*-DDP proceeds with k_1 and k_2 of 2×10^{-5} M^{-1} s^{-1} and 3×10^{-5} M^{-1} s^{-1}, respectively.[72] It is believed that bisaquation converts the neutral *cis*-DDP to the positively charged hydroxy species which in turn, is attracted to the negatively charged DNA target via electrostatic interactions.



The rate-limiting hydrolysis is then followed by nucleotide binding, for which the rate of dissociation is extremely slow. In light of this, binding of *cis*-DDP to its target sequences has been assigned as a kinetic rather than thermodynamically driven process.[66a]

More recent studies by the Reedijk and Sadler groups[73,74] have shown that thiol and thioether containing peptides and proteins may play an equally important role in the activation and transport of *cis*-DDP to its ultimate target. Sulfur ligands possess a much higher affinity for Pt(II) than do most nitrogen ligands, and this principle has been used to great therapeutic advantage in the removal of toxic Pt(II) upon administration of therapeutically designed sulfur nucleophiles.[75] In contrast to this, the Sadler group has recently demonstrated that platinated

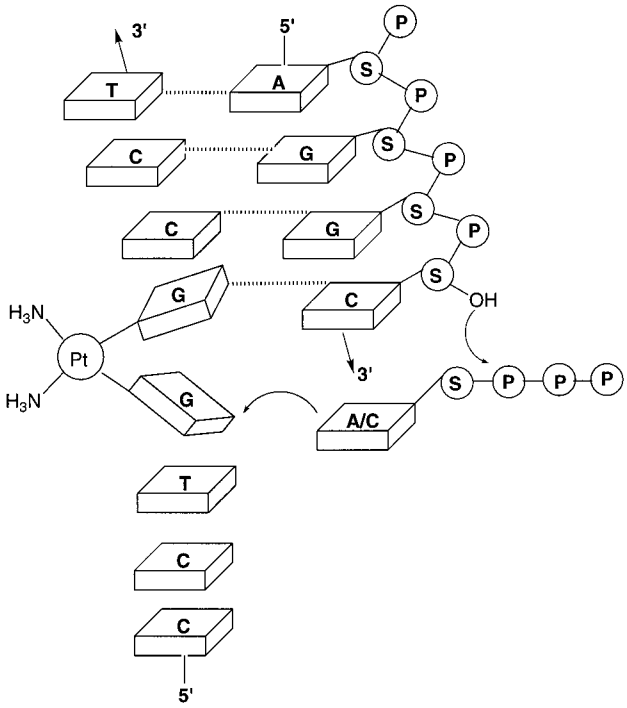
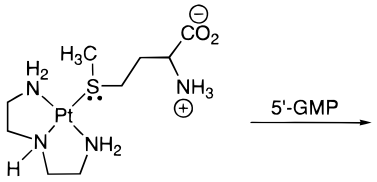


Figure 15. Mechanism of polymerase driven G to T transversion at 5′dG of *cis*-DDP modified $^{5'}GG^{3'}$.[78]

methionine **75** was capable of selective platinum transfer to the N^7 of 5′-guanosine monophosphate (5′ GMP) to afford **76**.[74] Attempts to effect Pt(II) transfer to any of the other three deoxynucleotides (dA, dT, or dC) yielded no conversion of **75** to the unplatinated amino acid. Additionally, reactions involving both methionine and 5′ GMP showed that generation of **75** was preferred over the direct N^7 platination of 5′ GMP but that over time **76** is formed as the thermodynamic product thus releasing the unbound thioether. Reedijk et al. have demonstrated that upon release of methionine in the conversion **75** to **76**, it is likely that platination may occur again and the cycle repeated thus suggesting that the process may be catalytic with respect to the thioether.[73]



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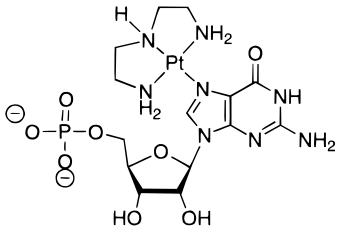
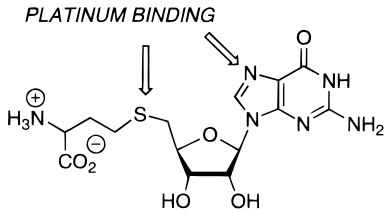


76

This is suggested based upon the intramolecular platinum transfer from the sulfur of S-guanosyl-L-homocysteine **77** to the guanosyl N^7 followed by a second platination at the previously occupied sulfur.



77

The initial kinetically driven thioether platination of **77** proceeds with a $t_{1/2}$ of 2 h, while the isomerization to N^7 is considerably slower ($t_{1/2}$ = 10 h). That migration proceeds via an associative mechanism (five-coordinate transition state) is supported by the second-order rate constants observed by Sadler et al. in the intermolecular conversion of **75** to **76** and is typical of many known substitution reactions of square-planar Pt(II) complexes.[76]

These data taken together comprise a meaningful (albeit very recent) picture of how *cis*-DDP may be transported to its nucleic acid target sequences. This mechanism is enticing too in that different cell types possess vastly different electrolyte compositions which could have bearing on the effectiveness of the bisaquation route to *cis*-DDP activation. For instance, liver cells possess [Cl^-] in excess of 150 mM which would hinder platinum hydrolysis thus limiting DNA adduction.[74] The viability of *cis*-DDP activation via sulfhydryl or thioether complexation, however, may afford a means of both delivery and activation toward DNA binding. Such a motif may involve the platination of select cysteines or methionines within a monoclonal antibody or small peptide targeted to a specific tissue or protein.

Although the intrastrand lesion is by far the major and perhaps the most interesting adduct, the induction of interstrand cross-links has also received considerable attention. The predominant DNA adducts (intrastrand) obtained upon treatment with *cis*-platin are the *cis*-[$Pt(NH_3)_2${d(GpG)-N^7(1),-N^7(2)}] (G*G*), *cis*-[$Pt(NH_3)_2${d(GpNpG)N^7(1),N^7(3)}], and *cis*-[$Pt(NH_3)_2${d(ApG)-N^7(1),-N^7(2)}] (A*G*) accounting for >90% of the DNA platination sites.[77a] The N^7-N^7 intrastrand cross-link at $^{5'}$GpG$^{3'}$ comprises roughly 65% of the adduction while the N^7 atoms of adjacent purines $^{5'}$ApG$^{3'}$ account for another 15%.[77b] The remaining products consist of the intrastrand 1,3 cross-link at $^{5'}$GNG$^{3'}$ as well as the interstrand lesion between the opposing N^7 atoms of the sequence $^{5'}$-GpC$^{3'}$.[77c] Although all intrastrand lesions have been shown capable of inducing mutations in the genome, it is widely recognized that the $^{5'}$GpG$^{3'}$ adduct is extremely lethal and is probably the source of *cis*-DDP cytotoxicity. Platination of $^{5'}$GpG$^{3'}$ induces upon the DNA polymer a high degree of structural distortion as evidenced by the destacking of these adjacent dG residues to afford a dihedral angle between the ring planes of 76−87°.[78] Both $^{5'}$GpG$^{3'}$ and $^{5'}$ApG$^{3'}$ intrastrand lesions bend the helix of duplex DNA by 32−35°, and it is known that the $^{5'}$GpG$^{3'}$ event bends the helix in the direction of the major groove despite retention of the hydrogen bonding network to the complementary deoxycytosine residues.[78,79] Helix perturbation is found to be much more profound with respect to the 5′-side of the principal $^{5'}$GpG$^{3'}$ target in that this G undergoes a G to T transversion (Figure 15) upon introduction of the $^{5'}$GpG$^{3'}$ *cis*-DDP lesion into bacteriophage substrates.[78] That the 3′ dG inflicts no such mutation in the replication of the original DNA substrate indicates that like apyrimidinic/apurinic (AP) sites in DNA, the platinated 5′-dG represents a "noninformational" or rather a "misinformational" site to the DNA polymerase responsible for complementary strand synthesis.[80]

Polymerase insertion of deoxyadenosine at such a site follows with the established default mechanism and ultimately results in the net G to T transversion.[78,80] That the intrastrand $^{5'}$GpG$^{3'}$ *cis*-DDP represents a mutational hot-spot detracts from the lesion's ability to halt polymerase activity altogether. Polymerase seizure is believed to be the source of cytotoxic activity while mutational activity is felt to cause development of secondary tumor formation upon therapy with *cis*-DDP. As such, efforts have focused on the generation of Pt(II) agents capable of shutting down polymerase and other enzymatic processes while avoiding detrimental mutation events. Emphasis has been placed upon the ability to form dinuclear Pt complexes which optimize DNA *inter-*

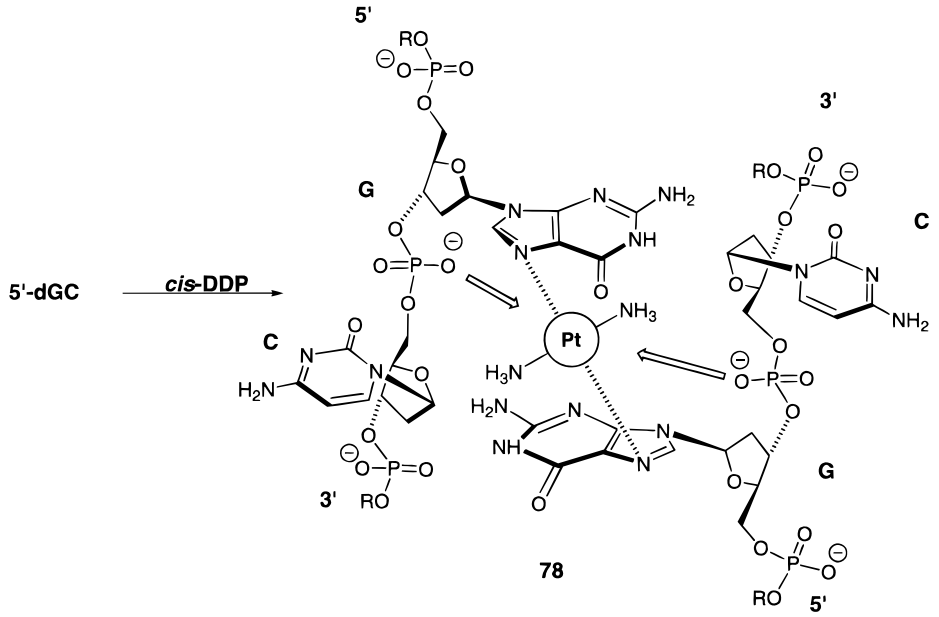


Figure 16. Structural distortion of $^{5'}$GpC$^{3'}$ upon *cis*-DDP-mediated ISC formation (Drobny and Hopkins[79]).

strand cross-linking events while avoiding intrastrand distortional processes.[81]

The high degree of target distortion induced upon DNA modification with *cis*-DDP is not limited to the intrastrand event. The interstrand lesion produced upon binding the diagonally displaced N^{7}s of $^{5'}$GpC$^{3'}$ accounts for 5−10% of the total platination products and has been shown to produce significant conformational perturbations within the substrate DNA. These distortions have been recently probed with respect to *E. coli* RNA polymerase (prokaryotic) and wheat germ RNA polymerase II (eukaryotic) elongation reactions involving the inter- and intrastrand platination events. Leng et al. have shown that DNA substrates bearing either the *cis*-d(G*TG*) intrastrand or *cis*-d(G*C/G*C) interstrand cross-link irreversibly shut down transcription at the site of platination.[70a] Termination was also observed several nucleotides ahead of the interstrand lesion suggesting that the presence of the *cis*-DDP lesion was not the sole reason for transcription blockage. That some DNA perturbation was induced by the lesion and was the cause of this "pre-lesion" block was supported by the efficient polymerase bypass of the same DNA substrate bearing monoadduction with chloro(diethylenetriamine)platinum(II). Severe structural distortion at the interstrand $^{5'}$GpG$^{3'}$ cross-link site had been suggested based upon molecular modeling, chemical hyperreactivity probes, and gel electrophoresis (DNA conformational analysis) but had not yet been demonstrated to alter enzymatic processes dependent upon the modified DNA.[77b,c,82]

Drobny and Hopkins have recently reported the solution structure of the *cis*-DDP interstrand cross-linked substrate [d(CATAGCTATG)$_2$].[79] Confirming earlier reports that the interstrand lesion induces an 80° unwinding of the DNA target and that considerable bending is induced as well, they also uncovered structural features never previously shown to result from small molecule−DNA interactions. Of particular interest was the determination that at interstrand cross-linked $^{5'}$GpC$^{3'}$ both cytosines are flipped completely out of the duplex leaving each N^7 platinated deoxyguanosine completely unpaired (Figure 16). Additionally, the NMR data generated indicate that the *cis*-DDP bridge resides in the minor groove of the duplex and that a highly localized change from right-handed B-DNA to left-handed Z-DNA occurs at this same Pt(II) bridge. This is in stark contrast to previous models for the *cis*-DDP interstrand cross-link, which place the bridge in the major groove and allow retention of hydrogen bonding/π stacking of the modified bases within the constraints of B-form DNA. Stabilization of the Z-DNA structure induced upon *cis*-DDP binding relies heavily upon electrostatic interactions of the phosphate oxyanions of each GC linkage (O−Pt distance = 3.2 Å) as depicted by Figure 16. In addition to the structural elucidation of the interstrand lesion, the NMR-derived prediction that DNA bending be in the direction of the minor groove versus the major groove (as previously assigned based upon modeling studies placing the Pt(II) core in the major groove) was confirmed utilizing a T4 DNA ligase assay. It is known that A tracts induce an approximate 20° bend (in the center of the A tract) in DNA toward the minor groove and that this distortion gives rise to electrophoretic retardation.[82]

To determine the direction of groove bending induced by *cis*-DDP, the ODN I (Figure 17) was interstrand cross-linked with *cis*-DDP and then ligated into both ODN II and ODN III each of which bear A tracts. Ligation into ODN II would afford a 17 bp separation between cross-link site and the bend site inherent to the A tract while ligation into ODN III would yield a 22 bp separation. These account for bends 1.5 and 2 helix turns removed from each other, assuming roughly 10.4 bp per helical turn.

Thus, sites of interest are either "in phase" with each other (the 22 bp situation) or "out of phase" with each other. That *cis*-DDP-mediated DNA bending was in the direction of the major or minor groove

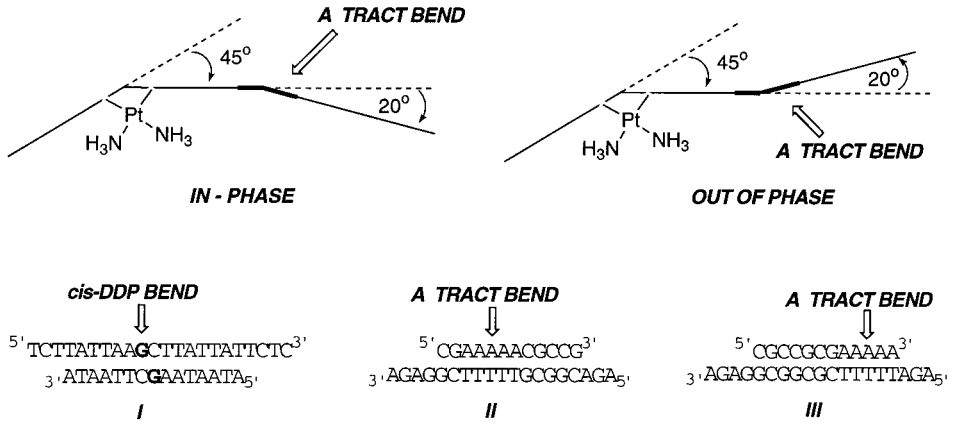


Figure 17. T4 DNA Ligase experiment with A tract bends "in phase" or "out of phase" with *cis*-DDP induced helix bending.

could thus be ascertained since bending (in phase substrate) in the same direction as that known for the A tract would afford a much more bent (electrophoretically retarded) structure than that seen for the "out of phase" case in which the two types of bends cancel each other. Additionally, bending toward the major groove would afford conformationally "neutral" ligation adducts in the "in phase" case resulting in minimal gel retardation or perhaps mobility enhancement depending on the extent of *cis*-DDP curvature induction. Comparison of electrophoretic mobilities between the "in-phase" and "out of phase" ligation adducts revealed that, in fact, the "in phase" products were considerably more gel-retarded than the " out of phase" ODNs. The ensuing minor groove assignment was thus consistent with the NMR-derived structure and further supported its relevance.

Most importantly, this work points out numerous mechanistic questions pertaining to the efficacy of *cis*-DDP and its analogues. Particularly intriguing, and very relevant to DNA cross-linking agents in general, are the issues of (1) the stepwise bond formation involved in going from monoadduct to cross-link, (2) conformational changes within the DNA target necessary for this conversion, and (3) the potential detrimental properties of an interstrand lesion or intrastrand lesion owing to perturbed DNA structure (i.e., mutational events), not inflicted by a simple monoadduction.

2.8. Dimeric DNA-Binding and Cross-Linking Agents

Chemical cross-linking of DNA by small molecules suffers from several drawbacks. First, most small molecules possess recognition motifs dependent upon only 2 or 3 bases of the target DNA sequence.[12] This lack of selectivity in many cases means that modification may occur not only in a region or genome of interest, but may also inflict undesired damage upon an otherwise innocuous substrate. Second, the means by which these molecules are attracted to their targets is somewhat limited due to the limited number of favorable drug−DNA interactions possible owing to the small size of the drug. A vivid example of this is the considerably lower binding affinity (k_a) of peptide fragments of a DNA binding protein versus that of the intact protein for a given DNA operator (recognition) domain.[83] Third and most importantly is the much slower rate of reaction for the second DNA modification event versus that of the initial DNA monoadduction.[84]

A strategy used commonly by nature in increasing site specificty and binding affinity of nucleic acid binding proteins is that of dimerization, both covalent and noncovalent.[85] Noncovalent dimerization in proteins often involves a "dimerization domain". In addition to the presence of a recognition domain these "domains" are responsible for extremely high binding affinities typically on the order $10^9 - 10^{12}$ M^{-1}.[85]

2.8.1. Sandramycin, Luzopeptins, and Isochrysohermidin

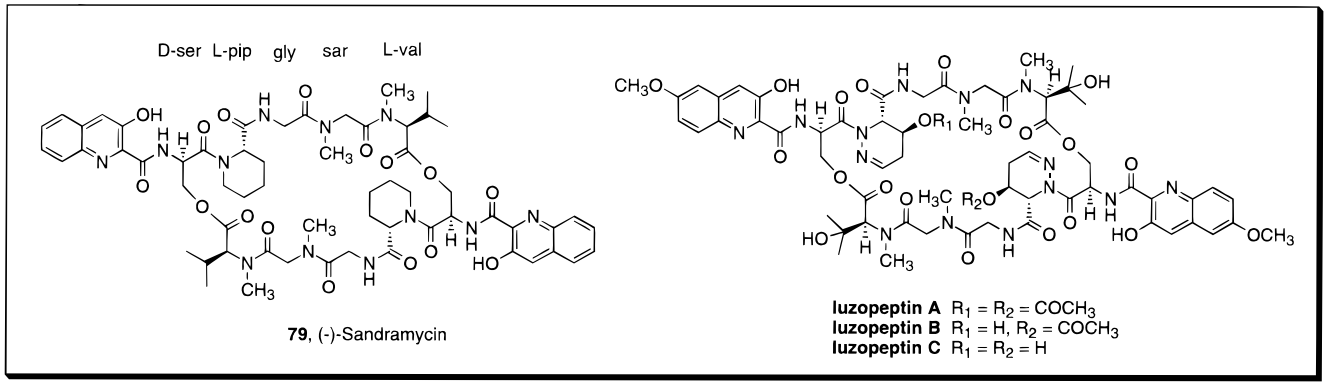
In addition to exploiting dimerization as a means of effecting efficient DNA binding by proteins, nature has also provided numerous examples of dimeric natural products which, despite their small size, bind DNA with high affinity and often surprising specificity. Many of these compounds bear a peptidic backbone to which is appended aromatic systems capable of DNA interaction via π-stacking interactions. These natural products usually display two unique modes of DNA interaction: intercalation via the appended aromatic systems, and extensive hydrogen bonding networks very often targeted to the DNA minor groove.

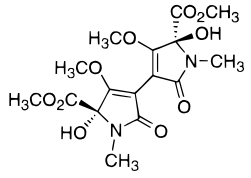
Representative of these agents are the recently isolated antitumor antibiotic sandramycin **79** and members of the luzopeptin class of bis-intercalators some of which are shown in Chart 2.[86a] These compounds possess a 2-fold axis of symmetry and preferentially target $^{5'}$TpA$^{3'}$ boxes within duplex DNA. This is in contrast to the closely related octadepsipeptides echinomycin and triostin A which prefer $^{5'}$CpG$^{3'}$.[86b,c] This sequence preference alteration is likely the result of the differing amino acid sequence of the decadepsipeptide backbone of sandramycin and the luzopeptins from that of the bicyclic octadepsipeptides of echinomycin and triostin A. It is noteworthy that regardless of such backbone differences, all these compounds benefit from the ability to bisintercalate into DNA at contiguous sites in a syn fashion.[86] This is a direct result of the dimeric nature of these peptidic natural products.

Significantly, Boger and co-workers have developed a very useful synthetic route to (−)-sandramycin

Chart 2

Bis-intercalative DNA Binders





80, isochrysohermidin

which allowed the dissection and assignment of energetic contributions to DNA binding by the different functionalities of sandramycin.[86a] In line with earlier studies of echinomycin,[86b,c] it was found that the largest contributor to favorable DNA interactions is the decadepsipeptide ($\Delta G° = -6.0$ kcal/mol).[86a] The aromatic chromophores substantially favor binding also, as evidenced by the incremental increase in affinity by 3.2 and 1.0 kcal/mol for the first and then second intercalation events, respectively. As such, bisintercalation favors DNA binding by approximately 4.0 kcal/mol.[87]

In contrast to the plethora of dimeric natural products which interact with nucleic acids noncovalently, isochrysohermidin, **80** has been shown to be an avid DNA interstrand cross-linking agent.[88] Although, the lesion resulting from DNA alkylation has not been fully characterized, mechanistic data suggest a likely pathway to covalent DNA bisalkylation (Figure 18).[88]

Boger and Baldino in presenting a novel total synthesis of isochrysohermidin reported the slow pH dependent formation of DNA interstrand cross-links into linearized φX174 DNA.[88] That *d,l*- and *meso*-isochrysohermidin all demonstrated equivalent cross-linking efficiencies under acidic (pH 4.2) or basic (pH 8.7) conditions, which were markedly lower near neutral conditions (pH 6−7) suggests the generation of the ring-opened amido ketones **81** and **83** as the electrophilic agents. Formation of the acylimminium **85** via dehydration and subsequent quenching by a nucleobase is unlikely in that basic conditions would deter such a process. Likewise, generation of **86** via methoxy group assistance of one pyrrole to the other is unlikely due to the poor leaving group ability of hydroxide and the preferential donation into the neighboring amide carbonyl of the same pyrrole unit. Experimentally, the pH dependence observed argues against the importance of **86** as a relevant intermediate.[88]

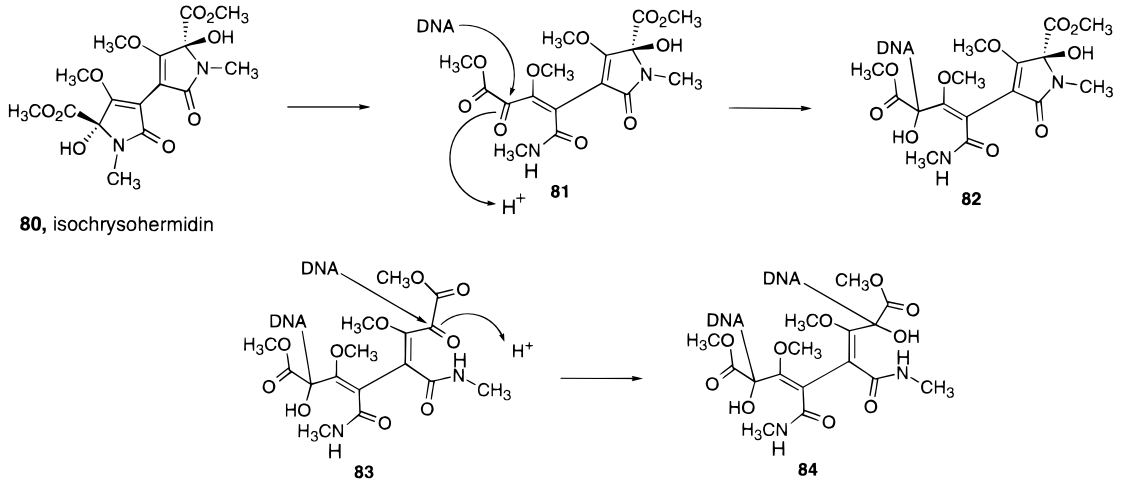
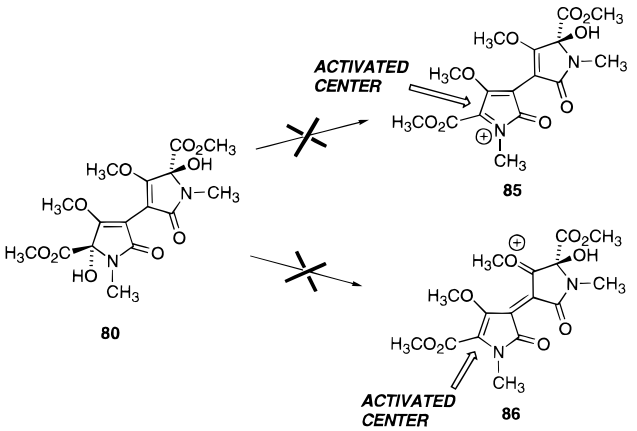


Figure 18. Proposed mechanism of DNA cross-linking by isochrysohermidin. [88]



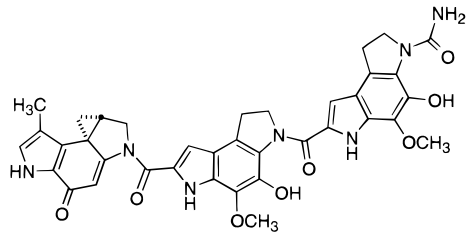
It is clear that the dimerism exhibited by this and other natural products affords dual reactivities not otherwise possible. In this manner, the dimerism of naturally occurring DNA monoalkylating agents leads to compounds which very likely possess vastly enhanced biological activities over the corresponding monomers. This has not only led to the study of dimeric natural products, but has been a likely inspiration for the synthesis and study of synthetically derived dimeric DNA binding substances.

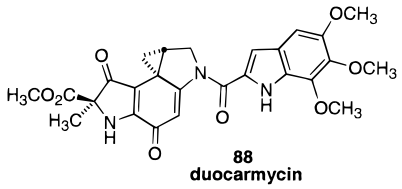
2.8.2. Biselezin (U-77,779) and Related Structures

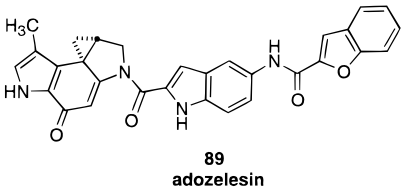
The cyclopropylpyrroloindoles (CPIs) have received considerable attention as extremely potent DNA monoalkylating agents. Of particular notoriety are CC-1065 **87**, duocarmycin **88**, and adozelesin **89** all of which induce selective adenine N^3 alkylation (Figure 19). Indeed, the mechanism of DNA alkylation by these and other related molecules (most of which are synthetically derived analogues of **87**) has been excellently reviewed by Boger and Johnson.[89a]

It is well established that S_N2 attack upon the cyclopropane methylene unit generates the phenol adduct **91** (Figure 19).[89b] This reversible process, in turn, gives rise to base- and heat-labile lesions leading to DNA strand scission.[89] Exploitation of these agents as potential interstrand cross-linkers was originally reported by Mitchell et al. in which generation of **93a**−**k** was achieved by reaction of **92** (2 equiv) with 1 equiv of the bisimidazole of the required diacid.[90]

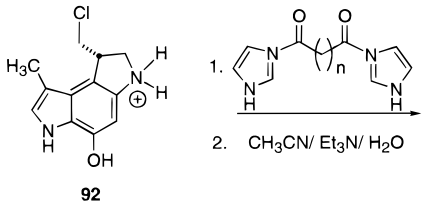
Additionally, dimers enantiomeric at one or both CPI units were constructed. Of these dimers, all enantiomeric derivatives were devoid of cross-linking activity while only **93 b**, **d**, **e**, and **g** gave rise to the

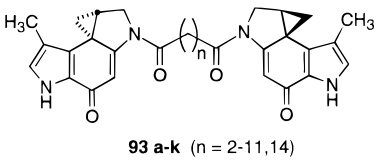






gel-retarded restriction fragments[91] indicative of cross-link formation.





Notably, **93b** and **d** at 1.7 μM concentrations afforded comparable amounts of cross-linked material to that achieved with 17 μM of trimethylpsoralen. Reaction of the restriction fragments of interest with **93e** and **g** yielded considerably less cross-linked material (as assayed by alkaline agarose gel electrophoresis) but still demonstrated the novelty of CPI dimerization in altering the mode of action of the parent core structure.

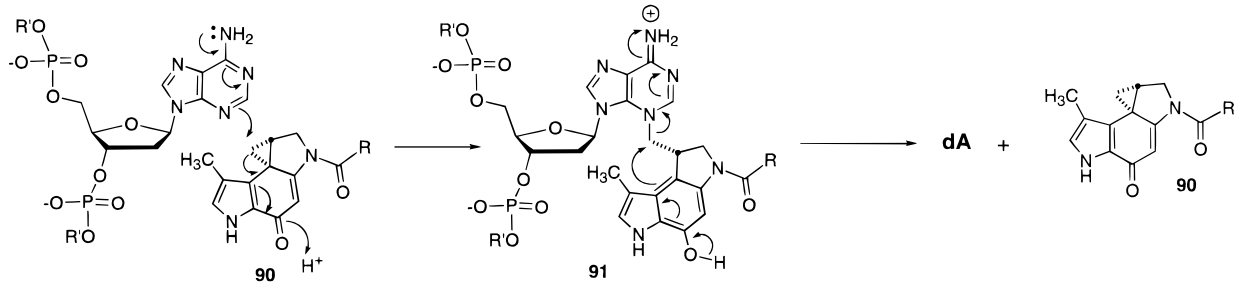
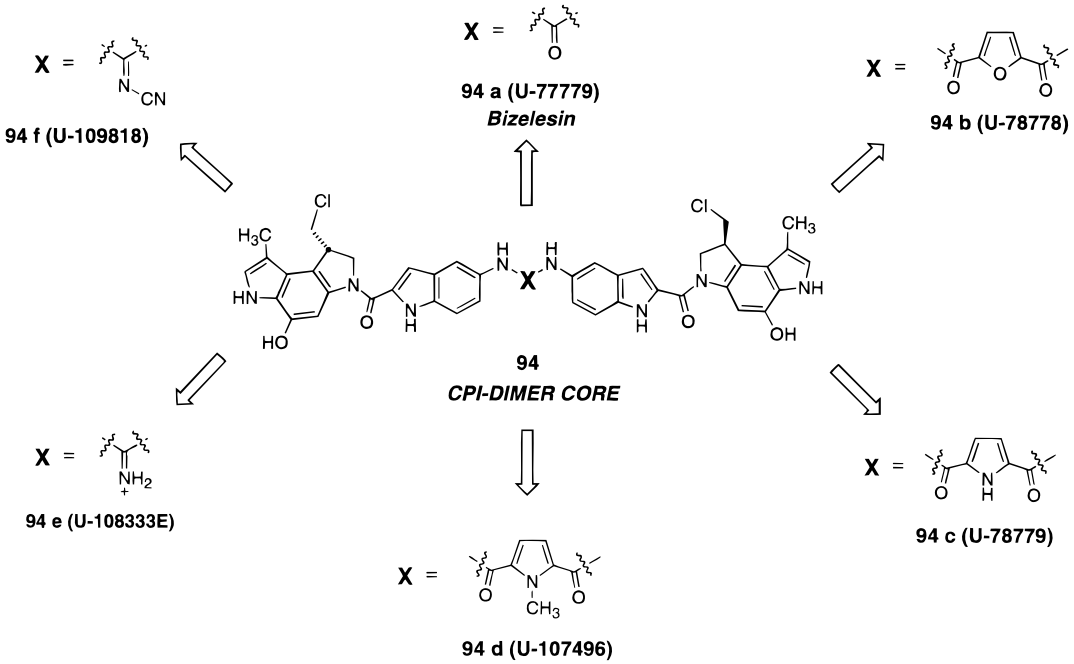


Figure 19. Reversibility of DNA alkylation by cyclopropylpyrroloindoles

Scheme 2



A wide array of second-generation CPI dimers have been investigated with respect to both interstrand cross-linking and monoalkylation properties. By far the most extensively examined of these is bizelesin (**94a**) which is currently in phase I clinical trials.[93,94] Indeed, many of the members of this generation have been used as probes for the mechanism of DNA recognition and modification by **94a**. Interestingly, the furan and pyrrole-based substances **94b** and **94c** are considerably more cytotoxic than the "parental" dimer **94a** (Scheme 2).[94b]

Unlike the methylene-tethered CPI dimers, the second generation compounds all possess the rigid bis(indolecarboxylic acid) linker which allows a 6 to 7 bp span between alkylation sites while occupying the intervening minor groove between the terminal alkylation sites. Like CC-1065 (**87**), the CPI units selectively alkylate the adenine N^3 of the sequence $^{5'}$TTA$^{3'}$. As such, bizelesin preferentially cross-links the sequence $^{5'}$*TAATT**A**$^{3'}$ (**A** indicates one of the cross-linking sites while * indicates the thymine opposite the other cross-linked adenine), although other sequences which place the two adenines of interest the same distance from each other have been noted.[93]

Hurley et al. have shown that bizelesin may also cross-link the sequence $^{5'}$TTAGTTA$^{3'}$ at the same residues observed with the shorter substrate lacking the central dG-dC base pair.[94a] Surprisingly, this sequence undergoes cross-linking preferentially to the shorter $^{5'}$*TAATT**A**$^{3'}$ despite a considerably greater distance between the targeted adenine N^3 residues.

^{1}H NMR analysis and inosine substitution (for the central dG) reveals that the exocyclic N^2 hydrogen of guanosine (not H-bonded to the complementary cytosine) allows an extremely strong hydrogen bond with the ureylene carbonyl which benefits from coplanarity with the dG exocyclic amine.[94a] Once established, this bond shows quantitative occupancy within the duplex adduct (even at 500 K) and essentially locks the bizelesin linker moiety within the minor groove. Additionally, twisting of the urenyl linker almost 90° out of the bisindole plane was found to induce considerable curvature of the dimer thus facilitating the minor groove fit. Given this, it has been postulated that upon monoalkylation, bizelesin undergoes hydrogen bonding to the central dG (via N^2 interactions). This, in turn, pulls bizelesin down into the minor groove allowing distortion of the alkylated adenine toward the center of the duplex thus reducing the distance between the respective adenine N^3 residues. This perturbation in target structure mediated by the urenyl linker affords an N^3 to N^3 distance in the 7-bp system comparable to that seen with the 6-bp target.[94a]

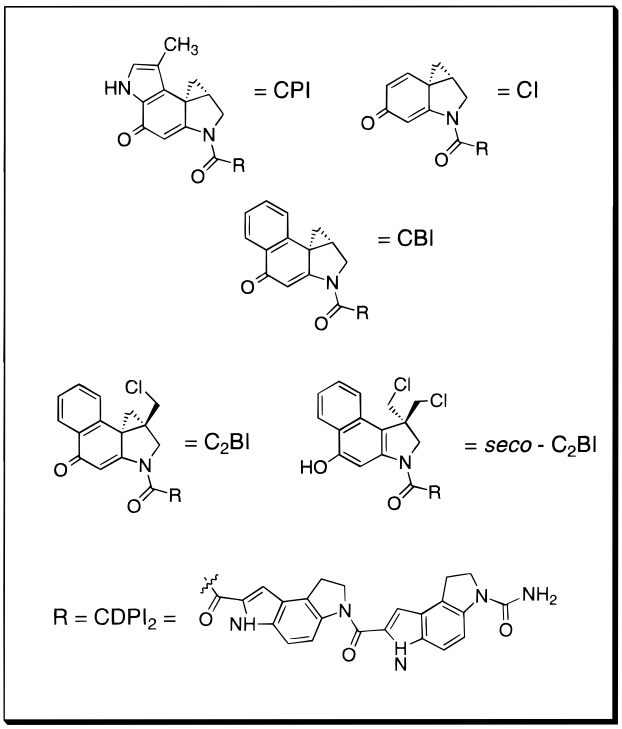
These results, coupled with studies aimed at understanding the principle of $^{5'}$TAATTA$^{3'}$ recognition, point out the importance of the dimerization motif chosen. Within the $^{5'}$TAATTA$^{3'}$ sequence, inherent instability of the central A·T step leads to base pair opening. Once opened, hydrogen bonding of the ureylene amido protons and the bases stabilizes the transient Hoogsteen and open base pair conformations thus facilitating ISC formation and specificity. That the ureylene moiety exerts such a strong influence upon the sequence selectivity of bizelesin suggests that dimerization offers enhanced specificity not due just to the compounding of monomer−DNA interactions, but also the result of linker−DNA interactions as well. Bizelesin represents a novel example of this concept and the importance of these interactions is reflected in the efficiency with which bizelesin selectively inflicts bisalkylation upon $^{5'}$TTAGTTA$^{3'}$. Hurley and co-workers have reported that bizelesin does not form detectable amounts of monoalkylated material upon reaction with $^{5'}$TTAGTTA$^{3'}$.[94a] Instead, only the direct conversion of native double-stranded DNA to covalently cross-linked substrate is observed for reaction aliquots analyzed between 0.5 and 72 h. That this has not been the case for

efforts involving the shorter 6 bp substrates lacking the central dG-dC pair bodes well for the importance of the dG-to-linker contacts observed with 5′TTAGAAT3′. Considerable quantities of monoalkylation adducts have been reported for other target sequences[93a] and thus point out less pharmacologically important targets given the relative ease with which monoadducts may be repaired[6a] and their inability to stop strand separation events.[9−11,13,14]

Significantly, Kelly et al. have shown that modification of the bis(indolecarboxylic acid) linker in **94a** to the pyrrole-linked **94c** provides a CPI dimer capable of mixed AT-GC DNA recognition−modification.[94b] Whereas **94a**,**b**,**d**−**f** all recognize predominantly AT-rich sequences, the pyrrole moiety of **94c** can hydrogen bond favorably with a centrally located CG base pair thus significantly altering binding properties.[94b] This is in similar accord to the rationale for enhanced cross-linking of 5′TTAGAAT3 relative to 5′TAATTA3′ observed for bizelesin.[94a] The theme of DNA structure recognition based partly on the choice of linker moiety has been further examined by these same workers. By designing a duplex containing a monoalkylation site immediately adjacent to a cross-linkable site, Kelly and co-workers have examined the ability of **94a**, **e**, and **f** to translocate within the DNA minor groove from the kinetically favored monoalkylation site to the thermodynamically preferred cross-link site.[94d] That the mechanism of "alkylation shifting" operates via a DNA translocation mechanism as opposed to free diffusion was supported by the unchanged alkylation properties of radiolabeled DNA in the presence and absence of both specific and nonspecific competitor DNAs. Significantly, it was found that **94a** and **94f** were readily capable of this translocation process, whereas the imminium species **94e** was not. This was attributed to highly favored hydrogen bonding of the immium moiety with the DNA phosphodiester backbone thus locking **94e** into the initially formed monoalkylation site. Taking this further, these workers have established hydrogen bonding interactions of the ureadiyl linker unit and differing target sequences.[94d] It has also been noted that whereas bizelesin reacts preferably with bent DNA targets to yield a straightened adduct, the GC-targeting pyrrole **94c** prefers to react with straight DNA to afford a significantly distorted product.[94b,c]

In addition to exploitation of cyclopropylpyrroloindoles (CPIs) within different dimerization motifs, the corresponding 1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (CBI) agents shown in Chart 3 have been studied.[89a,95] Boger et al. have shown that CBI-based agents display similar DNA alkylation specificities to those shown for the CPI counterparts.[95] Significantly, the rates and overall efficiencies of CBI-mediated DNA alkylation events are enhanced relative to the CPI analogues. These compounds also have the other beneficial attributes of improved chemical stabilities and greater biological activities.[95a] From a synthetic standpoint, they are also much more accessible than their CPI relatives.[95a] Examination and comparison of numerous CBI agents (shown in Chart 3 wherein the R group is $CDPI_2$ or some analogue thereof) revealed that C_2BI−$CDPI_2$ demonstrated substantially slower rates and intensities of DNA alkylation as compared to the analogous (i.e., R is mutually used for each conjugate) "monofunctional" CBI. This was the case with respect to comparison against both the (+)- and (−)-enantiomers of the CBI agent. The stereoelectronically controlled adenine N^3 addition to the least substituted cyclopropane occurs with a selectivitiy that represents the culmination of the two enantiomers of the corresponding monofunctional CBI agent. It was also found that variation of the $CDPI_2$ group exerted an influence upon the extent of alkylation observed. Not unexpectedly, as the size and corresponding DNA-binding affinity of the R group went down, so did the extent of alkylation.

Chart 3

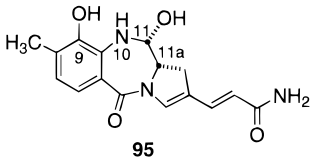


Unlike the CBI derivatives, C_2BI-based compounds were proposed to be capable of ISC formation. Examination of the achiral conjugate *seco*-C_2BI−$CDPI_2$ under conditions similar to those used for monoalkylation studies showed that, in fact, this agent is effective at ISC generation. At drug concentrations of 10 and 100 mM, cross-linking efficiencies of 20% and 98%, respectively, were obtained.[95b] Somewhat surprisingly, cross-linking efficiencies were closely related to those for monoalkylation under very similar reaction conditions. Notably, the overall DNA alkylation efficiencies of the "difunctional" C_2BI agents are much lower than those observed with the CBI agents. This was found not only from in vitro DNA degradation studies, but was also suggested by relative potencies derived from cytotoxic assays with L1210 cells. These authors suggest that the additional C9a chloromethyl substitutent sterically inhibits adenine N^3 access thus slowing the rate of alkylation. From these studies, however, it is clear that dimerization need not be the only means by which a monoalkylating agent can be converted to

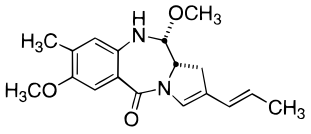
one capable of interstrand cross-linking. Indeed, the strategy of placing additional functional sites very close to the naturally occurring electrophilic center was pursued in early studies of DNA cross-linking pyrrolobenzodiazepines.[95]

2.8.3. Pyrrolobenzodiazepine Dimers

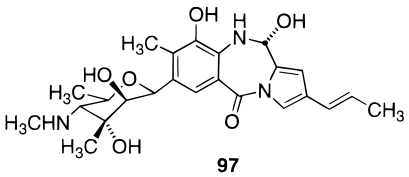
The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) comprise a class of antitumor antibiotic natural products which includes anthramycin **95**, tomaymycin **96**, and sibiromycin **97**.[96a]



95
anthramycin



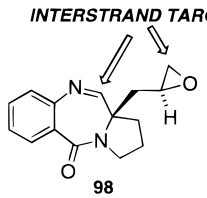
96
tomaymycin



97
sibiromycin

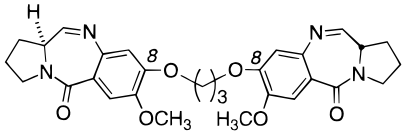
Conversion of the carbinolamine at C-11 to the corresponding imine affords reversible alkylation in the minor groove of the exocyclic N^2 amine of deoxyguanosine within duplex DNA. Minor groove binding is promoted by the PBD twist resulting from the *S* configuration at the bicyclic C11a position and related congeners bearing the phenolic 9-OH further benefit from hydrogen bonding with the O^2 of the cytosine complementary to the alkylated guanosine.[96b−d] Sibiromycin lacks the *S* stereochemistry at C-11a (due to the pyrrolic nature of this moiety) but the amino sugar compensates for this by providing attractive electrostatic interactions with the anionic backbone of DNA.[97] PBD monomers are efficient inhibitors of DNA and RNA synthesis[98a] and have proven to be highly sequence selective with a preference for purine-G-purine triplets.[98b] These agents have also received considerable attention as DNA anchoring systems for nonspecific DNA cleaving agents such as Fe^{II}−EDTA.[99] Additionally, conjugation to the 5′-AATT-3′ minor groove binding agent netropsin has been pursued as a means of enhancing the specificity of DNA modification by anthramycin.[100]

Bifunctional PBDs such as **98** have been prepared with the intention of effecting bisalkylation but have met with only minimal success.[95] Considerably more

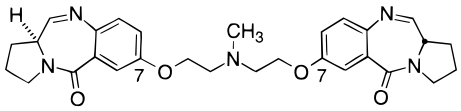


98

fruitful has been the generation of PBD dimers resulting from linker connection to the phenolic moieties of two PBC cores. Hartley and Thurston have shown that the dimers involving PBD linkage through their C8 positions via the flexible 1,3-alkanediyldioxy ether are extremely efficient interstrand cross-linkers.[101] Particularly effective is the propane−diyldioxy ether linked dimer **99**. After only 2 h at 37 °C, **99** induces detectable cross-linking (as assayed by alkaline agarose gel electrophoresis using linearized PBR322 DNA) down to 10 pM concentration (drug:nucleotide ratio = 0.025) while at 0.4 μM (drug-to-nucleotide ratio = 1.0) greater than 90% of the linear substrate is cross-linked. By comparison, **99** is 50 times more efficient than either mechlorethamine or *cis*-DDP and rivals the alkylation efficiency of the CPI dimers described above. More interestingly, cross-linking was found to be irreversible. This is in stark contrast to the weaker binding C7-linked dimer **100** examined by Suggs et al.[102]



99



100

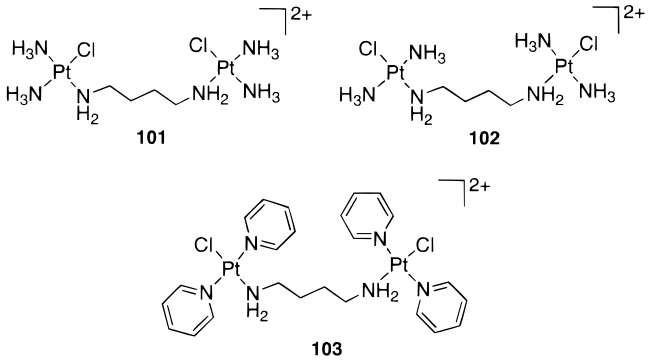
Thus, in the case of **99**, dimerization enhances the efficacy of each PBD monomer not only by increasing drug−DNA affinities, but also by preventing loss of the relevant lesion to hydrolytic decomposition. Additionally, it is interesting to note that the altered means of dimerization (C7 versus C8 connectivities) led to different reactivities with respect to DNA alkylation.

Although **99** gave rise to DNA cross-links that migrated analogously to double-stranded standards, **100** afforded DNA adducts which migrated considerably slower than psoralen cross-linked standards. That such adducts were attributable to interhelical events seems a likely explanation for this phenomenon and points out a potentially useful mechanistic difference which is potentiated by the chosen means of dimerization.[103]

2.8.4. Dinuclear *cis*-DDP Analogues

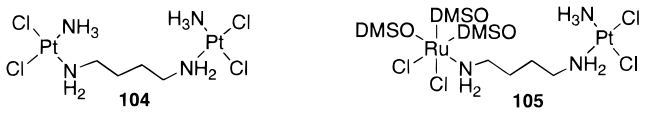
As mentioned previously, the interstrand lesion resulting from reaction of *cis*-DDP with 5′-GC-3′ results in massive distortion of the B-DNA target which very likely limits the utility of this compound as an interstrand cross-linking agent. Similarly, the intrastrand lesion while forming much more readily than the interstrand lesion, induces mutational events via the distortion of its nucleic acid target. These detrimental issues have been addressed by the generation of dinuclear Pt complexes which possess two Pt(II) centers each of which carries only one chloro ligand thus relegating each Pt(II) capable of only one coordination event.[81a] Farrell et al. have

recently synthesized the dinuclear complexes **101**−**103** and studied their respective interactions with calf thymus DNA and a small 49 bp ODN.[81a]



Owing to the presence of the pyridyl ligands, **103** induces a much higher degree of DNA unwinding than that seen with either of the ammonia-bound complexes, as well as the mononuclear *trans*-[$PtCl_2(py)_2$]. Similarly, **103** stabilizes B-form DNA while the other complexes induce B−Z transitions upon DNA binding. These alterations likely involve the ability of **103** to undergo π-stacking interactions upon DNA association which in turn, disfavors the Z-DNA conformation. Importantly, ISC formation is very efficient for all three complexes with yields of 80%, 52%, and 41% for **101**, **102**, and **103** respectively. Further, only **102** (the *trans*-isomer) was capable of intrastrand adduction. That the diamine chain in **102** can be rotated around the two Pt square planes to afford simultaneous dG N^7 binding in the sequence $^{5'}$GGCC$^{3'}$ is the proposed origin of the intrastrand capability of **102**.[16] The *cis* placement of the chlorides (as in **101**) with respect to the diamine linker prohibits (after the initial DNA connection) the butanediamine chain from achieving an orientation necessary for the intrastrand connectivity. As such, interstrand cross-linking has been very successfully achieved while avoiding the undesired distortional processes inherent to the mononuclear complexes.[81a] Importantly, such dinuclear Pt(II) complexes have been shown to be extremely effective against tumor cell lines which have become resistant to monomeric *cis*-DDP.[104]

The ability to form heterodimers of mono- or dichloro Pt(II) amine complexes and dichloro Ru(II) has allowed the generation of agents which interstrand or intrastrand cross-link a given DNA substrate with one end while giving rise to protein adducts at the other end.[16a] This methodology has been elegantly demonstrated en route to the formation of DNA−protein cross-links between the repair enzymes UvrA and UvrB and a synthetic ODN treated with the homodimers **102**, **104**, or the heterodimeric **105**.[16a]



The UvrABC nuclease system acts to initiate the process of nucleotide exicision repair leading ultimately to DNA repair of damaged nucleotides.[6a] UvrA and UvrB form a 2:l complex in solution that has been shown to travel along DNA in an ATP-dependent fashion. Arrival at a site of damage induces structural reorganization of the trimeric assembly resulting in loss of the UvrA dimer. Retention of the resulting UvrB-DNA complex signals to UvrC the precise location of damage. UvrC, in turn, induces dual incisions upon the phosphodiester backbone bracketing the lesion of interest. Demonstration that both UvrA and UvrB are in intimate contact with the DNA and its initially formed Pt(II) lesion (as the recognized site of damage) manifests itself in the observed DNA−protein cross-links obtained using **102**, **104**, and **105**. Specifically, cross-linking of both the UvrA/UvrB complex and lone UvrB to the ODN was obtained upon treatment with either **104** or **105**. Cross-linking of the UvrA dimer to the platinum-modified ODN ensued using any three of the dinuclear complexes thus further demonstrating the versatility of this approach. In this fashion, the dimerization of transition metal complexes represents a novel means of not only effecting DNA interstrand cross-links, but of also inducing site-specific suicide DNA lesions en route to enzyme inactivation.

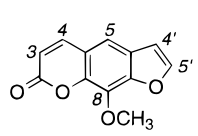
The versatility of the dinuclear metal complexes brings to light an additional strength to the strategy of dimerization; that being diversity. The mixing and matching of dimerized moieties not only allows the previously discussed issues to be addressed (with respect to ISC formation), but also allows elaboration on the themes of specificity and affinity. As evidenced with the metal complexes above, even basic mechanisms of action can be radically altered based upon the choice of the monomeric units involved in dimer construction.

3. Photoactivated Cross-Linking Agents

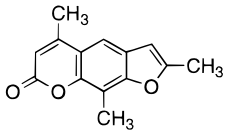
3.1. Psoralens

To date, the psoralens are the only class of compounds known to induce ISCs upon photolysis. This naturally occurring class of aromatic compounds consists of a furan ring fused to a coumarin.[105] They are found predominantly in plants from the *Umbelliferae*, *Rutacea*, and *Leguminosae* families, but have also been isolated from microorganisms including fungi.[106] The two most heavily studied psoralens, 8-methoxypsoralen (8-MOP, **106**), and 4,5′,8-trimethylpsoralen (Me_3psoralen, **107**) are found as fungal metabolites.[106] Clinically, the psoralens are employed in the treatment of psoriasis, vitilago, and cutaneaous T-cell lymphoma (CTCL).[107] They have also proven efficacious for the treatment of diseases associated with autoimmune disorders, organ rejections, and the AIDS-Related Complex.[107]

It is proposed that in addition to ISC generation, these agents express favorable activity by inducing A to C mutations which enhance immunogenicity in tumor cells.[108,109] The sequence $^{5'}$GGGATTTC**A**CCC$^{3'}$ is an essential enhancer motif in murine lymphoma cells but not in the host HeLa cells. Its binding to the transcription factor NF-kβ results in activation



106
8-MOP



107
Me_3psoralen

of transcription; an action traditionally associated with proliferation of nontumorigenic cells. Briegel et al. have shown that in the case of the IL-2 gene of murine lymphoma cells, mutation of the boldface **A** to C in this sequence converts a weak binding site (for the T-cell restricted NF-kβ) into a strong one resulting in release of NF-kβ from the inhibiting factor Ikβ.[108] Induction of the transcription factor gives rise to increased production of nontumorigenic cells thus allowing for an increased immunological response. This represents a unique application of mutation induction by a DNA reactive agent. Unlike *cis*-DDP which induces detrimental mutations, the psoralens may express the majority of their preferred biological activity via a mutational pathway which enhances the immune status of host cells.

In addition to the clinical applications, the psoralens have been used extensively in probing the secondary and tertiary structures of DNA and RNA.[106] Similarly, psoralen−DNA monoadducts have been used as probes for DNA−protein interactions via the generation of DNA−protein cross-links.[110] Solid-phase synthesis of psoralen-functionalized ODNs has been achieved upon generation of psoralen-tethered phosphoramidites[111] Additionally, biotinylated psoralens have been used for nonisotopic labeling of DNA.[112] They have also been used to inactivate viruses and other pathogens via irreversible nucleic acid interations which spare the function of proteinacious cellular subunits.[113]

Induction of ISCs into duplex DNA requires three distinct steps. Initially, the planar tricycle intercalates into the duplex between the two bases of either 5′-TA or 5′-AT. Such binding to synthetic ODNs is particularly strong with dissociation constants (K_d) on the order of 6.6×10^{-6} M.[114] Upon the absorption of a quantum of light, either the 4′,5′-furan double bond or the 3,4-pyrone double bond can undergo photocycloaddition to the 5,6-double bond of a thymidine.[115] Reaction of the pyrone destroys the coumarin nucleus thus resulting in a low lying triplet state for the resulting monoadduct. This state is unreactive toward the second photocycloaddition due to its rapid relaxation to the ground state. Thus, loss of the coumarin nucleus abrogates ISC formation. However, retention of the pyrone (via the initial photocyclization involving the furan) allows the intact coumarin to absorb a second quantum of light leading to local excitation of the 3,4-double bond to the excited triplet (π to π* transition) which cyclizes with the 5,6-double bond of the adjacent pyrimidine thus affording the ISC.

The photoreaction of thymidine with psoralens may occur from either the 3′-side or 5′-side of the thymidine base depending upon the displacement of the psoralen (pso). Monoadduct formation within either cross-linkable site $^{5'}$TpA (3′ face) or $^{3'}$TpA (5′ face)

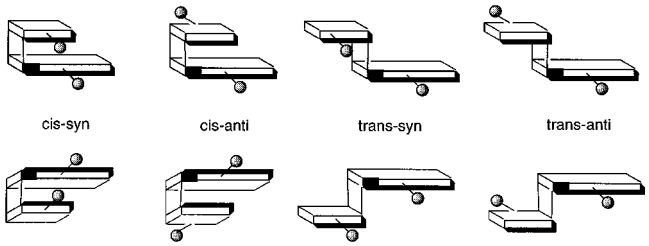
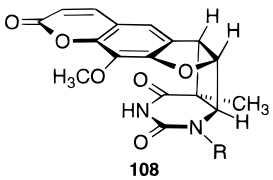


Figure 20. Representation of the eight possible configurational isomers for 8-MOP-thymine monoadducts. The large slabs represent 8-MOP with the furan end designated with the black marker and the C8 methoxy group as the shaded handle. Thymine is represented by the smaller slabs with the shaded handle representing N1. Isomers on the top row are mirror images of the isomers on the bottom row.[106]

results in the generation of enantiomeric thymine adducts. While a number of isomers are possible as shown in Figure 20, the *cis-syn* configuration is most readily formed due to severe restrictions on the modes of psoralen intercalation imposed by the duplex conformation.

Chirality of the deoxyribosyl unit renders these monoadducts as a mixture of diasteromers. Exemplary in this stereoselectivity is 8-MOP **106**. Reaction of **106** with $^{5'}$TpA$^{3'}$ or $^{5'}$ApT$^{3'}$ yields two furan-side monoadducts **108** and **109**, with concomitant generation of only one pyrone adduct **110** (C-8 methoxy not shown for clarity).[106] Additionally, thymidine-8-MOP-thymidine cross-links have been isolated as a mixture of two diastereomers each of which bears the *cis-syn* sterochemistry as per **111**. This stereochemical relationship has been observed for numerous psoralens and is very likely general to all linear furocoumarins.[116]



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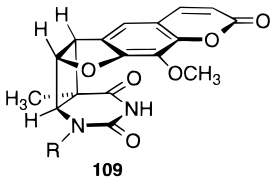

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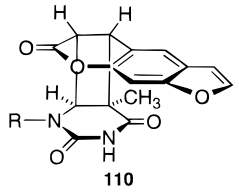

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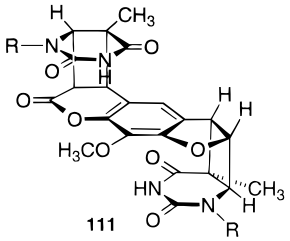


111

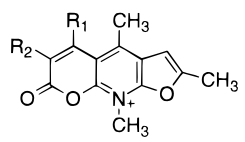
Psoralens have been used to noncovalently increase ODN binding affinities en route to triple helix motifs. Likewise, psoralen specificity has been enhanced via triple-helix strategies. Such instances of covalent attachment of a psoralen-tethered ODN to a given DNA target illustrates the symbiotic relationship between the two halves of the conjugate.[117] For instance, the Thuong group has demonstrated that psoralen conjugation to $^{5'}$TTTTCCTCTCCCCTCT$^{3'}$ (C = 5-methylcytosine) affords an ODN capable of inhibition of gene expression.[118] The functionalized polypyrimidine binds (via Hoogsteen base pairing) to the site (operator sequence) normally occupied by the

transcription factor NF-kβ which activates transcription from the IL-2Ra promoter. Photolysis at 365 nm selectively anchors the ODN proximal to the operator and thus increases the localized concentration of the ODN to its target sequence. Inhibition of transcription by the ODN−pso conjugate occurred upon in vitro photoinduced cross-linking followed by electroporation/transfection into HSB2 T-cell tumor lines. Additionally, in vitro transcription assays were performed with the cross-linked plasmid (IL-2Ra CAT) as were in vivo assays in which the ISC was induced following introduction of the ODN−pso conjugate into the cell line. Both assays revealed a high degree of ISC induction (70−80%) and subsequent transcription inhibition.

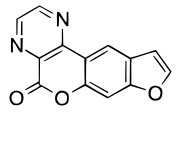
Thuong et al. have applied a similar approach toward targeted interstrand cross-linking of HIV proviral DNA.[119] Psoralen conjugation to the 5′ phosphate of $^{5'}$TTTTCTTTTCCCCCCT$^{3'}$ afforded a conjugate capable of triplex formation with two 16 bp homopurine runs of the HIV proviral genome. Each homopurine run overlaps the recognition/cleavage site of Dra I restriction endonuclease and subsequently, represented a novel target for ODN−pso conjugate binding. Construction of pBT1 and pLTR plasmid DNAs resulted from insertion of HIVBRUCG proviral fragments into the commercially available pUC19 and pBR322 plasmids. The pLTR substrate contained four Dra I sites ($^{5'}$TTTAAA$^{3'}$) one of which extends three base pairs into the triplex region. Plasmid pBT1 contained 14 Dra I sites of which two were within the triplex domain. Efforts involving Dra I cleavage of pLTR revealed that the conjugate (unphotolyzed) was capable of slight Dra I inhibition at short incubation times, but that over time, complete cleavage of the substrate occurred. The reversibility of triple helix formation responsible for this observation was averted upon photolysis. Irradiation at 20, 40, and 330 s resulted in irreversible ODN−pso binding as evidenced by complete Dra I blockage at the cleavage site in question. Similarly, the same phenomenon was observed with the pBT1 substrate. Additionally, reaction of this substrate with the conjugate (following irradiation) afforded an unanticipated Dra I inhibition site resulting from triplex formation involving only eight consecutive base triplets. This was ultimately attributed to the inability of contiguous cytosines to undergo protonation at the experimental pH of 6.9. Thus, a plasmid site capable of triple helix formation with only the $^{5'}$TTTTCTTT$^{3'}$ portion of the ODN−pso conjugate was detected via unintentional photoinduced ISC formation.[119]

The ability to fine-tune psoralen reactivity via synthetic manipulations has been demonstrated. The general trends observed place particular importance upon functionalization at carbons 3, 4′, 5, and 8. Methylation of the 4, 5′, and 8 positions enhances the intercalative binding affinity, as well as, the quantum yield of cycloaddition over that of the unsubstituted case.[120] However, methyl ether incorporation at C8 dramatically slows the photochemistry, hence, the much slower DNA alkylation by 8-MOP versus **107**.[120b] Methyl group placement at C4 results in near quantitative monoadduct formation at the furan side of the chromophore in contrast to 8-MOP which gives up to 20% pyrone monoadduct. This is attributed to steric clashes involving the C4 methyl and the thymidine C5 methyl with which the pyrone side would normally react.[121] Strong electron-donating or withdrawing groups at C5 and C8 greatly reduces or completely obliterates reactivity with DNA.[120b] Analogous substitution at C3 has a similar result.[122] However, positively charged groups placed at C4′ and C5 enhance both noncovalent DNA associations as well as covalent modification events.[123]

Recent synthetic efforts have focused on generation of psoralens with enhanced light absorption properties, those being longer lived and more easily accessed excited states and greater water solubility characteristics.[114] This has involved the synthesis of heterocyclic analogues in which sulfur or selenium replaces the endocyclic oxygens,[124] as well as, the construction of *N*-methylazapsoralens of the form **112a** (R_1 = CH_3 or H, R_2 = CH_3 or H) possessing vastly improved water solubility.[125a] Additionally,



112a



112b

the ring-extended psoralen **112b** bearing fusion of a pyrazine to the pyrone double bond has been synthesized and its 4′,5′-furan reactivity studied with several model olefins.[125b] Despite these considerable synthetic efforts, extensive study of the DNA modification chemistry by these agents has not yet appeared.

4. Oxidatively Activated Agents

The cyctochrome P-450 group of monooxygenases is a superfamily of enzymes which consists of more than 153 genes represented in 27 families, 10 of which occur in mammals.[126] Oxidative transformations catalyzed by cytochrome P-450 include carbon hydroxylation, heteroatom hydroxylation, heteroatom dealkylations (via aldehyde generation), oxidations of π systems, and conversion of aldehydes or alcohols to the corresponding acids.[127] In a general sense, the thermodynamic parameters of the oxygenation event may be represented by eq 3.

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + NADP^+ + H_2O \quad \text{(EQ 3)}$$

This tranformation involves two extremely high energy barriers, namely, dissociation of the dioxygen bond (460 kJ/mol), and dissociation of the carbon−hydrogen bond of the substrate (420 kJ/mol).[128] By breaking down the overall process into several discrete steps, cytochrome P-450 affords the net transformation requiring only 40−70 kJ/mol.[128] This is largely due to the weakening of the O−O bond upon binding of dioxygen to the heme iron in the active site. As depicted in Figure 21, the mechanism of substrate oxidation involves: (1) binding of the

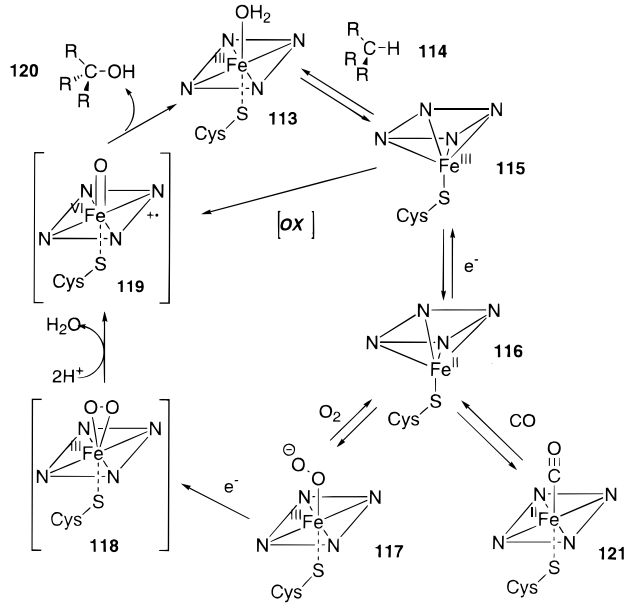


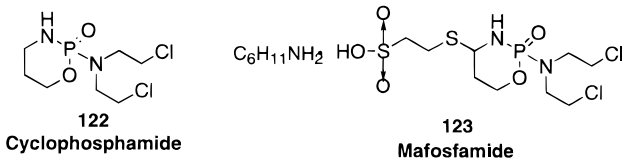
Figure 21. Proposed mechanism of xenobiotic oxidation by cytochrome P-450.[129]

substrate **114** to the enzyme resulting in conversion of the low-spin six-coordinate species **113** to the high-spin five-coordinate **115**; (2) one electron reduction of the iron by the flavoprotein NADPH cytochrome P-450 reductase to yield **116**; (3) reaction of the ferrous iron with O_2 to yield the unstable $(FeO_2)^{+2}$ complex **117**; (4) addition of a second electron from either NADPH-cytochrome P-450 reductase or cytochrome b_5 affording **118**; (5) heterolytic scission of the FeO−O(H) bond to yield the (FeO) compound **119**; (6) oxidation of the substrate (regenerating Fe^{3+}); and (7) release of the oxidized substrate **120** from the enzyme.[129] Alternatively, binding of carbon monoxide instead of O_2 may occur in step 3 thus yielding the monoxide bound species **121**.

Significantly, numerous enzymes are capable of oxidative transformations.[130] It is generally agreed however, that P-450 cytochromes are the predominant species responsible for hepatic activation of DNA alkylating agents. As a consequence of their versatility and natural abundance, the vast majority of studies dealing with oxidative activation of drugs have directly addressed issues pertinent to P-450. These include mechanistic issues such as substrate stereochemistry and corresponding rates of oxidation, P-450 overexpression, and P-450 inhibition (either by enzyme inactivation or overexpression of competing reductases). Detailed below are the three principal classes of interstrand cross-linking agents whose efficacy is cytochrome P-450 mediated.

4.1. Cyclophosphamide

Cyclophosphamide (CP, **122**) was among the first agents recognized as requiring metabolic activation culminating in biomolecule modification. Since its discovery in 1958, it has become the most widely used chemotherapeutic of the alkylating class.[131] Cyclophosphamide and related oxazaphosphorines undergo oxidation at the C4 position (adjacent to the cyclic nitrogen) to yield the corresponding 4-OH-CP **124**, which spontaneously undergoes reversible ring opening to afford the biologically active aldophosphamide **125** (Figure 22).[132] Once generated, the AP intermediate **124** may undergo several different transformations.



122 Cyclophosphamide

123 Mafosfamide

Alcohol dehydrogenase-mediated oxidation of **124** yields the amide **128** and has been shown to be a means by which DNA alkylation is shut down. Oxidation (via aldehyde dehydrogenase ALDH1) of the ring-opened **125** to the corresponding acid **130** also represents a nonproductive metabolic pathway with respect to ISC formation. Alternatively, elimination of the phosphoryl moiety from aldehyde **125** affords the mixture of phosphoramide mustard **126** and acrolein. Hydrolysis of the phosphoramide mus-

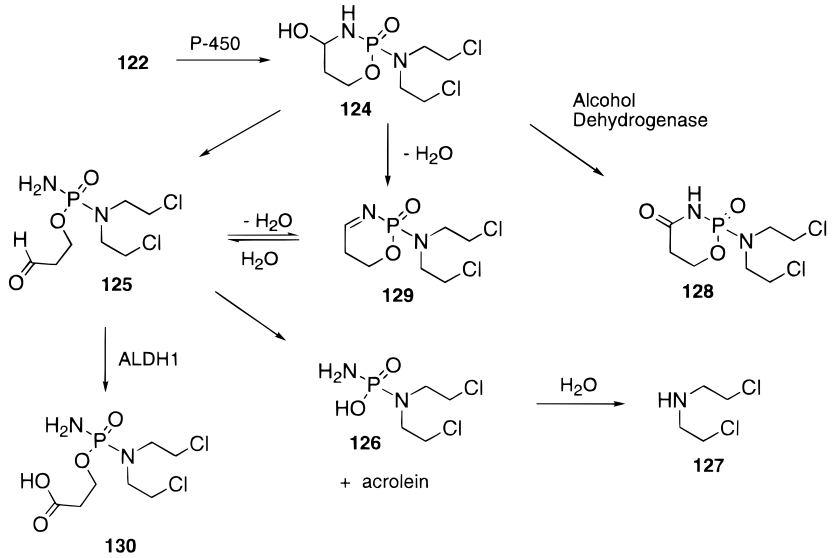
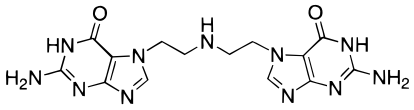


Figure 22. Mechanism of oxidative degradation of **122** to DNA reactive metabolites.

tard yields the secondary amine version of mechlorethamine **127** commonly referred to as nor-HN2.

Elucidation of the interstrand lesion formed upon reaction of activated CP with DNA revealed **131** as the relevant interstrand lesion.[133] Originally believed to arise from cross-linking by nor-HN2, Colvin et al. have shown that phosphoramide mustard **126** is the actual cross-linking agent formed upon CP metabolism.[134] Additional studies by Hemminki have shown nor-HN2 to be a very poor alkylating agent thereby further substantiating the Colvin findings.[134]

In addition to elucidation of the metabolic activation of CP, efforts to define other mechanistic aspects of CP's mechanism of action have been undertaken. The specific cytochrome P-450 enzymes responsible for the in vivo activation of CP have been identified.[135] These include CYP2B1, CYP2C6, CYP2C11, and CYP3A1 in the case of adult rat liver.[135] Similarly, CYP2B6, CYP3A4, and several of the CYP2C class enzymes have been implicated in the human liver activation pathways.[135] Additionally, it is proposed that only the oxidized 4-OH-CP can cross cell membranes, whereas the native "prodrug" cannot.[136] In vivo studies have demonstrated that oxidation/metabolism of the *S* enantiomer of CP proceeds at a faster rate than CP bearing the *R* configuration at phosphorus although ALDH1 inactivation of both stereoisomers of 4-OH-CP via conversion to the keto form proceeds at the same rate for both isomers.[137]



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Recent efforts have focused on the issues of drug resistance and activation/deactivation pathways via gene overexpression. It is well-established that one means of resistance to biological alkylating agents is the increased production of glutathione and higher levels of glutathione S-transferase over those seen in normal cells.[138] Normally present intracellularly at concentrations up to 5−10 mM, this tripeptide very often competes with DNA for drug binding. Importantly, increased levels of glutathione S-transferase (GST) have been found in tumor cell lines which have developed resistance to CP, as well as other alkylating agents such as the N-mustards and *cis*-DDP. Dirven et al. have shown that GSH readily forms the adducts shown below in Figure 23.[138] Additionally, GST enhances the formation 4-glutathionlylcyclophosphamide 2−4 times over that of the nonenzymatic rate. These efforts have suggested that CP resistance may, in part, result from overexpression of GST in tumor cell lines.

Studies by the Dalla−Favera group have demonstrated induction of mafosfamide resistance upon in vitro overexpression of ALDH1.[139] Cloning of the full-length human ALDH1 cDNA and introduction via retroviral vectors into human (U937) and murine (L1210) hematopoietic cell (HPC) lines endowed these cell lines with resistance to the activated CP congener **123**. Increases in mafosfamide resistance were 4−10 times above control transduced HPCs. As such, this

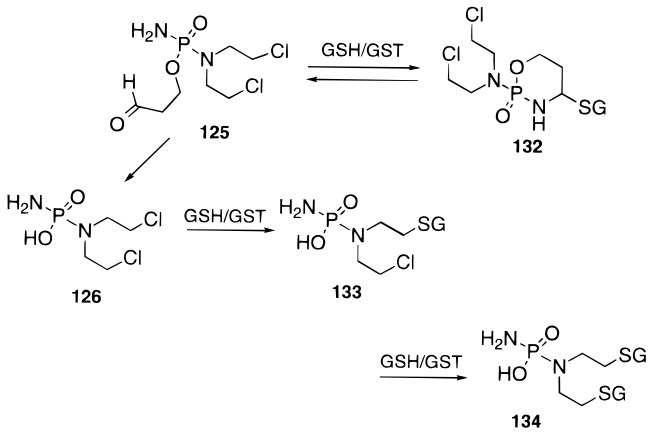


Figure 23. Mechanism of cyclophosphoramide inactivation by glutathione/glutathione S-transferase. GSH = glutathione; GST = glutathione S-transferase.

gene strategy revealed that the ALDH1-mediated conversion of **124** to the keto intermediate is likely implicated in the development of drug resistance not only to CP, but to other oxidatively activated drugs as well. This identifies the ALDH1 gene as a previously unidentified member of the drug-resistance gene family that includes the DHFRr and MDR1 genes.[140] More importantly, this methodology may provide a means of increasing CP resistance in cell lines which undergo undesired CP reactions upon clinical administration. For instance, the introduction of genetically engineered bone marrow cells expressing genes of therapeutic relevance has been suggested as a means of inhibiting myelosuppression in CP therapies.

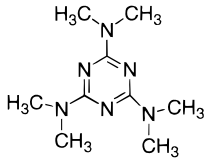
Gene overexpression techniques have been used to probe the avenues by which CP resistance is induced. These same strategies have also been exploited in connection with selective drug activation. The effectiveness of the oxazaphosphorines is limited by the hematopoietic, renal, and cardiac toxicity that accompanies the distribution of the liver-activated metabolites. A method of bypassing this problem has been demonstrated by Waxman and Chen.[135] Incorporation of the liver cytochrome P-450 gene CYP2B1 into human breast MCF-7 cancer cells was found to induce a high degree of cytotoxicity toward both CP and its analogue ifosfamide (IFA). This is particularly significant since MCF-7 cells possess little or no endogenous CYP enzyme activity, but instead express high levels of NADPH cytochrome P-450 reductase. Cell cycle analysis revealed that CP-arrested the CYP2B1 transfected cells but not the CYP2B1-negative cells. That CP-mediated cell cycle arrest was CYP2B1-mediated was further supported by the loss of cytotoxicity upon cell treatment with metapyrone, a known heme binding ligand. Cytotoxicity to adjacent tumor cells lacking the P-450 gene was also observed. This antitumor affect was attributed to transfer of toxic CP metabolites through cell−cell contacts as well as through the medium. Additionally, this gene expression, when treating MCF-7 tumors grown in nude mice gave rise to a 15−20-fold greater CP in vivo cytotoxicity.

In addition to breast cancer cell lines, Chen and co-workers have applied this same strategy toward

selective targeting of glioblastomas.[141] The most common malignant brain tumor, glioblastoma is considered incurable, with multimodal treatment (surgery, radiation, chemotherapy) increasing survival times by only a matter of months. Overexpression of P-450 2B1 gene (C6−P-450) in C6 glioma cells afforded cells highly sensitive to CP both in vitro and in vivo. Diffusion/transfer of toxic CP metabolites occurs as in the MCF-7 case with CP (500 μM)-induced tumor cell death observed after 3 h. This represents a novel tumor-killing strategy wherein target selectivity arises not only from the drug itself, but also from the selective expression of "activating" enzymes. Further, the Chen studies have shown the relevance of a "bystander" effect by which toxic drug metabolites generated in one tumor cell may inflict damage upon another nearby tumor cell. Thus, the need for quantitative transfection of the gene to be expressed is not necessary to elicit the desired therapeutic response.

4.2. *N*,*N*,*N*′,*N*′,*N*′,*N*′-Hexamethylmelamine and Related Structures

Hexamethylmelamine (HMM, **135**) is an antitumor agent shown to be effective against a number of different human tumor cell lines. It is active against metastatic breast cancer, lymphoma, cervical cancer, and bladder cancers.[142] Additionally, it has received considerable attention in the treatment of ovarian cancer and to a lesser degree, lung carcinomas.



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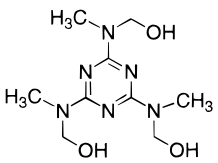

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Upon hepatic oxidation by cytochrome P-450, N-demethylation occurs via initial hydroxylation followed by dehydration (Figure 24).[143] The resulting imminium is a potent electrophile and is likely the active species responsible for DNA alkylation. Little is known about the actual stepwise activation−demethylation process and its relationship to DNA alkylation. It is likely, however, that multiple hepatic oxidations proceed prior to drug-mediated DNA ISC formation (Figure 24). Stemming from these observations has been the generation and examination of N^2,N^4,N^6-trimethylmelamine (trimelamol, **136**), a "preactivated" analogue of **135**.[143]

The trishydroxylated analogue **136** is much more water soluble than its predecessor and circumvents the need for oxidative activation. That trimelamol mimics the mechanism of action of hexamethylmelamine and structurally related congeners has been stipulated by the known metabolic pathway leading to demethylation of each trimelamine exocyclic nitrogen via P-450-mediated carbinolamine formation.[143] Little is known about the mechanism of action of the parent compound HMM; however, some data (in vitro) have been generated pertaining to the reaction of trimelamol with linearized plasmid PBR322 DNA.[144]

Hartley and Thurston have addressed the concern that ISC formation by trimelamol (and HMM by induction) may result not from covalent attachment of the drug to DNA, but rather from formaldehyde-mediated cross-linking (resulting from deformylation of **137**, Scheme 3).[144] That formaldehyde cross-links $^{5'}$ApT$^{3'}$ via methylene bridging of the two N^6 exocyclic amines of deoxyadenosine has been demonstrated by Hopkins et al.[145] Indeed, an analogous situation has recently been disclosed in the case of ISC formation by the anthracycline drugs doxorubicin and daunorubicin (section 5.3).

The viability of interstrand cross-linking via formaldehyde was probed in two ways.[144] First, it was demonstrated that in the presence of thiophenol, trimelamol gave rise exclusively to the trisubstituted thioether adduct. No deformylation adducts were obtained nor was any methylene-bridged bisthiophenol product detected. Additionally, reactions of the 5′-^{32}P end-labeled PBR322 with concentrations of formaldehyde analogous to those that would be present in trimelamol reactions, wherein ISC formation was readily detected, were completely devoid of cross-linked material. Taken together, these findings strongly implicate trimelamol as the DNA binding agent.

Preliminary evidence has suggested that the drug−DNA covalency is minor groove dependent since piperidine reactions of cross-linked material failed to reveal any base-labile sites resulting from facile

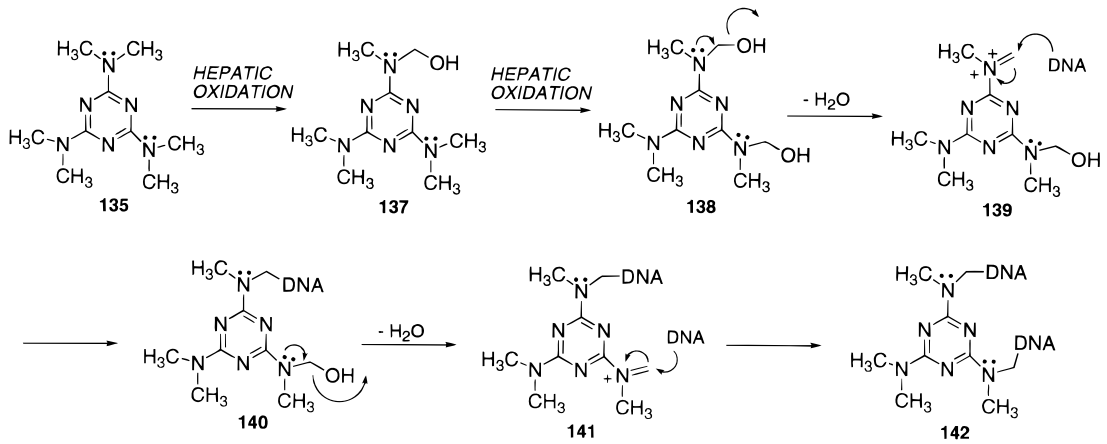
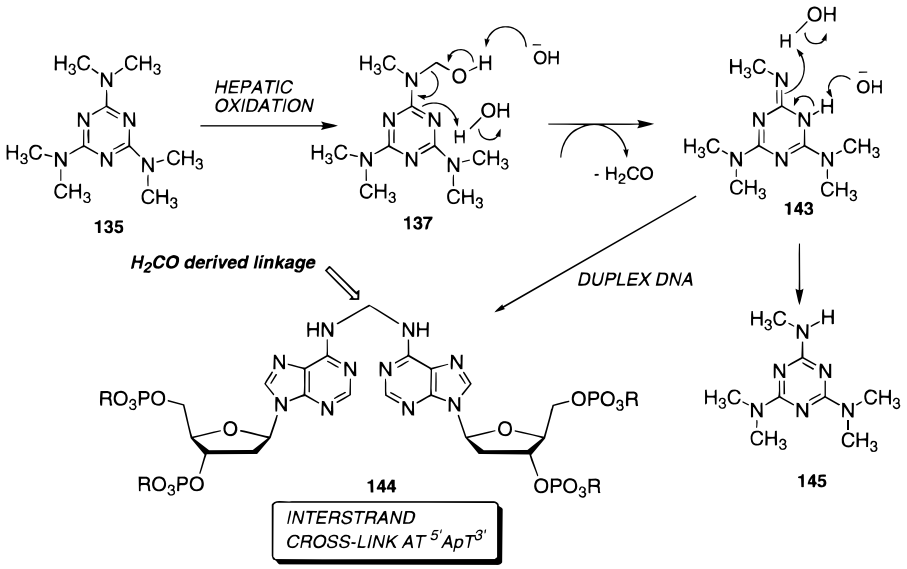


Figure 24. Mechanism of activation of hexamethylmelamine and subsequent DNA modifications.

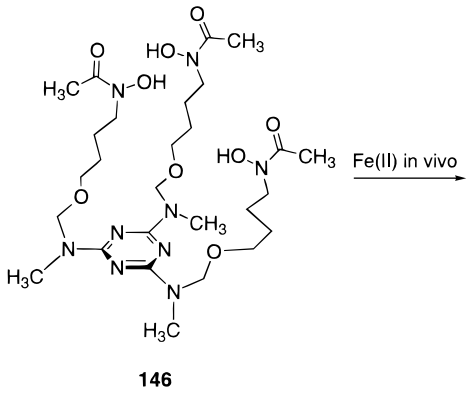
Scheme 3



depurination or depyrimidation (facilitated by base alkylation). This is in contrast to many of the alkylating agents which selectively alkylate the deoxyguanosine N^7 position (the DNA nitrogen bearing the greatest negative character) thus giving rise to base-labile lesions in a fashion paralleling that used in Maxam−Gilbert DNA sequencing.[146] That the two alkylation events leading to ISC generation proceed in rapid succession and perhaps simultaneously has been suggested. Like mechlorethamine, the second alkylation (typically rate limiting for many cross-linking agents) appears too fast to be measured, thus suggesting that monoalkylation brings the highly activated imminium species of the "second arm" in close enough proximity to the second strand such that almost immediate reaction occurs, presumably with little or no structural perturbation of the target duplex.[144]

The high degree of efficiency with which trimelamol inflicts ISCs upon duplex DNA has provided the impetus for improved delivery systems of this drug. While approaches such as antisense, triplex, and peptide conjugations have received attention with other cross-linking agents, these strategies have not yet been applied to trimelamol or its oxidative predecessor HMM.

An alternative strategy has recently been reported by Miller and Ramurthy wherein synthesis of the trimelamol siderophore **146** was achieved.[147] Iron-sequestering siderophores are readily recognized and actively transported into cells which might not otherwise allow delivery of the molecule in the absence of the siderophore. Once inside the cell, iron is released for use by the organism and the siderophore is likely metabolized and discarded.[148] As such, the incorporation of a DNA damaging moiety into such a molecule allows active transport into the cell followed by nucleic acid modification. Such a strategy converts drugs which had previously relied on passive diffusion (and to which resistance maybe developed) into much more interactive agents. Specifically, Miller et al. envisioned that once iside the cell, the trimelamol would release the sideophore tether



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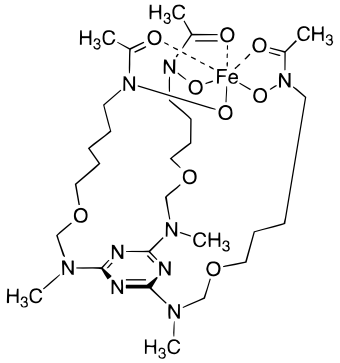

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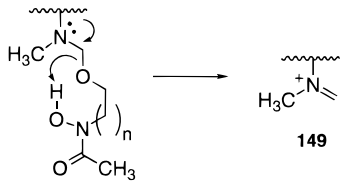
and in so doing would generate the DNA reactive imminium species **149**. Initial studies with **146** have



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revealed that growth delay of *E. coli* X580 cells is induced by the siderophore conjugate. This is consistent with other siderophore conjugates (not ISC

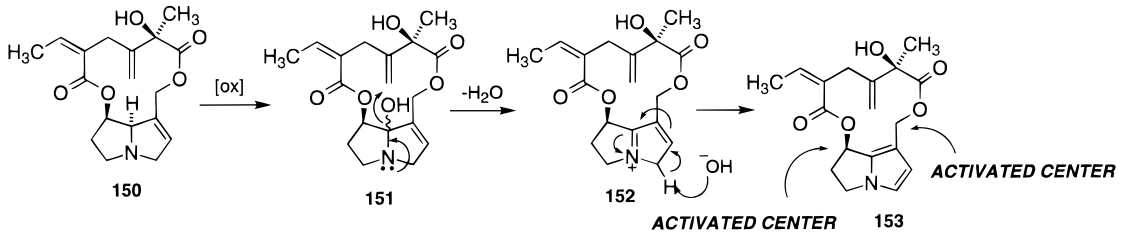


Figure 25. Mechanism of oxidative PA activation.

agents) studied by the Miller group and is suggestive of iron complex recognition by the outer membrane receptors followed by transport into the cell and subsequent DNA damage. To date, no further details have been reported on this pathway to ISC generation.

4.3. Pyrrolizidine Alkaloids

The pyrrolizidine alkaloids comprise one of the most abundant classes of biologically active natural products. As constituents of over 6000 species of plants with wide morphological and geographical diversity, these compounds (of which over 200 varieties have been reported) express poisonous activities of endemic proportions.[149] Particularly common to tropical regions such as the Carribean and Africa, poisonings have also been reported in the United States. An interestingly rich source of poisoning events in the United States has been through the use of herbal medicines. Despite their poisonous traits, these agents have provided a fruitful avenue by which to examine oxidative, selective DNA bisalkylation. An additional means by which these compounds express biological activity is through the formation of DNA−protein cross-links.[150] Ironically, indicine *N*-oxide (**161**) possesses potent antitumor activites against acute refractory leukemias and various solid tumors, despite the fact that it does not efficiently cross-link DNA.[151] That the degree of hepatotoxicity displayed is minimal compared to other pyrrolizidine alkaloids is likely due to a preference for DNA−protein cross-link formation in lieu of ISC formation (after hepatic oxidation).

Cytochrome P-450 oxidation affords two major products: the reactive, highly toxic pyrrole and the less toxic *N*-oxide. Generation of the pyrrolic moiety is proposed to occur as outlined by the oxidative transformation of **150** to **153** (Figure 25). This results in electrophilic activation at the C-7 and C-9 ester substituents via conjugation with the pyrrole nitrogen lone pair. That such a species is extremely reactive was recently demonstrated by Niwa et al.[152] Upon oxidation of monocrotaline **157** to dehydromonocrotaline **166** it was found that the pyrrole was completely hydrolyzed within 1 min in aqueous media at room temperature.

DNA cross-linking has been demonstrated with monocrotaline **157**, and its oxidation product dehydromonocrotaline **166**, as well as the pyrrolic metabolites of retronecine **162**, senecionine **156**, seneciphylline **150**, and ridelline **155** and the diacetates **167**−**169**. In general, those PAs bearing the α,β-unsaturated 12-membered macrocyclic necic diester

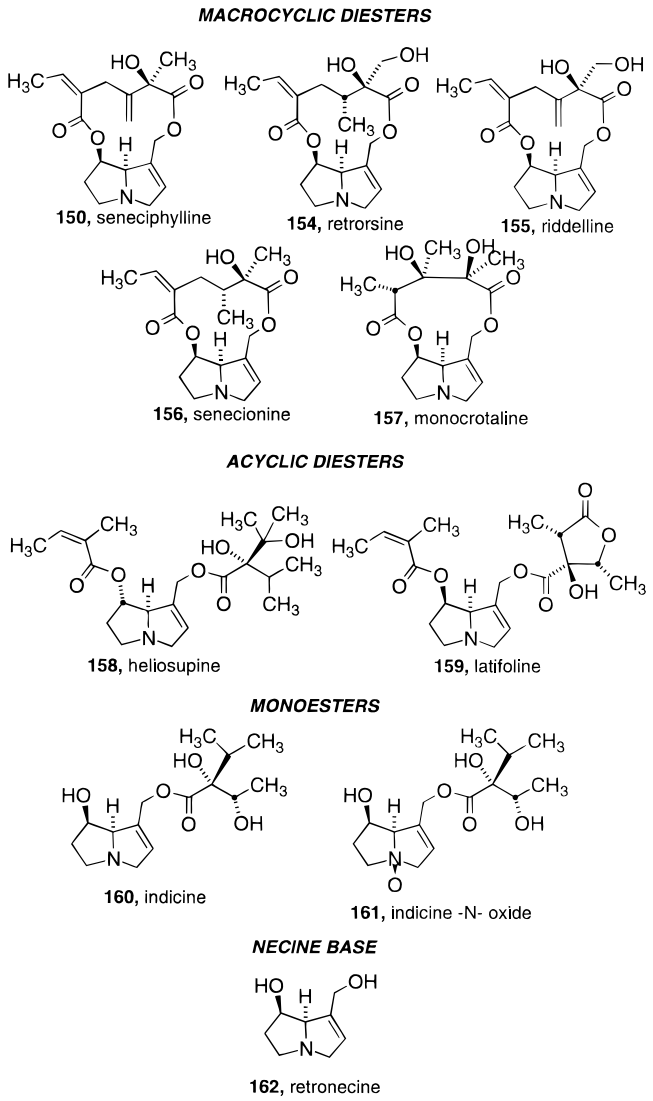
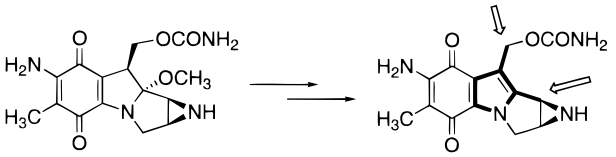


Figure 26. Some of the representative biologically active pyrrolizidine alkaloids.[151]

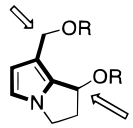
are more potent cross-linkers than PAs lacking analogous cyclic structure and/or the α,β unsaturation.[151] The origin of this preference is unknown, although it is possible that DNA modifications other than those involving C7 and C9 are possible. Particularly intriguing is the possibility for 1,4-addition to the α,β-unsaturated ester portion of such macrocycles (see structures in Figure 26).

The structural similarities between the mitomycins and pyrrolizidine alkaloids and their repercussions on DNA base specificity were first suggested by Iyer and Szybalski some 30 years ago.[153] This was in line with the proposed specificity for reductively activated

mitomycin C for which the alkylating "business end" of the molecule closely resembles that of the activated dehydro-PAs. Placement of the carbamate at the indoloquinone C10 and the presence of the aziridine at C1 parallels the leaving group placements in the activated pyrrolizidine alkaloids.

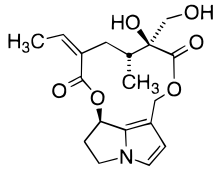


Mitomycin C, **163** **163**-derived indoloquinone

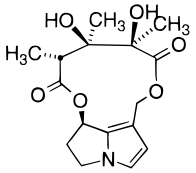


164

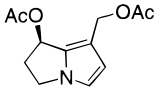
Only recently, however, has data been generated to support the hypothesis that activated pyrrolizidine alkaloids target the same sequences of DNA as those targeted by mitomycin C.[154] By utilizing dehydroretrorsine **165**, dehydromonocrotaline **166**, dehydroretronecine diacetate **167**, and 2,3-bis(acetoxymethyl)-1-methylpyrrole **168**, Hopkins et al. have demonstrated that ISC formation by these representative PAs proceeds with a high degree of selectivity for $^{5'}$CpG$^{3'}$.[154]



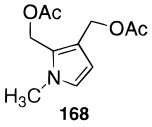
165 **dehydroretrorsine**



166 **dehydromoncrotaline**



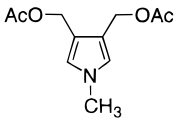
167 **dehydroretronecine diacetate**



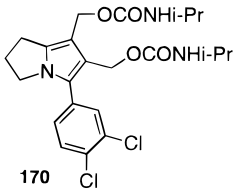
168

For **167** and **168**, Fe^{II}−EDTA footprinting revealed the interstrand cross-links to involve drug connectivities involving the adjacent deoxyguanosines of $^{5'}$CpG$^{3'}$. Additionally, **167** was found to be capable of ISC formation at the sequence $^{5'}$GpC$^{3'}$ and the **168**-mediated cross-linking event showed very little, if any, dependence upon the flanking bases of the $^{5'}$CpG$^{3'}$. For instance, in the case of methylpyrrole **168**, cross-linking of $^{5'}$TCGA$^{3'}$ and $^{5'}$GCGC$^{3'}$ proceeded with similar efficiencies. This is in stark contrast to the sequence preferences observed for mitomycin C wherein only $^{5'}$CpG$^{3'}$ serves as the template for ISC formation, with a strong dependence upon the base sequence on either side of the $^{5'}$CpG$^{3'}$ motif. That **167** and **168** both showed preference for $^{5'}$CpG$^{3'}$ suggests similar connectivities for these lesions and those induced by the mitomycins. However, the added sequence promiscuity of the simpler agents suggests that they lack some of the molecular recognition constituents inherent to the mitomycins. Thus, while preferred connectivities may be conserved on the basis of reactivity arguments, the molecular recognition elements pertinent to the enhanced specificity of the mitomycins over the PA analogues in this study represent candidates for a contribution to, but not solely responsible for, the mitomycin lesions formed with $^{5'}$CpG$^{3'}$.

More recently Hopkins and Woo have addressed the issue of regiochemistry involved in cross-linking of $^{5'}$CpG$^{3'}$ within small synthetic ODNs.[155] In addition to the methylpyrrole **168**, the regioisomeric pyrrole **169** was examined as was the diurethanyl compound **170**. By utilizing a series of inosine-



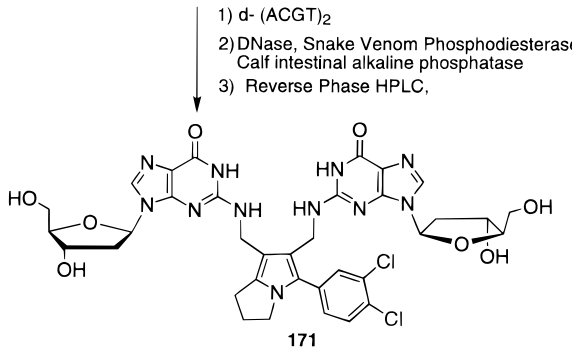
169



170

substituted ODNs and large scale preparation of **170** cross-linked 5′-(ACGT)$_2$ the importance of the exocyclic N^2 amines of the dGs contained within $^{5'}$CpG$^{3'}$ was revealed. Simply stated, incorporation of inosine at one or both dG residues within the self-complementary ODN $^{5'}$TATAATA**CG**TATTATA$^{3'}$ completely abrogated ISC formation upon treatment with **168**−**170**. In combination with the earlier Fe^{II}−EDTA fragmentation results, this demonstrated the necessity of dG N2−drug interactions en route to DNA bisalkylation. Alternatively, reaction of **170** with 5′-(ACGT)$_2$ followed by enzymatic degradation down to the nucleoside−drug adduct followed by HPLC mass spectrometry and UV analysis revealed the **170**-mediated cross-link to result from the lesion **171**. Although not a natural product, **170** had been shown to possess excellent antitumor activities, likely resulting from structural and mechanistic analogies to the pyrrolizidine alkaloids.[156]

170



171

Spectrophotometric analyses (UV and MS) of the resulting lesion was consistent with the structure assignment **171** as verified by comparison to a

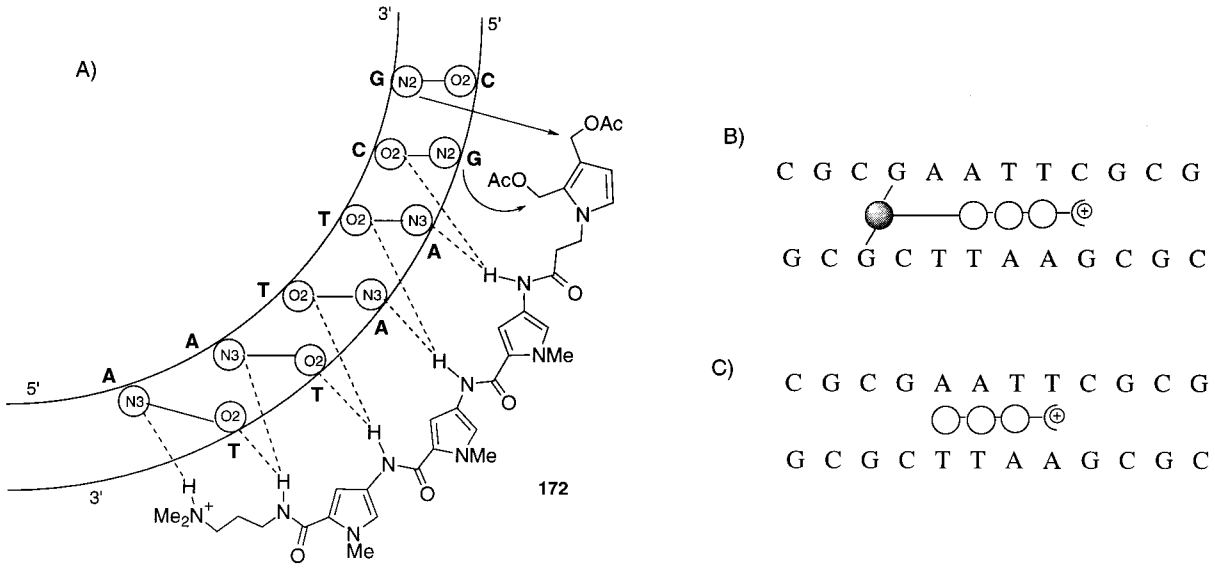


Figure 27. (A) Binding motif of distamycin−pyrrole conjugate **172** to 5′-$(CGAATT)_2$; (B) DNA base-to-conjugate contacts for distamycin−bis(hydroxymethylpyrrole) cross-linked d$(CGCGAATTCGCG)_2$ as elucidated by ^{1}H NMR; (C) DNA base-to-distamycin contacts for noncovalent interaction with d$(CGCGAATTCGCG)_2$. For panels B and C open circles denote *N*-methylpyrole units, dark circle denotes alkylating unit, and the positively charged half circle denotes the cationic dimethylamine terminus.[157b]

synthetically derived sample of **171**. Both the enzymatically obtained material and the synthetic sample displayed identical retention times by reverse-phase HPLC and migrated as one distinct entity following coinjection. Importantly, this represented the first isolation/elucidation of the bisnucleoside adducts responsible for DNA interstrand cross-linking by any members (or analogues thereof) of the pyrrolizidine alkaloid class. Further, it substantiated the assertion that the pyrrolizidine alkaloids interstrand cross-link DNA in a fashion similar to the mitomycins.

The elucidation of the mechanistic pathway by which simple pyrroles interstrand cross-link DNA serves as a convenient model system for the pyrrolizidine alkaloids. Studies directed at the eventual exploitation of these agents via conjugation strategies has led to the development of pyrrole conjugates.[157] Concomitant with the enzymatic degradation and subsequent characterization of cross-linked ODNs bearing the lesion **171**, was the synthesis of the polypyrrole conjugate **172**. Sigurdsson et al. demonstrated that conjugation of **168** to the 5′-AATT-3′ minor groove-binding element of distamycin led to a conjugate (**172**) whose rate of ISC induction was 1000 times greater than that of the untethered 1-methyl pyrrole **168**.[157a]

Initial studies were performed using *Eco*RI linearized plasmid pBluescript II KS which bears some 27 runs of four sequential A or T residues proximal to $^{5'}$CpG$^{3'}$ boxes. Utilizing this substrate, efficient ISC formation was obtained at concentrations as low as 10 nM (conjugate:bp ratio of 0.03). Subsequent efforts with several ODNs revealed that the sequence 5′-$(GATCGAATTCGATC)_2$ gave optimal cross-linking with an astounding 67% yield of the ISC. Inosine substitution at either dG residue flanking the $^{5'}$AATT$^{3'}$ abrogated ISC formation thus suggesting retention of the known alkylation pathway exerted by the methyl pyrrole. That DNA connectivity to the cross-linking moiety was conserved in the conjugate was verified upon scale-up and subsequent enzymatic digestion.[157a] All spectral data generated for the conjugate-mediated lesion were consistent with the regiochemical assignments previously made with the untethered **168**.

More recent work by Hopkins, Wemmer, and coworkers has addressed cross-linking of d$(CGCGAATTCGCG)_2$ by a conjugate closely related to **172**. It was shown by ^{1}H NMR that incorporation of bis(hydroxymethyl)pyrrole and subsequent cross-linking shifts the preferred DNA−distamycin network of contacts one nucleotide away from the alkylated dG residues (panels B and C, Figure 27).[157b]

Although the conjugation methodology presented provides a highly efficient and selective means of ISC induction, such strategies have not yet been *knowingly* applied to *oxidatively activated conjugates*. This likely represents the next frontier in the selective delivery and bioactivation of various drugs. Initial efforts with mitomycin C conjugated oligonucleotides have shown this to be a viable means of delivery for redox-activated drugs.[158a−c] Additionally, Ruoslahti has shown highly selective cell-killing by angiogenic-targeting peptides to which was tethered doxorubicin, a purported reductively activated agent which has recently been shown to be oxidatively activated en route to electrophilic activation (section 5.3).[158d]

5. Hypoxia-Selective Agents (Reductively Activated)

The proposed mode of action for radiation-based therapies (still the most successful noninvasive means of treatment for most cancers) relies on the abundance of oxygen within the tissue of interest.[159]

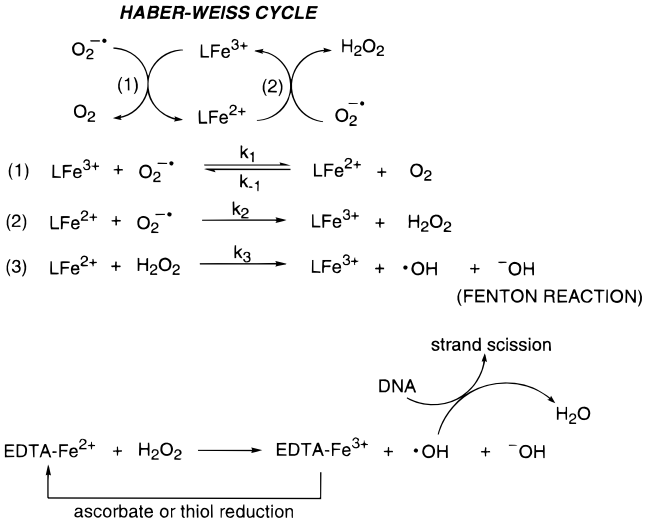


Figure 28. Fenton/Haber−Weiss redox cycling outline.

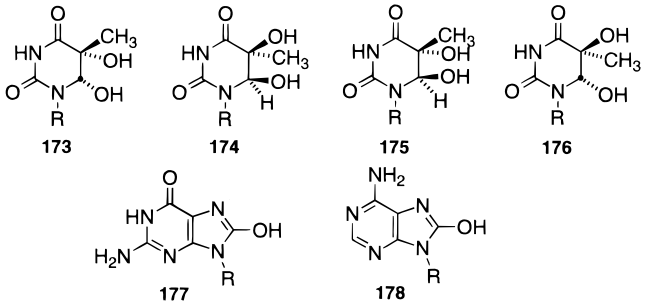


Figure 29. Common base oxidized nucleotides resulting from reaction with hydroxyl radical. R = deoxyribosyl connection to DNA polymer.[161]

Exposure of oxygenated tissues to ionizing radiation affords superoxide which in turn produces the highly reactive hydroxyl radical species via iron-mediated dismutation of hydrogen peroxide and subsequent Fenton chemistry.[160] Alternatively, direct generation of hydroxyl radical mediates cellular damage and provides another entry to Fenton/Haber−Weiss redox cycling (Figure 28).[160] Diffusion of the highly reactive hydroxyl radical inflicts damage upon phospholipids, proteins, and most notably, the nucleic acids. Reaction with DNA affords a wide array of base-oxidized nucleotides (Figure 29)[161] and leads also to indiscriminate strand scission of the phosphodiester backbone.[162]

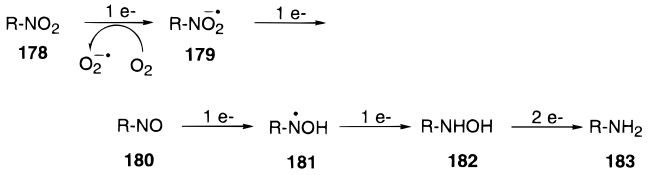
Superoxide dismutation is facilitated by a wide array of iron complexes and represents the only kinetically significant step in the net conversion of dioxygen to hydroxyl radical. The most heavily studied Fe^{II}−EDTA system possesses a rate-limiting k_1 of 76 M^{-1} s^{-1} at neutral pH affording a steady state of Fe^{II}−EDTA since $k_2 > k_{-1} > k_3 \gg k_1$.[163] Radiation therapy, although a highly effective means of treating diseases inherent to oxygenated tissues, often suffers from decreased efficiency upon the development of solid tumors.

It is well-established that hypoxic cells (those lacking oxygen) exist in solid tumors and that these cells are resistant to the cytotoxic effects of ionizing radiation. The gene expression and subsequent secretion of angiogenesis factors in connection with the development of a solid tumor's own blood suppy is necessary for tumor growth.[164] The inefficiency of this process mandates that many of the tumor cells are greater than 150 μm from the nearest blood vessel and are therefore oxygen defficient. This "diffusion-limited" hypoxia results from the rapid metabolism of oxygen by cells proximal to the blood vessel thus depleting the supply for the more distal tumor cells.[165] Alternatively, the closing off of blood vessels (either host vessels or tumor-generated vessels) by simple constriction due to tumor growth leads to "perfusion hypoxia".[165] Importantly, the generation of hypoxia is not limited to neoplastic cells. Anaerobic bacteria and other parasitic organisms have been proposed targets for hypoxia selective agents (HSAs) which like neoplasms, maintain the presence of reductive enzymes such as cytochrome P-450 reductase, DT diaphorase and others while possesing much lower than normal levels of oxygen.[166]

Most HSAs rather than undergoing selective reduction, undergo reduction events in all cells which are reversible in the presence of molecular oxygen (via superoxide generation). Hence, in the absence of O_2, the activated reduction product is long-lived enough to inflict macromolecular damage resulting in cytotoxicity. It has been demonstrated that agents possessing reduction potentials in the range −300 to −450 mV are accessible to enzymatic reduction in vivo.[167] These can be categorized into three classes: (1) nitro and aza aromatics, (2), quinone-containing agents, and (3) transition metal complexes. Application of these reducible functionalities has been applied to the design (both man-made and naturally occurring) of the following "prodrugs".

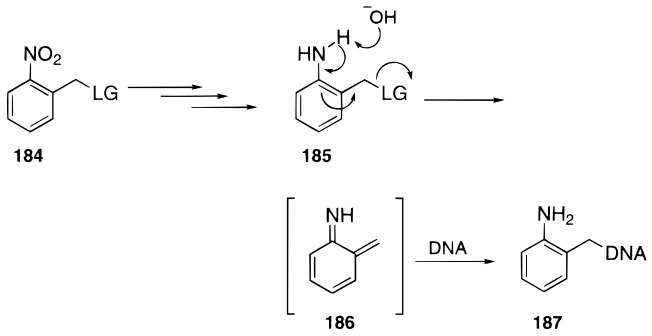
5.1. Masked Nitrogen Mustards

Owing to their structural simplicity and high degree of reactivity with nucleic acids, the nitrogen mustards have received considerable attention with respect to prodrug design. As with cyclophosphamide (essentially an oxidatively activated N-mustard derivative), the tethering of mustard moieties to redox active organic functionalities has allowed the generation of highly specific DNA cross-linking agents. Particularly successful has been the employment of nitro and aza aromatic chemistry. Several reports of nitroaromatic alkylating prodrugs have appeared, all of which undergo reduction via the conversion of **178** to **183**.[168]

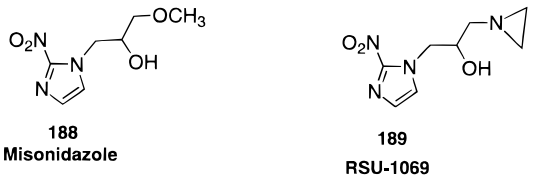


Nitrobenzyl compounds bearing leaving groups at the benzylic position have shown hypoxic selectivities in cell culture and are proposed to behave as nucleic acid alkylating agents via generation of the iminoquinone methide **186**.[169]

Additionally, the conversion of the electron-withdrawing nitro group to the electron-donating amine



or intermediate hydroxylamine has been used as a means of reductively activating substituted nitroimidazoles toward DNA mono- and bisalkylation.[170] Misonidazole **188** is a known DNA monoalkylating agent whose cytotoxicity is reductively driven. Unlike other nitroaromatics, the critical intermediate is not the free amine but rather the partially reduced hydroxylamine.[171]

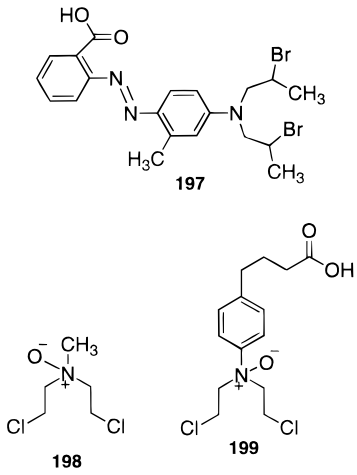


The identity of the lesion is not known but likely involves alkylation of the hydroxylated side chain via assistance from the hydroxylamine moiety. Within the realm of ISC generation, RSU 1069 (**189**) undergoes initial ring opening of the aziridine to afford monoadducts under aerobic conditions.[170] Once anchored to the DNA, reduction of the nitro functionality gives rise to a second electrophilic site which undergoes nucleophilic attack by the complementary strand to yield interstrand cross-linked material.[170]

With respect to the "protection" of nitrogen mustards, many derivatives of the nitroaniline chloromustards have been examined.[172] Conjugation of the nitrogen mustard lone pair with the *p*-nitro group abrogates aziridinium formation required for efficient ISC formation (Scheme 4).

However, conversion of the nitro group to either the hydroxylamine or the fully reduced amine deconjugates this lone pair thus facilitating the formation of the requisite aziridinium. Similarly, the nitroquinoline **194** is an extremely stable compound. Six-electron reduction of the nitro group considerably alters the electron density of the quinoline ring system.[173] A reflection of this electronic change is the considerably enhanced basicity of the cyclic nitrogen. Intramolecular base-catalyzed rearrangement after nitro group reduction facilitates elimination of phosphoramide mustard which, as discussed previously, is the DNA-reactive intermediate involved in cyclophosphoramide cytotoxicity (Figure 30).

In addition to nitroaromatic conjugation methodologies, the N-mustards have been tethered to aza aromatics in order to achieve similar results. One such example reported by Wilman et al. is depicted by **197**.[174] Conceptually, reduction of the azo functionality was envisioned to yield the free aniline mustard which, by analogy to the nitro systems, would effect selective DNA bisalkylation. This was in fact the case for the Wilman study in which **197** and structurally related analogues demonstrated selective liver cytotoxicity.[174]



The design of hypoxia-selective N-mustards has not been relegated only to nitro or aza aromatics. The conversion of nitrogen mustards to the corresponding *N*-oxides has been pursued. N-Oxidation consider-

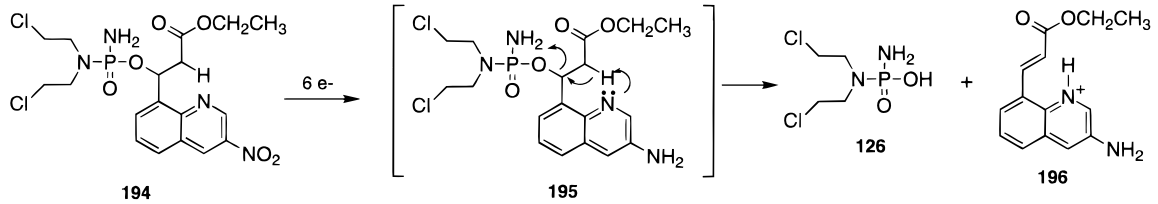
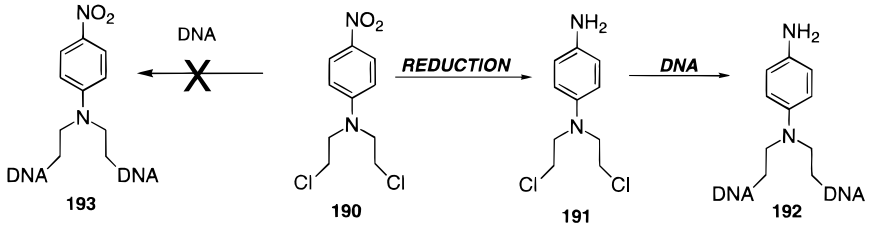


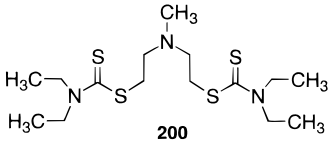
Figure 30. Reductive activation of **194**.

Scheme 4



ably lowers the pK_a of an amine (roughly 5 units) and effectively deletes nucleophilic characteristics. In line with this concept, compounds such as **198** and **199** have been designed as reductively activated prodrugs of mechlorethamine and chlorambucil, respectively. Connors et al. have shown that nitromin **198** undergoes enzymatic reduction (under anaerobic conditions) with resultant increases in unscheduled DNA synthesis of JB1, BL8, and Walker 256 carcinoma cell lines.[175]

Additionally, anaerobic enzymatic reduction by rat liver microsomal extracts followed by trapping of the biselectrophilic mustard with diethyldithiocarbamate (DDC) afforded the adduct **200**. Under aerobic



200

conditions, little, if any, nitromin was DDC derivatized while the DDC−mechlorethamine adduct was obtained in >90% yield. The reducing microsomal system examined can use either NADH or NADPH to effect reduction. Analogously, purified cytochrome P-450 reductase was able to reduce nitromin in the absence of any other microsomal proteins.

Taken together, these findings strongly suggest that *N*-oxide functionalization of mechlorethamine provided an effective means of masking the reactivity of the parent compound while allowing a reductive "unmasking" route to selective activation. Conversely, Shervington and Mann have shown that this strategy fails with the chlorambucil analogue **199** which shows no hypoxia selectivity en route to DNA modification.[176]

5.2. Transition Metal Complexes

Activation of HSAs ensues upon one electron reduction, but may also follow in the case of two electron reduction. Examples of two electron reductases include NAD(P)H:quinone oxidoreductases (DT diaphorase) and xanthine dehydrogenase both of which reduce quinones and nitroheterocycles under aerobic conditions.[177] Such a case is deleterious to the reductive selectivity of such agents since the whole concept of selectivity is based upon facile reoxidation of the one electron reduced species with concomitant generation of superoxide. The two electron reduction scenario bypasses this oxygen-reversible stage thus compromising selectivity.

One means of avoiding inadvertent drug toxicities resulting from irreversible two electron reduction processes has involved the construction of transition metal complexes which are capable of only one electron reduction events. Denny et al. have shown that Co(III) complexes are particularly attractive in this manifold.[178] The d^6 low-spin electronic configuration of octahedral Co(III) complexes renders them kinetically inert. For instance $Co^{III}(NH_3)_6$ possesses a half-life of 6×10^9 s with respect to aquation.[179] Alternatively, one electron reduction (reduction potentials typically in the range of −200 to −400 mV)

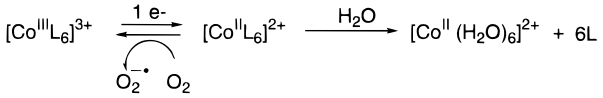
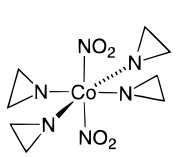


Figure 31. Reduction-promoted ligand release from Co(III) complexes.

results in a highly labile Co(II) complex capable of rapid ligand exchange (Figure 31).[180]

However, this process is essentially irreversible since, the resulting $[Co(H_2O)_6]^{2+}$ is extremely stable with respect to back-oxidation ($E° = +1800$ mV).[178b] The ability to control the substitutionally labile $Co^{II}L_6$ relies heavily upon the nature of the ligands used, with monodentate ligands demonstrating extremely facile exchange thus giving rise to the oxidatively inert hexaqua species. Indeed, initial studies by the Denny group[178c] revealed that the complexes **201** and **202**, while capable of reductive activation toward DNA alkylation, showed no selectivity for hypoxic cells due to the insufficient stability of the $Co^{II}L_6$ complex.



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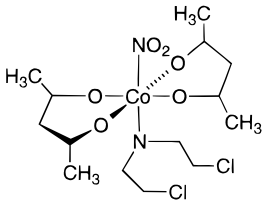
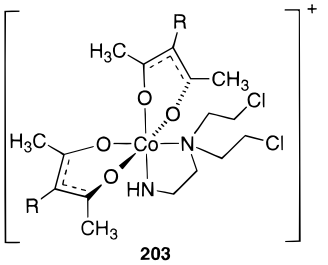


202

More recent accounts reveal that the use of polydentate ligands stabilizes the reduced Co(II) species thus allowing the oxygen reversibility needed to achieve hypoxic selection.[178b] The design of metal complexes **203** and **204** follows from the concept of N-oxidation of reactive mustards in that coordination of the nitrogen lone pair to Co(III) shuts down aziridinium formation.[178b] Variations of the R groups from H, to methyl, to ethyl, or to propyl was observed to considerably alter the reduction potentials of the respective analogues which in turn influenced the cytotoxicity properties observed.



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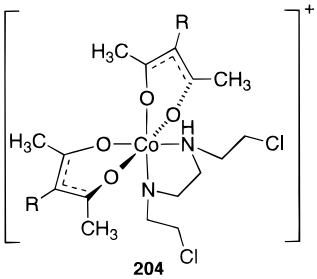
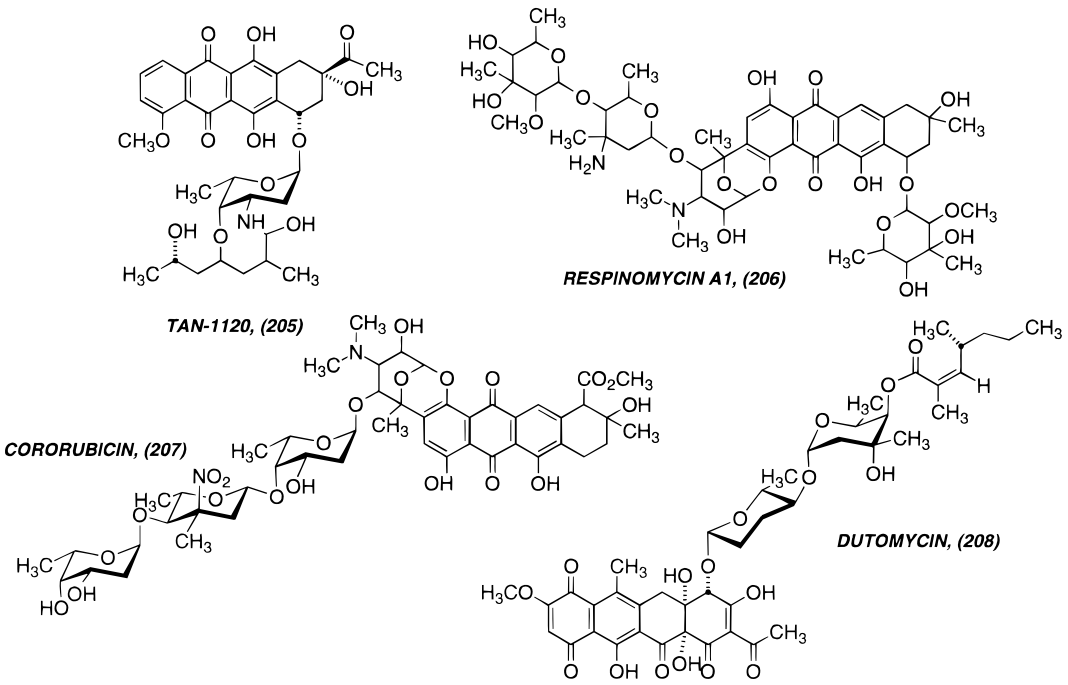


204

Surprisingly, only the parent compound **203** (R = H) possessed a reduction potential ($E° = -310$ mV) within the physiological range previously stated, with the methyl, ethyl, and propyl analogues possessing reduction potentials of −42, −460, and −500 mV, respectively. The methyl, ethyl, and propyl congeners of **204** possessed reduction potentials of −305, −350, and −385 mV, respectively, while the unsubstituted case was well outside the potential window with a potential of −235 mV. The hypoxic selectivities of these compounds was determined in UV4 cell

Chart 4



cultures. Although **203** possessed minimal potency in this assay, the methyl-substituted analogue was very selective toward hypoxic cells despite possessing a reduction potential outside the physiological window. All compounds of the general structure **203** were considerably more potent than the **204**-based compounds likely owing to the ability of the *N*,*N*-bis-(2-chloroethyl)ethylenediamine (DCE) ligand to induce monoadducts and ISCs via the mechlorethamine pathway.[181] That this is in fact the case has not been shown and the authors postulate that the enhanced cytotoxicities observed are due to more facile DCE release upon Co(III) reduction versus that observed with the *N*,*N*′-bis(2-chloroethyl)ethylenediamine (BCE) complexes. In either event, the utility of $Co^{III}L_6$ complexes as a means of selectively targeting hypoxic cells has been demonstrated and represents a novel approach toward reductive drug activation.

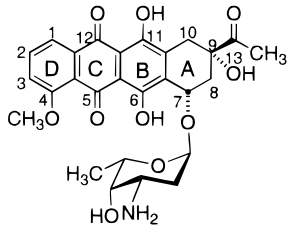
5.3. Anthracyclines

Possessing the tetracyclic quinophenolic ring system with associated carbohydrate moieties, the anthracyclines represent a structurally diverse and clinically important class of antitumor antibiotics.[182] The investigation of synthetic derivatives of doxorubicin (**210**, also named adriamycin) alone has resulted in the synthesis of more than 2000 compounds over the last 25 years.[183] Additionally, isolation from fermentation broths continues to reveal the multitude of natural products belonging to this class.

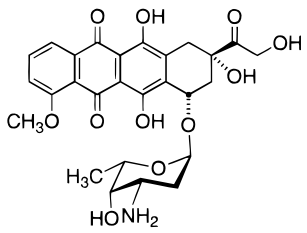
Recently isolated additions to the anthracycline group of compounds include TAN-1120 (**205**) (from *Streptomyces triangulatus*),[183a] respinomycin A1 (**206**)[183d] (from *Streptomyces* sp. RK-483), cororubicin (**207**) (from *Micromonospora* sp. JY16),[183c] and dutomycin (**208**) (from *Streptomyces* sp. 1725) (Chart 4).[183b] All of these compounds demonstrate potent cytotoxicities against several tumor cell lines, but most striking is the ability of cororubicin to generate superoxide in N18-RE-105 hybridoma cells (rat retina x mouse neuroblastoma cells) as detected by in vitro nitro blue tetrazolium (NBT) reduction.[183c] It is clear that the members of the anthracycline class of natural products exhibit vastly different chemistries and that these likely potentiate the biological significance of the individual anthracyclines. Common to all the anthracyclines, however, is the ability to intercalate between the base pairs of DNA.

That the anthracyclines are avid DNA intercalating agents was first demonstrated crystallographically by Wang et al. in 1987.[185] The structure of a 2:1 complex between daunorubicin **209** and 5′-d(CGTACG)$_2$ was reported at near atomic (1.2 Å) resolution and showed drug intercalation of the extended chromophore between the $^{5'}$CpG$^{3'}$ steps at both ends of a distorted B-DNA duplex. The chromophore penetrates the double helix with the D-ring protruding into the major groove and the amino sugar lying in the minor groove. Later studies revealed the relevance of this binding motif to several anthracyclines and also demonstrated the importance of certain drug−DNA interactions inherent to this mode of binding.[186]

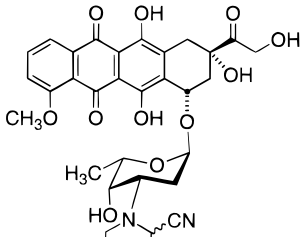
Particularly intriguing was the finding that anthracyclines devoid of the C9 hydroxyl were not biologically active.[186] This was proposed to be a reflection of lower binding affinity due to the absence of the critical hydrogen bonding network involving the C9 hydroxyl hydrogen and the N^3 and N^2 nitrogens of the deoxyguanosine adjacent to the aglycon. The importance of an intercalative binding mechanism has been shown by numerous workers and has been suggested as the predominant means of DNA association. Additionally, the intercalative mode of binding is very often accompanied by attractive DNA



209
Daunorubicin



210
Doxorubicin
(Adriamycin)



211
cyanomorpholino-adriamycin

contacts involving the tethered aglycons in a fashion reminiscent of enediynes such as calicheamicin and other amino sugar antibiotics such as chromomycin.[187]

Evidence suggests that the anthracyclines operate via several different modes of action. Impairment of topoisomerase II activity, membrane-related effects and redox-mediated DNA damage have all been implicated as manifestations of anthracycline biological activity.[188] Of these, topoisomerase impairment has received the most attention. Inhibition of enzyme activity follows from self-inflicted endonucleolytic DNA double-strand breaks. Binding to a given duplex, topoisomerase II induces strand scission of each strand by forming a transient covalent bond with the 5′ phosphoryl end of the broken strand and a tyrosine of each subunit of the 170 kDa homodimeric protein.[189] Formation of this "cleavage complex" allows another intact duplex to pass through the opening created in the topoisomerase II bound strand.[189] Subsequent ligation of the broken strands ensues leading to enzyme release. Stabilization of the cleavage complex by the anthracyclines prohibits ligation and thus affords the arrested drug−DNA complex as a dead end in the supercoiling process.[188] That this occurs at clinically relevant concentrations has been demonstrated for doxorubicin and is proposed to be pertinent to the mechanism of action of numerous other anthracyclines.

Membrane effects associated with the anthracyclines result from drug affinities for the negatively charged phospholipids and resultant alteration of membrane functions. These interactions (not involving radical damage or intercalative processes) at subcytotoxic levels serve to promote cell growth in both human and murine tumor cell lines. However, higher drug concentrations inhibit growth.[190] This has been attributed to excessive growth rates resulting from overstimulation of the membrane-mediated signaling mechanism responsible for growth; in effect, out-stripping of cellular resources leads to ultimate growth inhibition and eventual cell death. Surprisingly, despite some 20 years of clinical use and research, little is known about the specifics of this mechanism of cytotoxicity for the anthracyclines.

Anthracycline-mediated oxidative DNA damage has been implied due not only to the detection of nonprotein-associated strand scission products, but also to the tendency of the anthracyclines (specifically doxorubicin) to induce severe cardiomyopathy.[188] Heart tissue is known to activate doxorubicin at several sites including the cytosol, mitochondria, and sarcoplasmic reticulum.[191] Additionally, these tissues possess very low levels of the peroxide detoxification enzyme catalase. These facts coupled with the known inactivation of glutathione peroxidase (another peroxide-countering enzyme) by doxorubicin, demonstrate the origin of anthracycline induced cardiomyopathy. Not only does the drug stimulate superoxide generation, but it also shuts down (or avoids) the repair mechanisms by which oxidative damage is typically averted.[191]

Perhaps the most interesting chemistry demonstrated by the anthracyclines lies in their ability to undergo redox conversion to quinone methides. The inefficiency of aerobic reduction stems from the rate-limiting quinone reduction ($E° = -300$ mV) and the almost immediate conversion of this species back to the quinone with concomitant superoxide formation (rate constant $k = 10^8\ M^{-1}\ s^{-1}$).[188] However, as stated previously, the absence of oxygen facilitates retention of the activated species. Elegant studies by Koch et al. have shown that anaerobic reduction of daunomycin (**209**) with sodium dithionite affords a wide array of adducts as shown in Figure 32.[192] This anaerobic chemistry is highly substituent-dependent especially with respect to the 11 position. Indeed, the quinone methide stemming from reduction of 7,11-dideoxidaunomycin is considerably less reactive with protic solvents than is the analagous compound bearing the C-11 hydroxyl and is thus much longer-lived than the hydroxylated congener. That these compounds dimerize upon reduction has been demonstrated and is very often the predominant in vitro reaction pathway.[193]

Dimerization is proposed to result from one of two mechanisms. The possibility of radical generation at C7 followed by recombination has been suggested as has the condensation of two activated quinone methides wherein one nucleophilically adds to another (Figure 33). The ability of these dimeric compounds to induce biological activity has not been disproved. However, that the quinone methide generated upon aglycon release is an efficient electrophilic trap (with selective addition to the C7 position) for biologically relevant nucleophiles has been shown by several groups.

Fisher and Ramakrishnan have shown that anaerobic reduction of daunomycin with substoichiometric amounts of NADPH provides the quinone methide which is efficiently trapped by thiolate nucleophiles such as *N*-acetyl-L-cyteine, N-(*tert*-butoxycarbonyl)-L-cysteine, and 1-thio-β-D-glucose providing the pair

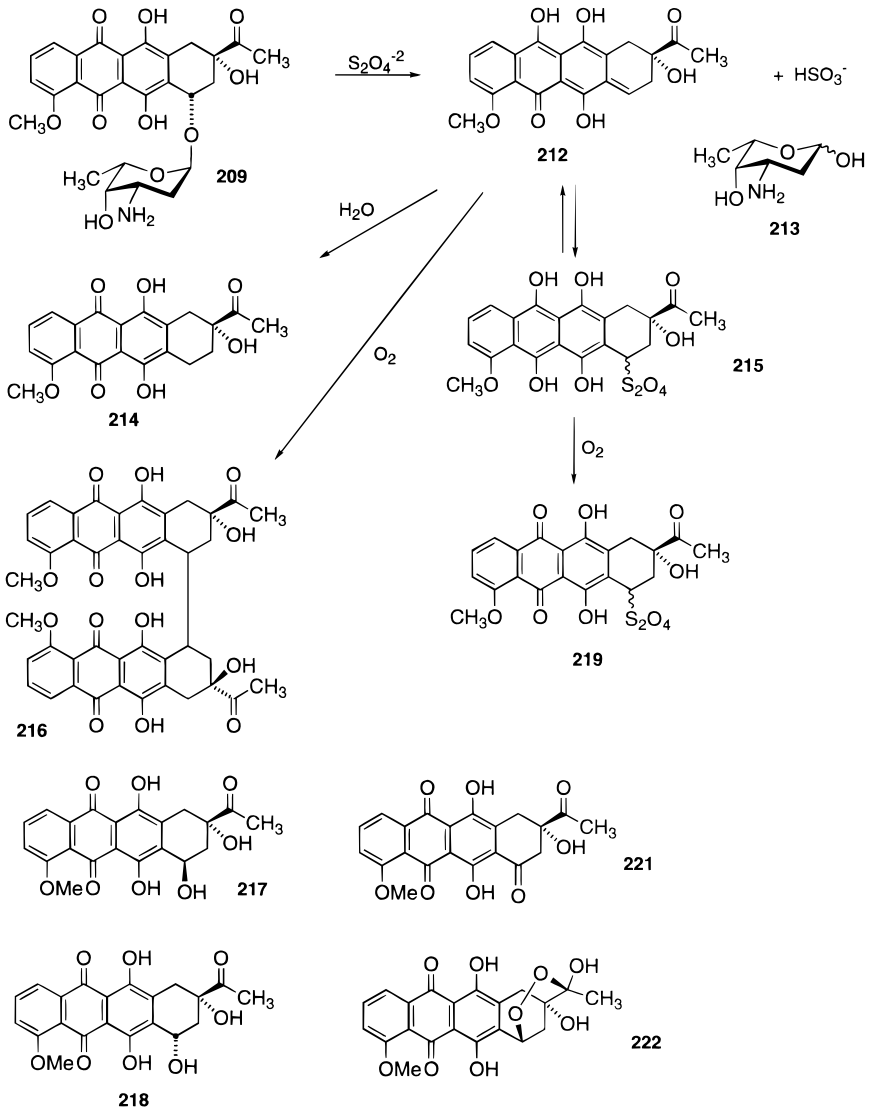


Figure 32. Product distribution of daunomycin reduction by sodium dithionite.

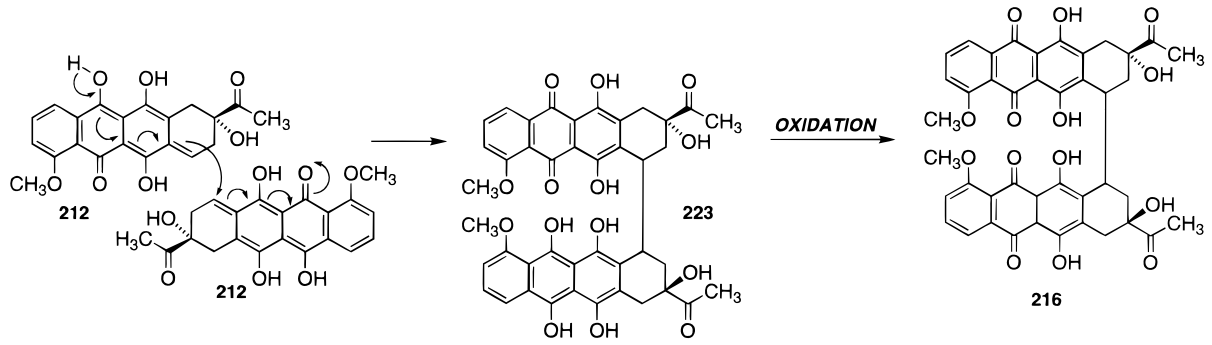
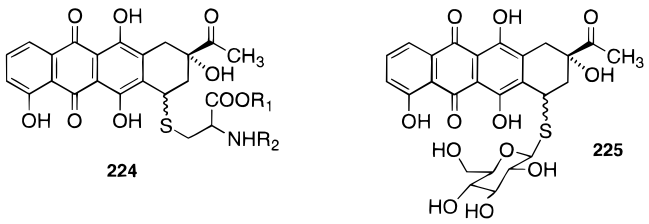


Figure 33. Mechanism of quinone methide dimerization en route to daunomycin dimer **216** (Figure 32).

of C7 diastereomers for the respective products **224** and **225**.[194]



The resulting thioethers were typically obtained in 65% yield with the major products bearing the (S)-stereochemical configuration at the 7 position. Nucleic acid alkylation studies by Koch and Egholm revealed that reduction of menogaril (**229**) with the one electron reducing agent bi(3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl) (DHM-3 dimer, **226**) in the presence of deoxyguanosine yields the C7 nucleoside-substituted **233** (Figures 34 and 35).[195]

Interestingly, none of the dG N^7-alkylated material was found nor were any of the nucleoside hydroxyls

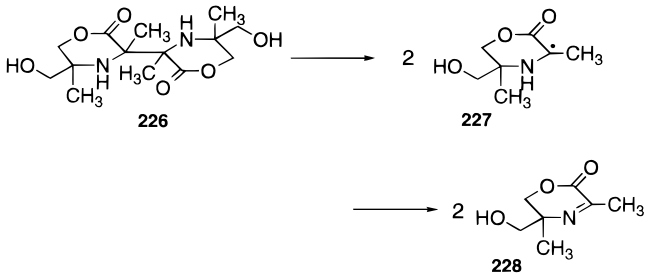


Figure 34. Free radical dissociation of DHM-3.

tethered to the anthracycline chromophore. Additionally, similar reaction conditions in the presence of the other purine nucleoside deoxyadenosine failed to afford any drug−nucleoside conjugates.

Studies by Skladanowski and Konopa have revealed that several members of the anthracycline class antibiotics induce interstrand cross-links into DNA.[196] Incorporation of [^{14}C]methylthymidine into the cellular DNA of HeLaS_3 cells was used as a means of detection for DNAs with abnormal renaturation kinetics following a cell−drug incubation period of 3 h at 37 °C. From these studies it was found that members of both the class I and class II anthracyclines inflicted ISCs upon HeLa cellular DNA (Figure 36).

The class I anthracyclines are nucleolar nonselective, inhibiting both DNA and nucleolar precursor ribosomal RNA (NO-RNA) synthesis at approximately equivalent concentrations while the class II compounds inhibit NO-RNA synthesis at concentrations 200−1300-fold lower than those required to inhibit DNA synthesis.[197] That members of either class express biological activity via ISC formation has led to intense efforts aimed at the elucidation of the resulting lesions and the mechanisms responsible for their generation.

Reductive quinone methide formation explains the ability of the anthracyclines to induce monofunctional binding, but is not obligate for ISC induction by all anthracyclines. One case in point originally disclosed by Westendorf et al. and recently elaborated upon by Phillips and Cullinane is that of (cyanomorpholino)doxorubicin **211**.[198] By utilizing a degradative exonuclease III digestion assay, **211** was found to induce intrastrand cross-links at the sequence $^{5'}$GpG$^{3'}$ on either strand of the alkylated duplex ODN (enzyme stop sites at the sequence $^{5'}$CpC$^{3'}$ was considered diagnostic for alkylation of the $^{5'}$GpG$^{3'}$ of the complementary strand).[198b] The intrastrand lesion is preferentially formed over the interstrand (involving alkylation at both dG residues of $^{5'}$GpC$^{3'}$) bisalkylation by a factor of 10 and both events have been shown to involve alkylation of the N^2 of each deoxyguanosine residue.[199]

Cross-linking of *Eco*RI linearized plasmid pSP64 with **211** did not require reductive conditions and afforded heat-labile adducts which readily decomposed upon heating to 60−70 °C for 10 min.[199] The observed decomposition was attributed not to DNA strand scission, but rather to reversible release of the anthracycline chromophore from the duplex substrate. More important was the *ability to induce ISCs without any exogenous reductants.* This substantiated earlier studies by Begleiter et al. in which DNA cross-links were isolated from drug-treated L5178Y murine lymphoma cells.[200] Notably, the in vitro plasmid reactions called for relatively high drug concentrations (micromolar), whereas the cellular studies required only nanomolar concentrations, thus suggesting the importance of metabolic activation. That the **211**-induced ISCs were obtained without reductive activation and show a high degree of reversibility at higher temperatures was suggestive of an adduct resulting from aminal formation between the drug and the dG N^2. Additionally, retention of both the anthracycline chromophore and the morpholino moiety supports the proposal that the

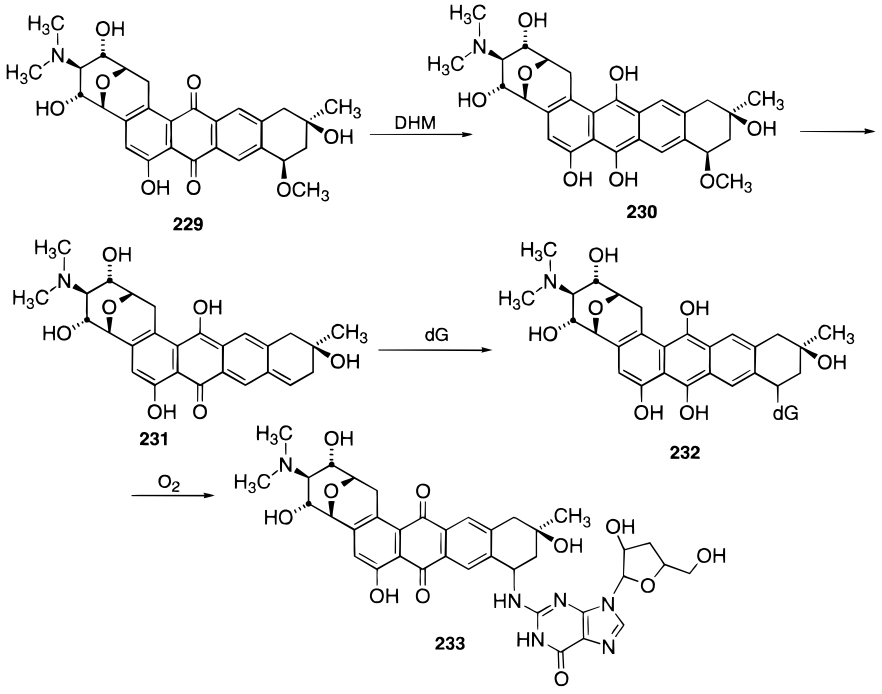
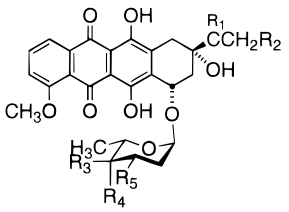


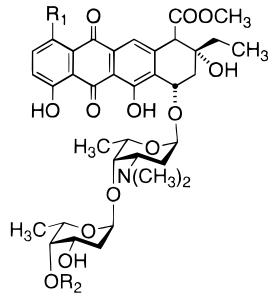
Figure 35. Reductive activation of menogaril (**229**) with DHM.

CLASS I ANTHRACYCLINES



Compound	R_1	R_2	R_3	R_4	R_5	% ISC
Adriamycin	=O	OH	H	OH	NH_2	72.6
Daunomycin	=O	H	H	OH	NH_2	53.6
Epirubicin	=O	OH	OH	H	NH_2	50.0
Iodorubicin	=O	OH	H	I	NH_2	76.9
4'-amino-Adria	=O	H	H	NH_2	OH	10.0
Rubidazone	O	H	H	OH	NH_2	25.0
	$=N-N-C(=O)-C_6H_6$					

CLASS II ANTHRACYCLINES



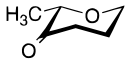
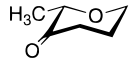
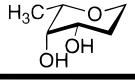
Compound	R_1	R_2	% ISC
Aclacinomycin	H		66.7
Cinerubin A	OH		35.0
Marcellomycin	H		> 70.0

Figure 36. Representative class I and class II anthracyclines known to interstrand cross-link $HeLaS_3$ cellular DNAs.[196]

ISC results partly from cyano displacement followed by addition of dG N^2 to the resulting imminium species.[199] Although the viability of this mechanism is clear (based on analogy to DNA alkylation by saframycin A), it only accounted for one of the two alkylation events necessary for ISC production. The second event has been proposed to involve simple Schiff base formation involving the C-13 ketone and the N^2 of the other deoxyguanosine involved in cross-linking. Given that the extended chromophore remains intercalated between the bases of interest, it is likely that the drug functionalities responsible for DNA alkylation be placed above and below the plane of the tetracycle. This, in combination with the relative reactivities of drug functional groups suggests that it is these "side chains" that endow **211** with interstrand cross-linking activity. However, this issue has very recently been clarified as discussed below. Significantly, this clarification shifts emphasis from the C-13 ketone functionality to the previously noted C-9 hydroxyl unit as being the source of critical drug−DNA contacts.

Studies by the Phillips group have addressed the ability of doxorubicin (**210**) to induce similarly unstable cross-links into synthetic ODNs.[183,201] Contrary to the morpholino-substituted anthracycline, *doxorubicin displays the absolute requirement of an exogenous reductant in order to efficiently cross-link DNA*. Concentrations as high as 5−10mM are required with reducing agents such as dithiothreitol, glutathione, and β-mercaptoethanol.[183] Additionally, the rate of cross-link formation is dependent upon the concentration of Fe(III).[201b] This has been attributed to the inability of intercalated anthracyclines to undergo reduction due to steric constraints inherent to the DNA-bound species.[188] However, formation of the extremely stable anthracycline-Fe(III) complex affords a species still capable of intercalation, but now possessing a redox shuttling system.[188] It has been proposed that reduction of the drug bound Fe(III) to Fe(II) gives rise to the intramolecularly reduced semiquinone radical which is then electrophilically activated. Again, recent evidence suggests a critically different role for the $Fe^{II}-Fe^{III}$ redox system involved in DNA cross-linking by doxorubicin and structurally related compounds.

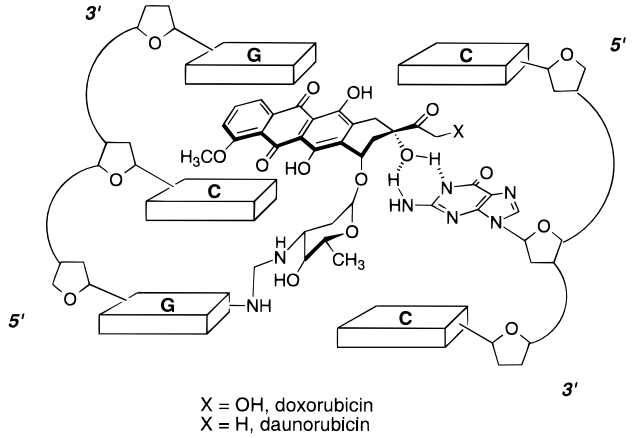


Figure 37. Proposed "virtual cross-link" of doxorubicin or daunorubicin intercalated into DNA via one covalent bond (resulting from adventitious formaldehyde generation) and two C-9 hydroxyl-dependent H-bonds with the central dG residue of the opposing strand.

Doxorubicin-mediated ISCs have been mapped to the sequence $^{5'}GpC^{3'}$ using λ-exonuclease and are dependent upon the exocyclic N^2 amines of the deoxyguanosine residues on each strand of the duplex.[201b,c] However, the lesion is unstable to isolation and has been difficult to characterize. Recently, the structure of the lesion has been solved through the efforts of Wang[201d] and Koch and associates[201e−g] for doxorubicin and daunorubicin as shown in Figure 37. Significantly, Wang and co-workers[201d] reported the X-ray crystal structure of a daunomycin-formaldehyde adduct with $(CG)_3$ in 1991. Subsequently, Koch and co-workers[201e−g] have now shown that the primary functional role of the ("perceived") reductive activation is to produce superoxide and hydrogen peroxide which then produce formaldehyde via Fenton-type chemistry with constituents in the medium (which apparently includes the drug itself). The

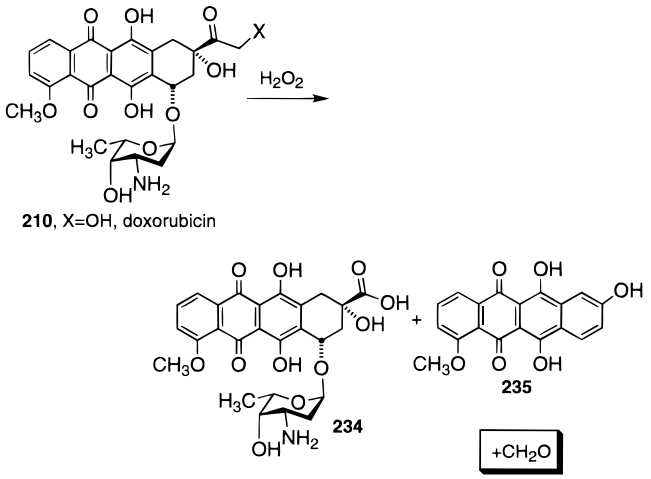


Figure 38. Baeyer−Villiger oxidation of doxorubicin with hydrogen peroxide furnishes formaldehyde as proposed by Koch.[201e]

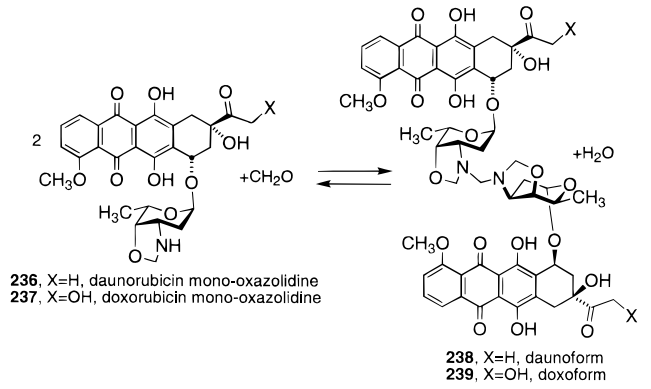


Figure 39. Formation of daunoform (**238**) and doxoform (**239**) from the monooxazolidines of daunorubicin and doxorubicin, respectively.

adventitiously produced formaldehyde then condenses with the 3′-amino group of the intercalated drug and forms an aminal linkage with the 2-amino group of the proximal deoxyguanosine (Figure 37).[201f]

Further, Koch has presented preliminary evidence[201e] that Baeyer−Villiger oxidation at the C-13 position with hydrogen peroxide furnishes formaldehyde and the byproducts **234** and **235** (Figure 38) showing that, in fact, the anthracycline drugs daunorubicin and doxorubicin are most likely oxidatively and not reductively activated as previously thought.

Continuing, these workers have gone on to characterize the formaldehyde adducts of daunorubicin and doxorubicin (called daunoform (**238**) and doxoform (**239**), respectively; Figure 39).[201g] Both formaldehyde adducts are labile to hydrolysis back to the parent drugs but displayed significant cytotoxicities against both adriamycin-sensitive and -resistant cell lines. Doxoform proved to be 150 times more toxic to MCF-7 human breast cancer cells and 10 000 times more toxic to MCF-7/ADR resistant cells. This enhanced level of cytotoxicity is attributed to enhanced lipophilicity of these substances and is felt to be independent of adventitious redox cycling and the ensuing formaldehyde production seen with the parent drugs. These workers have thus shown that the DNA alkylation and cross-linking events apparently *do not involve the incipient quinone methide* which had been the accepted mode of action until these papers appeared.

5.4. Aziridinylbenzoquinones

The aziridinylquinones represent perhaps the simplest of the mitomycin C-like prodrugs. Reduction of the quinone results in the transition from a nonaromatic quinone to the aromatic semiquinone or hydroquinone (one and two electron reductions, respectively). The resulting altered electronic distribution no longer invokes conjugation of the nitrogen lone pair electrons with the respective carbonyls.[202] As such, this substantially enhances the basicity of the aziridinyl nitrogens thus facilitating protonation of each tertiary amine.[202] This activation process vastly enhances aziridine electrophilicity thus affording a species capable of facile DNA alkylation. Following alkylation, autoxidation ensues thus restoring the quinone moiety (Figure 40). Importantly, autoxidation of the bisalkylated species back to the quinone (as in the anthracycline case) is favored over the parent hydroquinones due to the increased electron donation from the acyclic amines into the aromatic hydroquinone as opposed to that of the ring-strained aziridinyl case.[202]

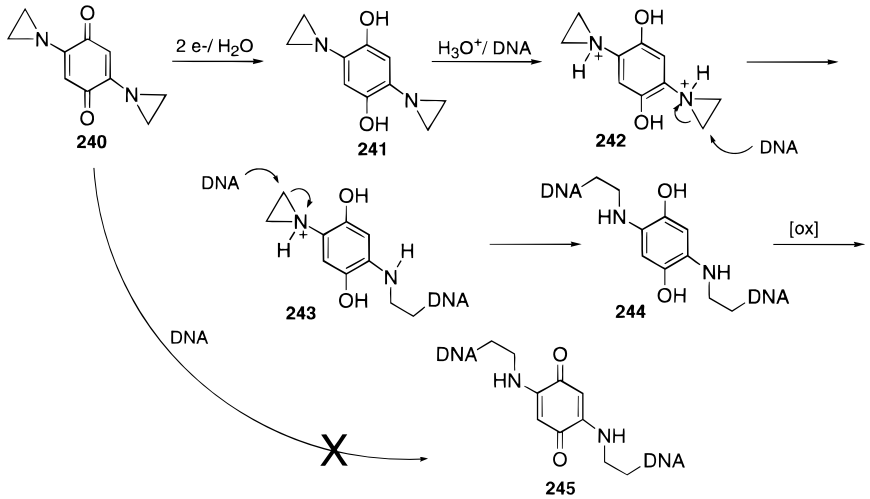


Figure 40. Reductive mechanism of DNA interstrand cross-linking by the diaziridinylquinones.

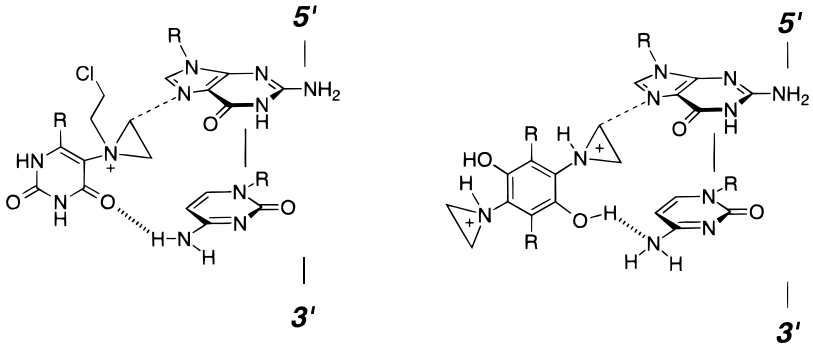
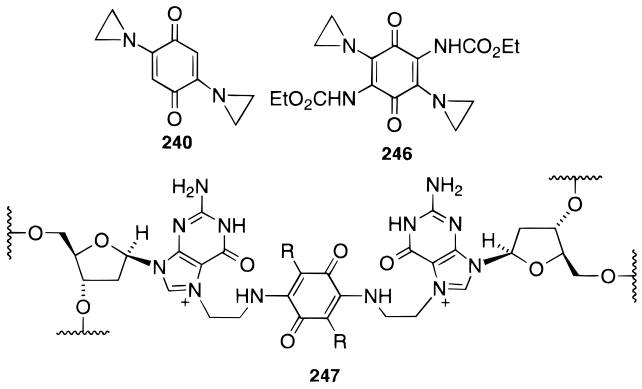


Figure 41. Proposed $^{5'}GpC^{3'}$ binding motifs for activated uracil mustard (**242**) and activated DZQ (**234**).[205]

Of the diaziridinylquinones, the most heavily studied are the simple unfunctionalized diaziridinylquinone **240** (DZQ) and the more clinically promising 2,5-bis(1-aziridinyl)-3,6-bis(carbethoxyamino)-1,4-benzoquinone **246** (AZQ). AZQ is particularly interesting since it is one of the few therapeutic agents known to be active against human brain tumors.[203]

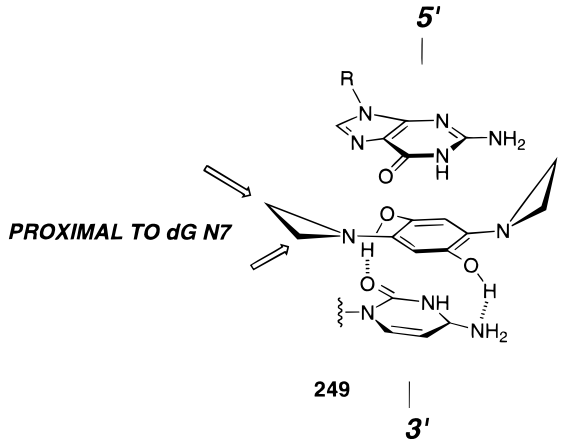
Hopkins and co-workers have recently described the lesion structure resulting from reaction of various synthetic ODNs bearing the sequences $^{5'}GN_nC^{3'}$ (n = 0, 1, or 2) with both DZQ and AZQ.[204]

Enzymatic digestion followed by HPLC, UV, and mass spectral analyses reveal the previously assigned structures to be consistent with those of the isolated lesions resulting from connectivities to both N^7 atoms of the two cross-linked dG residues as shown by **247**.



Concurrent with elucidation of these covalencies, studies have been directed at understanding the noncovalent interactions inherent to the reaction of these agents with DNA. For instance, DZQ (**240**) when in the quinone oxidation state, demonstrates DNA alkylation patterns very similar to most structurally related analogues.[205] However, reduced **240** reacts exclusively at $^{5'}GpC^{3'}$ with a particularly strong preference for $^{5'}TGC^{3'}$.[205] The nature of this radical transition in sequence selectivity has not been unambiguously defined. However, the analogous selectivities of DZQ and uracil mustard (**248**) in which both share a high degree of selection for $^{5'}PyGC^{3'}$ has prompted the proposed noncovalent motif shown in Figure 41.[205] Both cases involve ISC formation via major groove N^7 alkylations of the opposing deoxyguanosines on each strand of the duplex and indeed this model is consistent with at least one N^7 alkylation. The fact that unreduced DZQ (structurally more similar to uracil mustard than the reduced form) did not show the same specificity as the reduced form has raised questions pertaining to the motif shown in Figure 41 and issues pertinent to this have been carefully reviewed.[205]

An alternative rationale derived from molecular modeling studies has shown that the activated DZQ core readily undergoes hydrogen bonding between the hydroquinone hydroxyls and the O^2 and C^4-NH_2 of the cytosine to the 3′ side of the guanosine target base as in the complex **249**.[205,206] In this fashion, the



reactive carbon of the aziridine is positioned within bond-forming distance of the dG N^7 above the plane of the hydroquinone. That only the hydroquinone of DZQ could undergo such a binding mechanism suggests the validity of this view, although an unambiguous demonstration of this motif has not been reported.

In addition to alkylation of the major groove N^7 of deoxyguanosine, some instances of adenine alkylation have been noted.[206] Enzymatically reduced DZQ alkylates not only deoxyguanosine, but also shows a preference for adenines within $^{5'}$(A/T)**AA**$^{3'}$ runs. The methylated 2,5-dimethyl-DZQ (**250**, Figure 42) readily monoalkylates $^{5'}GpC^{3'}$ which is in line with most DZQ-based structures. However, reduction with either ascorbate or DT-diaphorase affords a species highly selective for $^{5'}T$**AA**$^{3'}$. The resulting lesion gives rise to base-labile sites as demonstrated through piperidine digestion. More precisely, it appears that the major groove adenine N^7 is the modified moiety since the presence of 10mM Mg^{2+} abrogated adenine alkylation (via competitive major groove N^7 coordination to Mg^{2+}). The origin of this altered selectivity has not been determined, but dG alkylation with such

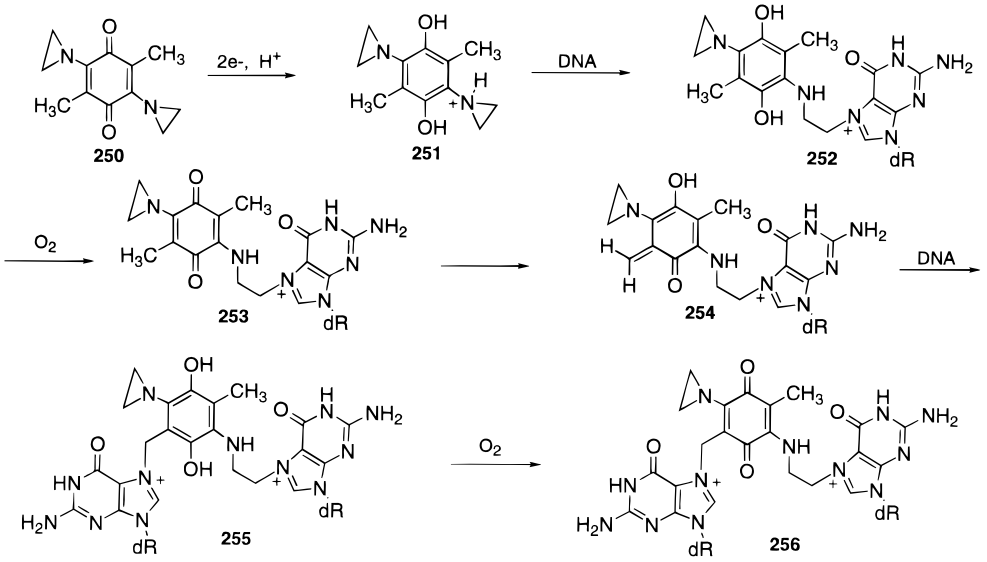
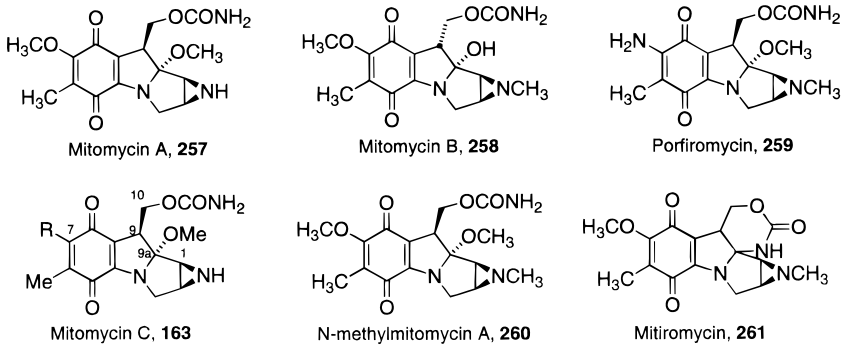


Figure 42. Proposed mechanism of 2,5-dimethyldiaziridinylquinone reaction with DNA via dual aziridine ring opening followed by oxidation and quinone methide formation.

Chart 5



analogues has been found to result partly from quinone methide formation.[202]

Butler et al. have shown that **250** is capable of ISC formation at the sequence 5′T**G**C3′ and that this process requires the initial reduction of the quinone.[202] Computer modeling has revealed that aziridine ring opening by the first dG brings the para-situated methyl group much closer to the N^7 of the opposing dG. Oxidation followed by quinone methide formation ensues and subsequent Michael addition by the second strand affords the cross-linked adduct **256** shown in Figure 42. That oxidation of the initially formed monoadduct is required for ISC generation by **250** strongly supports this theory, although structural elucidation of the resulting lesion still has not been reported. Additionally, the fate and/or role of the second aziridine ring is not known in this quinone methide route to bisalkylation.

The diaziridinylquinones have received considerable attention with respect to the design of 2,5-substituted analogues, but for the most part, little attention has been paid toward the design of peptide or DNA conjugates. As with the anthracyclines, this is likely due to the complexity of such a conjugate which requires not only the desired drug−target interactions, but which must still be capable of the desired activation process. Surprisingly, the mitomycins, for which the diaziridinylquinones may be thought of as mechanistic and structural predecessors,[207] have received attention with respect to such conjugation methodologies despite their considerably more complex structures.[158a−c]

5.5. Mitomycin C and Related Structures

The mitomycins display a wide array of substitution patterns inherent to the core pyrrolo[1,2-*a*]indole structure. Originally isolated in Japan by Hata et al., mitomycins A and B were quickly recognized as potent antibacterial and antitumor agents.[208] The 1956 isolation of these compounds from broths of *Streptomyces caespitosis* was followed two years later by the isolation of mitomycin C (MC, **163**) from the same bacterium at the Kyowa Hakko corporation. In 1960, the aziridinyl N-methylated analogue of MC, porifomycin **259** was isolated from *Streptoverticillatium ardus*. In 1962, the same four compounds were isolated from broths of *Streptomyces verticillatus* as was the biologically inactive pentacyclic mitiromycin.

More recent additions to the mitomycins include *N*-methylmitomycin A (**260**) reported in 1981[212a] and the equilibrium mixture of isomitomycin A (**262**) and albomitomycin A (**263**) which ultimately affords mitomycin A (**257**) as the thermodynamically favored isomer (Chart 5, Figure 43).[212b]

In addition to the isolation of mitomycin congeners from "native" broths, the doping of these broths with biosynthetic "synthons" has yielded a wide array of

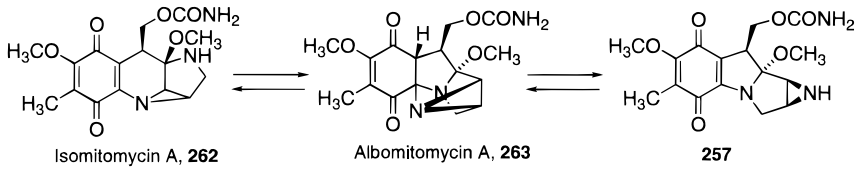


Figure 43. Interconversion of isomitomycin A and mitomycin A through albomitomycin.

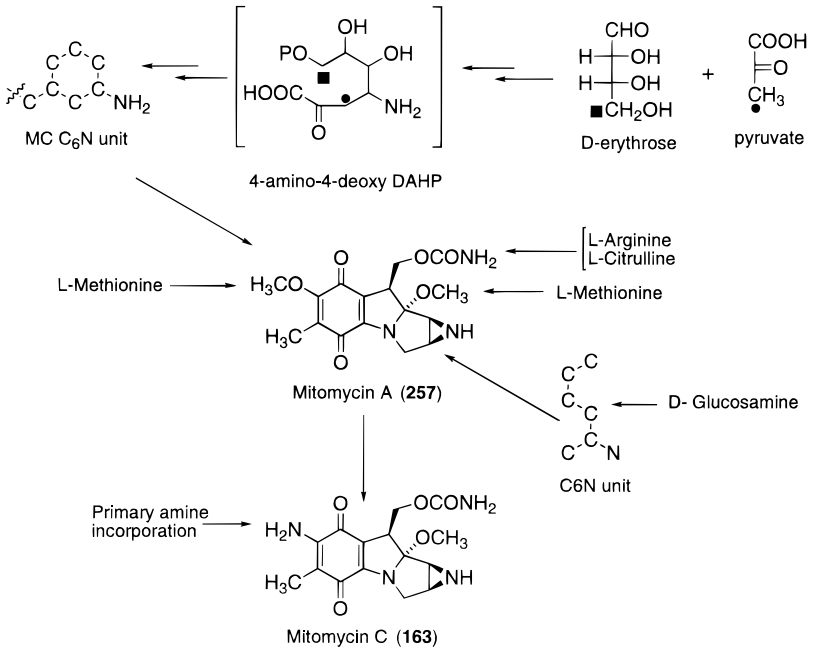


Figure 44. Proposed biosynthetic pathway to mitomycin C.[214]

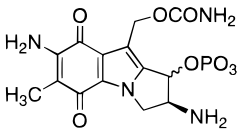
"unnatural" mitomycins. Bush et al. have demonstrated that supplementing the normal fermentation medium for *Streptomyces caespitosis* with various primary amines results in two types of mitomycin analogues.[213] The first type is analogous to mitomycin C with the exception that primary amine incorporation at the quinone C7 position is observed. Alternatively, the mitomycin isolated is analogous to mitomycin B with the primary amine incorporated once again at the C7 position. Broth supplementation with methylamine, ethylamine, propylamine, propargylamine, and 2-methylallylamine gave rise to mitomycin C analogues all of which possessed greater activities against L1210 lymphatic leukemia cells in mice than the related mitomycin B congeners.

As outlined in Figure 44, biosynthetic studies have shown that the six-carbon chain from C10 to C3 containing the aziridine of the natural product is derived from D-glucosamine.[214a] The carbamate derives from citrulline and the O-methyl group at C9a is donated from S-adenosyl methionine.[214b] The quinone is believed to originate from glucose via pyruvic acid.[214b] Importantly, mitomycin C is derived by way of mitomycin A via the biosynthetic route proposed by Hornemann.[215] As such, broth supplementation has been viewed as a viable means of continued generation of new mitomycin analogues, particularly with respect to C7 functionalization.

Like the other quinone-containing DNA interstrand cross-linking agents, the mitomycins benefit from good chemical stability in the absence of exogenous reductants. However, quinone reduction affords a species capable of facile DNA alkylation as well as superoxide production and subsequent oxidative DNA damage.[216] Indeed the vastly enhanced reactivity of mitomycin C upon reductive activation has earned MC the reputation as the prototypical reductively activated chemotherapeutic. The activated "aziridinomitosene" core has been shown to react principally with deoxyguanosine resulting in a wide array of possible lesions.[217]

Preliminary mechanism of action studies revealed that the mitomycins shut down DNA synthesis and also induced phosphodiester strand scission within cellular DNAs. Particularly interesting were the Iyer and Szybalski studies which demonstrated that cell death was not a consequence of the strand scission events.[153] Rather, it was attributed to the formation of interstrand cross-links as evidenced by the anomalously fast renaturation kinetics of mitomycin C-treated bacterial DNA.[153]

Fragmentation studies with mitomycin-modified DNAs revealed that cross-linking was extremely rare with only one ISC per 20 000 base pairs of DNA.[153] Additionally, the failure to induce ISCs in vitro without the presence of cell lysates suggested that cellular activation was required for drug action.[153] Indeed, Iyer and Szybalski demonstrated that the normally inert mitomycin was capable of DNA cross-linking upon treatment with chemical reductants such as H_2/Pd/C, $NaBH_4$, or sodium dithionite and that this reaction demonstrated differing efficiencies with different DNA substrates.



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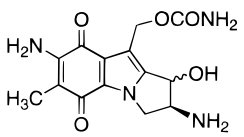

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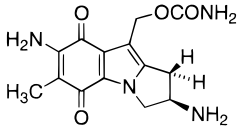
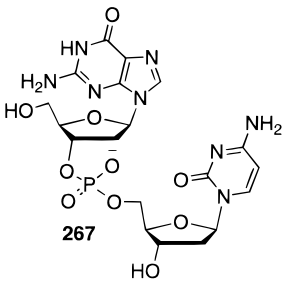


266

The different CG content of *S. lutea* (a high GC content genome) versus *C. johnsonii* (34% CG) DNAs led to much more efficient cross-linking of the former substrate.[153,218] The first adducts isolated from the reductive activation of mitomycin C were reported by Tomasz and co-workers.[219] Reduction of MC with rat liver microsomes and NADPH in phosphate-buffered aqueous solutions afforded the aziridine ring-opened adducts **264**−**266**.

The preference for GC runs of DNA was elaborated upon via the elegant studies of Tomasz and Nakanishi in which enzymatic digestions of the mitomycin-modified dinucleotide dGC (**267**) and subsequent HPLC purification revealed **268** as a presumed monoalkylated intermediate in the pathway to cross-link formation.[220] This represented the first documentation of an MC−nucleotide adduct although subsequent studies[219] mandated its regiochemical and stereochemical reassignment as **269**.



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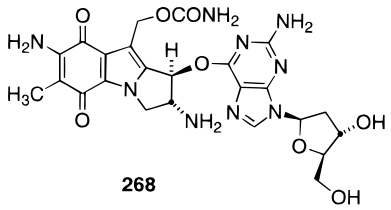

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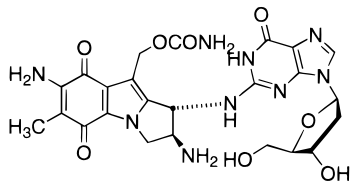
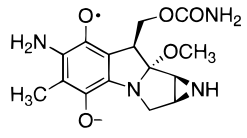


269

Later studies by Nakanishi et al. revealed that MC could alkylate DNA via acid-catalyzed pathways in addition to the reductive methodologies previously demonstrated.[30,221] Acidic activation requiring pH ≤ 5 induced both monofunctional binding and cross-linking. Generation of 1-substituted 2β,7-diaminomitosenes was effected with a remarkable change in selectivity from dG N^2 alkylation to the dG N^7 event. The imidazolium formed upon N^7 alkylation gave rise to not only the deribosylated lesion **271** (leading to DNA strand scission), but also the N-formylated adducts arising from hydrolysis at the purine C8 position. Both furanose (**272**, **273**) and pyranose (**274**) adducts were found (Figure 45).

The reductive activation pathway for the mitomycins has been the topic of intense study and considerable insight has been gained largely through the use of synthetic derivatives and various reductants. Until recently, the mechanism of DNA modification by MC entailed initial one or two electron reduction of the quinone moiety to either the semiquinone radical anion (**283**) or the hydroquinone (**275**).[216,238i,242] As depicted in Figure 46, generation of the hydroquinone allows facile expulsion of the angular methoxy group and subsequent tautomerization to afford the intermediate **277**. In a fashion analogous to that seen with the pyrrolizidine alkaloids, the resulting core structure is activated toward nucleophilic attack at both C1 and C10 positions. As such, a pathway continuing from **277** to **281** was proposed to result in DNA bisalkylation with some degree of reversibility possible at both sites of drug−DNA connectivity.[248b] Oxidation back to the quinone, however, could be viewed as prohibiting release of either DNA-bound site thereby stabilizing the ISC.

The amenability of the semiquinone radical anion **283** to undergo this chemistry in vivo has been a topic of particular interest and some debate. This is due



283

largely to the realization that under aerobic conditions, electron transfer from the semiquinone to O_2 results not only in superoxide production, but net inactivation of the pharmacaphore.[216] Danishefsky, Crothers, and co-workers have suggested, however, that anaerobic conditions could allow retention of the one electron reduced *N*-methylmitomycin A (**260**).[242] That both fully reduced **260** and its semiquinone congener could efficiently cross-link synthetic ODN duplexes was supported via stepwise $Na_2S_2O_4$ (two electron reductant) reduction of **260** followed by addition of Fe^{3+} as a one electron oxidant. From these studies, it was further suggested that the one electron reduced **260** species presented the more reactive of the two possible C10 centers. This was based largely on the observation that reduction (presumed to afford complete conversion to the hydroquinone of **260** with a 3−4-fold molar excess of $Na_2S_2O_4$ gave rise to efficient ISC formation (yield = 35%). However, the ISC yield could be significantly increased by addition of Fe^{3+} in reactions where reductant:oxidant:drug ratios of 1:1:1, 1:2:1, and 2:2:1 were investigated. This led to the theory that both hydroquinone and semiquinone were capable of ef-

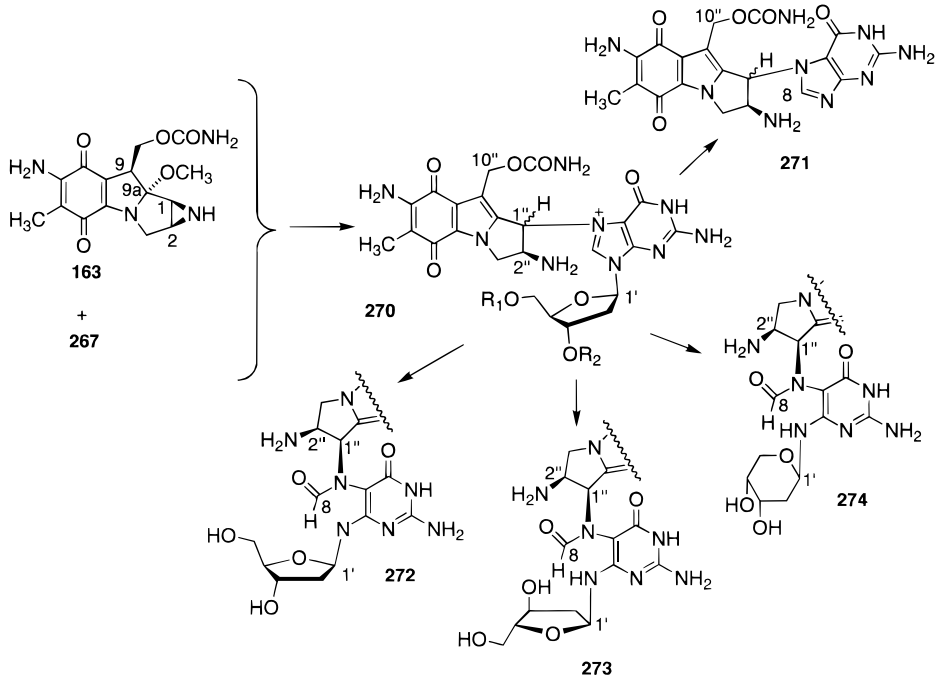


Figure 45. MC−DNA product distribution under acidic activation conditions.[30]

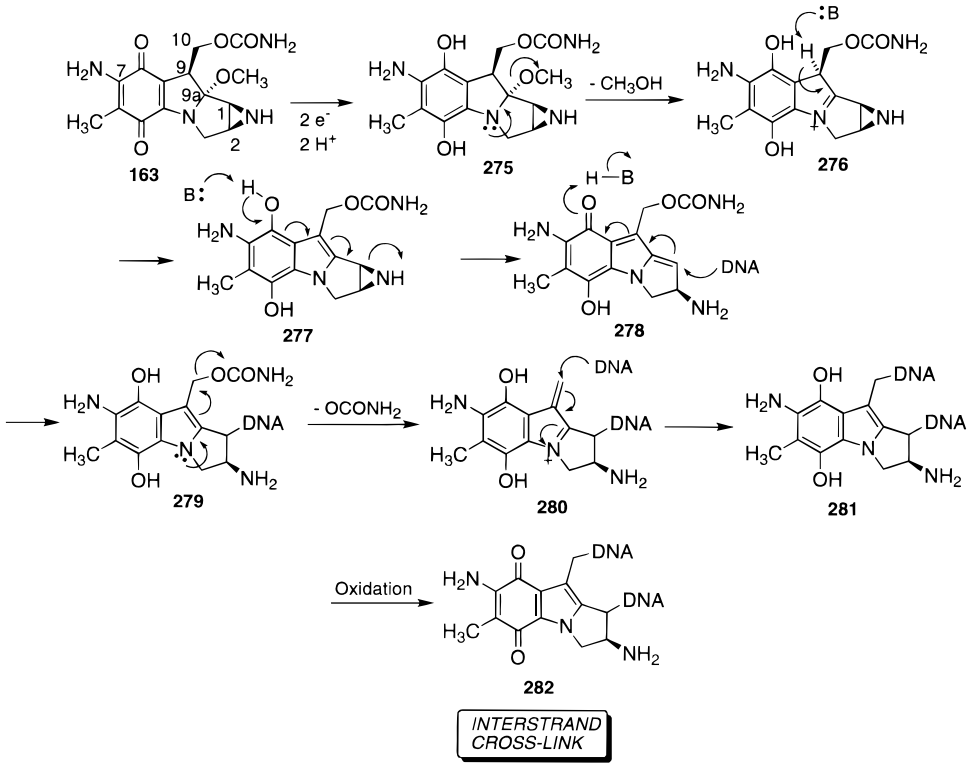


Figure 46. Proposed mechanism of DNA cross-linking by two electron reductive activation of mitomycin C.

fecting ISC formation but that the semiquinone (resulting from one electron oxidation of the hydroquinone) actually played a much more significant role in bisalkylation than the corresponding two electron reduced **260** species. This was reasoned to result from more facile loss of the C10 carbamate in the semiquinone case than in the hydroquinone. These data supported the generally accepted mechanism for reductive activation and ensuing DNA alkylation shown in Figure 46 with the caveat that a one electron reduced species was probably the better bisalkylating species although was applicable only under anaerobic conditions. These findings provided further support for earlier findings by Egbertson and Danishefsky wherein the semiquinone radical anion of **260** afforded C1 and C10 xanthate esters and bifunctional drug−xanthate conjugates following reductive activation with $Na_2S_2O_4$ in aqueous pyridine.[238i] The rate of formation of fully reduced **260** could not account for this species as the primary alkylating agent. Rather, it suggested that a partially reduced **260** analogue (i.e., semiquinone) played a significant

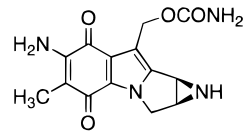
role in product formation with the added nucleophile potassium xanthate under these specific conditions.

Whether a one or two electron reduced species (or some combination of both) is responsible for interstrand cross-linking is still an issue of some controversy. It is noteworthy, however, that the vast majority of literature regarding this issue, portrays the reduced intermediate as a fully reduced hydroquinone or some equivalent thereof.

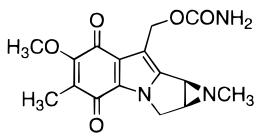
Mitomycin-dependent DNA alkylation mechanisms have traditionally placed an emphasis on the retention of the reduced state of mitomycin throughout the cross-linking pathway. Contrary to this, recent efforts by the Kohn group suggest that reductive activation of the mitomycins may only be necessary for loss of the C9a angular substituent so as to afford the core indoloquinone.[222a] Quite significantly, these workers found that the oxidized aziridinomitosenes **284a** and **284b** both react with a 129bp *Bst*N1−*Eco*R1 restriction fragment from pBR322 plasmid DNA in the absence of reductant, and that the DNA product profiles closely resemble that for reductively activated MC.[222a] Both **284a** and **284b** displayed a high degree of selectivity for $^{5'}CG^{3'}$ as did MC (following reduction with xanthine oxidase/NADH). This selectivity, however, was severely compromised upon addition of $Na_2S_2O_4$ to the alkylation reactions. More importantly, this added reductant inhibited DNA alkylation by the **284a** and **284b**. Taken together, these observations suggest that it is the oxidized 7-aminoaziridinomitosene **284a** (following reduction, expulsion of the C9a methoxide group, and subsequent restoration of the quinone moiety) that is the active alkylating species formed during reduction-dependent DNA alkylation by MC. In this sense, an alternative explanation for the Danishefsky findings with **260** and Fe^{3+} could envision the full conversion of not all, but some, of the fully reduced **260** back to the quinone oxidation state as opposed to the semiquinone. This would imply, however, that a species such as **284a** could interstrand cross-link, a point which remains to be determined.

Additionally, it was found that reductive activation of *N*-methylmitomycin A (**260**) and its corresponding indoloquinone **284b** led to a pronounced loss of both alkylation efficiency and specificity as compared to unreduced **284b**. On the basis of this, Kohn et al. have suggested that reductively activated **260** alkylates DNA through an intermediate other than **284b** and that likely candidates are the *N*-methyl-7-methoxyleucoaziridinomitosene **284c** or the aziridine ring-opened quinone methide **284d**. Both possible intermediates are suspected to be much more reactive and to thus demonstrate diminished DNA alkylation selectivities. Additionally, Kohn and Schiltz have previously shown that fully reduced mitomycins prefer to undergo conversion to 2-aminomitosenes (as in **266**) via C1 protonation rather than undergoing nucleophilic attack (as in the very early lesions isolated by Tomasz et al., **264** and **265**).[222a,b]

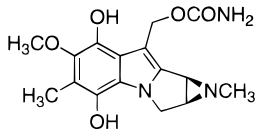
It is important to note that these data are the result of DNA alkylation patterns as obtained by UVRABC nuclease incision methods. As such, the relevance of these experiments within the context of



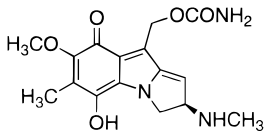
284a



284b



284c



284d

DNA cross-linking has not yet been determined. Given the integral role of C1 alkylation en route to ISC formation, these results have a clear and important bearing on the understanding of mitomycin mechanisms of cross-link formation. As will be discussed later in this section, mitomycin C1 alkylation is the first DNA-drug connectivity formed en route to ISC formation and thus, the steps leading to and following this event have a large influence on the overall cross-linking process.

Extensive molecular modeling of both interstrand cross-linked $^{5'}CpG^{3'}$-containing oligodeoxy-ribonucleotides and monoalkylated ODNs has been performed. Molecular mechanics simulations of the $^{5'}CpG^{3'}$ cross-link in the computer-generated decamer d(GCATCGATGC)$_2$ was carried out by Tomasz and Verdine (Figure 47).[30] This model showed the comfortable fit of the MC-derived mitosene within the minor groove of B-DNA. No distortions of the sugar−phosphate backbone were detected and all glycosidic torsion angles proximal to the cross-link conformed to the ususal anti configuration.[30] Additionally, the C−G base pairing was maintained in both drug-modified pairs although slight alteration of the propeller-twisting of each alkylated base was observed.[30] Twisting of the C10″-bound dG was reduced and twisting of the C1″-bound dG was increased relative to B-DNA. This is believed to be a function of the approximate 0.3 Å difference in bonding distances between the mitosene and the intraduplex dG N^2−dG N^2 box resulting in slight spreading apart of the duplex at the expense of propeller twisting.

Within the decamer examined, the mitosene N2 ammonium function appears to be involved in considerable hydrogen bonding interactions with three atoms on the same strand to which the C1″ is attached.[30] Particularly noteworthy are contacts with the N^3 and $O^{3'}$ of the A7 residue and the $O^{4'}$ of the T8 residue one base removed from the C-1″ bound dG nucleotide (Figure 47). The base pairing interactions of both strands are intact, but some degree of reorganization has occurred with respect to the C1″ bound strand. This is facilitated by the proximity to the mitosene N2 ammonium group. The minor groove is compressed to widths of 8.1 and 9.5 Å (as determined by phosphorus-to-phosphorus distances) immediately below the cross-link and returns to the normal values of 11.1−11.9 Å in the rest of the duplex.[30]

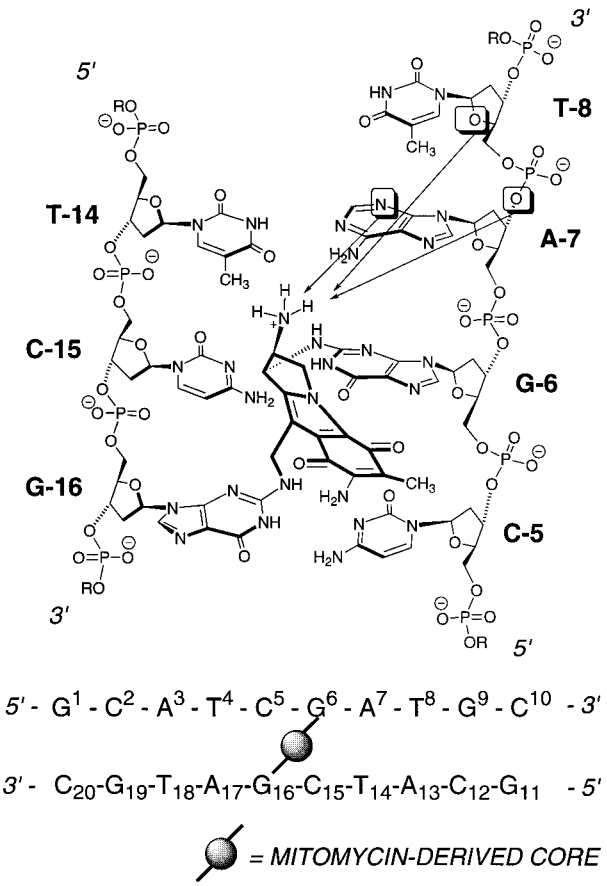


Figure 47. Mechanical modeling of MC cross-linked $d(G^1C^2A^3T^4C^5G^6A^7T^8G^9C^{10})_2$. Only partial structure of the DNA duplex is shown, and interstrand hydrogen bonding interactions are not shown for the purposes of clarity. Intrastrand DNA−drug contacts are highlighted involving the aziridinyl N2.[30]

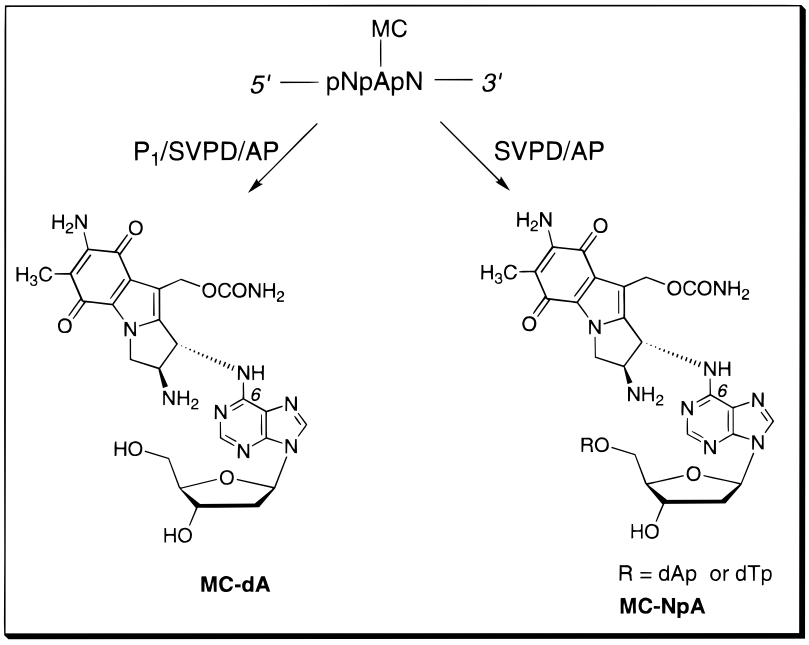
Recent ^{1}H NMR examination of MC cross-linked 5′-$d(T^1A^2C^3G^4T^5A^6)$ has more rigorously addressed the solution structure of the MC cross-link than was possible via the Verdine modeling study.[223] Like the modeling study, interproton distance constraints obtained by Tomasz and co-workers verify the retention of all H-bonding interactions between the two DNA strands and also confirm the anti conformations of the glycosidic bonds.[223a] Strikingly, the ^{1}H-derived data suggest that the substrate DNA does, however, deviate from that of normal B-DNA. The sugar protons on adjacent nucleotides are 5 Å apart in B-DNA and as such nOes are not found between sugar H1′ protons on adjacent residues. However, nOes were detected between the sugar H1′ protons of G10 and T11. A similar result was obtained involving the same protons of G4 and T5.[223a] As such, MC cross-link formation was found to be facilitated, in part, by alteration of the phosphodiester backbone of the G10-T11 and the G4-T5 steps which brings the sugar rings at these positions into closer proximity than expected for retention of the B-form structure. Strikingly, measurement of phosphorus resonances revealed that MC complex formation results in distortions in the phosphate backbone also at the G10-T11 step and its complementary G4-T5 step.[223a] This, in combination with the recognized alignment of the MC aromatic chromophore toward the G10-T11 step of the T^7-A^8-C^9-G^{10}-T^{11}-A^{12} strand rather than with the center of the minor groove, allows for slight widening of the minor groove in contrast to preceding mechanical modeling studies.

Like the MC interstrand cross-link, the solution structure of MC monoalkylated DNA also undergoes nonsymmetrical stacking of the chromophore ring system with principally one of the two DNA strands.[223d] Indeed many of the traits inherent to the interstrand cross-link are also observed with the monoadduct. One notable difference is that the MC monoadduct displays rapid exchange of the MC dG exocyclic amino protons.[223d] This is in stark contrast to the very slow exchange rate seen for the cross-link. As such, it has been reasoned that the MC dG monoadduct is much more solvent accessible than in the case of bisalkylated DNA substrates. The monoalkylated MC chromophore is positioned in the minor groove and the indoloquinone system lies at an approximate 45° angle with respect to the helix axis. The DNA substrate examined (in this case a 9-mer self-complementary ODN 5′-d(ICACGTCIT)$_2$) possessed base pairs displaced by approximately −3.0 Å toward the major groove thus allowing the minor groove positioning of the MC derived mitosene. This is consistent with the interstrand structural data in that retention of the hydrogen bonding network between the two strands was observed and that the O-10″ atom of the MC carbamate side chain forms a critical H-bond with the exocyclic amino group of the nonalkylated dG residue proximal to the alkylated deoxyguanosine.[223d]

The vast majority of studies pertaining to interstrand cross-linking by the mitomycins have exploited chemical reducing agents such as sodium borohydride or sodium dithionite under anaerobic conditions. Such assays have revealed that ISC formation proceeds almost exclusively at $^{5'}$CpG$^{3'}$ sequences with some variation in efficiency based upon the flanking bases on either side of the two-base sequence.[224] Additionally, cytosine methylation of the $^{5'}$CpG$^{3'}$ enhances the extent of cross-linking.[224b] Absolutely critical to mitomycin-induced cross-linking is the presence of the two exocylic N^2 amines of the adjacent deoxyguanosines on either strand.[217,218]

The Tomasz group has recently described the very interesting and potentially significant finding that deoxyadenosine alkylation by reductively activated MC occurs in the presence of calf thymus DNA and other synthetic AT-rich ODNs.[225] Reductive activation of MC was performed using three different systems: substoichiometric $Na_2S_2O_4$, H_2/PtO_2, and NADH/NADH-cytochrome *c* reductase. In all three activation scenarios, adenine−MC adducts were obtained bearing connectivity of the mitosene C1 center to the exocyclic N^6 of adenosine. The substoichiometric $Na_2S_2O_4$ activation method was exploited with several AT-rich synthetic ODNs. These reactions afforded two different species following different digestive workup procedures. As shown in Scheme 5, digestion with SVPD followed by *E. coli* alkaline phosphatase (AP) afforded the mixture of MC−NpA adducts where N represents either dA or dT nucleotides. Alternatively, a related digestion (inclusive

Scheme 5



of nuclease P_1) of the same alkylated substrates afforded only the single nucleoside−MC adduct. The observed 5′-phosphodiester resistance to SVPD for the MC−DNA adduct is in stark contrast to the MC−dG monoadduct which is readily cleaved by SVPD but is resistant to 3′-hydrolysis by nuclease P_1. An explanation for this potentially important difference in reactivity has not been achieved.

Formation of the three different MC-dA lesions was found to depend on not only the enzymatic analysis method, but also dG content and sequence context of the specific DNA substrate. The general trends of reactivity dictated that adenines within poly-dA runs were roughly 2-fold more reactive toward activated MC than those in alternating AT boxes. Additionally, as the number of readily alkylated dG sites increased within a given substrate, the efficiency of dA alkylation decreased, due to preferred consumption of activated MC by dG residues. Important to note, is that enzymatically digested MC-alkylated DNA substrates revealed no dA-mediated interstrand cross-links under any circumstances.

Perhaps most significant about these disclosures, is that the in vitro generation and characterization of the MC−dA lesion has been exploited to identify a previously unknown adduct (substance **X**) formed in EMT6 mouse mammary tumor cells that had been treated with ^{3}H-labeled MC.[225] These workers note that further tests are necessary to confirm the identity of **X**; however, it is noted that the in vitro derived MC−dA adduct and **X** do coelute by HPLC. Interestingly, it had been noted that the in vivo proportion of **X** (relative to dG-based adducts) could be increased and that these increases significantly enhanced the cytotoxicity of MC. Given these findings, it appears that MC and its relatives may display biologically important mechanisms of action which do not rely solely upon the established mode of deoxyguanosine alkylation pathways.

Contrary to results utilizing chemical reduction, enzymatic reduction by one electron reductants such as xanthine oxidase or NADPH-cytochrome *c* reductase results in monoadduct formation accounting for more than 90% of the DNA-bound drug.[226] Additionally, aerobic chemical reduction methodologies result in base-labile lesions via presumed dG N^7 alkylation involving aziridine ring cleavage and subsequent C1 substitution.[227] Gibson and co-workers have shown that dG N^7 alkylation occurs not only upon acidic activation, but may also be facilitated by DT-diaphorase (DTD)-mediated reduction (Figure 48).[228] Under aerobic conditions, this two electron reductase has

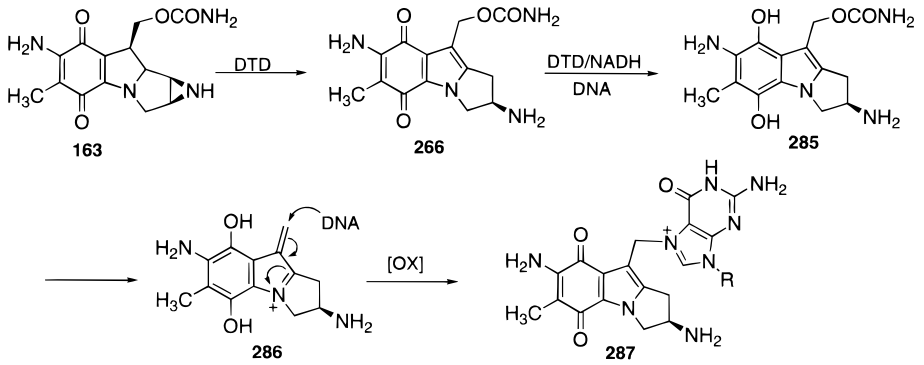


Figure 48. DT-diaphorase (DTD)-mediated reduction of mitomycin C.

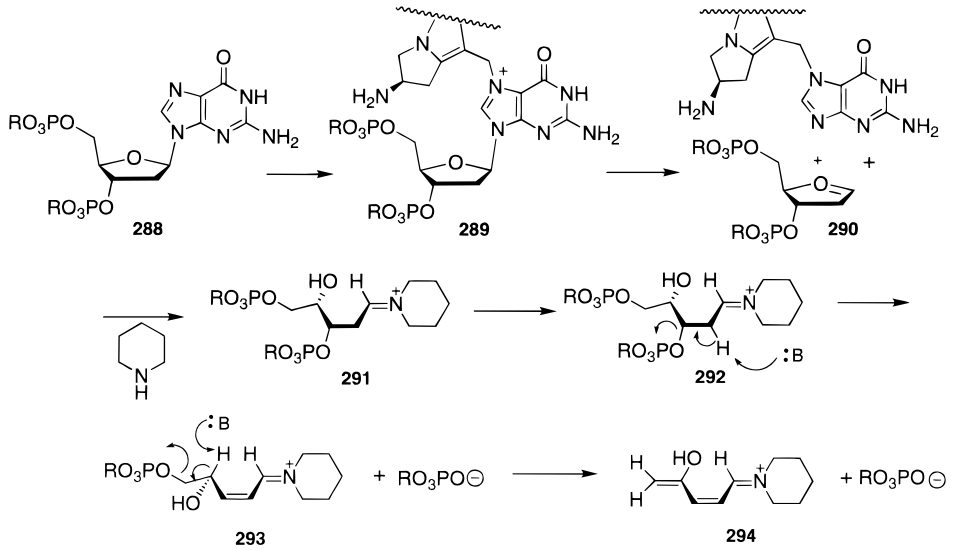
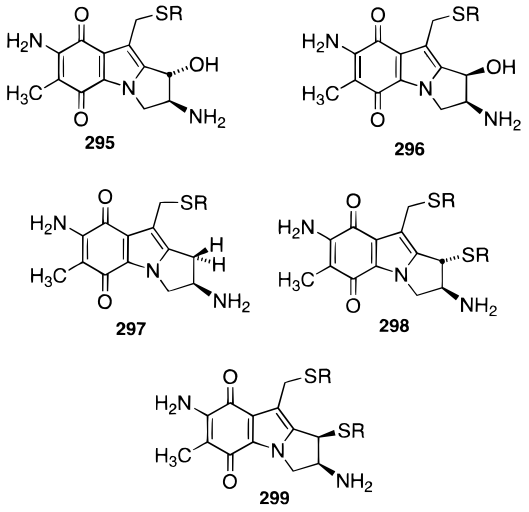


Figure 49. Mechanism of depurination followed by base-mediated DNA strand scission.

been implicated in the DNA mono- and bisalkylation events, as well as, glutathione conjugation reactions with mitomycin C.[228] Surprisingly, the major product of DTD metabolism is the 2,7-diaminomitosene **266**.

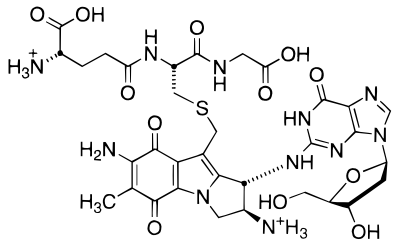
Substrate **266**, upon reduction with DTD is capable of only monoalkylation via loss of the carboxamide to afford the Michael acceptor **286**. No longer capable of cross-linking (due to destruction of the aziridine) **285** readily alkylates dG N^7 moieties preferentially within $^{5'}$GpG$^{3'}$ and $^{5'}$GTC$^{3'}$ sequences thus giving rise to base-labile lesions (Figure 49).[228a] The reaction required both DTD and NADH and showed a marked dependence upon pH with optimal alkylation observed at pH 5.8. Proton-assisted loss of the carbamate has been proposed as the root of the observed pH dependence. The DNA modification chemistry of **266** has been further elaborated upon by Tomasz and co-workers and the actual base−drug lesion (resulting from heat-induced deribosylation off of the substrate DNA) isolated and characterized.[228b] *Importantly, the noted requirement by both the Gibson and Tomasz groups that **266** be reductively activated to undergo C10 decarbamoylation and subsequent DNA alkylation underscores that the DNA alkylation patterns observed with indoloquinones **284a** and **284b** are likely indicative of only monoalkylation processes and may not necessarily be extrapolated to the intact cross-linking pathway.*

In addition to studies pertaining to sequence selectivities of the mitomycins (much of which will be discussed below), the reactions of mitomycin C with various nucleophiles of biological consequence have been explored. Tomasz and Sharma have shown that reaction of reductively activated mitomycin C with glutathione, mercaptoethanol, and *N*-acetylcysteine affords mono- and bisthiolated compounds **295**−**299**.[229] The reactions absolutely required the presence of an additional reducing agent aside from the chosen thiols and in fact, the thiols studied were incapable of mitomycin reductive activation even at concentrations as high as 50 mM in thiol. Unreduced MC (**163**) was completely inert toward thiolation.[229]

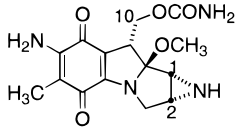


An interesting observation was that although incapable of MC reduction, glutathione was found to radically accelerate the rate of MC reduction by "slow" reductants such as cytochrome *c* reductases and H_2/PtO_2.[229a] Additional studies have shown that sodium dithionite reduction of MC in the presence of *M. luteus* DNA and glutathione affords the peptide adduct **300**.[229b] The same lesion was isolated following PtO_2 activation procedures, contrary to the belief that thiol poisoning of the catalyst would abrogate MC activation. Additionally, reduction of the monoalkylated ODN $^{5'}$ACACG*TCAT$^{3'}$ (G* denotes the mitomycin bound dG residue) in the presence of 50 mM glutathione also produced the peptide adduct **300** following enzymatic digestion and HPLC purification.[229b]

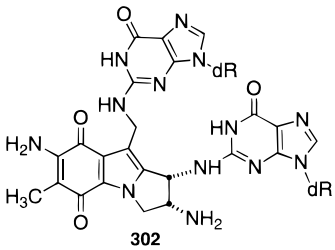
Concomitant with the thiol alkylation studies, the Tomasz group, in collaboration with Fukuyama, demonstrated that the $^{5'}$CpG$^{3'}$ selectivity displayed by the mitomycins is dictated largely by the initially formed C1″ α linkage to the first dG.[230] The solution structures of synthetic ODN duplexes containing both the $^{5'}$CpG$^{3'}$ interstrand cross-link and the monoadduct had been determined by 2-D NMR as well as molecular modeling.[222,223] Consistent with earlier proposals, the structures revealed that the monoad-



300



ent-MC, **301**



302

duct assumes an orientation within the minor groove in which the C10 carbamate is positioned in the 5′ direction (upstream) from the initially alkylated dG. Given the observed orientation of the monoadduct, only that dG N^2 of the complementary strand would be capable of C10 alkylation thus affording the cross-link. The alternative alignment wherein the carbamate is directed to the 3′ side (downstream) of the initially alkylated dG could give rise to the interstrand cross-link such that $^{5'}GpC^{3'}$ specificity was obtained. That such specificity had never been observed was attributed to the chirality of the drug C1″-α linkage to the N^2 atom of the first alkylated dG though a thorough examination of this was lacking. Alternatively, this specificity was proposed to result from favorable hydrogen bonding interactions involving the C10-oxygen atom of the MC C10 carbamate and the exocyclic guanosine N^2 in the nonbonding strand at the $^{5'}CpG^{3'}$ bonding site (Figure 50).[224,226,250]

To probe this issue more thoroughly, reactions involving the reductive activation and subsequent DNA alkylation chemistry of *ent*-MC (**301**) were pursued.[230] Surprisingly, the resulting interstrand cross-link was formed and found to bear the α-stereochemistry (giving the 1″,2″ *cis*-product **302**) at the C1″ position analogous to that seen with the native mitomycins.

This refuted the proposal that the aziridine stereochemistry of the natural products was responsible for the stereospecific α-face attack by the first dG residue. Assuming that no oxidation back to the quinone occurs following reduction and subsequent C9a demethoxylation, this suggests that the quinone methide **278** is the species to undergo monoalkylation in an S_N1 fashion (as per Figure 51). As such, the chiral minor groove of $^{5'}CpG^{3'}$ binds to both quinone methides (**278** and **304**) using a similar motif. This allows stereospecific attack upon the prochiral quinone methide C1 by deoxyguanosine on the α face of the mitomycins (Figure 51).[230]

Importantly, once in place, the C1″ stereochemistry sets the mitosene core within the $^{5'}CpG^{3'}$ sequence so as to allow only the carbamate upstream positioning (Figure 50). In this motif, $^{5'}CpG^{3'}$ behaves similarly to an enzyme active site via chirality induction upon an achiral center.[230] That the proposed carbamate−dG N^2 hydrogen bond exists has been shown by NMR studies,[223d] but equally important is the demonstration that substitution of the potential H bond donating dG amine with inosine (lacking N^2) affords retention of only the upstream orientation upon $^{5'}CpG^{3'}$ monoalkylation.[223a] As such, considerable evidence has been presented to suggest the importance of the stereospecific first addition to the activated mitosene with respect to interstrand

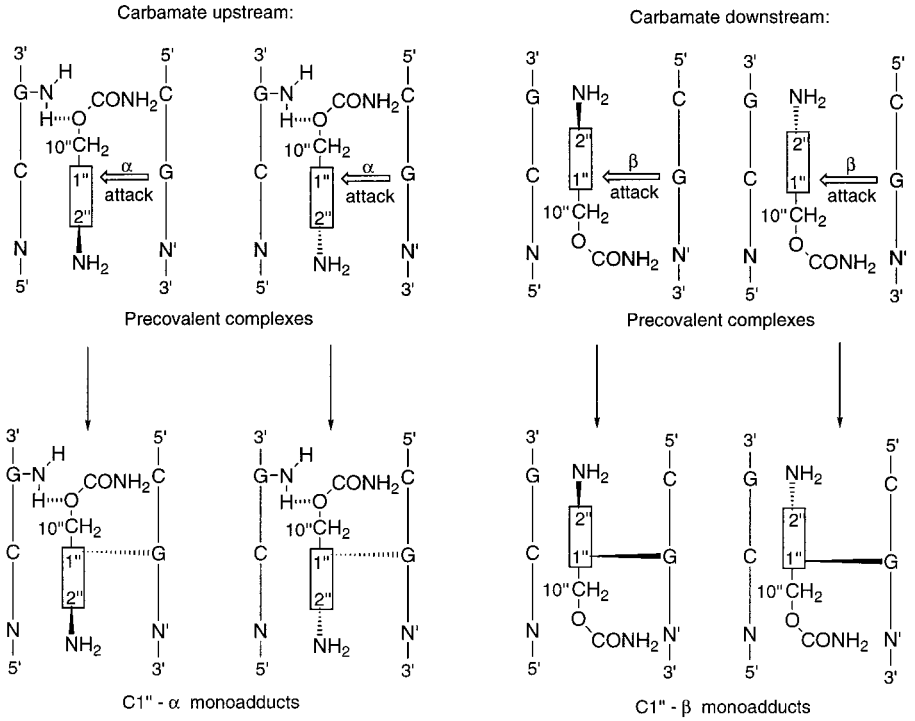


Figure 50. Noncovalent interactions of "aziridinomitosenes" within $^{5'}CpG^{3'}$ leading to stereospecific α face attack at C1. (C1″-*β*-mono- or bisalkylation adducts are not experimentally observed).[230]

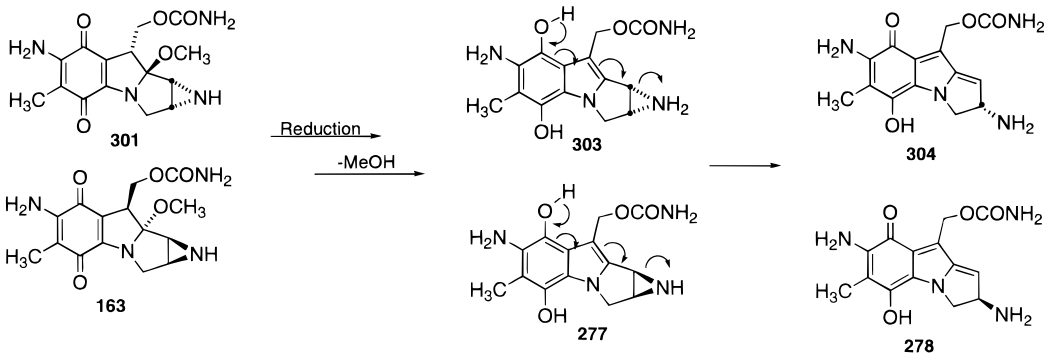


Figure 51. Generation of quinone methide species **305** from mitomycin C and *ent*-mitomycin C.

Figure 52. MC conjugate binding to single-stranded ODN.[158a]

cross-linking by the mitomycins (Figure 51).

The generation of synthetic analogues of the mitomycins has represented a fruitful area for study and the immense effort put forth warrants much greater consideration than is available within this discussion. The interested reader is referred to the synthetic endeavors described in refs 231 and 238.

As with many other redox-activated agents, the mitomycins have received little attention with respect to bioconjugate chemistry. To date, three instances of MC conjugation to DNA-binding ODNs have appeared. Tomasz and Maruenda have demonstrated that a number of 5′-functionalized ODNs were capable of reductively activated minor groove alkylation events.[158a] The single-stranded target ODNs underwent hybridization with the MC-tethered complements and upon treatment with NADPH-cytochrome *c* reductase readily formed the covalently bound duplex (Figure 52).

Target alkylation was found to occur exclusively at dG N^2 and was relegated to the 3 dG residues proximal to the MC core. The preferred alkylation site was G15 (third from the 3′ target terminus), although substitution of G-15 with inosine shifted the alkylation chemistry to G16 and G-18 despite the fact that neither one is involved in base pairing with the conjugate. Utilizing a similar approach, Tomasz and Maruenda have also demonstrated DNA−RNA cross-linking via an MC-tethered ODN.[158b]

Kohn et al. have shown that conjugation of various mitomycin core structures to phosphorothioate oligodeoxyribonucleotides could be achieved involving conjugation via the mitomycin C10 position to afford a conjugate capable of facile C1 alkylation.[158c] This was in contrast to the Tomasz studies wherein the MC unit was tethered through the C7 position so as to leave the DNA−alkylation chemistry at C1 and C10 intact.[158a,b] The use of oligodeoxyribonucleotides containing phosphorothioate units instead of the phosphodiester backbone was sought as a means of inhibiting exo- and endonuclease activities.

Several strategies were envisioned by which to effect conjugation of the MC chromophore to the ODNs of interest. The first involved decarbamoylation of MC (**163**) with sodium methoxide to afford **306** (Figure 53). This was followed by functionalization of the aziridinyl nitrogen with dimethyl sulfate or methyl chlorothiolformate to yield the N-substituted mitomycins **307** and **308**. Conversion of **307** and **308** to the carbonylimidazoles **309**−**312** was effected upon treatment with either 1,1′-carbonyldiimidazole or 1,1′-thiocarbonyldiimidazole.

Alternatively, conjugation of the aminohexyl-functionalized oligodeoxyribonucleotides was envisioned to occur via ODN addition to the isothiocyanate **316** (Figure 54). Substrate **316** was derived from decarbamoyl MC (**306**) by mesylation of the C10 alcohol, displacement with sodium azide, and subsequent reduction to the amine (PtO_2,H_2) in pyridine followed by air oxidation to ultimately afford **315**.

Conversion of **315** to the isothiocyanate **316** was effected upon treatment with 2-pyridyl thionocarbonate (DPT). Conjugation efforts emphasized the reactions of **309**, and **312** with $H_2N(CH_2)_6P(S)(OH)$-GGCCCCGTGGTGGCTCCAT. Difficulties associated with these and the other activated carboimidazoles **310** and **311** ultimately led, however, to the

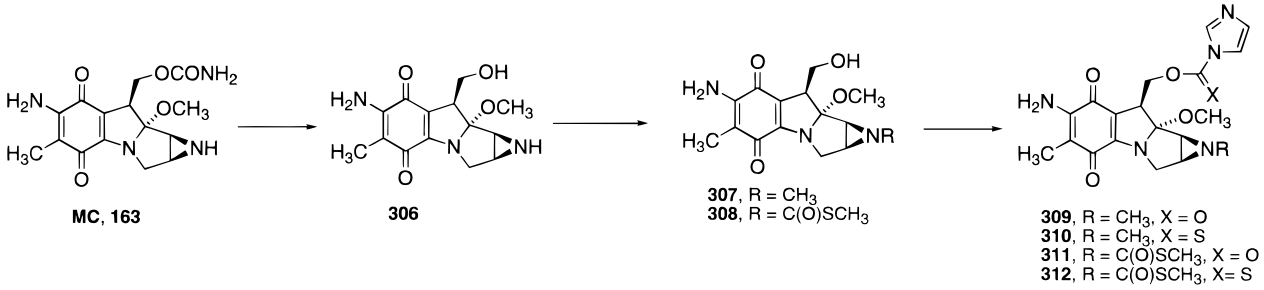


Figure 53. Decarbamoylation of mitomycin C and subsequent functionalization at C-10.

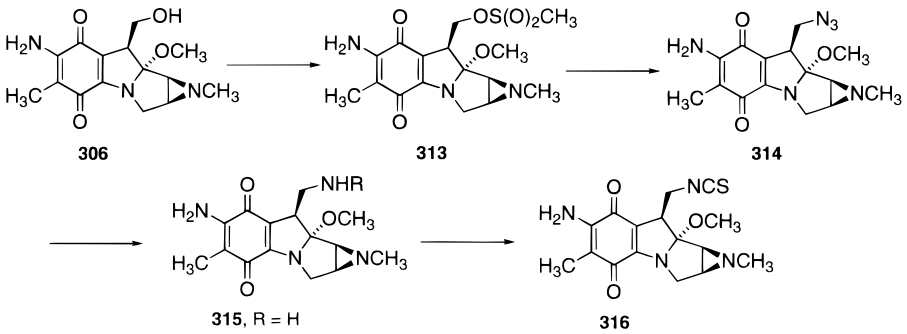
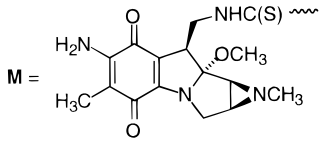


Figure 54. Synthesis of isocyanate **316**.

exclusive use of isothiocyanate **316** as the mitomycin derivative of choice for phosphorothioate conjugations.

Reaction of **316** with aminohexyl-functionalized ODNs of varying length and sequence composition gave rise to the conjugates **317**−**324** all of which were intended as antisense strands to a 30-bp-long region from the coding region of the human FGFR1 gene. Bearing the sequence $^{5'}$AGATGGAAAAGAAATTG-CATGCAG-TGCCG$^{3'}$, the sense strand of this region was intended as the target for conjugate-mediated inhibition of high-affinity bFGF binding to smooth muscle cells. This activity was in fact confirmed with all mitomycin-bearing conjugates inhibiting the proliferation of culture human aortic smooth muscle cells at concentrations as low as 1 μM. Although interstrand cross-linking was presumed to not be a likely mechanism of action for these conjugates, it was clear that conjugation of **316** to each ODN was capable of endowing the conjugate with inhibitory attributes far exceeding those of the untethered oligos.

ACCTTTTCTTTAACGTACGTCACGG-P(S)(OH)-$(CH_2)_6$NH-**M** **317**
TACCTTTTCTTTAACGTACGTCACG-P(S)(OH)-$(CH_2)_6$NH-**M** **318**
CTACCTTTTCTTTAACGTACGTCAC-P(S)(OH)-$(CH_2)_6$NH-**M** **319**
TCTACCTTTTCTTTAACGTACGTCA-P(S)(OH)-$(CH_2)_6$NH-**M** **320**
ACCTTTTCTTTAACGTACGTCACGG-P(S)(OH)-$(CH_2)_6$NH-**M** **321**
TACCTTTTCTTTAACGTACGTCACG-P(S)(OH)-$(CH_2)_6$NH-**M** **322**
CTACCTTTTCTTTAACGTACGTCAC-P(S)(OH)-$(CH_2)_6$NH-**M** **323**
TCTACCTTTTCTTTAACGTACGTCA-P(S)(OH)-$(CH_2)_6$NH-**M** **324**

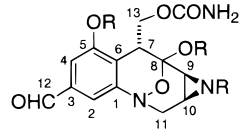


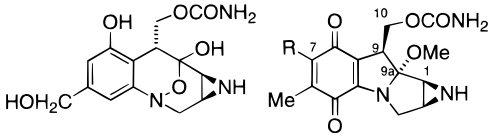
These studies have clearly shown the amenability of the mitomycins with respect to bioconjugate approaches to drug delivery. Although the specific mechanism of action of the conjugates was not examined, these efforts (in combination with the system examined by Tomasz) represent one of the more progressive aspects of mitomycin research. More intriguing however, is the mechanistic and structural analogies between the mitomycins and the recently discovered FR-900482 class of drugs detailed below. The discovery of the FR-900482 class of compounds may thus be viewed as representing the most recent chapter in mitomycin research efforts.

5.6. FR-900482 and Related Structures

5.6.1 Interstrand DNA Cross-Linking

The recently discovered antitumor antibiotics FR-900482 (**325**) and the dihydro congener, FR-66979 (**327**), obtained from the fermentation harvest of *Streptomyces sandaensis* 6897 at Fujisawa Pharmaceutical Co., Japan, bear close structural similarity to the clinically employed bioreductive alkylating agent mitomycin C (MC, **163**).[232] In preliminary clinical studies, it was shown that FR-900482 (4-formyl-6,9-dihydroxy-14-oxa-1,11-diazatetracyclo-[7.4.1.0.0]tetradeca-2,4,6-triene-8-yl methyl carbamate) and the derived triacetate, FK973 (**326**), are ~3-fold more potent than mitomycin C and have significantly lower toxicity.[233] FK973 (**326**) has recently been shown to form DNA−DNA interstrand cross-links and DNA−protein cross-links in L1210 cells.[234] Even more intriguing is that both FK973 and FR-900482 are active against multidrug-resistant (including MC) P388 cells.[233a,c]





Like the mitomycins, **325**−**327** also require reductive activation both in vivo and in vitro.[235] However, in contrast to mitomycin C, these compounds do not produce single-strand DNA breaks typically associated with superoxide production upon bioreduction.[233c,d,236] The inability of these agents to take part in Fenton/Haber−Weiss chemistry (leading to DNA strand scission) most likely abrogates other damaging events such as phospholipid oxidations.[237] The relatively low host toxicity of the Fujisawa drugs in clinical trials relative to MC may thus be correlated to the incapacity of these substances to cause indiscriminate oxidative damage to DNA and other cellular targets.[236] Since FK973 and MC both share the ability to cross-link DNA, it is very clear that the lack

Table 1. 325α:325β Ratios for Various pH Conditions[232a]

pH	buffer system	ratio (**325α**:**325β**)
1.0	0.1 N HCl	1:0
2.0	0.1 M NaOAc-HCl	12:1
4.0	0.1 M NaOAc-HCl	6:1
7.0	0.1 M KH_2PO_4	2.3:1
9.0	0.1 M Tris-HCl	3:1

of adventitious redox cycling chemistry inherent in the bioreductive activation of **325**−**327** has not compromised their potential efficacy as antitumor drugs relative to MC.

The inability to induce DNA single-strand breaks (SSBs) is likely due to the absence of the quinone functionality[233d,234a] obligate for the reductive unmasking of the potent, biselectrophilic mitosene from MC.[238] In the Fujisawa drugs, it appears that the reductively labile functionality is the N−O bond of the unique hydroxylamine hemiketal. Given the therapeutic potential of the these agents coupled with both their unusual structures and relationship to MC, a great deal of effort has been directed toward the synthesis of the natural products[239,240] as well as preliminary mechanistic studies concerning the biological mode of action of these secondary metabolites.[241]

FR-900482 and FR-66979 both exist as a mixture of diastereoisomers **325**α and **325**β.[232a] The tautomeric equilibrium resulting from the labile hydroxylamine hemiketal at C8 affords **325**α as the major constituent of the FR-900482 mixture.[232a] The equilibrium between **325**α and **325**β for FR-900482 is sensitive to pH but favors **325**α, particularly at low pH (Table 1). This results from an intramolecular hydrogen bond between the bridged hydroxylamine oxygen and the aziridine NH, which is not allowed in the β form (Figure 55).[232a] Conversely, the stereoisomeric mixture of acetates **326**α and -β equilibrates such that the β form is favored in a ratio of 79:7.

Goto and Fukuyama were the first to propose reductive activation of **325** as an obligatory step prior to DNA cross-linking (Figure 56) in a preliminary synthetic approach to these substances.[240i] Deriving from the known ability of the mitomycins to undergo electrophilic activation via either one or two electron reduction of the quinone moiety,[238,242] this proposal invoked a mechanism of action for the FR class of compounds analogous to that previously demonstrated for the mitomycins. Reductive cleavage of the N−O bond was proposed to generate aniline derivative **329** which was expected to rapidly cyclize (to **330**) and dehydrate to aziridonomitosene **331**. Related and extensive work[238] on structurally similar mitosenes generated from MC provided ample precedent for the expectation that interaction of **331** with DNA would afford the bisalkylated DNA species **332**. If this proposal proved correct, the $^{5'}$CpG$^{3'}$ cross-linking sequence specificity for mitosenes derived from MC would also be anticipated for the FR-900482-generated DNA interstrand cross-links.

Alternatively, Danishefsky and McClure proposed that activation of the FR-900482 class of compounds might follow a nucleophilically triggered motif (Figure 57).[240c,243] Addition of some biologically relevant nucleophile (presumably a proteinaceous thiol or amine) to the aromatic C2 was envisioned to induce heterolytic cleavage of the hydroxylamine hemiketal with concomitant loss of water. Following rearomatization or coinciding with it, the expected ring closure would yield carbinolamine **334**. Dehydration of **334** would then afford the highly reactive aziridinomitosene **335** capable of bisalkylation at the activated C1 and C10 positions (mitomycin numbering system). DNA alkylation by this species would yield a lesion similar to that invoked by the Fukuyama proposal with the notable exception of substitution

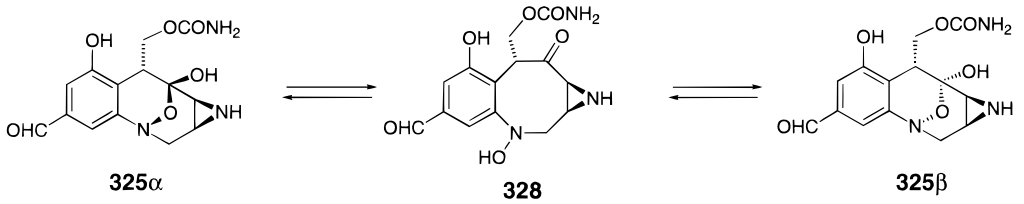


Figure 55. Interconversion of hydroxylamine hemiketal diastereoisomers **325**α and **325**β of FR-900482.

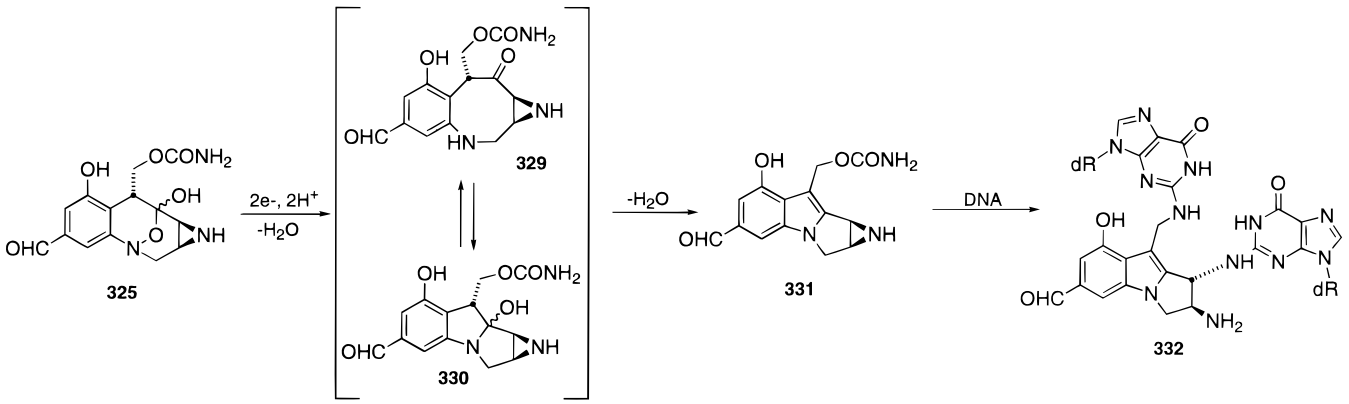


Figure 56. Fukuyama and Goto proposal for reductive activation and subsequent DNA interstrand cross-linking by the FR-900482 class of antitumor natural products.[240i]

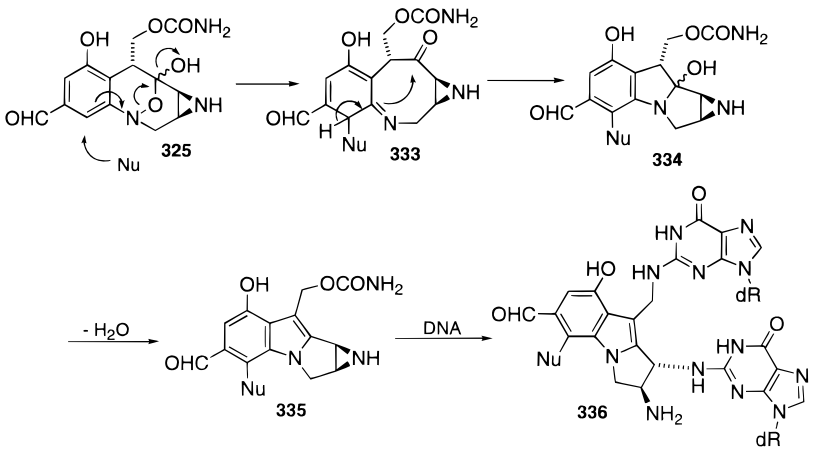


Figure 57. Danishefsky and McClure proposal for nucleophilic activation pathway to DNA interstrand cross-linking by FR-900482 and related compounds.[240c]

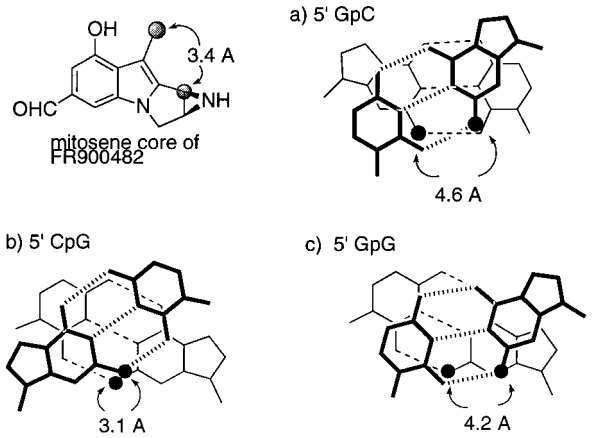


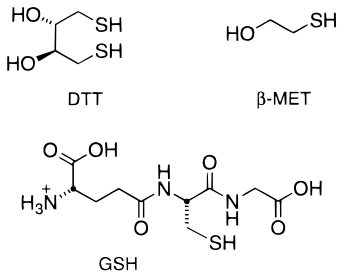
Figure 58. Molecular distances of adjacent guanosine exocyclic amines in sequences of interest.[30]

at the aryl C2 position.

Experimental work later reported by Williams and Hopkins demonstrated that the simple two electron reductive activation mechanism proposed by Fukuyama and Goto[240i] proved to be correct.[235,241] The Fukuyama and Goto proposal (Figure 56) invokes the generation of a mitosene (**331**) which was expected to display a preference for the $^{5'}$CpG$^{3'}$ cross-linking sequence specificity observed for mitosenes derived from MC. The $^{5'}$CpG$^{3'}$ cross-linking sequence specificity of mitosenes is based upon a good geometrical fit of the intramolecular distance between the two electrophilic centers of the mitosene (3.4 Å) and the distances between the two cross-linked bases of the duplex substrate (3.1 Å for $^{5'}$CpG$^{3'}$, Figure 58).

Paramount to efforts aimed at elucidation of the mechanism of action of the FR-900482 class of drugs was the ability to induce activation in vitro. Efforts by Fujisawa demonstrated quite clearly the ability of these agents to form DNA−DNA and DNA−protein cross-links in L1210 leukemia and other tumor cell lines.[233,234] However, FK-973 (**326**) formed no detectable interstrand DNA−DNA or DNA−protein cross-links when isolated nuclei of cells were exposed to the drug. This suggested that cytosolic (presumably reductive or oxidative) activation was required by the FR class compounds in order to inflict DNA damage.[233c,d,234b] The nature of the drug-induced lesions was known in only very general terms. Issues such as DNA−amino acid specificities and potential activation pathways were not addressed in any detail.

Reductive activation of FR-900482 and FR-66979 by thiols, and H_2−Pd was demonstrated by Williams and Hopkins[235] and the $^{5'}$CpG$^{3'}$ cross-linking sequence specificity was demonstrated by both groups through footprinting of the isolated cross-link. Williams and Rajski examined the cross-linking efficiency by FR-900482 in the presence of dithiothreitol and β-mercaptoethanol. Given the vastly different reduction potentials of the two agents,[244] it was expected that a reductively driven process would benefit from the more potent reductant DTT.

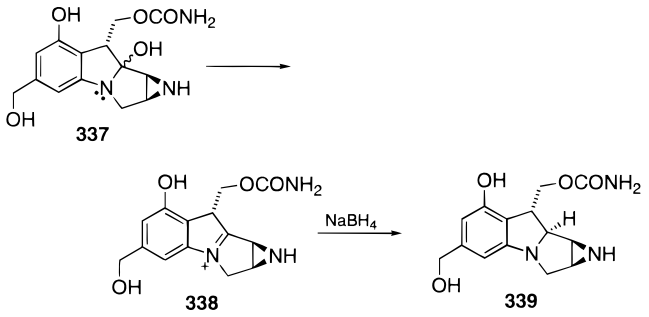


Comparison of dithiothreitol (DTT)- and β-mercaptoethanol (β-MET)-containing reactions revealed that, in general, the more potent reductant DTT ($E° = -0.36$ V) afforded the higher cross-linking efficiency. Reactions 1 mM in FR-900482 demonstrated ≥99% interstrand cross-link (ISC) formation in the presence of 10 mM DTT as opposed to 72.7% for the analogous mercaptoethanol ($E° = -0.207$ V) reaction.

Paz and Hopkins[235b] have shown that adventitous iron salts constitute a critical electron-transfer relay in the reductive activation of FR-900482. They were able to demonstrate by using efficiency of cross-linking as an assay, that thiols, such as DTT in the absence of iron(II) salts gave only ~5% of cross-link formation, whereas in the presence of added iron(II), the cross-linking efficiency was 55−62%. These authors further attributed the weak cross-linking observed with just DTT in the absence of metal to trace contamination in the DTT sample.

More recently, these workers have achieved the reductive trapping of an intermediate involved in

generation of the DNA-*cross*-linking species via a novel chelex-$NaBH_4$ procedure.[235c] Exploiting the requirement for Fe(II) within the reductive activation of FR-66979 in the presence of thiols, the generation of intermediate **337** (in equilibrium with the keto form) was effected followed by sequestering of the critical Fe(II) via passage over chelex resin. Shutting down of the first reduction (via Fe(II) removal) step allowed careful kinetics measurements to be carried out on the processes that follow **337** generation. Of particular interest was the conversion **337** to **338**. Significantly, it was found that the rates of **339** production (upon treatment of **338** and possible other Fe(II)-reduction products with excess $NaBH_4$) coincided closely with DNA interstrand cross-linking.



Similarly, the rates of decay for the reductively activated precursor of **339** and the corresponding decay in cross-linking activity were closely related. These findings supported the Hopkins and Paz proposal that a single-rate-limiting step accounts for both the production of dihydroindole **339** and DNA cross-link induction and that this step is quite possibly formation of the imminium **338** via loss of the C-8 hydroxyl unit in N−O reduced FR-66979. Additionally, these workers found that **339** results from a relatively stable ($t_{1/2}$ of about 1 h at physiological pH in the absence to Fe(II)) reduction product of FR-66979. From this, they suggest that the FR drugs may not need to be in close proximity to their DNA target upon activation and that in vivo activation processes may involve a metalloprotein.

Rajski observed similar results using added Fe(III) salts and proposed a mechanism for the reductive activation of FR-900482 in the presence of thiols and Fe(III)salts.[246] Given the importance of Fe(III) within Fenton/Haber−Weiss redox cycling and its role in the oxidation of thiols to the corresponding disulfides, a viable means by which to probe a possible redox

Table 2. Summary of Reactions of FR-900482 with Linearized pBR322 in the Presence of *β*-Mercaptoethanol (*β*-MET) and/or Fe^{III}-EDTA

entry	[FR-900482], mM	[*β*-MET], mM	[$FeCl_3$−EDTA]	% ISC[a]
1				
2	10			
3	10	10		
4		10		
5	10		1 μM	11.5
6	10		10 μM	33.5
7	10		100 μM	71.6
8	10		1 mM	≥99
9	10	10	1 μM	54.2
10	10	10	10 μM	≥99
11	10	10	100 μM	≥99
12	10	10	1 mM	≥99

[a] % ISC = % interstrand cross-link formation.

manifold involved alteration of Fe(III) concentrations in the cross-linking reactions. As shown in Table 2, reaction of **325** with Fe(III) in concentrations ranging from 1 μM to 1 mM produced a marked increase in double-stranded material with progressively increasing Fe(III) concentration. This most likely resulted from thiol reduction of Fe(III) to Fe(II) followed by FR-900482 reduction/activation as per the Fukuyama model. This was found for not only those reactions involving *β*-mercaptoethanol, but was also visible in **325** + Fe^{III}-EDTA control reactions. The synergism thus witnessed in the thiol + Fe^{III}-EDTA reactions offered strong evidence that FR-900482 activation followed a reductive pathway. Two facts supported this reasoning. First, the thiol mediated reduction of Fe(III) to Fe(II) is known to effect the reduction of nitroaromatics to the corresponding amines.[247] Second, were nucleophilic thiol additions responsible for drug activation, then the addition of Fe^{III}/EDTA would have greatly reduced the amount of ISC induction due to increased conversion of the nucleophilic thiol to the "inert" disulfide thereby quenching the activating agent.

The ability of **325** to efficiently interstrand cross-link DNA in the presence of Fe^{III}-EDTA alone (Table 2, entries 5−8) indicates that FR-900482 and structurally related compounds may undergo some degree of "self-activation" in the presence of metal ions. Fe(III) readily undergoes reduction by hydroxylamines to afford Fe(II) complexes. Thus, a mechanism wherein Fe(III) is reduced to Fe(II) which then reduces FR-900482 to the mitosene **331** (Figure 59)

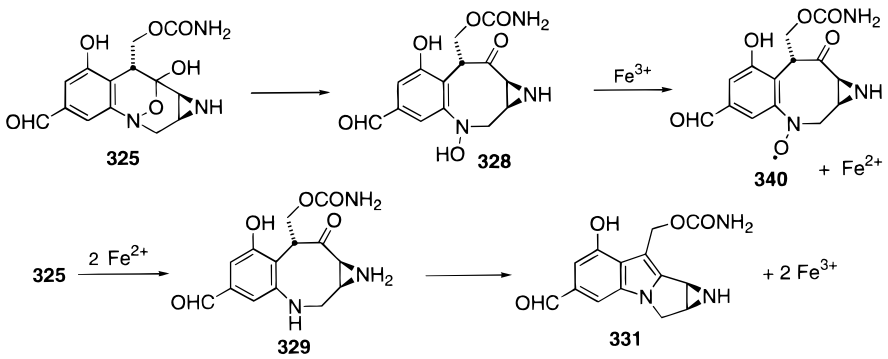


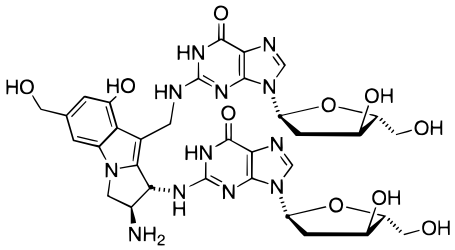
Figure 59. Proposed model for Fe(III)-mediated reductive activation of FR-900482.[246]

is completely consistent with the literature and seems viable on the basis of the available data.

The dihydro derivative FR-66979 has been the primary substrate examined since initial efforts by Williams and Rajski[241a,b] demonstrated that of the two natural products FR-66979 was by far the more efficient DNA interstrand cross-linking agent. Concomitantly, Hopkins, Woo, and Sigurdsson[241c,d] were investigating the ability of FR-900482 and FR-66979 to cross-link various self-complementary DNA substrates. Both laboratories concluded that the FR-900482 class compounds displayed a strikingly similar specificity for 5′CpG3′ demonstrated for MC. Collaborative efforts[235a] ultimately demonstrated that both FR-900482 and FR-66979 require reductive activation to cross-link DNA; the lesion from both drugs has been fully characterized.[241c] It has also been demonstrated that both natural products, as well as FK973, display sequence selectivity for 5′CpG3′ in which a number of guanosine residues preside. Additionally, monoalkylation of DNA by FR-66979 appears to require similar, yet distinct "precovalent" associations deemed necessary for efficient substrate alkylation (typically yields ≥ 10%) by MC, and that these compounds give rise to separable orientation isomers of the respective interstrand cross-links.[245]

The substrate 5′TTTATTAA**CG**TAATGCTTAAT**CG**-CAATGGGATT3′ (**template 1**) and a modified version bearing inosine (guanosine lacking the N^2 exocyclic amine) at the G-10 position (**template 2**) and their mutual complement were synthesized. Cross-linking of both substrates followed by isolation and Fe^{II}-EDTA digestion rendered the cleavage patterns consistent with minor groove association of the drug and N^2 to N^2 cross-linking. Native DNA subjected to Fe^{II}-EDTA cleavage affords an equimolar assortment of all fragment sizes up to and including the full-length strand, whereas analogous treatment of cross-linked DNA yields short fragments corresponding to cleavage at or to the radiolabeled side of the alkylated residue. As such, the observed cleavage patterns showed that for cross-linking of the unsubstituted duplex, G-10 is the principal site of alkylation, whereas cross-linking and digestion of the duplex bearing inosine at the G-10 position reveals G-23 as the primary alklation site. This "cleavage shift" is the result of deletion of the exocyclic amine (N^2) at the G-10 residue. Installation of inosine at this position halts cross-link formation and thus implicates the N^2 residues of adjacent guanosines (within only the 5′CpG3′ step) exposed in the minor groove, as the sole functionalities responsible for interstrand cross-linking by **325**−**327**. This is in accord with the reasonable expectation that interaction of **325**−**327** with DNA should occur in the minor groove of DNA and supports the hypothesis that FR-900482, FR-66979, and FK-973 cross-link DNA via the same mechanism.

Hopkins and Woo have isolated and characterized the FR-900482-derived lesion responsible for the cross-link by NMR and mass spectral analysis[241c] and found it to be identical to that derived from reductively activated FR-66979. The self-complementary 14-mer 5′ATAATA**CG**TATTAT3′ was interstrand cross-linked with crude **327** from hydrogenation. The interstrand cross-linked product was isolated from DPAGE and enzymatically digested. The resulting mixture was then compared by HPLC with the corresponding hydrolysate from the same DNA cross-linked ODN obtained using dithionite-activated FR-66979 prepared by sodium borohydride reduction. Rigorous structural characterization had proven the latter to contain substance **341**, fully consistent with the reductive activation hypothesis in Figure 56.



341

Williams and Rajski examined the 32P-5′ end-labeled substrate 5′TGTTGAATACTCATA**CG**TC-TCTTGCTGAGGG3′ (**template A**) which was cross-linked by FR-66979 and revealed the presence of two bands on DPAGE corresponding to cross-linked product possessing only one 5′CpG3′ site.[245,246] The presence of another interstrand site was considered highly unlikely but, on the basis of the available results, could not be ruled out. To probe these issues two additional ODNs were synthesized. One substrate replaced the 5′GAGGG3′ run with the sequence 5′TTAAT3′ (**template B**) while the other substrate had the cross-linkable 5′CpG3′ site replaced with 5′TpT3′ (**template C**). As a control, the self-complementary ODN 5′TATTAAAATTAATACGTATTAA-TTTTAATA3′ was synthesized. Treatment of the respective duplexes with FR-66979 and DTT revealed the two band pattern not only for the known **template A**, but also for the substrate lacking the dG-rich 3′ terminus (**template B**).

As expected, the substrate lacking the 5′CpG3′ (**template C**) site gave no reaction thus ruling out the possibility of another interstrand cross-linkable site and the self-complementary substrate yielded only one band corresponding to cross-link (as resolvable by 20% denaturing polyacrylamide gel electrophoresis). Retention of the two-band pattern between substrates with and without the 5′GAGGG3′ terminus suggested that, the observed two band pattern was not due to over-alkylation, but rather the separation of orientational isomers. Each cross-linked species was isolated and subjected to electrospray mass spectral analysis. The slow orientational isomer had an observed molecular weight of 19 261.39 ± 10.78; the faster orientational isomer had an observed molecular weight of 19 259.73 ± 9.36 (calculated MW = 19 259.75). Following isolation, each orientational isomer was subjected to Fe^{II}-EDTA digestion, which revealed that, each band corresponds to the singly cross-linked species. The minor groove nature of the cross-link was verified by digestion of each orientation isomer with piperidine which failed to reveal any major base-labile (major

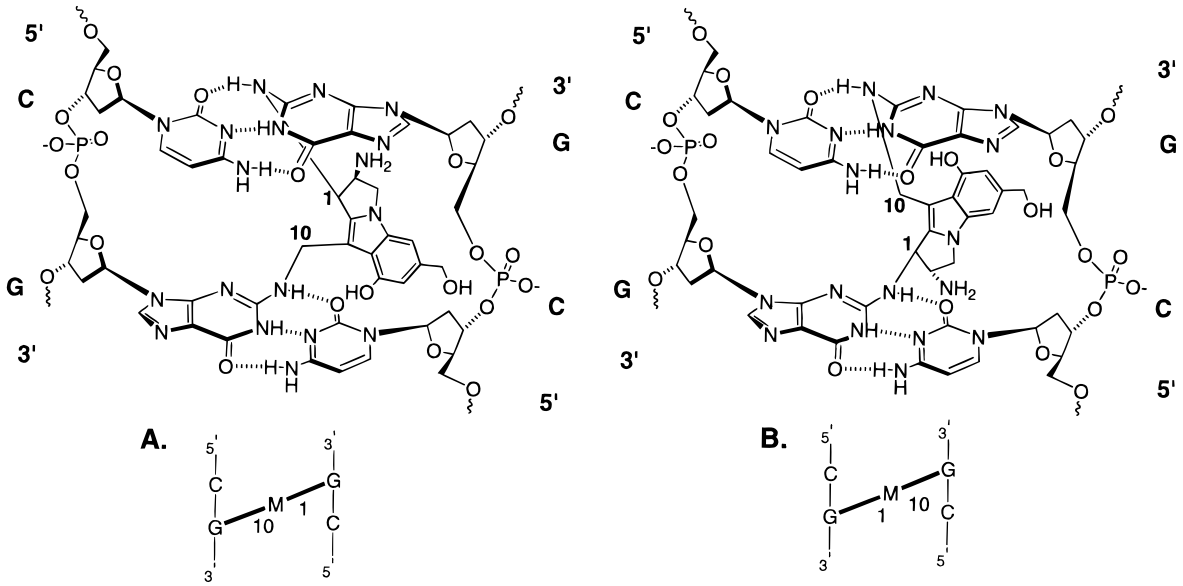


Figure 60. Orientational isomers of a FR-66979-derived cross-link.[245]

groove) lesions involved on either strand of the cross-linked duplex (Figure 60).[245]

Efforts to determine if one orientational isomer of cross-linked **template A** was subject to reversion to the other orientational isomer or a mixture of both was carried out by separating the two products and subsequently exposing each to denaturing conditions. Analysis by 20% DPAGE revealed that heat denaturation did not generate the other orientation isomer but instead yielded hydrolysis products. Cross-linked adducts in which **template A** was 5′-labeled and its complement unlabeled were purified and then subjected to heating at 80 °C in 200 mM Tris buffer (pH = 7.5) for 20 min and then cooled to room temperature. Upon heating the slow orientational isomer, low molecular weight material, was produced corresponding to native ssDNA, and a large percentage corresponding to monoalkylated material. By contrast, upon heating the faster orientational isomer, only a tiny fraction of monoalkylated product was produced, the majority being ssDNA (5′-labeled **template A**).

The reversibility of alkylation of mitosenes derived from MC is more facile (under reductive conditions) at C-1 versus C-10.[248] Thus, this suggested that the slow orientational isomer of the FR-66979 cross-link is that corresponding to alkylation in which the radiolabeled substrate $^{5'}$TGTTGAATACTCATACGTCTCTTGCTGAGGG$^{3'}$ is connected to the C-10 of the mitosene core of FR-66979. Likewise, the faster of the two orientational isomers results from connection of this strand at C-1, the more labile toward hydrolytic DNA release. To further verify and corroborate this result, 5′-radiolabeling of the complementary strand to **template A** was performed and the assay repeated. This experiment completely verified the preceding result. These experiments permitted an assignment of the relative connectivity of the mitosene core in each orientational isomer. This behavior can likely be correlated to which orientational isomer exhibits the lower melting temperature (T_m) for the cross-linked substrate. Orientational isomers of 4,5,8-trimethylpsoralen cross-linked oligonucleotides have been shown to be easily separable due to base pair disruptions in the vicinity of the cross-link.[249a] The fact that an analogous difference in migration between the two orientational isomers derived from **327**, as well as from **325** and **326** suggests that the cross-link formed results in one orientation giving rise to the fully base-paired cross-link while the other orientational isomer compromises the Watson−Crick base pairing network resulting in the mobility difference observed. Cross-linking of a self-complementary substrate with FR-66979 results in only one band by DPAGE. This is due to the presence of a C_2 axis of symmetry throughout the cross-linked adduct. However, in cases giving rise to separable cross-linked products, the DNA substrates are asymmetric with respect to bases flanking the $^{5'}$CpG$^{3'}$ and thus, the two orientation isomers are not chemically equivalent. Also noteworthy is the finding that cross-linking of **template A** with reductively activated MC gave rise to separable orientational isomers of similar electrophoretic mobilities to those seen with the FR-derived cross-links. It is possible that perturbation of the cross-linked DNA derives from a functionality common to both the mitomycins and the FR-900482 class antibiotics, this in all likelihood being the C2 primary amine derived from the aziridine. Molecular modeling of both orientational isomers indicated that, in each isomer, an N2 hydrogen of the FR-66979 derived mitosene core is within H-bonding distance of the C2 carbonyl oxygen of the thymine 3′ to the alkylated guanosine residue.[245]

The ability of the Fujisawa drugs to give rise to different cross-linked species at the same site with the same connectivity raises numerous questions with regard to the physical basis for the observed electrophoretic differences. An issue of particular significance is the possibility that the observed orientational isomers may exert unique effects on processes of biological importance. The well-defined structural constraints of numerous protein−DNA recognition motifs suggested that such interactions

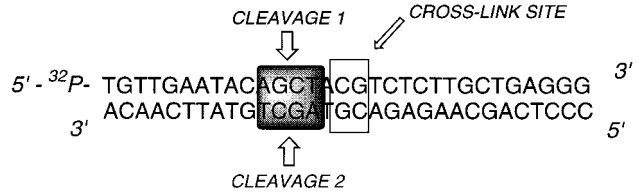


Figure 61. Template A modified with an Alu I cleavage site one base pair upstream of the cross-linkable $^{5'}$ACGT$^{3'}$. Alu I recognition domain is depicted by shaded region.

might easily be affected by the DNA distortions induced by one or both orientational isomers. An initial probe of this was performed using the restriction endonuclease Alu I which recognizes the palindrome $^{5'}$AGCT$^{3'}$ and cleaves both strands of the duplex through the central G-to-C phosphodiester linkage (Figure 61).

Given the ease with which separation of FR-66979-derived cross-link orientational isomers of $^{5'}$TGTTGAATACTCATACGTCTCTTGCTGAGGG$^{3'}$ could be achieved, Williams and Rajski followed the protocol described by Tomasz[249b] in which an Alu I site was incorporated one base to the 5′-side of the cross-linkable $^{5'}$ACGT$^{3'}$ run.[245] Substrate alkylation and purification of the resulting orientational isomers followed by incubation of each isomer, as well as a standard duplex control, with four units of Alu I for various times was carried out. Each deoxyoligonucleotide duplex was 5′ labeled on the sequence detailed below.

The faster mobility orientational isomers inhibited Alu I cleavage of the radiolabeled strand while the other orientational isomer showed very little inhibition versus the standard duplex. This was consistent with related findings by Tomasz[249b] (pertaining to MC) which invoked that one orientational isomer orients the bulky aromatic ring toward the Alu I cleavage site thus inhibiting strand cleavage by the protein while the other orientational isomer has this group oriented away from the Alu I site thus exerting minimal influence upon the degree of Alu I cleavage.

These observations, taken in conjunction with the reversibility studies, indicate that the faster of the two orientational isomers is that in which C-1 of the FR-66979-derived mitosene core is covalently attached to the radiolabeled strand $^{5'}$TGTTGAATACAGCTACGTCTCTTGCTGAGGG$^{3'}$. Conversely, the slower orientational isomer requires C-10 connection of the mitosene to the radiolabeled strand. Interestingly, Williams and Rajski found[245] that cleavage 1 (Figure 61) is selectively inhibited while cleavage 2 proceeds at about the same rate for each orientational isomer. This is in contrast to the findings of Tomasz[249b] which demonstrated inhibition of cleavage event 2 for the MC orientation isomer in which C-1 was connected to the cleavage 1 target strand. These workers verified the Tomasz results with mitomycin C utilizing the electrophoretic assay and found the electrophoretically derived results consistent with the HPLC results originally reported.[249b] As such FR-66979 cross-link orientation isomers do indeed demonstrate an extremely interesting departure from the known mechanism of Alu I inhibition intrinsic to mitomycin C.

Tomasz has shown that upon bioreductive activation, loss of the carbamate functionality is the major obstacle to cross-link formation by MC;[226,248,249b] the presence of this moiety however, is obligate for MC to efficiently alkylate the $^{5'}$CpG$^{3'}$ site. Hydrogen bonding between the C-10′−O of the activated form of MC and one of the exocyclic amines within the $^{5'}$CpG$^{3'}$ site aligns the drug properly for ring opening of the aziridine by the "nonassociated" guanosine of the opposing strand, directing the initial monoalkylation within the $^{5'}$CpG$^{3'}$ site; an event that ultimately results in interstrand cross-linking.

Experimental results reported by Williams and Rajski[245] suggest however that the carbamoyl H-bonding interactions deemed necessary for MC to efficiently alkylate or cross-link DNA is not obligatory for target modification by FR-66979. These workers propose that one possible explanation for this is the ability of the phenolic proton of **327** to H-bond with N^3 of the guanosine opposite to that responsible for connectivity to C-1 of the activated mitosene. Li and Kohn[227,250] have carried out modeling studies on MC in which they proposed H-bond contacts for the reduced hydroquinone form of **163** as an obligate parameter for DNA monoalkylation. The importance of such a contact was later questioned by Tomasz given that upon deletion of N^2 from the "precovalently" associated guanosine, the yield of monoadduct at $^{5'}$CpG$^{3'}$ was decreased more than 10-fold under both reductive and acidic activation conditions (in which MC is reported to be in the quinone form thus lacking the C-8 OH group). However, such an interaction might be considerably more important for FR-900482 due to the availability of the phenolic proton to not only become hydrogen bonded to the dG N^3 but to also undergo deprotonation resulting in formation of the quinone methide species depicted in Figure 62.

To determine the importance of such interactions with FR-66979, reactions were carried out with 5′-^{32}P-end-labeled $^{5'}$TGTTGAATACTCATACGTCTCTTGCTGAGGG$^{3'}$ (**template A**) annealed to the following complementary strands in which various guanosines were replaced by inosine.[245] The data collected on these substrates clearly show that use of strands **CS 1**, **CS 2**, or **CS 3** as the complement resulted in nearly identical patterns of monoadduct formation. Reaction of the **4** duplex resulted in greatly diminished yields of monoadduct and surprisingly, significant monoalkylation was observed in the single-strand control reaction.[245]

CS 1 5′CCCTCAGCAAGAGACGTATGAGTATTCAACA3′

CS 2 5′CCCTCA**I**CAA**I**A**I**ACGTAT**I**A**I**TATTCAACA3′

CS 3 5′CCCTCAGCAAGAGAC**I**TATGAGTATTCAACA3′

CS 4 5′CCCTCA**I**CAA**I**A**I**AC**I**TAT**I**A**I**TATTCAACA3′

The ability to form the interstrand cross-link with **CS 2** in comparable yields to those using **CS 1** demonstrated that denaturing of substrate **CS 3** was not a factor in these reactions. Substitution of deoxyinosine for deoxyguanosine at the $^{5'}$CpG$^{3'}$ site had no effect on the relative amounts of monoalky-

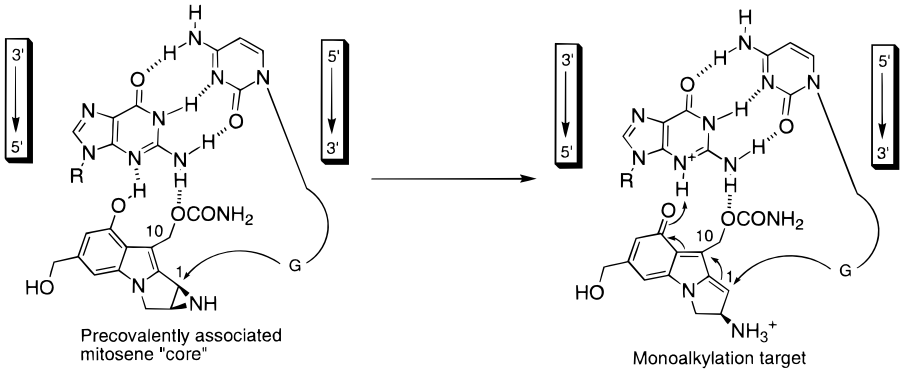


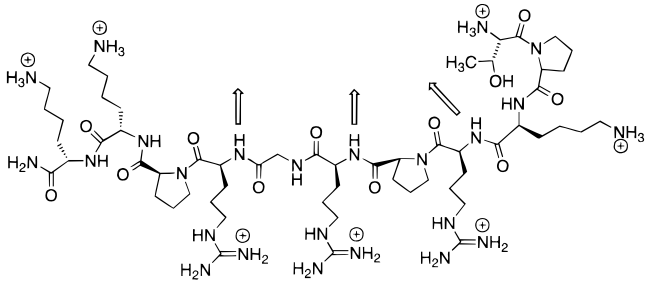
Figure 62. Potential H-bonding network leading to monoalkylation of DNA by activated FR-66979.[245] Note that the guanosine N^3 to phenol contact represents not only a means of precovalent association but may facilitate collapse to the aziridinyl ring opened monoalkylation target depicted above.

lation; this represents an interesting departure from the mitomycin C pathway with respect to noncovalent, preassociation of the drug with the DNA. These results suggest that in the case of FR-66979, alternative noncovalent, preassociation phenomena might be operative which bear greater influence in sequestering the drug to the DNA target than that exerted by the carbamoyl-to-N^2 interactions strongly implicated in MC−DNA interactions.

Structure elucidation and Fe^{II}-EDTA footprinting of cross-linked and alkylated DNA product studies have thus demonstrated that members of the FR-900482 class of antitumor antibiotics interact with DNA in a fashion very similar to that of mitomycin C. Like the mitomycins, FR-900482 and congeners are therefore "prodrugs" that must be activated reductively both in vitro and in vivo to produce the potent, electrophilic mitosene species that alkylate and cross-link DNA. Of particular interest is the role played by the phenolic portion of the FR compounds versus the quinone/semiquinone of reductively activated MC. Evidence has been presented that the phenol functionality may play a crucial role in the ability of these compounds to very efficiently monoalkylate DNA thus facilitating ultimate DNA−DNA and potentially DNA−protein cross-links.[245] It is clear from the results presented that the guanosine N^2-to-C-10-O contact involving the activated mitosene core of FR-66979 plays a much smaller role than does the analogous (and necessary) contact with activated MC. It is likely that the presence of the phenol OH plays an infinitely more important role in directing the FR-900482 pharmacophore to its initial monoalkylation target than does the C-7 carbamate. The ability of the FR compounds to not only shut down strand separation events (via cross-linking) necessary for transcription, but to also inhibit DNA−protein recognition motifs clearly provides additional insight into the mechanism by which these agents inflict cellular damage. Such information should prove invaluable in the design of second generation FR-900482 congeners and will no doubt shed further light on the mechanism of action of the clinically employed mitomycins.

5.6.2 DNA–Protein Cross-Linking by FR-66979

Very recently, Rajski and Williams reported on the covalent cross-linking of FR-66979 (**327**) to DNA and



342, *HMG I Binding Domain (BD) peptide*

Figure 63. Structure of the binding domain (BD) peptide of the HMG I/Y protein.

the binding domain (BD) of the High Mobility Group I/Y (HMG I/Y) peptide substrate **342** (Figure 63).[251] The BD peptide **342** was constructed which was identical to that examined by Wemmer for which the DNA−peptide contacts were identified by elegant ^1H NMR studies.[252]

These authors note that, facile cellular excision repair mechanisms dictate that DNA monoalkylation is significantly less important in the expression of therapeutic activity[253] and that DNA−protein cross-linking can be presumed to result in lesions of vastly greater importance than monoalkylation adducts. The importance of DNA−protein cross-links relative to that of DNA−DNA interstrand cross-links is not yet known. Research aimed at the elucidation of DNA−protein cross-linking mechanisms has been hampered by the generally lower yields of such adducts relative to the interstrand events and the radically more complex and diverse structural possibilities inherent to nucleic acid−protein cross-links.

Significantly, the HMG proteins are preferentially expressed in rapidly proliferating undifferentiated cell lines such as those of cancerous origin. The HMG I/Y proteins contain three runs of the BD sequence per protein.[252] The AT-hook motif inherent to TP-KRPRGRPKK results from the synergism of the N-terminus TPK hook and the *trans*-proline-induced crescent conformation of the PRGRP palindrome.[252] The N-terminal hook results from an AsnX turn resulting from intramolecular hydrogen bonding between the threonine hydroxyl and the lysine-to-proline amide hydrogen.[252] This hook fits tightly into the widened minor groove of AT/GC junctions and has been implicated as a major factor in DNA

binding. Notably, the BD peptide binding to the preferred sequence $^{5'}$AAATTT$^{3'}$ occurs via minor groove contacts in a fashion similar to that known for netropsin and Hoechst 33258.[252]

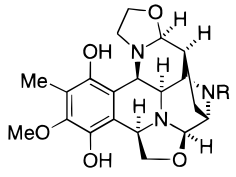
Molecular modeling studies of the proposed DNA−FR-66979−peptide adduct revealed that one of the two arginine moieties (of PRGRP) placed a guanidine nitrogen proximal (within 2−3 Å) to the mitosene C10 position of a FR-66979 monoalkylated deoxyguanosine residue abutting the BD recognition sequence $^{5'}$AAATTT$^{3'}$. On the basis of these studies it appeared that a likely structure with the best geometrical fit (permitting DNA and peptide connectivities to the FR-derived mitosene) would invoke the N-terminal arginine.[251] This suggested that a sequence bearing the FR-66979 alkylation "hot-spot" $^{5'}$CpG$^{3'}$ to the 5′-side of $^{5'}$AAATTT$^{3'}$ would result in a high probability for reaction between an essential arginine and the mitosene C10. Thus, these workers examined the ODN $^{5'}$CCCACATCACTATACACGCGCGAAATTTCTCACTATC$^{3'}$ (**ODN 1A**) and its inosine-substituted complement $^{5'}$GATAGTGAGAAATTTCICICITGTATAGTGATGTGGG$^{3'}$ (**ODN 1B**) as substrates for protein−DNA cross-linking. It was found that optimal reaction conditions involved initial alkylation of the DNA with the drug followed by rigorous desalting of the alkylated substrate via size exclusion chromatography (Sephadex G-50) and repetitive precipitation from ethanol. It was observed that binding was not only drug-dependent, but unexpectedly occurred with both strands of **template 1** indicative of peptide−drug interactions not restricted to the anticipated dG N^2 of the DNA-bound FR-66979-derived mitosene. Inosine incorporation across from the $^{5'}$CpG$^{3'}$ run proximal to $^{5'}$AAATTT$^{3'}$ of **ODN 1A** was anticipated to facilitate identification of the HMG−DNA cross-link by abrogating interstrand cross-linking and accentuating the formation of the peptide−DNA cross-link.

The covalency of the adduct was corroborated by ligand−DNA exchange reactions. Purified radiolabeled DNA−drug−peptide adducts with each strand of **template 1** were incubated with unlabeled competitor **template 1**. Incubation of each drug-mediated complex with increasing amounts of competitor DNA at 37 °C for 24 h failed to exchange peptide **342** from either radiolabeled strand. Indeed, complete retention of each labeled complex even in the presence of a >1000-fold molar excess of unlabeled duplex was observed. In addition, the covalent nature of the complex was further supported by negative ion electrospray mass spectral analysis of the isolated and gel-purified DNA−drug−peptide conjugate.

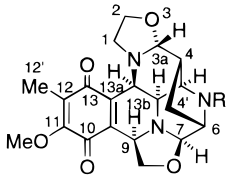
DNA interstrand cross-linking agents figure prominently in current chemotherapeutic approaches to the treatment of cancer due to their ability to inhibit strand separation processes. Their ability to induce DNA−protein cross-links is beginning to be recognized and the biochemistry of such lesions merits additional scrutiny. Several DNA cross-linking agents have been reported to induce such cross-links, including the psoralens and mono- and dimeric cisplatin-based metalloagents.[254] Mitosene/pyrrole-based agents have also been reported to form DNA−protein cross-links, but no defined DNA-binding protein system has been reported in which this occurs. It is thus highly significant that FR-66979, whose mechanism of DNA modification chemistry closely parallels that of the mitomycins, pyrrolizidine alkaloids and simple pyrroles form DNA−protein cross-links between the BD of HMG I/Y proteins and a synthetic DNA bearing the protein recognition sequence $^{5'}$AAATTT$^{3'}$. It can be expected that, an increasing number of reports will be appearing in the literature dealing with the structural chemistry of drug-mediated DNA−protein cross-links. The ability to target small molecule drugs for DNA-binding proteins unique to tumor cells may play an important role in improving the therapeutic window of antitumor drugs.

5.7. Bioxalomycin α_2 and Related Structures

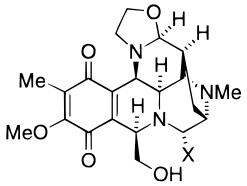
Recent efforts by Williams and Herberich have revealed that bioxalomycin α_2 efficiently cross-links duplex DNA bearing the sequence $^{5'}$CpG$^{3'}$.[255] Possessing the intricate heptacyclic core structure shown below, the bioxalomycins are isolated (as a mixture of compounds **343**−**346**) from *Streptomyces viridostaticus* subsp. "litoralis" and bear striking structural resemblance to the naphthyridinomycins and cyanocyclines.[256] Importantly, bioxalomycin α_2 (**344**), the main component of the mixture, possesses activity against Gram-positive and Gram-negative bacteria including potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA).[257] These substances also exhibit significant antitumor activities closely paralleling those previously observed with naphthyridinomycin. Early work on naphthyridinomycin (**347**) and cyanocycline A (**348**)[258] demonstrated that these substances inhibit DNA synthesis via alkylation of DNA in the minor groove in GC rich regions.[259]



343, Bioxalomycin α_1, R = H
344, Bioxalomycin α_2, R = Me



345, Bioxalomycin β_1, R = H
346, Bioxalomycin β_2, R = Me



347, Naphthyridinomycin, X = OH
348, Cyanocycline A, X = CN

This alkylation event was believed to result in only monofunctionalization of deoxyguanosine residues within duplex DNA.[259,260] In addition, reduction of the quinone moiety to the semiquinone radical anion, leads to superoxide generation and ensuing Fenton−Haber−Weiss redox cycling (Figure 28) culminating in oxidative DNA and protein damage.[261]

Significantly, it has been shown that naphthridinomycin is an artifact of bioxalomycin isolation wherein hydrolysis at C7 leads to ring opening of the

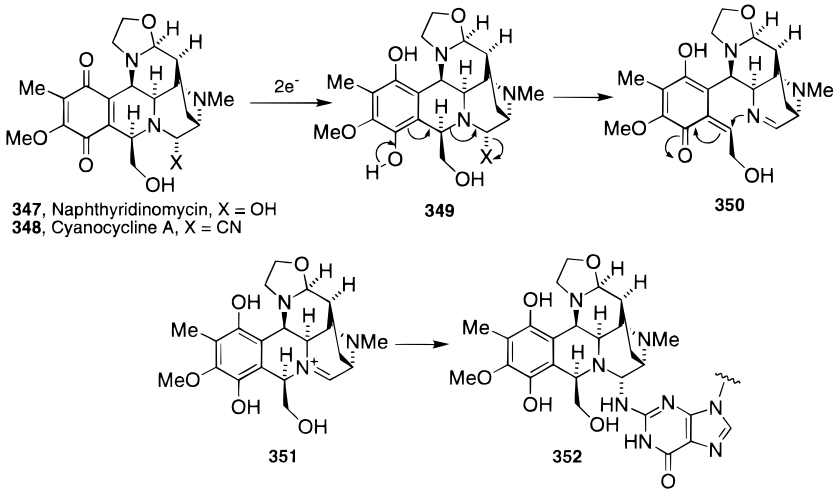


Figure 64. Proposed mechanism of reductive activation and ensuing DNA alkylation by naphthyridinomycin and cyanocycline.

bioxalomycin β_2 oxazolidine.[255] This observation has provided considerable insight into the DNA alkylation chemistry of the bioxalomycins since dG alkylation by naphthyridinomycin has long been supported by molecular mechanics calculations.[260] These molecular mechanics studies led to the proposal that naphthyridinomycin covalent monoadduct formation in the minor groove could be precipitated by two electron reduction of the quinone moiety to the corresponding hydroquinone (as in Figure 64). This conversion was proposed by Zmijewski[259] to facilitate expulsion of the cyano group (in the case of **348**) or water (in the case of **347**) from C7 to form the highly reactive iminium species **351** with ensuing nucleophilic addition by the exocyclic amine of guanine providing the monoadduct **352** with the *R* stereochemistry at C7. Additional modeling of the possible formation of covalent adducts at C3a and C7 led to the assertion that "The geometry of naphthyridinomycin does not permit interstrand cross-linking involving both C3a and C7, but formation of cross-link to protein appears possible."[260b] As such, the recent findings of Williams and Herberich represent a significant advance in the understanding of how the bioxalomycins and related molecules express biological activity.

DNA−DNA interstrand cross-linking by bioxalomycin α_2 was demonstrated initially by incubation of duplexed DNA **template 1** (5′TTTATTAA**CG**-TAAT**GC**TTAAT**CG**CAATGGGATT3′) with bioxalomycin α_2 at 37 °C for 12 h. This resulted in products of slower electrophoretic mobility (under denaturing conditions) attributable to interstrand cross-linking. Following isolation and recovery of these drug-bound DNA bands, Fe^{II}-EDTA footprinting was performed revealing that sites of DNA alkylation corresponded to the 5′CpG3′ box proximal to the 5′-terminus of DNA **template 1**. That the *exocyclic N^2 amino group of at least one of the cross-linked deoxyguanosine residues* participates in the drug−DNA interstrand covalency was demonstrated by substitution of one strands' 5′CpG3′ guanosine with 2′-deoxyinosine. Incubation of the substituted duplex with **344** failed to afford the interstrand adduct thus implicating the minor groove N^2 of each deoxyguanosine as critical

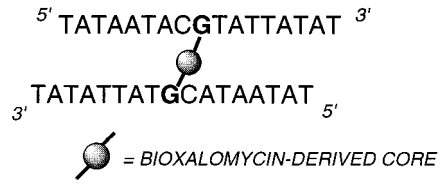


Figure 65. Bioxalomycin cross-linked d(TATAATACG-TATTATAT)$_2$.

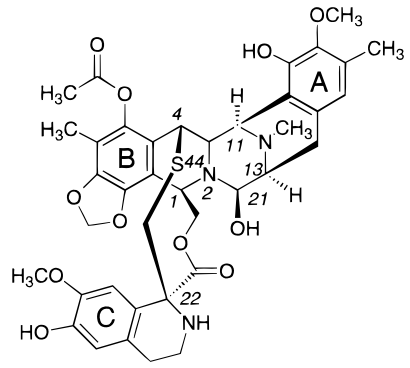
for bioxalomycin-mediated cross-linking. That in fact, *each guanosine participates* in bioxalomycin-mediated cross-linking via its exocyclic amine was supported by piperidine digestion of independently radiolabeled cross-linked species. The inability to detect any alkali-labile lesions (on either strand of the cross-linked duplex) substantiates the contention that DNA−DNA interstrand cross-linking occurs via minor groove covalencies (specifically involving each dG N^2) and does not involve major groove connectivities as these would have given rise to alkali-labile sites.

In addition to the disclosed Fe^{II}-EDTA footprinting studies, mass spectral data further support the covalent nature of the observed drug−DNA interaction. Isolation of the bioxalomycin α_2 cross-linked self-complementary DNA substrate represented by Figure 65 followed by negative ion electrospray mass spectrometry afforded an observed mass of 10 732 ± 5.5 (theoretical mass = 10 766) consistent with actual covalent linkage of the two strands via the bioxalomycin-derived pharmacaphore. The difference in the calculated and observed mass corresponds to loss of the hydroxymethyl moiety at C9. This fragmentation has been observed in related hydroxymethyl-substituted isoquinolines[262] and is observed as the predominant fragmentation for cyanocycline.[255] Indeed, given that cyanocycline (quinone oxidation state), readily undergoes net deformylation at C9 as the major fragmentation, it seems likely that cross-linked bioxalomycin is also in the quinone oxidation state and necessarily would have to have undergone oxidation either during or after cross-link formation. Given this, it is significant to note that these workers demonstrated interstrand cross-linking of duplex DNA **template 1** by cyanocycline. More importantly,

cross-linking by cyanocycline displayed the absolute requirement for reductive activation by dithiothreitol. As such, it would appear that bioxalomycins α_1 and α_2 represent "preactivated" cyanocycline/naphthyridinomycin congeners with respect to DNA cross-linking activity. Thus a large number of molecules belonging to the bioxalomycin and related classes are, in all likelihood, reductively activated DNA alkylating species.

The complexity of the bioxalomycins suggests that nucleophilic drug trapping by deoxyguanosine could occur at several positions. Alkylation at C3a or C7 of bioxalomyicin α_2 (cross-link **357**) has been proposed by Zmijewski[259] via a mechanism similar to that shown in Figure 64. Alternatively, Moore proposed that naphthyridinomycin-mediated cross-linking could occur via quinone methide (**356**) formation resulting from dihydroquinone deprotonation.[263] Such a scenario with bioxalomycin would afford C13b and C9 as the preferred sites of DNA connectivity. Additional possibilities involve a lesion structure corresponding to cross-link **359**. Drug−DNA connectivity involving C9 and C3a is possible via activation processes represented in structure **355**. However, such a lesion structure, is unlikely given the known importance of the carbinolamine (C7 for bioxalomycin) and functionally equivalent structures in DNA alkylation by these compounds.[264] On the basis of the results with reductively activated cyanocycline and preliminary modeling studies, Williams and Herberich suggest that generation of the *o*-quinone methide species **354** would result in alkylation at C7 and C13b (affording cross-link **358**). This presumes electrophilic activation to benefit from bioxalomycin being in the dihydroquinone oxidation state as opposed to the quinone oxidation state. This is clearly in line with the results of cyanocycline studies. Additionally, molecular modeling revealed that these centers are ideally presented to the critical DNA residues when the aromatic chromophore is partially intercalated into DNA proximal to sites of modification.[255]

It is highly significant that examination of these recently isolated compounds has revealed not only their own unique DNA cross-linking/binding properties but also has shed light upon biologically important activities likely possessed by other structurally related compounds. Among such compounds are the recently isolated ecteinascidins of which ecteinascidin 743 (**361**) is representative.[265] Along with the structural characterization of the dinucleoside-bioxalomycin lesion, it will be interesting to see if **361** or some activated derivative of it is capable of effecting DNA cross-links. Given its extreme potency and unusually wide spectrum of anticancer activities in clinical trials it would seem that a mode of biological interaction other than simple DNA monoalkylation may be operative. Additionally, the structural homologies of the ecteinascidins and bioxalomycins suggest too, that **361** may well be capable of DNA interstrand cross-linking under "activating" conditions.



Ecteinascidin 743, **361**

6. Conclusion

The DNA interstrand cross-linking agents represent one of the most potent categories of antitumor antibiotics. This is due largely to the ability of such compounds to shut down DNA strand separation events crucial to replication and transcription. In addition to unique DNA sequence recognition properties resulting from structural considerations, many of these agents preferentially target specific regions based upon "activation" processes which are unique to a physiological region of interest. In addition to synthetic manipulations of the drug core structure aimed at enhancing or changing such activation processes, molecular biology has also come to play a role in selective drug triggering. The methodology of selective drug activation via protein overexpression is perhaps the most progressive field of study with

Table 3. Summation of DNA−DNA Interstrand Cross-Linking Agents and Their Interactions within Duplex DNA

agent or class thereof	agent	interstrand cross-link specificity	related analogues	DNA groove occupancy
N-mustards	**1**−**5**	dG N^7−dG N^7	**14**−**18**	major
chloroethylnitroso-ureas (CNU)	**19**	dC N^3−dG N^1	**26**	major
1,3-dialkyl-3-acyltriazene	**27**	dC N^3−dG N^1	**38**, **41**	major
Busulfan	**45a**	dG N^7−dG N^7	**45b**−**f**	major
clomesone	**46**	dC N^3−dG N^1		major
glycidaldehyde	**55**	not known		not known
diepoxybutane	**56**	dG N^7−dG N^7		major
carzinophilin/azinomycin B	**58**	dG N^7−dG N^7 dG N^7−dA N^7		major
cis-diammine-dichloroplatinum(II)	**70**	dG N^7−dG N^7	**71**−**74**	major
isochrysohermidin	**80**	not known		not known
cyclopropylpyrrolo-indole dimers	**93a**−**k**	dA N^3−dA N^3	Scheme 1	minor
bizelesin	**94a**	dA N^3−dA N^3	**94b**−**94f**	minor
pyrrolobenzo-diazepine dimers	**99**, **100**	dG N^2−dG N^2		minor
dinuclear *cis*-DDP dimers	**101**−**103**	dG N^7−dG N^7	**104**, **105**	major
8-methoxypsoralen	**106**	dT $C^{5,6}$−dT $C^{5,6}$		major
4,5′,8-trimethylpsoralen	**107**	dT $C^{5,6}$−dT $C^{5,6}$		major
cyclophosphamide	**122**	dG N^7−dG N^7	**123**	major
hexamethylmelamine	**135**	not known	**136**, **146**	minor
pyrrolizidine alkaloids	**150**, **154**−**162**	dG N^2−dG N^2	**168**−**172**	minor
RSU-1069	**189**	not known		not known
aza aromatic N-mustards	**197**	dG N^7−dG N^7		major
nitromin	**198**	dG N^7−dG N^7		major
chlorambucil *N*-oxide	**199**	dG N^7−dG N^7		major
Co(III) mustards	**201**−**204**	dG N^7−dG N^7		major
daunorubicin	**209**	dG N^2−dG N^2	Figure 36	minor
doxorubicin	**210**	dG N^2−dG N^2	Figure 36	minor
cyanomorphilinodoxorubicin	**211**	dG N^2−dG N^2	Figure 36	minor
AZQ/DZQ	**240**, **246**	dG N^7−dG N^7		major
mitomycin C	**163**	dG N^2−dG N^2	**257**−**261**	minor
FR-900482	**325**	dG N^2−dG N^2	**326**, **327**	minor
bioxalomycin α_2	**344**	dG N^2−dG N^2	**343**−**348**	minor

respect to the DNA cross-linking agents. Also important has been the design/discovery of cross-linking agents based upon the concept of dimerization. The ability of the synthetic chemist to mix and match monoalkylating subunits of a dimeric agent clearly represents an important area for future research. Despite some 30 years of intense research, the interstrand cross-linking agents still represent an area of great therapeutic promise and intense interest. The interstrand cross-linking agents discussed herein are summarized in Table 3.

7. Abbreviations

Glossary

ADR	adriamycin
ALDH1	aldehyde dehydrogenase
BCE	*N*,*N*′-bis(2-chloroethyl)ethylenediamine
BD	binding domain
bp	base pairs
BPDE	7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene
CBI	1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one
CENU	chloroethylnitrosourea
cis-DDP	*cis*-diamminedichloroplatinum(II)
CPI	cyclopropylpyrroloindole
CTCL	cutaneaous T-cell lymphoma
Cys	cysteine
DCE	*N*,*N*-bis(2-chloroethyl)ethylenediamine
DDC	diethyl dithiocarbamate
DHM-3	one electron reducing agent bi(3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl)
DPAGE	denaturing polyacrylamide gel electrophoresis
DPT	2-pyridyl thionocarbonate
DTD	DT-diaphorase
DTT	dithiothreitol
en	ethylenediame
GMP	guanosine monophosphate
GR	gastrin receptor
GSH	glutathione (reduced form)
GST	glutathione S-transferase
HMG	High Mobility Group (class of proteins)
HSA	hypoxia selective agent
IC_{50}	concentration at which 50% inhibition of growth is observed
ISC	interstrand cross-link
β-MET	β-mercaptoethanol
MRSA	methicillin-resistant *Staphylococcus aureus*
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NBT	nitro blue tetrazolium
NO-RNA	nucleolar precursor ribosomal RNA
nt	nucleotide
ODN	oligodeoxyribonucleotide
PA	pyrrolizidine alkaloids
PBD	pyrrolobenzodiazepine
pso	psoralen
py	pyridine
SSB	single-strand break (as applies to DNA)
SVPD	snake venom phosphodiesterase
T_m	melting temperature

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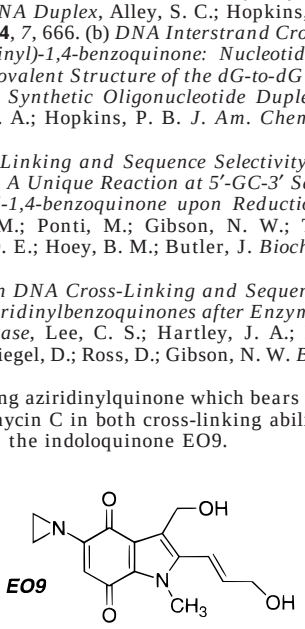

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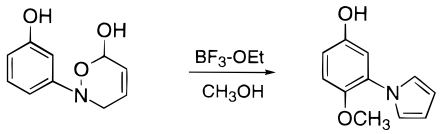

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