Photocleavage of Nucleic Acids

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Received July 21, 1997 (Revised Manuscript Received January 21, 1998)

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I. Introduction

The explosive growth in molecular biology has opened doors through which scientists from many different disciplines have passed in recent years. Along the way they have applied the tools and knowledge of their respective fields to addressing questions of fundamental and applied biological importance. One of the areas in which chemists have made substantial contributions is in the design and development of nucleic acid cleavage agents for use as structural probes and therapeutic agents. While natural enzymes have been extremely useful in many applications, their large size and/or limited range of sequence-recognition capabilities prevent their general use. For example, restriction enzymes, which are so critical in the synthesis of recombinant DNA, will only cleave DNA at specific 4−8 base pair (bp) sequences that are usually palindromic (i.e., the sequence is the same on both strands). An application which demands cleavage with a higher level of sequence selectivity (>8 bp) or at nonpalindromic sequences requires a new cleavage agent with expanded sequence recognition features. Alternatively, RNA structure probing experiments typically involve partial digestion of the RNA substrate with a series of ribonuclease enzymes, which differ in their sequence and secondary structure preferences. Development of new RNA cleavage agents that cleave at any single-stranded region, regardless of sequence, could greatly simplify these experiments. These shortcomings of natural nucleic acid cleavage agents provide opportunities for chemists to introduce synthetic cleavers.[1]

The topic of this review is a class of synthetic nucleic acid cleavage agents that are activated photochemically. One appealing characteristic of photocleavage agents is the fact that all components of a system to be studied can be mixed together without initiating the chemical reaction until the sample is irradiated. The ability to control light, in both a

S0009-2665(96)00428-1 CCC: $30.00
Published on Web 03/27/1998

Bruce Armitage was born March 1, 1966, in Niagara Falls, NY. He graduated from the University of Rochester with a Bachelors of Science degree in Chemistry and performed research under the supervision of Professor David G. Whitten (UR) and Drs. Samir Farid and Ian Gould (Eastman Kodak Co.). Bruce then moved to the University of Arizona for graduate school and completed his Ph.D. in Chemistry in 1993 under the supervision of Professor David F. O'Brien, studying photoinduced electron transfer and energy-transfer and polymerization reactions in lipid bilayers. Bruce then joined Professor Gary B. Schuster's group as a postdoctoral associate, first in the Department of Chemistry at the University of Illinois and, subsequently, at the Georgia Institute of Technology. During this time, Bruce was introduced to photochemical cleavage of nucleic acids, sparking many of his current interests. After a three-month visit to the University of Copenhagen to work with Professors Peter E. Nielsen and Henrik Nielsen on sequence-specific recognition of RNA by DNA analogues, Bruce moved to Carnegie Mellon University in August of 1997 as Assistant Professor of Chemistry. His current work focuses on the study of ligand–nucleic acid interactions and supramolecular photochemistry.

spatial and temporal sense, would be advantageous for applications ranging from the time-resolved probing of various biochemical processes, such as transcription or translation, to therapeutic agents which are activated in vivo by laser sources coupled into the body by fiber optics. Perhaps the most important feature of photoinduced cleavage is the fact that light can be a very selective cofactor in a chemical reaction. Provided the chromophore of the photocleaver is sensitive to light at wavelengths longer than 300 nm, nucleic acids and most proteins will be transparent, thereby allowing selective excitation of the photocleavage agent. This is critical in limiting the number of side reactions in the system as well as in analyzing the reaction mechanisms.

An excellent review on photocleavers was written in 1990 by Dunn and Kochevar;[2] the interested reader is referred to that article for a comprehensive presentation of early contributions (i.e., through 1988) in the field. In addition, a thorough review on photocleavage mechanisms was written by Paillous and Vicendo in 1993.[3] The present article reviews the literature from 1988 through 1997. Section II contains a brief description of the analytical techniques typically used to study nucleic acid cleavage and discusses some of the mechanistic information that can be obtained. Sections III and IV present the DNA photocleavers and are divided into groups on the basis of their likely sites of reactivity on the nucleic acids: namely, sugar (III) or nucleobase (IV). While this has not been conclusively determined in most cases, the observed sequence selectivity and other chemical characteristics of the cleavage can be reliable indicators for not only the site of reaction but also the mechanism of damage. In addition, there are photocleavers that are able to target more than one of these sites, depending on either the wavelength of excitation or on the mode by which the photocleaver is bound to the nucleic acid. Wherever possible, quantum yield and other photochemical data will be presented, as it is one goal of this review to present a useful comparison of the many photocleavage agents that have been reported in the past decade. A few additional photocleavage agents for which mechanistic data is lacking are collected in section V. Since there have been relatively few examples of RNA photocleavers, they are all dealt with in one section (VI).

Synthetic nucleic acid cleavage agents are often referred to as "nucleases". In the strictest sense, the term "photonuclease" should be reserved for those compounds that react directly with a nucleic acid while in an electronically excited state and cause an immediate scission of the nucleic acid chain. Moreover, the cleavage agent should not be consumed in the process, permitting it to react catalytically with the nucleic acid. It should be clear after reading this review that in reality, very few compounds actually meet these criteria. The vast majority of compounds discussed below will be referred to as "photocleavers" or "photocleavage agents", defined as *those compounds whose excited states can initiate a series of chemical reactions which ultimately lead to nucleic acid cleavage*. Thus, compounds which photochemically generate ground-state, reactive intermediates, such as radicals, carbenes, and carbocations, which, in turn, initiate cleavage of the nucleic acid, will be discussed, as will compounds which damage the nucleic acid without causing an immediate strand break. In these cases, the nucleic acid must be subjected to a secondary treatment, such as incubation with hot piperidine or aniline, to fully reveal the sites and extents of damage. By generating diffusible reactive species, such as hydroxyl radicals, a photocleavage agent need not even bind to the nucleic acid in order to induce cleavage. Care will be taken to point out whether the cleavage is spontaneous or requires further chemical manipulation for each photocleavage agent.

An additional feature of nucleic acid cleavage that should be addressed here involves the factors that determine the sequence selectivity of cleavage. Observation of preferential cleavage at particular sequences could be due either to preferential binding or to preferential reactivity at that site. For example, a cleavage agent which functions by abstracting hydrogen atoms from sugar residues might be expected to cleave at any sequence since a sugar residue is present within each nucleotide. However, if the cleavage agent has a higher affinity for one site (or subset of sites), then preferential cleavage may result. Alternatively, binding might occur with comparable affinities at all sites, but orientation of the cleavage agent on the nucleic acid at certain sites could lead to more or less efficient reaction at those sites. These issues are magnified given the relatively short amount of time available for reaction to occur

since the cleavage agent must react prior to returning to the ground state, a process which can be as short as a few hundreds of picoseconds. This can severely limit the number of orientations that the photocleaver can explore during its reactive lifetime.

Finally, I hope that this review proves useful not only to the practicing photochemist who wishes to move into biological chemistry but also to the molecular biologist looking for new tools for studying the structure of nucleic acids and their complexes with proteins and drug molecules.

II. Detection of Nucleic Acid Photocleavage and Mechanistic Implications

Photocleavage of nucleic acids typically involves an initial oxidative reaction with either a nucleobase or sugar residue. The damaged nucleotide then degrades either spontaneously or after incubation of the nucleic acid in hot piperidine (DNA) or aniline (RNA), yielding shorter DNA/RNA strands and small molecule byproducts such as sugar fragments or nucleobases. The nucleic acid fragments are generally separated using electrophoresis through agarose or polyacrylamide gels while nucleotide fragments may be identified by HPLC and mass spectrometry. The information supplied by these diagnostic techniques can be quite useful in determining the mechanism by which the photocleavage agent reacts with the nucleic acid.

A. Electrophoretic Analysis of Photocleavage

Two electrophoretic methods are generally used to detect DNA fragments produced by photocleavage.[4] In one case, supercoiled DNA is the target. Single-strand (ss) cleavage (i.e., "nicking") converts the supercoiled DNA to a relaxed, circular form while double-strand (ds) cleavage produces linear DNA. The three forms are readily separated on an agarose gel and detected by fluorescent staining. This assay is highly sensitive since a single ss or ds cleavage event occurring anywhere in the molecule is sufficient to cause the change to circular or linear forms. However, no information is obtained from this method with regard to the preferred cleavage sequences.

A more informative method for analyzing nucleic acid photocleavage involves the use of end-labeled targets. In these cases, the nucleic acid is enzymatically labeled at one terminus of one strand with a radioactive, fluorescent, or chemiluminescent tag. A sequencing ladder of the labeled nucleic acid can be obtained by either chemical or enzymatic methods. Photocleavage of the nucleic acid produces shorter strands that will migrate faster than the uncleaved target in polyacrylamide gel electrophoresis (PAGE). By running the sequencing ladders in adjacent lanes on the gel, the nucleotide at which the cleavage event took place can be identified. For example, photocleavage of a ^{32}P-end-labeled DNA target with no sequence preference will lead to a "ladder" of bands of equal intensity after exposing the gel to autoradiography film (Figure 1A). However, if there is preferential cleavage of certain sequences, then only a subset of those bands will appear on the film, i.e., rungs will be missing from the ladder (Figure 1B).

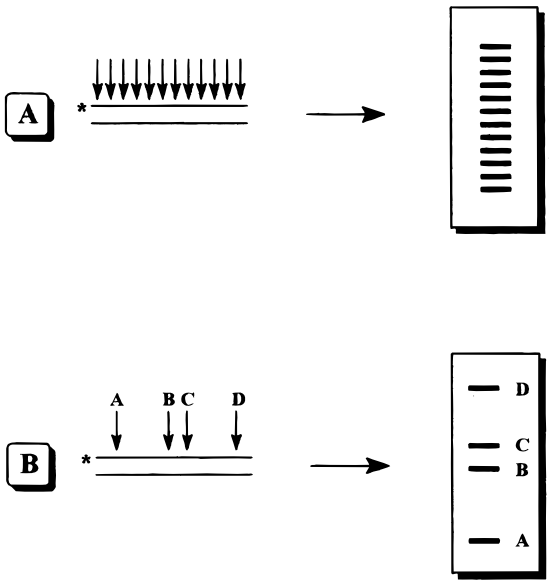


Figure 1. Illustration demonstrating how the sequence selectivity for cleavage is visualized by PAGE/autoradiography. The asterisk (*) denotes a radioactive end label on one strand of the duplex. (A) The photocleaver exhibits no sequence selectivity, leading to a "ladder" of cleavage bands after electrophoresis and autoradiography. (B) The photocleaver exhibits a high level of sequence selectivity, leading to only a few, discrete cleavage bands on the film.

In addition to pinpointing the cleavage site, end-labeled nucleic acids can provide considerable insight into the photochemical mechanism of cleavage. The photocleavers reported to date react with either the nucleobases or with the sugars. Observation of spontaneous cleavage is strong evidence that the photocleavage agent initiates hydrogen atom abstraction from the sugar residues since attack at the nucleobase typically requires a piperidine or aniline treatment in order to cleave the backbone. In all but a few cases, photocleavers that attack the nucleobases do so selectively at guanines. Thus, observation of cleavage bands at guanines only after piperidine/aniline treatment is indicative of nucleobase-targeted chemistry. The end-labeling/PAGE procedures can also be applied to the study of RNA cleavage and many of the same principles apply.

When photocleavage is initiated by H-abstraction from deoxyribose in DNA, bands are typically observed which comigrate with the sequencing ladders, where fragments are terminated by 5′- or 3′-phosphates. Often, additional cleavage bands will be observed which migrate between the phosphate-terminated fragments. For example, when the sugar is oxidized by abstraction of hydrogen from C-4′, the sugar radical can decompose to form fragments with 3′-phosphate (the entire nucleotide is lost) or 3′-phosphoglycolate (a 2-carbon piece of the deoxyribose moiety remains linked to the phosphate) termini. The latter products migrate faster than their 3′-phosphate analogues, due to the extra negative charge, but slower than fragments with one less nucleotide. In a high-resolution polyacrylamide gel, a doubling of the bands will be observed and is good evidence in support of a sugar oxidation mechanism. Comigration of the extra bands with those produced by Fe^{II}-EDTA cleavage of DNA (which is known to abstract

H from C-4′) provides strong support for a C-4′ oxidation mechanism. Extra bands can also be observed when C-3′ or C-5′ is oxidized.

In comparing the methods using supercoiled DNA/agarose gels and end-labeled DNA/polyacrylamide gels, it is clear that the former is considerably faster and less expensive. However, the amount of mechanistic information available in the latter format more than compensates for the added difficulty in terms of equipment, time and chemicals. This is particularly true when one considers the fact that several photocleavage agents give spontaneous cleavage of supercoiled DNA but cleavage sites on end-labeled DNA are only observed at G sites after piperidine treatment. Thus, the spontaneous cleavage observed in the first experiment would indicate that the sugar was being degraded but the sequence selectivity and requirement for piperidine argue strongly in favor of nucleobase attack. The high sensitivity of the supercoiled DNA relaxation assay can produce misleading data if a low quantum yield process leads to spontaneous cleavage while a higher quantum yield process requires a piperidine treatment in order to cleave the strand.

B. Analysis of Nucleotide Degradation Products

The nucleotide degradation products arising from attack at either the nucleobases or sugar residues have been well-characterized in most cases and are discussed in detail in the reviews by Burrows[5] and Tullius,[6] respectively, in this volume. Briefly, products that contain the nucleobase can be detected by HPLC with UV absorption or electrochemical detection. The observation that the intact nucleobases are released during cleavage indicates that the sugar is degraded by the photocleaver, particularly if the quantum yields for base release and strand cleavage are approximately equal. On the other hand, modified nucleobases can be detected by HPLC. For example, one of the most common modified bases is 7,8-dihydro-8-oxoguanine (8-oxo-G), a byproduct of oxidative damage to guanine. After irradiation, the DNA is degraded enzymatically to individual nucleosides, then the mixture is analyzed by HPLC. Although the nucleosides 8-oxo-dG and dG can have similar retention times in HPLC, use of electrochemical detection can selectively detect one or the other since their oxidation potentials differ by ~0.4 V.[7]

The sugar moieties are generally not released intact by photocleavage, even if the nucleobase is the initial site of damage. However, the fragmented products can often be detected by mass spectrometry, with some products being traceable to initial H-abstraction from a specific carbon atom on the sugar.

C. Photochemical Studies

In contrast to the product analysis methods described above, there has been very little characterization of photocleavage agents by standard photochemical procedures. There are many potential reasons for this shortfall of mechanistic information, but the most important is likely the extreme difficulty of interpreting the results one might obtain. For example, a standard issue dealt with by photochemists is whether an observed reaction involves the singlet or triplet excited state of the chromophore. One method for resolving this issue is to use a triplet sensitizer that generates the triplet excited state of the chromophore of interest by energy transfer, effectively bypassing the singlet state. In principle, the same method could be applied to the study of photocleavage agents. Complications arise since the majority of photocleavage agents spontaneously bind to the nucleic acid and the binding mode and site can have substantial effects on the quantum yield for cleavage. Thus, in cases where preassociation is involved, observation of a decreased quantum yield for cleavage via triplet sensitization could indicate that the cleavage arises from singlet-state chemistry, but it could also reflect a change in the binding constant or binding site for the photocleavage agent. These issues are particularly daunting given the heterogeneity of potential binding sites in DNA and RNA.

D. Detection of Reactive Intermediates

Since photocleavage of nucleic acids typically occurs by oxidative mechanisms, opportunities exist for either direct observation or trapping of one electron reduced or oxidized species arising from the photocleavage agent and/or the nucleic acid. In a few cases, transient absorption spectroscopy has been used to study radicals produced by electron-transfer reactions between photocleavage agents and DNA. Experiments such as these are critical because they provide insight into the earliest steps in the photocleavage process, as opposed to the product analysis studies, which only demonstrate which pathways were followed without explaining why one reaction was favored over another. In other cases described below, ESR spectroscopy was used to evaluate the mechanism of cleavage by metal complexes.

Finally, there has been very little investigation into the photodegradation pathways for RNA; consequently much of the presentation which follows will describe experiments involving DNA. A later section (VI) will detail the few RNA photocleavage reports.

III. DNA Photocleavage Arising from Deoxyribose Oxidation

Oxidation of deoxyribose due to hydrogen atom abstraction from the sugar furanose ring is quite often the key step in DNA cleavage. As described elsewhere in this volume,[6] the resulting sugar radicals can decompose by a variety of pathways to yield small molecule byproducts and DNA fragments.[8] The hydrogen atoms on carbons 1′, 3′, 4′, and 5′ are all thermodynamically reasonable targets for abstraction due to the presence of heteroatoms at the α-positions. The fact that identical deoxyribose residues are found at every step along the DNA duplex means that cleavage by hydrogen abstraction is inherently nonselective with respect to sequence. Thus, the chemistry can occur at any position, but observing sequence selectivity depends on the local structure of the DNA and the physicochemical properties of the

abstraction agent. For example, a photocleaver that initiates cleavage by direct H-abstraction from deoxyribose can be highly sequence selective if it only binds to one or a few sequences within the DNA target. The ability to alter the sequence selectivity of cleavage by varying the structure of the photocleavage agent is a key component in the design of new DNA cleavage agents. This, and other features in molecular design, will be highlighted in this section.

A. Photocleavage by Metal Complexes

1. Uranyl Ion

The uranyl ion (UO_2^{2+}) was first introduced as a photocleavage agent by Nielsen and co-workers in 1988.[9] They observed that irradiation of UO_2^{2+} in the presence of supercoiled DNA could introduce nicks into the DNA. The cleavage occurs spontaneously in both supercoiled and linear DNA targets. Mechanistic studies indicate that the cleavage occurs by an H-abstraction pathway, although it is not clear at this time if the uranyl directly oxidizes the sugar or if a diffusible intermediate is generated. The intact nucleobases are observed as byproducts of the photocleavage and the quantum yield for their production ($\sim 10^{-4}$) matches the quantum yield for plasmid nicking, consistent with a mechanism in which the damage is targeted to the sugar and not the base.[10]

UO_2^{2+} binds to the DNA by coordinating backbone phosphates across the minor groove with an affinity on the order of 10^{10} M^{-1}. Substitution of a methylphosphonate linkage for a phosphate inhibits uranyl photocleavage at the site of modification, providing strong evidence in support of the proposed binding mode. Binding via phosphate coordination would be expected to be sensitive to the minor groove width, a prediction verified by Nielsen and co-workers, who found that the cleavage efficiency for UO_2^{2+} was greatest in A/T-rich sequences, where the minor groove of B-form DNA tends to be the most narrow.[11] Thus, the uranyl ion is capable of discerning variation in the interstrand phosphate separation distance, making it a valuable probe of local DNA structure. Uranyl has also been used for analyzing the structure of bent DNA,[12] for probing metal ion-binding sites in a DNA four-way junction,[13] and for mapping the electronegative potential of DNA.[14]

While the cleavage efficiency of uranyl is dependent on the DNA minor groove width, the range of cleavage efficiencies is rather small, meaning that cleavage occurs readily at all positions in B-form DNA. However, if a ligand such as a protein or drug molecule is bound to the DNA in a manner that blocks uranyl binding, cleavage at that site will be inhibited. Polyacrylamide gel electrophoresis and autoradiography of radiolabeled DNA cleaved by uranyl in the presence of the ligand will produce regions of protection in the otherwise uniform ladder of cleavage bands. When sequencing ladders are also run on the gel, these "footprints" effectively pinpoint the location of the ligand binding sites on the DNA (Figure 2). Uranyl photofootprinting has been successfully applied to the imaging of DNA triplexes,[15] RNA polymerase/promoter complexes,[16] and gene expression regulator/DNA complexes.[17]

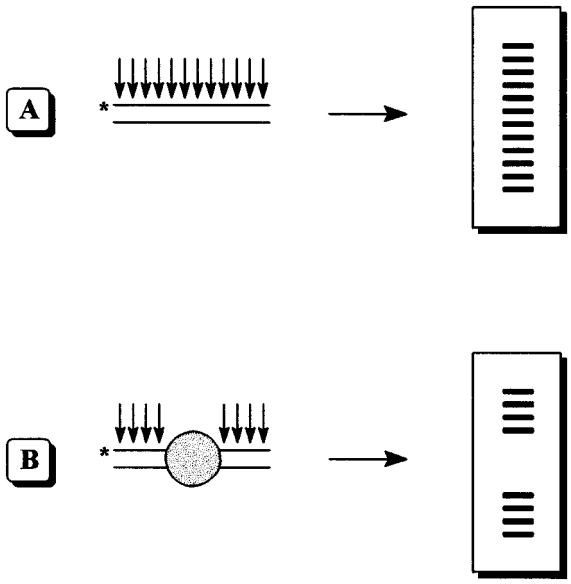


Figure 2. Illustration depicting footprinting of a DNA-bound ligand. (A) In the absence of the ligand, photocleavage occurs at all sites. (B) Binding of the ligand physically blocks access of the cleavage agent to some sites; electrophoresis and autoradiography effectively mark the ligand's position on the DNA.

Overall, UO_2^{2+} has been successfully employed to probe a wide variety of nucleic acid structures and protein−nucleic acid complexes. To date, it is the most useful photocleavage agent developed and can be readily utilized in virtually any lab, with only a fluorescent lamp required to perform the irradiations. The few disadvantages of UO_2^{2+}, such as the need to work at neutral or acidic pH to prevent the uranyl from forming insoluble uranyl hydroxide aggregates, and the preclusion of phosphate buffers on the grounds that the inorganic phosphate anions compete with the DNA for coordination of UO_2^{2+}, are minor and have not prevented the use of uranyl for a wide range of applications.

2. Rh(phi) Complexes

Photocleavage of DNA by rhodium(II) complexes containing the phi ligand (phi = 9,10-phenanthrenequinonediimine, Chart 2) has been studied in great detail by Barton and co-workers.[18] Irradiation of solutions containing Rh(phi) complexes in the presence of DNA results in spontaneous cleavage of the DNA. Free bases and base propenoic acids are detected as small molecule byproducts of the reaction, while the DNA fragments are terminated by 3′-phosphates and, in some cases, 3′-phosphoglycaldehydes. These products correspond to those expected from H-abstraction from the 3′-carbon of deoxyribose. The quantum yields are in the range of 10^{-3}−10^{-4}.[19]

The Rh(phi) complexes bind to B-form DNA by intercalation of the phi ligand from the major groove. Barton and co-workers propose that the excited state of the complex directly abstracts a hydrogen atom from C-3′ at the intercalation site. The 3′-phosphate termini are produced by anaerobic decomposition of

the 3′-radical while the 3′-phosphoglycaldehyde arises from trapping of the C-3′ radical by molecular oxygen prior to sugar decomposition and backbone scission. The two termini migrate at different rates in a polyacrylamide gel, leading to a doubling of each cleavage band. When photocleavage is performed with $[Rh(phen)_2phi]^{2+}$, only the 3′-phosphate band is observed, but both the phosphate and phosphoglycaldehyde bands are produced when $[Rh(phi)_2bpy]^{2+}$ is used, presumably reflecting the increased access of molecular oxygen to the radical when the smaller bpy ligand is present relative to the phen ligands.

DNA cleavage by Rh(phi) complexes exhibits a wide range of sequence selectivities. Barton and co-workers have explored the role of the ancillary (i.e., nonintercalating) ligands on the cleavage selectivity. For example, the Δ-isomer of $[Rh(en)_2phi]^{3+}$ cleaves DNA with high selectivity for 5′-GC-3′ dinucleotide steps.[20, 21] (The same preference is observed for the achiral $[Rh(NH_3)_4phi]^{3+}$ complex.) However, the λ-isomer exhibits cleavage at all sites, with some preference for A/T sites. Molecular modeling indicates that the dramatic difference in the two cases arises because the en ligands in the Δ-isomer can follow the right-handed helicity of the DNA while they cannot in the λ-isomer. The possibility that the nitrogen atoms in the ligands were forming specific hydrogen bonds to DNA was explored by synthesizing two macrocyclic complexes. The $[Rh([12]aneN_4)phi]^{3+}$ complex produces similar cleavage selectivity as the Δ-isomer of $[Rh(en)_2phi]^{2+}$, whereas cleavage by the corresponding $[Rh([12]aneS_4)phi]^{3+}$ complex, where the Rh-coordinating nitrogen atoms in the macrocyclic ligand are substituted with sulfurs, which would form much weaker hydrogen bonds to the DNA, is similar to the λ-isomer of $[Rh(en)_2phi]^{2+}$. Taken together, the results indicate that at least one of the nitrogen atoms in the nonintercalated ligands participates in a hydrogen bond with one of the DNA functional groups, presumably O-6 of guanine. In fact, methylation at O-6 inhibits cleavage at that site, whereas replacement of G with 7-deazaG had no effect, supporting the proposal of a specific hydrogen bond.

In addition to exploring the role of hydrogen bonding in DNA recognition by the Rh(phi) complexes, Barton and co-workers have studied the contribution of van der Waals' interactions by adding methyl groups to the Rh-coordinating ligands.[22] For example, the presence of the two methyl groups on the nonintercalating Me_2trien ligand of $[Rh(Me_2$-trien)phi$]^{2+}$ (see Chart 2) leads to a 2−3-fold enhancement in the cleavage at 5′-TGCA-3′ sites over all other 5′-XGCY-3′ sites, leading the authors to propose that the methyl groups make van der Waals' contacts with the methyl groups on thymine residues at the ends of the palindromic recognition site. It would be interesting to see if the preference is lost for 5′-UGCA-3′ sites (U = uracil), which lack the thymine methyl groups.

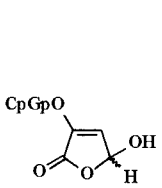
One potential pitfall of using photocleavage data to determine binding site locations and structural information is the possibility that the cleavage efficiency is not directly proportional to the binding affinity. There is no reason, a priori, that the quantum yield for cleavage should be independent of sequence, particularly for a reaction like H-abstraction, which has relatively stringent geometric requirements regarding the alignment of the abstracting atom with the hydrogen. In the example given above, the possibility exists that methylation of guanine at O-6 introduces a new reaction pathway, such as electron transfer, for deactivation of the rhodium complex excited state. (Substitution of 7-deazaG for G would not have such an effect since it would likely result in a weaker donor.) This new reaction path would probably not lead to spontaneous cleavage even though the complex could bind at the site with comparable affinity as to the unmodified G. Thus, to avoid ambiguities, it is important to provide binding data when attempting to rationalize cleavage selectivities in terms of binding affinities. This can be done in some cases by footprinting of the bound photocleaver, an approach which Barton and co-workers took in a subsequent report.[23] The Δ-$[Rh(dpb)_2phi]^{2+}$ complex (Chart 2) photocleaves DNA with high selectivity for 5′-CTCTAGAG-3′ sites. Meanwhile, footprinting experiments with the iron−EDTA-based reagent, MPE-Fe^{2+}, shows very clear regions of protection at the same sites, providing the necessary correlation between binding and reactivity.

The Rh(phi) complexes represent an excellent example of how the cleavage selectivity can be determined not by the chemical mechanism, but rather by the binding selectivity of the photocleaver. The ability to alter the cleavage selectivity by varying the ancillary ligands on the complexes provides a level of control unavailable in most other systems. Finally, major groove binding and chemistry is quite rare for small synthetic ligands, despite the fact that most of the information (i.e., functional groups) in DNA is located there. Thus, while most DNA-binding proteins make extensive contacts with the major groove, most synthetic ligands interact more closely with the minor groove. The Rh(phi) complexes provide a scaffold on which to build more elaborate structures for the recognition of DNA major groove functional groups, as well as a photochemical mechanism for reporting on that binding.

3. Co(BLM) and Analogues

In 1982, Chang and Meares first reported that a cobalt(III)−bleomycin complex could cleave DNA photochemically.[24] Subsequently, Saito, Meares, Hecht, and co-workers studied the photocleavage characteristics of the Co^{III}-BLM (GREEN) complex.[25] When the self-complementary dodecamer 5′-CGCTT-TAAAGCG-3′ was used as the target, selective cleavage was observed after alkali treatment at positions C-3 and C-11. Free cytosine was detected as a byproduct of the reaction by HPLC. In addition, fragment **1**, shown in Chart 1, was detected and attributed to cleavage at C-3 initiated by hydrogen atom abstraction from carbon-4′ of deoxyribose. These results clearly demonstrate that the site of attack by Co^{III}-BLM is deoxyribose, but they do not offer insight into the oxidizing species nor for its mechanism of

Chart 1



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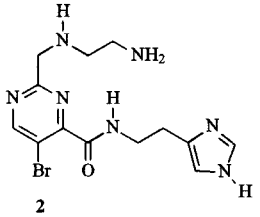

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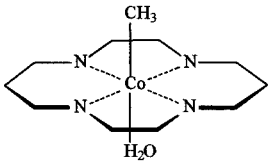

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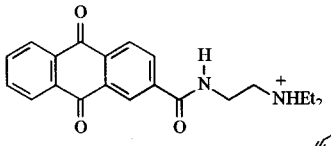

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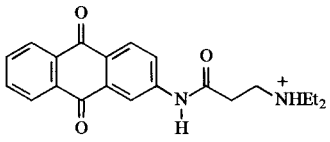

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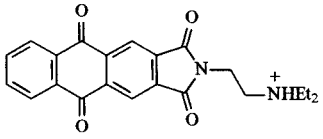

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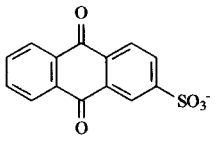

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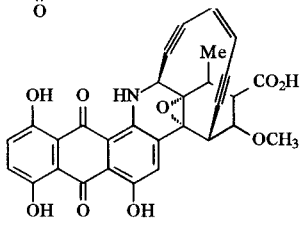

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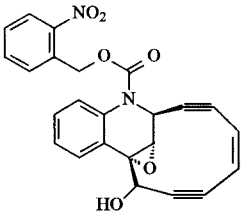

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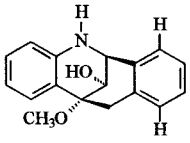

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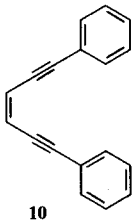
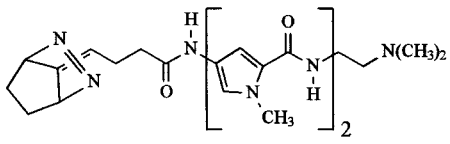
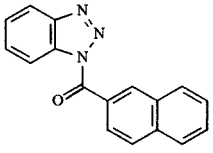


10



11



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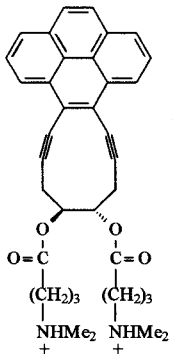

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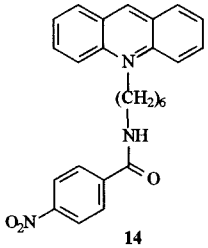

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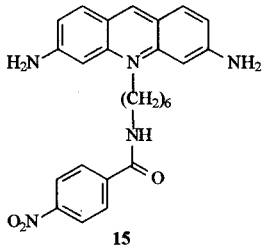

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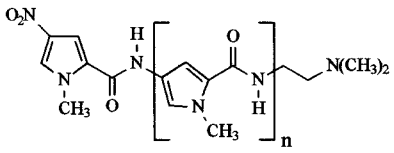

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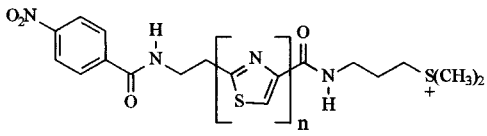
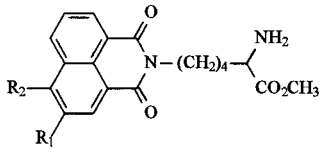
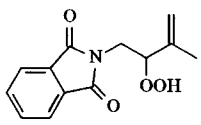


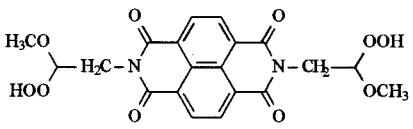
17



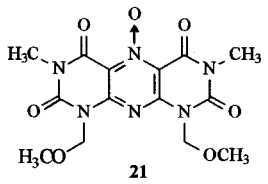
18a : $R_1 = NO_2$, $R_2 = H$
18b : $R_1 = H$, $R_2 = NO_2$



19



20



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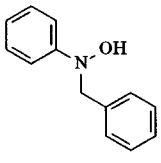
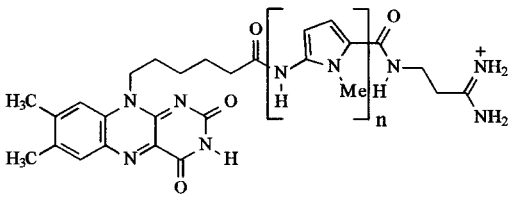
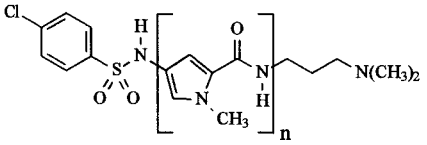


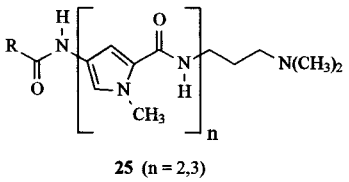
22



23 (n = 2,3)

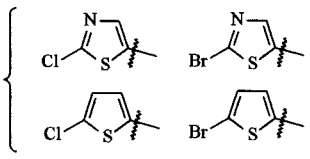


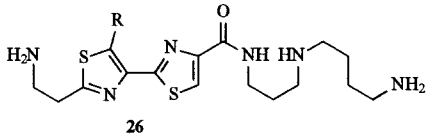
24 (n = 1-4)



25 (n = 2,3)

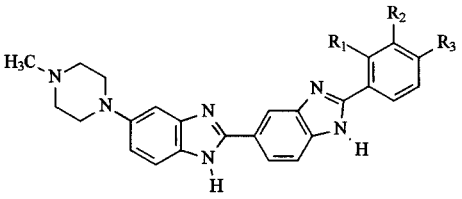
R =





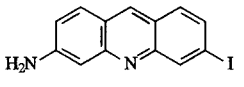
26

Chart 1 (continued)

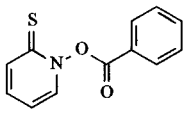


27

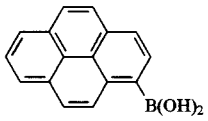
Iodo-Hoechst : R_1 = H, R_2 = I, R_3 = OH
ortho : R_1 = I, R_2 = H, R_3 = H
meta : R_1 = H, R_2 = I, R_3 = H
para : R_1 = H, R_2 = H, R_3 = I



28



29



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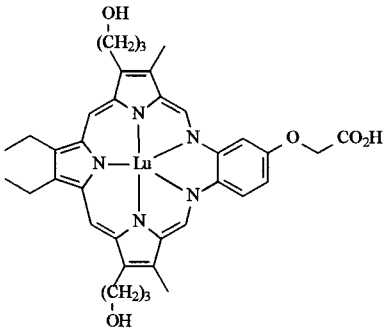
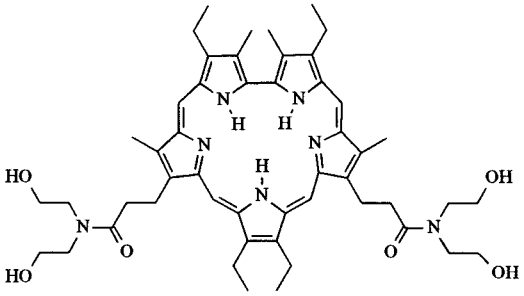
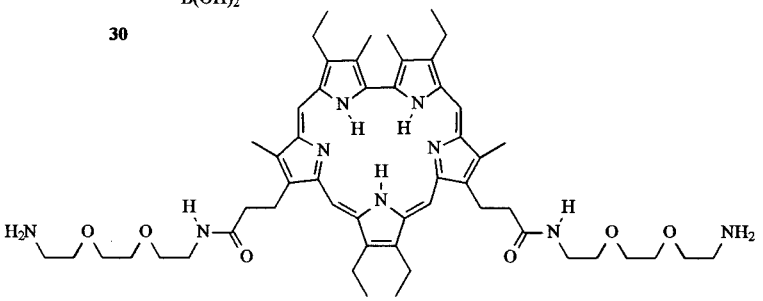


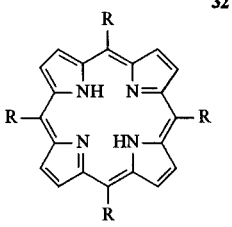
31

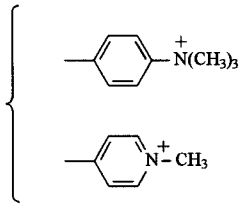


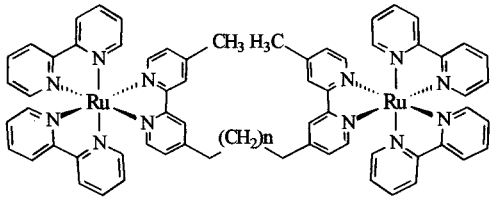
32



33

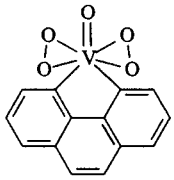


R = 



34

35



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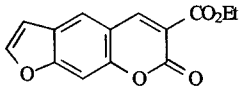
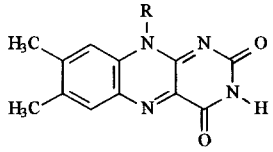
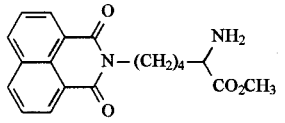


37

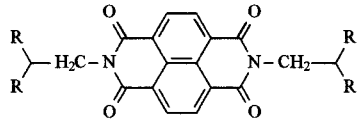


38

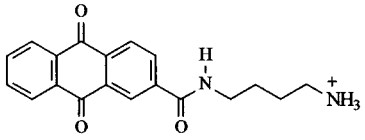


39

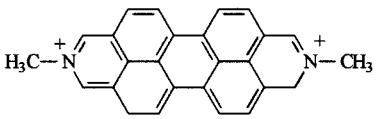
(R = D-ribo-2,3,4,5-tetrahydroxypentyl)



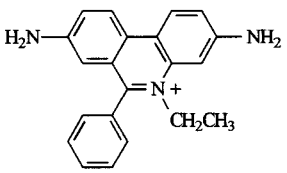
40a : R = OCH_3
40b : R = CH_3



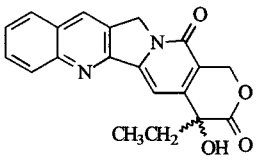
41



42



43



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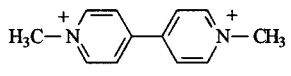
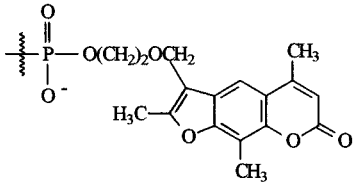


44



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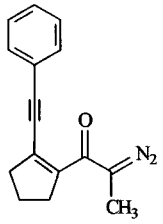
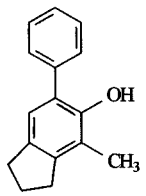
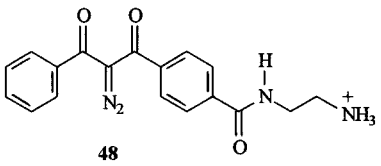


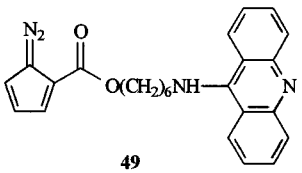
47



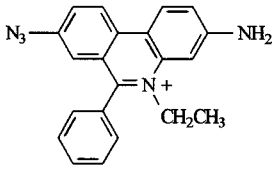
47a



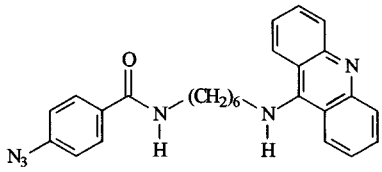
48



49



50



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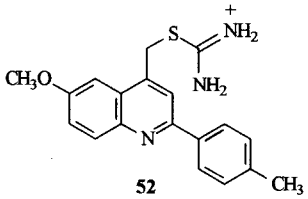
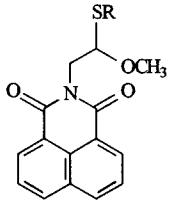
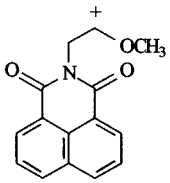


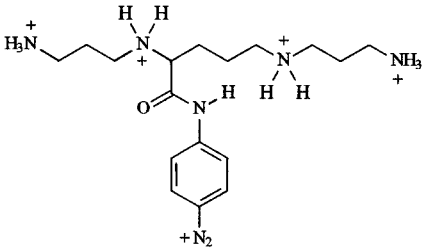
52



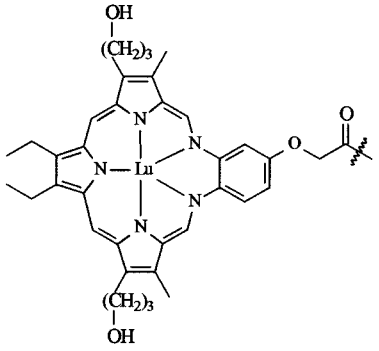
53



53a



54



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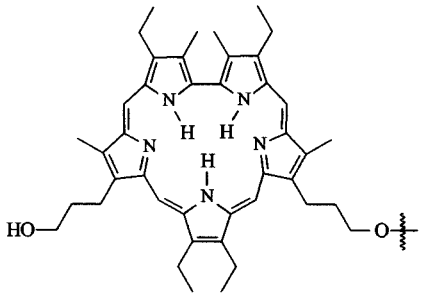
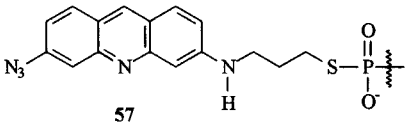
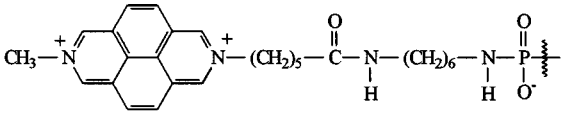


56



57



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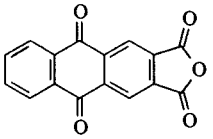

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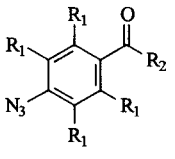

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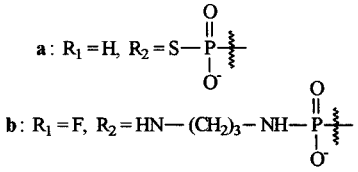

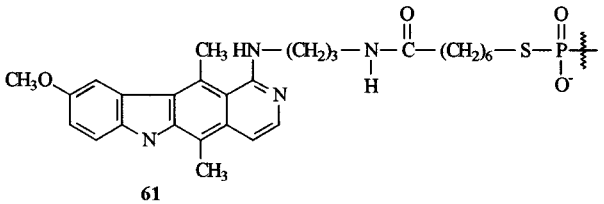

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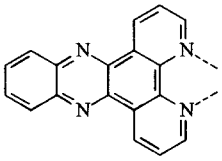
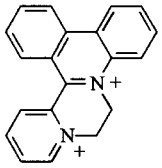


62



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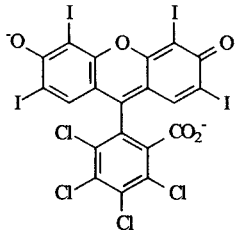

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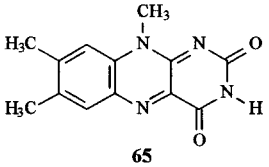

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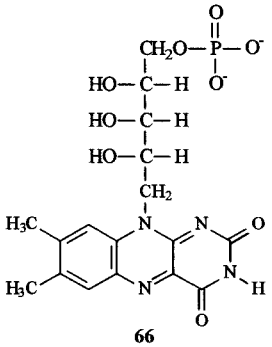


66

formation. Two Co^{III}-BLM model compounds were also synthesized by Saito and co-workers and found to cleave DNA with no sequence selectivity but no other mechanistic data was reported.[26]

A series of reports from Mascharak and co-workers has suggested an intriguing mechanism for DNA photocleavage by Co^{III}-BLM.[27] Coordination of cobalt by the PMAH ligand (**2**) yields a Co^{III}-BLM analogue that lacks the DNA-binding bithiazole moiety of the natural ligand. $[Co(PMA)(H_2O)]^{2+}$ was initially found to photonick plasmid DNA.[28] ESR trapping experiments with the nitroxyl radical DMPO revealed that irradiation of $[Co(PMA)(H_2O)]^{2+}$ in dry acetonitrile under anaerobic conditions leads to formation of a ligand-based C/N radical.[29] If the experiment is repeated in the presence of water, the characteristic ESR spectrum of DMPO−OH is obtained, indicating that the C/N radical reacts with water to produce hydroxyl radicals, which are then trapped by DMPO. These experiments demonstrate the production of hydroxyl radicals by irradiation of $[Co(PMA)(H_2O)]^{2+}$ and suggest that the DNA cleavage arises not from direct reaction between the excited cobalt complex and the DNA but rather is mediated by the hydroxyl radical. This is consistent with the 4′-directed chemistry cited above, since other hydroxyl radical generating systems, such as iron−EDTA-based reagents, cleave DNA primarily through H-abstraction from C-4′ of deoxyribose. Further experiments using a short DNA duplex target revealed the presence of two bands for each cleavage site, another common feature of C-4′ cleavage chemistry.[30] Due to the strong structural similarity between these model complexes and cobalt−bleomycin, it is quite possible that Co^{III}-BLM functions by the same mechanism. Finally, a novel feature of these cobalt−bleomycin model systems is the fact that hydroxyl radicals are produced without involving molecular oxygen. (In fact, cleavage is slightly inhibited by the presence of oxygen.) Hydroxyl radicals generated by photocleavage agents are, in most other cases, derived from reduction of molecular oxygen to hydrogen peroxide and subsequent decomposition of the peroxide.

The cobalt(III) complex $[Co(cyclam)(H_2O)(CH_3)]$ (**3**), reported by Riordan and Wei, photonicks plasmid DNA.[31] Intact free nucleobases are detected by HPLC, indicating that cleavage arises from sugar oxidation. However, it is unlikely that this complex functions by an analogous mechanism to the cobalt−bleomycin analogues of Mascharak. Rather, cobalt(III) complexes with axial alkyl ligands are known to undergo a photodissociation reaction, releasing alkyl radicals. The authors proposed that irradiation releases a methyl radical which either attacks the DNA directly, or reacts first with oxygen to produce the methylperoxy radical which then initiates cleavage. While the oxygen-trapping reaction is quite fast in solution ($k = 10^9$ M^{-1} s^{-1}), the methyl radical could be very short-lived if it is produced in close proximity to its site of reaction due to binding of the cationic cobalt complex to the DNA. It will be interesting to determine which hydrogen is abstracted by the organic radical and if that property can be altered by using different equatorial ligands on the cobalt complex to vary the binding selectivity.

4. $Pt_2(pop)_4^{4-}$

The tetraanionic diplatinum complex, $Pt_2(pop)_4^{4-}$ (pop = pyrophosphito), has been studied by Thorp and co-workers as a sequence-neutral photocleavage agent. The complex is able to catalytically oxidize organic substrates by photoinduced hydrogen atom abstraction,[32] suggesting that it could be used to catalytically cleave DNA. $Pt_2(pop)_4^{4-}$ does indeed cleave DNA by hydrogen atom abstraction.[33] The DNA fragments that arise from cleavage possess 3′-phosphate and 3′-phosphoglycolate as well as 5′-phosphate and 5′-aldehyde termini. The two sets of termini are consistent with H-abstraction from both C-4′ and C-5′, respectively. H-abstraction occurs upon collision between an excited-state $Pt_2(pop)_4^{4-}$ complex and DNA and is strongly enhanced by inclusion of Mg^{2+} in the reaction buffer. (The magnesium cations presumably associate with the DNA phosphates and/or the platinum complex, effectively screening the electrostatic repulsion between the two reactants, thereby increasing the cleavage efficiency.) Turnover of the platinum complex in the photocleavage has not been demonstrated, however, since irradiation of $Pt_2(pop)_4^{4-}$ in aqueous solution leads to hydrolysis of the pyrophosphito ligand and release of mononuclear Pt(II) complexes which, in the presence of DNA, become covalently bound to the nucleic acid and must be removed by treatment with cyanide prior to electrophoresis.

$Pt_2(pop)_4^{4-}$ has been used to photofootprint the λ-repressor/DNA complex at high resolution.[33a] The lack of binding by the tetraanionic complex to the DNA is an appealing feature for a footprinting agent since there will be no competition between the photocleavage agent and the protein for binding sites. The results are comparable to those obtained from hydroxyl radical footprinting but offer the advantage of requiring only light as an additional cofactor. The low concentration of $Pt_2(pop)_4^{4-}$ required for the footprinting (<100 μM) essentially guarantees that DNA−ligand complexes can be probed with minimal perturbation of the interaction.

B. Photocleavage by Organic Compounds

1. Anthraquinones

A series of anthraquinone (AQ) derivatives studied by Schuster and co-workers exhibit DNA photocleavage by three distinct pathways that are differentiated by the binding mode of the AQ as well as the composition of the buffer. One mechanism, involving electron transfer from the DNA nucleobases to an intercalated AQ and leading to selective cleavage at guanines, will be discussed in detail in section IV.B.2. The other two pathways, discussed in this section, involve hydrogen atom abstraction from deoxyribose.

Anthraquinones possessing electron-withdrawing groups at one or more of the β-carbons are capable of oxidizing organic substrates, such as 2-propanol, by hydrogen atom abstraction, similar to $Pt_2(pop)_4^{4-}$.[34] The excited-state electronic configuration for these

molecules is n,π^*, meaning that a nonbonding electron is promoted to an antibonding π-orbital upon absorption of a photon. This leaves an unpaired electron in the nonbonding orbital located on one of the AQ carbonyl oxygens, permitting H-abstraction. The monocationic AQ derivative **4** intercalates into DNA and reacts with the nucleobases.[35] However, if the AQ is present in excess relative to intercalation sites, spontaneous sequence-neutral cleavage of the DNA results.[36] The mechanism of this reaction was studied by comparing **4** with the reversed amide analogue **5**. Inversion of the amide orientation changes the substituent from electron withdrawing to (weakly) electron donating. This gives the AQ an excited-state configuration of π,π^*, which is not capable of H-abstraction. AQ **5**, even when present in excess, fails to initiate spontaneous photocleavage of DNA, indicating that the cleavage observed by **4** is due to H-abstraction.

The preferential binding of monosubstituted AQ derivatives to DNA by intercalation limits their ability to abstract hydrogens from deoxyribose due to rapid electron transfer from the nucleobases which form the intercalation site, requiring saturation of the intercalation sites before H-abstraction by non-intercalated quinones can occur. However, AQ imide derivative **6** behaves quite differently. This AQ fails to intercalate into duplex DNA, instead binding in one of the grooves or by electrostatic association with the backbone phosphates.[37] This has a profound effect on the photochemical reaction with DNA: laser spectroscopy experiments demonstrate that electron transfer from the nucleobases to **6** is slowed more than 50-fold. Spontaneous, sequence-neutral cleavage occurs even when **6** is present at nonsaturating concentrations. These examples illustrate the critical role that the DNA-binding mode can play in the cleavage mechanism for photocleavage agents.

The two examples cited above for DNA photocleavage by AQ derivatives involve direct reaction between the AQ excited state and the DNA sugars. An additional pathway is available in which the excited AQ generates a diffusible, reactive intermediate which then abstracts H atoms from deoxyribose, leading to spontaneous cleavage. Early experiments involving DNA photocleavage by AQ derivatives were performed in a buffer which contained 10 mM sodium phosphate (pH = 7) and 100 mM sodium chloride. Photocleavage, as monitored by a decrease in the number of binding sites for ethidium bromide on the DNA, was attributed to direct reaction between an intercalated AQ and the DNA.[35] However, subsequent experiments revealed that the spontaneous cleavage observed in those experiments required the presence of chloride anion in the buffer.[38] Thus, repeating the experiments in fluoride, bromide, iodide, or perchlorate dramatically reduced the cleavage efficiency. Furthermore, the anionic AQ derivative **7**, which should bind to DNA with far lower affinity, if at all, compared to the cationic derivatives, cleaves DNA quite effectively in the presence of chloride. Mechanistic experiments, combined with consideration of early work on the photochemistry of anthraquinones in aqueous chloride solutions by Treinin, Linschitz, and co-workers,[39] indicate that AQ reacts with chloride in the aqueous phase by photo-induced charge transfer, leading to production of chlorine atoms which then either react directly with DNA by H-abstraction or generate secondary radicals which are responsible for the cleavage. The observation of faster migrating 3′-termini on the cleavage fragments suggests that at least part of the cleavage is due to abstraction of hydrogen from C-4′, as observed for hydroxyl radical chemistry. This minor groove targeted chemistry is effectively inhibited by binding of netropsin to the DNA, leading to high-resolution photofootprinting of the drug.

2. Enediynes and Related Compounds

The enediyne class of natural products bind to DNA and, upon activation, undergo elaborate rearrangements to produce diradicals (Figure 3). These compounds are potent DNA cleavage agents, capable of producing single- and double-stranded breaks of the DNA, suggesting their use in a variety of diagnostic and therapeutic applications. Considerable interest exists in developing enediyne mimics that can be triggered to rearrange to the diradical photochemically, due to the noninvasive, selective nature of light activation.

Early work by Sugiura and co-workers focused on the natural products esperamicin and neocarzinostatin.[40] These compounds are normally activated by reduction, but irradiation also leads to cleavage of supercoiled DNA. Preirradiation of the enediynes in the absence of DNA inhibits their ability to subsequently initiate cleavage and an organic radical can be trapped by the nitroxyl radical DMPO and studied by ESR. These observations are consistent with the proposal that irradiation triggers rearrangement of the enediyne to the diradical, DNA cleaving structure.

Sugiura and Shiraki also studied DNA photocleavage by dynemicin A (**8**).[41] Irradiation of this enediyne with visible light results in a mixture of single- and double-stranded cleavage of supercoiled pBR322 plasmid DNA. It is unclear how the rearrangement of the enediyne is triggered photochemically, although it is possible that the compound undergoes photoreduction by Tris [tris[(hydroxymethyl)amino]-methane] which was used to buffer the solution. The observation of double-stranded cleavage events is significant because it indicates that the enediyne does, in fact, rearrange to the diradical form, which subsequently abstracts two hydrogen atoms, one from each strand of the DNA.

Due to the structural complexity of the natural enediynes, a number of simplified model compounds

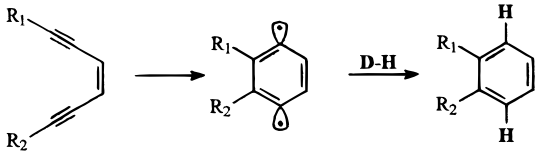


Figure 3. Cycloaromatization of an enediyne leads to formation of a 1,4-diradical, which abstracts hydrogen atoms from a donor (D−H) to yield a substituted benzene product.

designed to generate diradicals have been studied as photocleavers. Wender and co-workers synthesized the dynemicin model compound **9**.[42] Enediyne cyclization of **9** is triggered by photocleavage of the nitrobenzoyl group and subsequent epoxide ring opening. Irradiation of **9** in the presence of 1,4-cyclohexadiene results in formation of the hydrogen abstraction product **9a**. Compound **9** initiates both single- and double-stranded photocleavage of plasmid DNA, fulfilling the goals of its design.

Enediyne compound **10** initiates photonicking of plasmid DNA, presumably due to cyclization to form the 1,4-diradical.[43] Linearized plasmid, due to double-stranded cleavage, was not observed in this case. Radical scavengers such as mannitol and superoxide dismutase were able to inhibit the cleavage, demonstrating that the radicals are generated in solution and suggesting that oxy radicals such as superoxide or hydroxyl might also play a role in the cleavage. Cationic groups to promote binding of the enediyne to the DNA prior to irradiation would aid in clarifying the cleavage mechanism and would probably improve the efficiency for this reagent.

Compound **11** is clearly not an enediyne; however, it is considered here since it was designed such that photoextrusion of nitrogen would yield a diradical capable of cleaving DNA.[44] Photonicking of plasmid DNA is indeed observed for **11.** Further experiments involving radiolabeled restriction fragments revealed that the cleavage was selective for A/T-rich sequences, as expected for the netropsin-like component attached to the chromophore. Thus, the A/T preference for cleavage by **11** is most likely due to binding selectivity, not chemical selectivity.

Wender and co-workers used a similar strategy in developing a series of triazole compounds (such as **12**) as new photocleavers.[45] Irradiation of **12** in the presence of ethanol led to formation of hydrogen-abstraction products, consistent with a diradical intermediate. The triazoles photonick plasmid DNA and cleave radiolabeled restriction fragments spontaneously. There is some preference for cleavage at GG sites, as observed for many photocleavers that react with DNA nucleobases by electron transfer. However, the spontaneous nature of the cleavage and a distinct preference for cleavage at the 3′-G of the GG steps by the triazoles argues against cleavage by an electron-transfer pathway (see section IV.B.).

One additional report of enediyne-related photocleavage was given by Funk, Williams, and co-workers.[46] Noting that irradiation of the *o*-dialkynylpyrene derivatives in the presence of a hydrogen atom donor led to cycloaromatization, presumably via a 1,4-diradical, the authors synthesized **13**, wherein the cationic substituents were included to improve DNA binding affinity. Irradiation led to spontaneous nicking of supercoiled DNA and, while cycloaromatization in the presence of DNA was not demonstrated, a derivative which lacked the dialkynyl functionalities failed to nick the DNA, providing indirect support for a cleavage mechanism involving H-abstraction from deoxyribose residues by the phenyl radical. Very little linear DNA was observed, indicating that double-stranded cleavage by the 1,4-diradical was inefficient. This could simply reflect binding of **13** to the DNA in a conformation in which only one radical center is within reactive distance of an abstractable H atom. Dissociation of the resulting monoradical from the DNA would then preclude secondary H-abstraction.

3. Nitro-Substituted Photocleavers

Nitro-substituted organic compounds have been studied for 10 years as DNA photocleavage agents. The first group of compounds studied in this context were the acridine-linked nitrobenzamides (e.g., compound **14**), reported by Nielsen and co-workers.[47] These compounds nick plasmid DNA and spontaneously cleave restriction fragments with some preference for G and T sites. Piperidine treatment enhances the cleavage ~5-fold without influencing the selectivity. The acridine moiety is essential for effective cleavage, demonstrating the need to position the nitro group close to the DNA in order to ensure reaction within the excited state lifetime. The authors propose that the nitrobenzamide excited state abstracts hydrogen atoms directly from the DNA, in accordance with the known photochemistry of nitro compounds.

A curious feature of the nitrobenzamide−acridine conjugates is the finding that irradiation into the acridine absorption band also leads to cleavage of the DNA and that the cleavage products can be partially religated using DNA ligase.[48] This indicates that some of the products have 3′-hydroxyl termini. Irradiation into the nitrobenzamide absorption band with shorter wavelength UV light still leads to cleavage, but there are no ligatable fragments among the cleavage products. The efficiency of the reaction and the yield of ligatable fragments are sufficiently low that this process does not appear to have much promise with respect to use in synthesis of recombinant DNA. However, it remains the lone report to date of photochemical production of ligatable cleavage products.

Kuroda and Shinomiya studied a similar set of compounds in which the nitrobenzamide was linked to a proflavine intercalator rather than acridine (compare **15** with **14**).[49] These compounds also photonicked plasmid DNA and the proflavine moiety was required for activity. Unfortunately, sequence-selectivity data was not reported so a direct comparison with the acridine compounds cannot be made.

Buchardt and co-workers also studied the DNA cleavage properties of various peptides substituted with separate acridine (Acr) and nitrophenylsulfonyl (Npso) groups.[50] The peptide Acr-Aha-Lys(Npso)-Gly-$(Lys\text{-}Gly)_3$−OH (Aha = 6-aminohexanoic acid; Lys(Npso) = lysine with a *p*-nitrophenylsulfonamide linked to the ϵ-amino group) was synthesized by solid-phase methods. Significant retardation of DNA during electrophoresis in the presence of the peptide demonstrated strong binding of the peptide to the DNA. Single-stranded photocleavage of supercoiled and linear DNA targets occurred spontaneously with little sequence preference.

A series of nitro-substituted oligopyrroles (**16)** was prepared and studied by Shibuya and colleagues.[51]

The oligopyrrole group was included to enhance binding to DNA, mimicking netropsin. Irradiation of these compounds led to nicking of plasmid DNA. However, ESR experiments indicated the presence of both hydroxyl radicals and the nitro radical anion, while various radical scavengers such as phenol and benzoate inhibited cleavage. The subsequent report that an analogous series of compounds lacking the nitro group also cleaved plasmid DNA[52] cast further doubt on the mechanism for DNA cleavage by these compounds.

Kuroda and co-workers have studied a similar series of compounds in which a nitrobenzamide is linked not to an oligopyrrole group but rather to an oligothiazole, mimicking the DNA recognition moiety of bleomycin A_2 (**17**).[53] The uni- and bithiazole derivatives were able to photocleave radiolabeled duplex DNA spontaneously with a strong preference for A/T-rich regions. DNase I was unable to footprint these compounds, presumably because of weak binding and/or fast dissociation kinetics for the thiazoles. Extending the methylene chain linking the nitrobenzamide to the thiazole led to a shift of the cleavage sites by two nucleotides in the 3′-direction for some bands, indicating an orientational preference for binding of the photocleaver to those sites. The observation of spontaneous cleavage at these sites is consistent with photoinduced H-abstraction by the nitro group. Intriguingly, extension of the oligothiazole to three units completely changed the cleavage selectivity from A/T-rich sequences to GG sites, with cleavage only appearing after piperidine treatment.[54] This derivative, discussed in greater detail in section IV.B.5, once again demonstrates the dramatic alterations in cleavage chemistry which can be encountered in making what appear to be relatively minor structural changes.

Recently, Saito and co-workers reported the DNA photocleavage properties of a family of nitro-substituted naphthalimide derivatives (**18**).[55] After irradiation and piperidine treatment, compound **18a** was found to cleave duplex DNA with high selectivity for thymine residues. Irradiation of **18a** in the presence of 3′,5′-dibenzoyl protected thymidine led to oxidation of the thymine methyl group to an aldehyde (formyl-Uracil or ^{f}U) while prolonged irradiation resulted in decarbonylation, leaving uracil at that site. This demonstrates the ability of **18a** to directly oxidize the nucleobase rather than (or in addition to) the sugar. The authors also investigated the ability of **18a** to photocleave the single-stranded trimer 5′-d(ATA)-3′. Irradiation again led to oxidation of the thymine methyl group and piperidine treatment resulted in cleavage of the trimer at the oxidized T. Finally, irradiation of poly(dA)-poly(dT) also led to formation of ^{f}U, indicating that **18a** can oxidize the thymine methyl group in double-stranded as well as single-stranded DNA. The authors propose that the oxidation reaction is due to photoinduced hydrogen abstraction from the methyl group by the excited-state nitro group. Evidence in support of this hypothesis comes from the observation that **18b** cleaves duplex DNA with significantly lower selectivity for thymine residues. If the two compounds bind to DNA in similar orientations (presumably by intercalation), then the nitro groups will be projecting in different directions, leading to different H-abstraction efficiencies. Significantly, compound **39** does not cleave at thymine residues, but rather at GG sites (see section IV.B.5), demonstrating the importance of the nitro group for this type of cleavage.

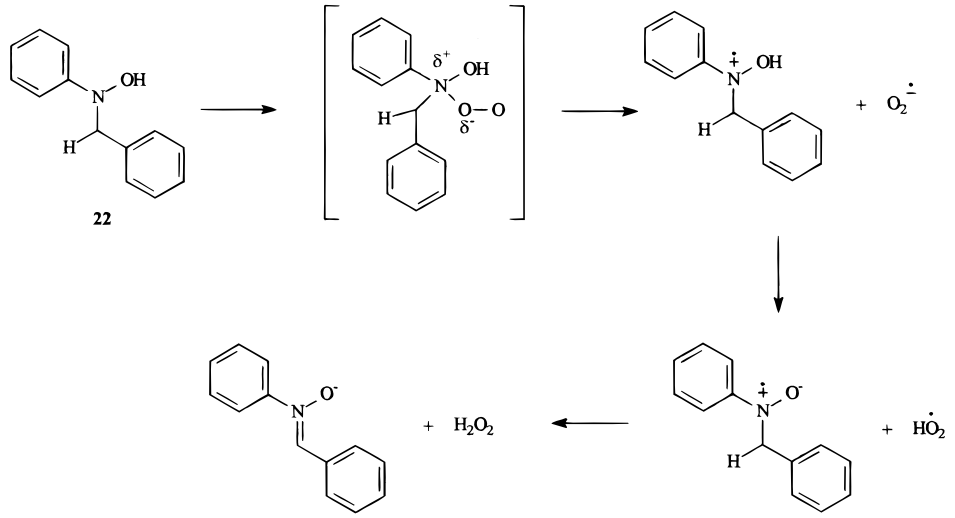
In summary, these reports indicate that nitro-substituted compounds are capable of cleaving DNA photochemically and that the nitro group likely abstracts hydrogen atoms from deoxyribose, leading to spontaneous cleavage, or from thymine methyl groups, resulting in piperidine-dependent, T-selective cleavage.

4. Hydroxyl Radical Generators

One of the most well-known DNA cleavage agents is the hydroxyl radical, which abstracts hydrogen atoms from deoxyribose to give sugar radicals and water. Several methods exist for production of hydroxyl radicals, the simplest of which involves irradiation of hydrogen peroxide with UV light, leading to O−O bond homolysis. MacGregor showed that irradiation of 100 mM H_2O_2 in the presence of DNA results in spontaneous, sequence-neutral cleavage of the DNA and that the method could be used for photofootprinting DNA-bound netropsin.[56]

Hydroperoxides such as **19** can be useful sources of hydroxyl radical.[57] Irradiation of **19** causes nicking of plasmid DNA while ESR experiments demonstrate that hydroxyl radicals are produced and trapped by DMPO. An important caveat in discussing these types of experiments is to note that the ESR studies are typically performed in the absence of DNA. While this undoubtedly simplifies the experiment (since DNA radicals will not be present), one must consider the possibility that the presence of DNA alters the photochemistry. This is probably not a concern for **19** since it probably binds to DNA with very low affinity. However, for other compounds, which have relatively high affinity for DNA, photochemical reaction between the DNA and the photocleavage agent could *precede* hydroperoxide degradation, significantly altering the cleavage mechanism. For example, the naphthalene diimide **20** was reported by Saito and co-workers to produce hydroxyl radicals on the basis of DMPO-trapping and ESR spectroscopy in the absence of DNA.[58] Photonicking of supercoiled plasmid DNA was also reported for this compound and the cleavage was inhibited by 1 mM sodium benzoate, consistent with a hydroxyl radical mediated cleavage mechanism. However, when the cleavage selectivity was studied with radiolabeled restriction fragments, damage was found to occur with high selectivity at the 5′-G of GG sites and required piperidine treatment. The need to perform piperidine treatment on the radiolabeled DNA in order to visualize cleavage sites whereas spontaneous cleavage was evident in the plasmid relaxation assays can be attributed to the much higher sensitivity of the latter technique. However, the sequence selectivity observed in the restriction fragment is inconsistent with hydroxyl radical mediated damage, which is typically sequence-neutral (unless the com-

Scheme 1



pound binds to the DNA sequence selectively). The numerous examples of photocleavers that exhibit the same cleavage selectivity but lack hydroperoxide groups (e.g., compound **39**) indicate that hydroxyl radicals are most likely not the agent responsible for this damage. (See section IV.B.)

An alternative source of hydroxyl radicals is the heterocyclic *N*-oxide derivative **21** synthesized by Maki and co-workers.[59] Irradiation of **21** in water results in production of 2 equiv of hydroxyl radical per *N*-oxide precursor, but radicals are not produced if the irradiation is performed in acetonitrile. The *N*-oxide was able to photonick plasmid DNA in a process that could be inhibited by the radical scavenger DMSO. However, production of hydroxyl radicals in the presence of DNA was not demonstrated. In subsequent work, the *N*-oxide was covalently linked to an acridine intercalator, but this failed to enhance the cleavage.[60] The sequence selectivity of the cleavage has not been reported.

More complex versions of the MacGregor experiment involve compounds that generate hydrogen peroxide photochemically. The peroxide can then decompose to form hydroxyl radicals under the irradiation conditions or after reduction by adventitious metal ions. Photocleavers of this type include the *N*-(arylalkyl)-*N*-phenylhydroxylamine **22**, which is proposed to undergo the O_2-dependent chemistry depicted in Scheme 1.[61] Experimental support for the mechanism comes from the observations that (i) hydrogen peroxide was detected after irradiation and (ii) the deprotonated amine radical cation was detected by ESR spectroscopy after spin trapping. Irradiation of **22** led to nicking of plasmid DNA, but the sequence selectivity of the cleavage was not reported.

Superoxide undergoes a spontaneous dismutation reaction in aqueous solutions, producing hydrogen peroxide and oxygen (eq 1):

$$O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

As mentioned above, hydrogen peroxide can decompose in the presence of UV light or after reduction by metal ions to produce hydroxyl radicals. Thus, any agent that is capable of reducing oxygen to superoxide can produce hydroxyl radicals.[62] There are many examples of natural[63] compounds which photocleave DNA by processes that are at least partially inhibited by radical scavengers. These photocleavers are also susceptible to inhibition by the enzyme catalase, which decomposes hydrogen peroxide to water and oxygen. Superoxide dismutase (SOD) is often used to detect superoxide in the reaction pathway; however, SOD simply catalyzes reaction 1, meaning that hydrogen peroxide production can be faster in the presence of SOD than in its absence. Thus, inhibition of DNA cleavage by SOD without catalase present could mean that superoxide plays a more significant and complex role in the cleavage mechanism than simply serving as a precursor to hydrogen peroxide. Complicating the situation even further is the fact that many of these compounds also can generate singlet oxygen, which is capable of initiating damage of the nucleic acid (see section IV.A).

The synthetic flavin-oligopyrrole compounds (e.g., **23**) reported by Giorgi-Renault and co-workers are interesting since they demonstrate spontaneous, sequence-selective cleavage of DNA in A/T-rich regions where the compounds are expected to bind.[64] The authors propose that the excited state of the flavin is initially quenched by electron transfer from the DNA and that the reduced flavin then transfers an electron to oxygen, forming superoxide and leading to production of hydroxyl radicals and cleavage of the DNA. Both superoxide and hydroxyl radicals are detected in solution in the absence of DNA if EDTA is included in the buffer as an electron donor. This report is interesting since the flavins are also known to damage DNA by electron transfer and have recently been shown to photocleave RNA (see sections IV.B.1 and VI.B, respectively). Thus the diversity of the nucleic acid cleavage pathways available to the flavins is impressive, although a direct H-abstraction reaction, a la the anthraquinones, has not been demonstrated.

5. Halogenated Organics

Halogenated organic compounds are of interest as photocleavage agents since irradiation can trigger carbon−halogen bond homolysis leading to production of halogen atoms and carbon-based radicals. Shibuya and co-workers extended their study of the oligopyrrole-based photocleavers (section III.B.3) to include halogenated derivatives. The *p*-chlorophenylsulfonamide derivatives **24** photonicked plasmid DNA and a significant decrease in the cleavage yield was observed for an analogue in which the chloro substituent was replaced by a methyl group.[65] The authors proposed that cleavage was due to H-abstraction by the phenyl radical resulting from C−Cl bond homolysis, but did not discount the possible involvement of the chlorine atom.

A second set of halogenated oligopyrroles was studied in which the halogen was placed on a terminal heterocycle (**25**).[66] Again, photonicking of plasmid DNA was observed, and the cleavage yield dropped significantly in the absence of the halogen. Interestingly, bromo-substituted analogues gave a higher yield of cleavage than did chloro-substituted analogues. If the chlorine atom were responsible for the cleavage, then one would expect the brominated versions to be far less active since the bromine atom will be a poor H-abstraction agent. Since the activity increased upon replacement of chlorine with bromine, it is likely that the aryl radical is responsible for the observed cleavage. However, since true quantum yield data (to account for differences in the absorption spectra of the compounds as well as potentially different bond homolysis efficiencies) is lacking, it is impossible to say definitively that the aryl radical is the sole cleavage agent.

Hecht and co-workers synthesized halogenated bithiazole reagents **26** based on the bleomycin structure.[67] The chlorinated derivative photonicks plasmid DNA at very low concentrations (10−50 nM). Interestingly, and in distinct contrast to the observations regarding the oligopyrroles, the cleavage efficiency is much higher with the chlorobithiazole than with the brominated version, implicating the chlorine atom in the cleavage mechanism. Thus, photooxidation of chloride anion by anthraquinones and photolysis of aryl chlorides are viable pathways for generation of chlorine atoms that subsequently cleave DNA by H-abstraction.

Aryl iodides have also been studied as DNA cleavage agents since photoinduced C−I bond homolysis also produces radicals that can abstract H atoms from deoxyribose. In these cases, however, there is little ambiguity with regard to the identity of the cleavage agent since the iodine atom is a most unlikely H-abstraction agent from deoxyribose. Martin and colleagues have synthesized iodinated analogues of the minor-groove binding drug Hoechst 33258 to probe DNA conformation and to map the drug binding sites by inducing DNA cleavage. Initially, this was accomplished with the use of I-125 labeled Hoechst, which cleaved DNA radiolytically. More recently, nonradioactive iodinated derivatives (**27**) have been synthesized and used as photocleavers.[68] These compounds photocleave radiolabeled restriction fragments in the expected A/T-rich sequences. Analysis of the termini of the cleavage fragments reveals that bands are observed which migrate slower than 5′-phosphates but which are converted into 5′-phosphates by piperidine treatment. This behavior is consistent with the proposal that at least some of the cleavage involves abstraction of a hydrogen from C-5′ of deoxyribose. Similar observations are made for the meta- and para-iodinated Hoechst analogues, whereas the ortho isomer only gives 5′-phosphates before and after piperidine treatment, indicating abstraction of a different hydrogen atom. This set of compounds illustrates how sensitive the DNA cleavage mechanism is to the structure of the photocleavage agent, primarily because of the geometrical requirements for H-abstraction reactions.

Another iodinated DNA binding ligand is the iodoacridine derivative **28**. Irradiation of DNA-intercalated **28** leads to nicking of supercoiled plasmids.[69] While photoinduced bond homolysis is efficient in solution, the authors propose that electron transfer from the nucleobases to the excited-state acridine occurs first in DNA on the basis of fluorescence quenching experiments. The resulting acridinyl radical anion then decomposes to form the acridinyl radical and iodide anion, with the former then presumably initiating cleavage by abstraction of hydrogen. Of course, some damage due to the oxidized base could also occur and should show up as piperidine-dependent cleavage at GG sites in restriction fragments; the sequence selectivity of cleavage by **28** has not been reported.

6. Additional H-Abstracting Photocleavage Agents

Two recent examples of photocleavage agents present similar strategies for generating reactive species to damage DNA. The *N*-(aroyloxy)-2-thiopyridones (e.g., **29**) studied by Theodorakis and Wilcoxen undergo N−O bond homolysis in the excited state to generate two free radicals.[70] The aroyl radical is stable to decarboxylation and can be trapped by H-donors such as thiols. Irradiation of these compounds in the presence of supercoiled DNA leads to spontaneous nicking of the DNA, suggesting that deoxyribose plays the role of H-donor for the aroyl radical.

Shinkai and co-workers also used photoinduced bond homolysis to initiate DNA cleavage.[71] They observed that irradiation of pyrenyl boronic acid (**30**) leads to formation of deuteriopyrene in the presence of D_2O or CD_3CN, indicating that irradiation leads to C−B bond homolysis and the remaining pyrenyl radical abstracts a deuteron from the solvent. Compound **30** binds to DNA, apparently by intercalation, with an association constant of 1.27×10^5 M^{-1}. Irradiation in the presence of supercoiled plasmids leads to spontaneous nicking of the DNA. This process is unaffected by sodium azide but is inhibited by the radical scavenging nitroxyl reagent TEMPO. It will be interesting to see if photocleavage by either the (aroyloxy)thiopyridones or pyrenylboronic acid reagents occurs with any sequence selectivity.

In conclusion, a variety of photocleavers function by abstracting hydrogen atoms from the deoxyribose

moieties of DNA. Cleavage by this process is inherently sequence neutral; sequence selectivity arises from discrimination between binding sites and/or conformations.

IV. Photocleavage Agents Which Target Nucleobases

Compounds that react with the nucleobases of DNA have relatively few pathways open for inducing permanent damage to the DNA in comparison with systems that react with the sugar residues. This class of photocleavage agents function by three distinct processes: (1) direct electron transfer from the base to the excited-state photocleaver; (2) triplet energy transfer from the excited photocleaver to O_2, producing singlet oxygen, which then reacts with the base; and (3) formation of an adduct with the base.

The oxidation potentials and reactivities of the four bases vary over a wide range, with guanine being the most easily oxidized as well as the most reactive toward singlet oxygen. Thus, in the first two mechanisms listed above, cleavage occurs almost exclusively at guanine, regardless of the binding site. On the other hand, adduct formation occurs with different selectivities, depending on the mechanism. In virtually all cases, damage of the base by these three mechanisms does not lead to spontaneous cleavage of the DNA; incubation of the irradiated nucleic acid with hot piperidine or aniline is usually required in order to reveal the damage. Mechanisms of cleavage arising from oxidative attack on nucleobases are discussed in detail in the review by Burrows.[5] Therefore, this section will focus on how the damage is initiated rather than on how the base degrades.

A. Singlet Oxygen Generators

Electronically excited compounds that intersystem cross to the triplet state and have a sufficiently high triplet energy can generate singlet oxygen by energy transfer to O_2. Singlet oxygen is a highly reactive species that preferentially adds to guanine.[72] The resulting oxidized guanine is sensitive to piperidine treatment which induces a strand break with little variation in cleavage yield at the various G sites in the DNA.[73,74] Performing the experiment in D_2O rather than H_2O can lead to a substantial increase in the cleavage efficiency since the lifetime of singlet oxygen is significantly longer in the former.[75]

Numerous systems have been reported to cleave DNA by a singlet oxygen mechanism. Unfortunately, G-selective cleavage was demonstrated in only a few cases. Rather, most experiments were done with supercoiled plasmid DNA and the observation of spontaneous cleavage that was enhanced in D_2O and/or inhibited by singlet oxygen quenchers such as azide or *N*-acetylhistidine were taken as evidence for a singlet oxygen pathway. One complication of this approach is that it is often difficult to distinguish between compounds that generate singlet oxygen and those that oxidize the DNA directly by electron transfer. For example, inhibited cleavage by azide could arise from reaction between the excited-state photocleaver and the azide prior to singlet oxygen production. Likewise, the excited states of some compounds increase in D_2O, potentially accounting for an enhanced cleavage yield.

1. Porphyrins

Numerous examples of G-selective photocleavage of DNA by porphyrin derivatives have been reported. Porphyrins are known singlet oxygen generators[76] and cleave DNA selectively at guanine residues.[77] Photocleavage of duplex DNA by porphyrins is reviewed in ref 77; this section will deal with photocleavage by "expanded" porphyrins as well as selective cleavage of nonduplex structures by porphyrins. A later section (IV.D) will review the numerous studies in which porphyrins have been covalently linked to a DNA oligonucleotide in order to obtain sequence-selective photocleavage agents.

Magda, Sessler, and co-workers reported the synthesis and DNA photocleavage properties of the expanded porphyrins **31** and **32**.[78] These compounds were found to initiate photonicking of supercoiled plasmid DNA upon irradiation at wavelengths above 700 nm. Cleavage by **31** was ~5−6-fold more efficient than for **32**. Sodium azide strongly inhibited cleavage as did oxygen removal by argon purging, indicative of a singlet oxygen mechanism. The effect of D_2O on the cleavage efficiency was not reported. The significance of this work lies in the long wavelength of irradiation which was used: far-visible/near-IR radiation can be used in vivo for photodynamic therapy applications of the expanded porphyrins.

The relatively modest cleavage efficiency for **31** was enhanced by linkage of the expanded porphyrin to cationic ammonium groups (e.g., **33**).[79] The substantial improvement in cleavage was attributed to stronger electrostatic attraction between the ammonium-substituted derivatives and the DNA target. Spectrophotometric analysis indicated that the cationic substituents increased the equilibrium binding constant by 10−20-fold.

Porphyrins can also be used to probe nonduplex structures in nucleic acids. One particularly interesting application was described by Leontis and coworkers.[80] Tetracationic porphyrins (**34**) were found to bind with high selectivity at a DNA three-way junction, then initiate photocleavage at guanine residues adjacent to the junction. It is unclear at this time just how the cleavage selectivity is partitioned between the selective binding of the porphyrins at the junction and selective reaction of the guanines present in the junction (as opposed to those in duplex regions which are likely to react more slowly with singlet oxygen). The porphyrins are potentially valuable probes because, while they can intercalate into duplex DNA, these results, as well as others described in section VI.C, indicate that the porphyrins may prefer to bind in regions which are somewhat more flexible.

2. Ru(II) Complexes

Polypyridylruthenium(II) complexes (Figure 4) are well-known photocleavers. Simple derivatives, such as the trisbipyridyl or trisphenanthroline complexes

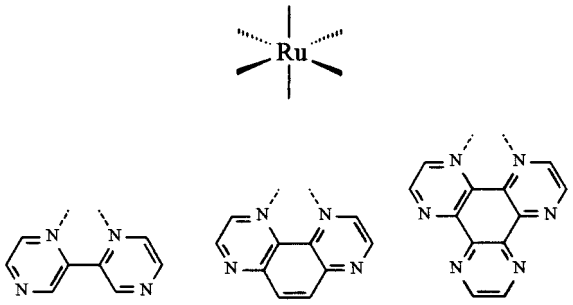


Figure 4. Commonly used ligands for Ru(II) photocleavage agents.

initiate cleavage by generating singlet oxygen.[81] Other Ru(II) complexes are reported to cleave DNA by the same mechanism,[82] although Paillous and co-workers reported that bipyrazyl (bpz, Figure 4) rather than bipyridyl (bpy) ligands caused a change in the cleavage mechanism, presumably to one in which the complex oxidizes the DNA by electron transfer.[83]

O'Reilly, Kelly, and Kirsch-De Mesmaeker covalently linked two Ru(II) complexes (**35**); the resulting bimetallic complex exhibited ~100-fold enhancement in binding and significantly more efficient photonicking of supercoiled DNA relative to the corresponding mononuclear analogues.[84] This intriguing study suggests the synthesis of more multinuclear metal complexes to study polyfunctional interactions with DNA.

Kelly and co-workers have also studied the interaction between Ru(II) complexes bearing strongly oxidizing ligands such as (hat) or (tap) with DNA (Figure 4).[85] These complexes react with DNA primarily by electron-transfer rather than by generation of singlet oxygen, as determined by time-resolved laser spectroscopy. In addition, the complexes can induce spontaneous nicks in supercoiled DNA and form photoadducts with guanine residues that are oxidized. The sequence selectivity of the cleavage has not been reported but could provide considerable insight into whether the observed electron-transfer chemistry leads to strand breaks (as described in the next section).

3. Vanadium(V) Complexes

DNA photocleavage by vanadium(V) bisperoxo complexes (vanadates) is worth noting because of the novel mechanism by which it generates singlet oxygen. Dabrowiak and co-workers initially demonstrated plasmid nicking by **36** and proposed the intermediacy of a vanadium(IV) complex as well as oxy radicals in the cleavage mechanism.[86] Subsequent work by Chan and co-workers revealed that a vanadium(IV) complex was in fact produced but that it was the result of photoinduced expulsion of one of the peroxo ligands as singlet oxygen, which then initiated DNA damage.[87] Several experiments provided support for this mechanism, including the observation of enhanced cleavage in D_2O, inhibited cleavage in the presence of azide and the generation of singlet oxygen even under anaerobic conditions. Thus, the complex provides its own equivalent of oxygen for the cleavage process. The vanadates differ from those agents that produce $^1O_2$ by energy transfer since they are not regenerated in the process and therefore cannot make $^1O_2$ photocatalytically. However, the interesting mechanism and insulin-mimetic properties of these compounds[88] certainly justifies the investigation of the photochemistry.

B. Electron-Transfer Agents

Oxidation of guanine (G) by an excited-state photocleaver (P) produces the G radical cation and the P radical anion (eq 2):

$$P^* + G \rightarrow P^{\bullet -} + G^{\bullet +} \qquad (2)$$

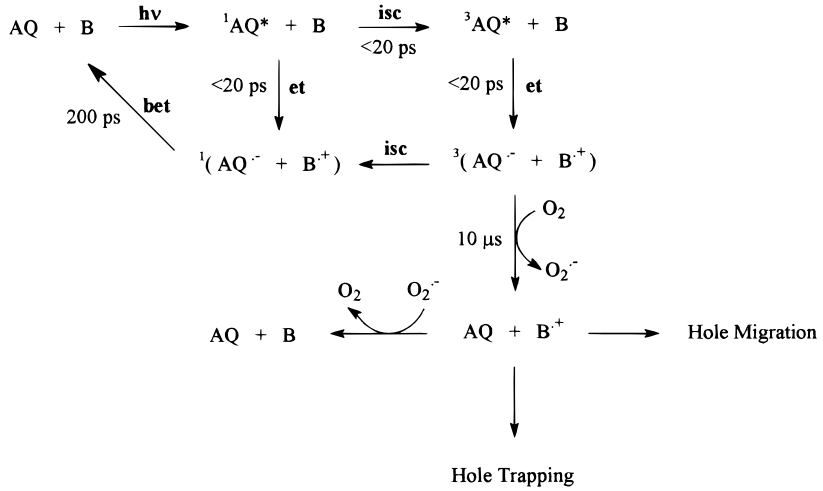
The subsequent reactivity of $G^{\bullet +}$ is complex and highly dependent on the secondary structure of the DNA, leading to both 8-oxo-G and oxazolone decomposition products.[89] 8-Oxo-G is often detected in conjunction with photocleavage at G by electron-transfer agents, but Cullis and co-workers have recently questioned whether this lesion is the one which is actually cleaved by piperidine treatment or is merely incidental.[90] In any event, photocleavage of B-form DNA by electron-transfer agents is highly selective for guanines but can be distinguished from singlet oxygen cleavage based on the observation that there is a wide variation in the efficiency of cleavage at different G sites depending on the flanking sequences. In particular, guanines located on the 5′-side of at least one other G are strongly preferred over all other cleavage sites, and even then, not all GG sites are cleaved equally. Whether this is due to unequal binding preferences for the photocleavage agent or to different reactivities of the sites remains to be determined.

An early report by Hélène and co-workers demonstrated that irradiation of 3-carbethoxypsoralen (**37**) in the presence of DNA followed by piperidine treatment resulted in selective cleavage at GG sites with a modest preference for the 5′-G.[91] Understanding of the mechanism was complicated by the observation of enhanced cleavage in D_2O and inhibited cleavage by azide. Thus, while the cleavage pattern was inconsistent with a singlet oxygen process, the mechanistic experiments were. It is quite possible that both singlet oxygen and electron-transfer pathways were operative. Another source of concern was the use of a Tris buffer, since earlier work demonstrated that irradiation of methylene blue and DNA in a Tris buffer led to formation of adducts between the Tris and the DNA at G residues.[92] While the results certainly do not permit conclusive determination of the cleavage mechanism, the observation of GG selective cleavage provides a useful starting point for discussion of this class of photocleavers.

1. Riboflavin and the Naphthalimides[93]

In 1993, Kawanishi and co-workers reported that irradiation of riboflavin (**38**) with UV light in the presence of DNA led to piperidine-dependent cleavage at the 5′-G of GG steps.[94] 8-Oxo-dG was detected as a byproduct of the chemistry, but no other mecha-

Scheme 2



nistic data was presented. An electron-transfer mechanism was suggested but the 5′-preference for the cleavage could not be explained.

As mentioned in section III.B.4, the dihydroperoxide **20** exhibits GG-selective photocleavage of DNA.[58] The observation that lysine-naphthalimide **39** cleaves DNA with the same preference demonstrates that the hydroperoxide functionality is not required for GG cleavage.[55] (Although the fact that diimides **40a** and **40b** fail to cleave DNA is curious.) Saito and coworkers performed mechanistic experiments in an effort to understand what factors led to cleavage at GG sites.[95] The quantum yield for cleavage of the hexamer duplex 5′-TTGGTA-3′•5′-TACCAA-3′ by **39** was determined to be 3×10^{-4} with an 8:2 preference for the 5′-G. Laser spectroscopic experiments demonstrated production of the imide radical anion with the same microsecond time scale kinetics as quenching of the imide triplet state, providing clear evidence for a photoinduced electron-transfer reaction.

Ab initio calculations have been used to try to explain the intriguing selectivity for cleavage at GG steps by electron-transfer agents. Sugiyama and Saito reported the results of calculations on all 10 of the possible stacked, base-paired dinucleotide steps which revealed that GG steps were by far the most easily oxidized of the 10 and that the highest occupied molecular orbital (HOMO) for GG was localized almost exclusively on the 5′-G (provided a "normal" B-form DNA conformation is used), both in the neutral and cation radical forms.[96] Prat, Houk, and Foote recently proposed an elegantly simple model to explain this localization based on electrostatic interactions.[97] They noted that positions N7 and O1 of guanine bear substantial partial negative charges. When located on the 3′-side of another guanine residue, these atoms are located in favorable positions to stabilize the positive charge which is present in the cation radical on the 5′-G. In particular, N7 is located directly beneath the six-membered ring of the 5′-G.

2. Anthraquinones

The hydrogen abstraction chemistry of anthraquinone (AQ) derivatives described in section III.B.1 requires that the AQ not intercalate. This restriction arises because when the AQ intercalates, it reacts very rapidly with the nucleobases by photoinduced electron transfer. Schuster and co-workers demonstrated that the radical anion of **41** is produced by electron transfer from a nucleobase less than 20 ps after excitation with a laser pulse, effectively precluding reaction by other pathways.[35] The radical anion subsequently decays by approximately 30% over the next 100−200 ps but is then stable for several microseconds, until it transfers an electron to oxygen, thereby producing superoxide and recycling the AQ (eq 3):

$$AQ^{\bullet-} + O_2 \rightarrow AQ + O_2^{\bullet-} \qquad (3)$$

Schuster and Breslin demonstrated that irradiation of intercalated AQ derivatives and subsequent piperidine treatment led to GG selective cleavage, as expected for an electron-transfer mechanism.[36] Reaction 3 could have considerable importance since it prevents the electron from returning directly from the reduced AQ to the oxidized base and allows the oxidized base to be trapped, ultimately leading to cleavage.

The quantum yield for cleavage of 5′-GCGCAATG-GAAA-3′•5′-TTTCCATTGCGC-3′ by **41** was determined to be 1.4×10^{-2} under atmospheric conditions, with a 9:1 preference for cleavage at G-7 versus G-8.[98] (No other cleavage products were observed.) In addition, the chemical yield in this experiment was greater than 80%, demonstrating the robustness of the AQ as a photocleaver. Rigorous removal of oxygen decreased the quantum yield more than 10-fold. Interestingly, oxygen saturation also decreased the quantum yield. Thus, some oxygen is required for the cleavage but too much becomes inhibitory. Importantly, addition of superoxide dismutase (to scavenge the superoxide produced by AQ recycling) increased the quantum yield to 2.0×10^{-2}, indicating that superoxide inhibits the cleavage.

The mechanism outlined in Scheme 2 is consistent with the laser spectroscopic and quantum yield data. The excited state, intercalated AQ has two reaction paths open to it: intersystem crossing from the

singlet to the triplet state, and electron transfer from a nucleobase. The former reaction can be quite fast (<20 ps) in AQ derivatives, meaning it can conceivably compete with the rapid electron-transfer chemistry. The fast but incomplete decay of the radical anion is attributed to return electron transfer within the singlet radical ion pair formed prior to intersystem crossing. The stable radical anion is then assigned to the product of electron transfer to the triplet state AQ. The resulting radical ion products cannot undergo return electron transfer since the unpaired electron on the AQ and base have the same spin. This stabilizes the radical anion until oxygen removes the electron from the helix, leaving the base radical cation time to be trapped by addition of water and/or oxygen and explains the decreased quantum yield in the absence of oxygen. The decreased quantum yield in O_2-saturated samples and the increased quantum yield in the presence of SOD are attributed to electron transfer from superoxide to the base radical cation, effectively repairing the damage initiated by electron transfer from the base to the AQ. Thus, too little oxygen cannot prevent return electron transfer from occurring while too much oxygen permits the concentration of superoxide to increase to the point where it can act as a relay in transferring electrons from reduced AQs to oxidized bases and compete with trapping of the radical cation.

Compound **42**, reported by Brun and Harriman,[99] provides an interesting comparison to the AQ intercalators. This compound also binds to DNA by intercalation and photooxidizes the nucleobases by electron transfer within 20 ps of excitation. In addition, back-electron transfer occurs within 100 ps but, unlike the anthraquinone intercalators, this reaction goes to completion. Thus, the "hole" introduced into the DNA rapidly recombines with the electron on the intercalator and no permanent damage to the DNA occurs. Perhaps significantly, intersystem crossing for **42** is $\sim 10^6$-fold slower than electron transfer, meaning that only singlet radical ion pairs are produced. These observations provide support for the idea that fast intersystem crossing to the triplet state is an important criterion in determining which photooxidants will actually initiate GG cleavage.

If superoxide does, in fact, transfer an electron to the oxidized base before the base is irreversibly trapped, then it implies that the trapping step requires more than a few microseconds, since this is the time scale on which transfer of the electron from the reduced quinone to oxygen occurs. An alternative reaction that could be available to the oxidized base on this time scale is to accept an electron from a nearby base, effectively transferring the "hole" one position further away from the AQ. The hole will then migrate through the DNA molecule by a series of electron-transfer reactions until it reaches a site that is of low oxidation potential and/or high trapping reactivity. Precedent for this type of migration of oxidative damage comes from the radiation chemistry and biology literature, where it has been demonstrated that oxidation of DNA under conditions where all bases can lose an electron leads to permanent damage of the DNA only at guanines. Of particular interest is the report by Steenken and coworkers that irradiation of DNA with ionizing radiation leads to selective cleavage of DNA at GG sites,[100] similar to electron-transfer photooxidants, indicating that the holes are mobile in both cases.

3. GG Cleavage by Covalently Linked Photooxidants

The concept that the hole, once injected into the DNA by the photocleavage agent, is free to migrate until it reaches a low oxidation potential GG site and is trapped, suggested that the photocleaver need not even be bound at the GG site. The ideal way to test this hypothesis would involve covalently linking a photooxidant at one position in the duplex and looking for photoinduced cleavage at GG sites which are too far away for the compound to contact directly. Barton and co-workers recently reported one such system in which a Rh(phi) complex was linked to the 5′-terminus of a DNA oligonucleotide which was then hybridized with a complementary strand.[101] Irradiation at 313 nm led to the normal hydrogen abstraction chemistry (see section III.A.2), demonstrating that the complex had access only to the last few base pairs of the duplex. However, irradiation at 365 nm led to GG-selective cleavage at sites that were far removed from the metal complex. A preference for the 5′-G at these sites and the observation that switching from a 5′-GGC-3′ to a 5′-GCG-3′ site eliminated that preference were taken as evidence in support of an electron-transfer mechanism and against a singlet-oxygen mechanism. These results indicate that the photocleaver can be bound at one site in a duplex and initiate damage at far away sites by an electron-transfer pathway.

The quantum yield for damage at the remote guanine sites by the tethered Rh(phi) complex is only 3×10^{-8}, nearly 6 orders of magnitude lower than that reported for the untethered AQ derivative. Whether this is due to the electron-transfer chemistry (i.e., more efficient back-electron transfer or less efficient forward electron transfer in the rhodium system) or to the relative efficiency of trapping the hole at the different GG sites studied in the two reports remains to be determined.

An additional intriguing feature of this system is the observation that the cleavage efficiency did not decrease with increasing distance. Thus, essentially equal cleavage was observed at the 5′-G of GG sites located $\sim$5 and 10 bp away from the rhodium complex. The authors considered two mechanisms for the lack of distance dependence in cleavage. The possibility exists that the electron is transferred directly from the GG sites to the rhodium complex over distances of tens of angstroms. Alternatively, the distance-independent cleavage could arise if the rate of trapping were significantly slower than the rate of hole migration. This would permit the hole to equilibrate throughout the DNA and be trapped at sites depending on their oxidation potentials and reactivity rather than on their proximity to the photocleaver. Distinction between these two models will require further experiments.

Subsequent work by Barton's group has focused on the role of DNA secondary structure on the efficiency

of remote GG cleavage by tethered photosensitizers. In one experiment, insertion of extra bases on one strand of the duplex between two GG sites led to reduction in cleavage at the distal site relative to the proximal site by factors of 1.2−4.0, depending on the number and identity of the bases constituting the "bulge".[102] The authors concluded that the disruption of the double-helix by the bulge impeded migration of the hole to the distal trap site. In another system, mismatched base pairs within the duplex caused a minor decrease in the cleavage efficiency at a downstream GG site.[103]

In addition to these experiments, Gasper and Schuster recently reported the effect of an abasic site (i.e., a hydrogen replaces a nucleobase at one position) on cleavage at remote GG sites by 5′-tethered anthraquinone photosensitizers. Insertion of such a "gap" had virtually no effect on photocleavage at either upstream or downstream GG sites. The conclusion to be drawn from these experiments is that bulges, mismatches, and gaps do not perturb the DNA secondary structure sufficiently to reduce the electronic coupling to a level where migration of the oxidative damage is completely inhibited.

A different type of experiment, also reported by Gasper and Schuster, demonstrates how one might actually trap a migrating hole.[104] They replaced the 5′-G of a proximal GG site with 8-oxo-G. This base still forms a stable base pair with C but its oxidation potential is ~0.4 eV lower than G, meaning that the proximal (8-oxo-G)G site is a much deeper trap for the hole to fall into than the distal GG site. In fact, irradiation of the tethered AQ results in effective cleavage at the 8-oxo-G position while cleavage at the distal GG site is dramatically reduced. One drawback to this experiment is that there are at least two explanations for why the hole is trapped at the 8-oxo-G: (i) the oxidation potential is so much lower than at any of the other bases, precluding further "hopping" of the hole or (ii) the reaction of the one-electron oxidized form of 8-oxo-G could be significantly faster than for the corresponding form of G. Nevertheless, it clearly demonstrates that the GG preference for cleavage can be overcome by a deep trap.

The experiments described above probe many of the characteristics of GG photocleavage by electron-transfer agents, but do not provide insight into the question of whether the electron is transferred directly from the GG step to the excited state photocleaver or if the hole migrates to the GG site by a hopping mechanism beginning with initial oxidation of an adjacent base by the photocleaver followed by a series of thermal electron transfers along the DNA helix until the hole is trapped at a GG site. Schuster and co-workers recently reported experiments that shed some light onto this question.[105] They synthesized a peptide nucleic acid (PNA)-AQ conjugate in which the AQ was positioned at the central position of a 19mer. (PNA is an analogue of DNA in which the sugar−phosphate backbone is replaced by a polyamide; see Figure 5.) The PNA-AQ conjugate hybridizes with its DNA complement to form a duplex in which the AQ is intercalated at the central position. GG sites are located on the DNA strand

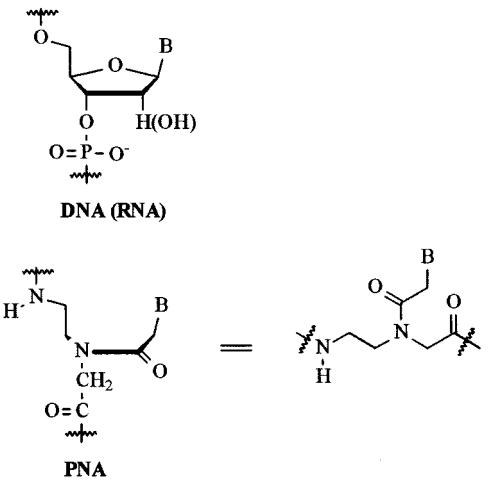


Figure 5. Comparison of DNA, RNA, and PNA (peptide nucleic acid) chemical structures.

three base pairs away in one direction (site A) as well as three and seven base pairs away in the other direction (sites B and C, respectively). Irradiation of the AQ results in selective cleavage at all three of these sites. Substituting the 5′-G at site B with an 8-oxo-G resulted in enhanced cleavage at site B but completely eliminated cleavage at site C, consistent with the observations of Gasper and Schuster. However, cleavage at site A was unaffected by the substitution of 8-oxo-G for G. These results argue strongly in favor of a mechanism by which the hole migrates to the GG sites by a series of discrete hops, since an instantaneous, long-distance electron-transfer event from the 8-oxo-G to the AQ should lead to inhibited cleavage at both sites A and C. While this data does not settle the issue regarding the mechanism involved in remote GG cleavage within a DNA−DNA duplex, it is worth noting that the base pairs in PNA−DNA should actually be better stacked than they are in B-form DNA, due to a smaller pitch for the PNA−DNA helix.[106] Thus, both the short- and long-range electronic coupling through the π-stack should be greater for PNA−DNA.

4. Cosensitization

One of the key components to successful cleavage of DNA by an electron-transfer mechanism is inhibiting the exergonic back electron transfer. For AQ derivatives, and most likely for the other compounds described above, this is achieved in large part by using compounds which can intersystem cross to the triplet state prior to electron transfer. Another approach is to use a cosensitizer, as described by Kochevar and co-workers.[107] Ethidium bromide (**43**) and methyl viologen (**44**) were simultaneously bound to duplex DNA and then irradiated with visible light, which only the ethidium can absorb. The ethidium does not react appreciably with the DNA by electron transfer but it can be oxidized by the viologen, leaving the ethidium radical cation and the viologen radical anion. The oxidized ethidium can then accept an electron from one of the nucleobases, retarding back electron transfer. Meanwhile, the reduced viologen can give the electron to oxygen, further separating the hole from the electron. This process leads to

G-selective cleavage of duplex DNA, although the GG-selectivity characteristic of electron-transfer chemistry has not been demonstrated.

5. Other GG-Selective Photocleavers

A surprising report was made by Kuroda and co-workers in conjunction with their study of nitro-substituted oligothiazole reagents (see section III.B.3). While the uni- and bithiazole reagents (**17**) apparently cleaved by hydrogen abstraction chemistry at their preferred binding sites, the terthiazole derivative cleaved at the 5′-G of GG sites and only after piperidine treatment.[54] One possible explanation for these results is that the terthiazole reagent follows a cosensitization mechanism similar to that described above. Irradiation could lead to photo-induced electron transfer from the terthiazole unit to the nitrobenzamide, with the oxidized terthiazole then abstracting an electron from the DNA.

It has also been reported that the DNA topoisomerase I inhibitor, camptothecin (**45**), leads to piperidine-dependent GG cleavage with a preference for the 5′-G.[108] Catalase was reported to inhibit the cleavage but mannitol did not, thus the mechanism for cleavage is currently unclear.

C. Other Nucleobase-Directed Photocleavage Agents

There are numerous chromophores that will form photoadducts with DNA. The sequence selectivity of these reagents varies considerably depending on the mechanism by which they react with the DNA. In some cases, such as the psoralens, the excited state compound reacts directly with the DNA while in other cases, such as aryl azides, the excited state undergoes an initial reaction to produce a ground-state, highly reactive intermediate which subsequently adds to the DNA. While the DNA is sometimes cleaved spontaneously, piperidine treatment generally is used to fully reveal the sites of adduct formation. Early work involving aryl azides was reviewed by Kochevar and Dunn.[2]

1. Psoralens

The psoralens are perhaps the most well-known class of compounds which react with DNA by forming cycloaddition products with the nucleobases, particularly thymine, although recent work has demonstrated that DNA cleavage occurs as well.[109,110]

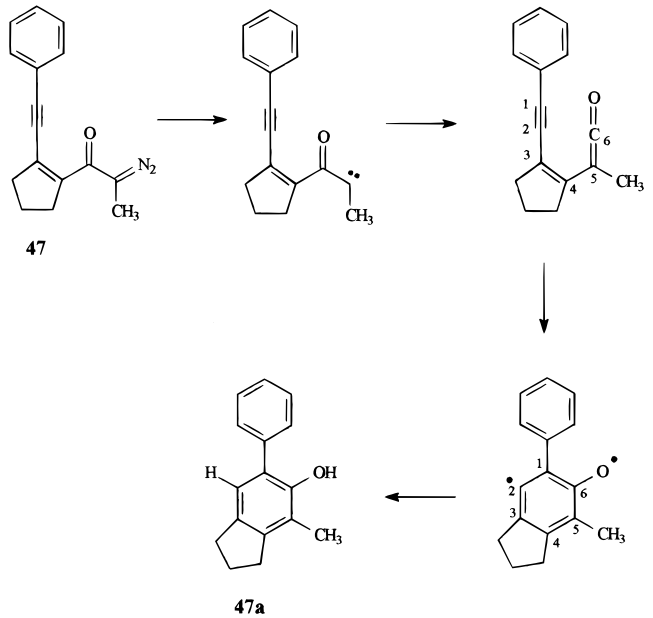
Hopkins and co-workers reported the covalent linkage of a psoralen derivative (**46**) to a DNA oligonucleotide and subsequent photo-cross-linking to complementary regions of single stranded DNA.[111] Treatment of the cross-linked products with sodium borohydride and aniline led to reversion of the cross-link and partial cleavage specifically at thymine residues adjacent to the psoralen position, thereby identifying the site of cross-linking. Compounds of this type could be envisioned as probes for single-stranded DNA regions; however, the oligonucleotide would have to be the right length in order to achieve hybridization and to position the psoralen at the appropriate distance from a thymine.

2. Diazo and Azido Compounds

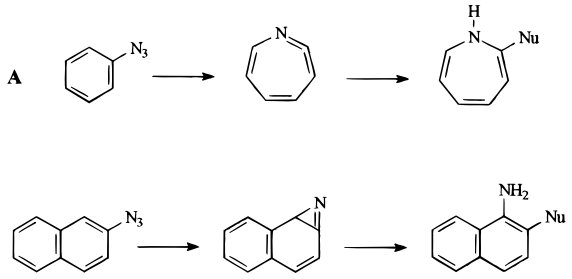
Saito and co-workers reported the DNA-cleaving properties of diazo compounds **47**[112] and **48**.[113] Irradiation of these compounds should initiate expulsion of nitrogen, leaving a carbene behind. When **47** is irradiated in toluene solution in the presence of the hydrogen donor 1,4-cyclohexadiene, product **47a** is obtained. The authors proposed that the carbene undergoes a Wolff rearrangement to form a ketene, which then cyclizes to form the diradical intermediate (Scheme 3). Abstraction of hydrogen from the cyclohexadiene then yields the isolated product, and it is not difficult to envision a similar process occurring in the presence of DNA. In fact, both diazo compounds nick supercoiled DNA. However, a radiolabeled linear DNA target revealed that compound **48** selectively cleaved at G residues and that the cleavage required piperidine treatment; the sequence selectivity of cleavage by **47** was not reported. Significantly, the photochemistry of **47** was investigated in an aqueous acetonitrile solution and no products due to cyclization could be found. The authors propose that the compound reacts with DNA from the carbene intermediate prior to undergoing the Wolff rearrangement. However, it is possible that the rearrangement occurs but that the ketene is trapped prior to cyclization. Ketenes can react with amines and the exocyclic amino group of guanine might be a trapping agent. Further characterization of the DNA cleavage products are required in order to elucidate the mechanism for cleavage by these diazo compounds; however, these reports point out the dangers in trying to apply product analysis studies performed in organic solution to draw conclusions regarding a DNA cleavage mechanism.

The cleavage results for **48** should be compared with those for the diazocyclopentadiene derivative **49**, originally reported by Nielsen and co-workers.[114] This compound cleaves DNA with virtually no sequence selectivity and has been used for photofootprinting. The drastically different cleavage properties of **48**

Scheme 3



Scheme 4



and **49** could be due to either differential binding preferences or to chemical reactivity differences. In particular, the carbene expected to arise from extrusion of nitrogen from **49** cannot undergo the Wolff rearrangement.

Azido compounds are often used as photoaffinity labels to probe binding of ligands to protein receptors. Rill and co-workers recently reported the use of azide substituents on the DNA intercalators ethidium and actinomycin D to investigate their preferred intercalation sites (e.g., **50**).[115] Irradiation of aryl azide compounds can initiate expulsion of nitrogen to leave behind a nitrene, which can rearrange to form either an azaheptatetraene or an aziridine (Scheme 4).[116] The propensity for either of these species to react with nucleophiles supports the idea that the azide compounds react with DNA by forming adducts with the nucleobases. Piperidine treatment was required in order to observe cleavage and some variation was noted in the base selectivity; however, the cleavage efficiencies varied over a range of only 2−5 for the two compounds so all four bases are effectively targeted. These results indicate that azido substitution and photocleavage could be used as a general method for detecting ligand binding sites on DNA.

In contrast to these results, the azidobenzoyl acridine compound (**51**) reported by Nielsen and co-workers induces photocleavage of DNA specifically at A and G sites. The damage is only observed after piperidine treatment and all adenines and guanines are cleaved with comparable efficiency. These results provide indirect evidence for adduct formation between the photocleavage agent and the nucleobase, since oxidative reaction with the bases should occur preferentially at guanines. Presumably, the difference in cleavage selectivity reported for the azidoethidium and **51** arises from the positioning of the azido group by the intercalator. Neither ethidium nor acridine have pronounced sequence selectivity in their DNA binding, a fact which is reflected in the cleavage by **50**. The close proximity of the azido group to the intercalating phenanthridinium ring restricts reaction to the intercalation site. On the other hand, the relatively long, flexible linker separating the azidobenzoyl group from the intercalating acridine in **51** should permit attack at the most reactive sites; i.e., the more electron-rich purine bases.

It should be pointed out that while the diazo and azido compounds described are successful photocleavers, very little has been reported with regard to the actual mechanisms of reaction. Much is known about the photochemistry of these functional groups in solution, but there is no guarantee that similar reaction pathways are followed in the presence of DNA, particularly if the compound is bound strongly to DNA. Thus, while there is certainly precedent for the idea that these compounds can attack DNA by forming adducts with the nucleobases, direct evidence that this is actually occurring in DNA has not been provided.

3. Carbocation Precursors

The quinolylmethylisothioronium salts **52**, reported by Buchardt and co-workers, are designed to generate carbocations upon irradiation.[117] UV−titration curves show that reagent **52** clearly binds to DNA. Irradiation leads to essentially equal cleavage at each G in a restriction fragment after piperidine treatment. The authors proposed that irradiation initiated heterolytic carbon−sulfur bond cleavage to produce a carbocation, which then adds specifically to guanine. The lack of a dependence on oxygen was taken as evidence against the involvement of singlet oxygen in the system.

Saito and co-workers reported the photoaddition chemistry of thioacetal derivative **53**, linked to the ever-useful naphthalimide chromophore.[118] Irradiation of **53** in the presence of calf thymus DNA led to the alkylation of adenine at N-7 as identified from a mixture of products by HPLC analysis. The adduct was released from the DNA only after heating the samples to 90 °C for 20 min prior to HPLC. Nicking of plasmid DNA was also reported although, given the complex array of chemistries exhibited by naphthalimide derivatives,[119] it is quite possible that the cleavage and alkylation reactions are independent pathways from the excited state.

In mechanistic experiments, the authors were able to trap cation **53a** with methanol, and proposed that it is formed not by direct bond heterolysis, but rather by initial electron transfer from the thioacetal to the naphthalimide, followed by C−S bond heterolysis to yield the carbocation and thiyl radical. Electron transfer from the reduced naphthalimide to the thiyl radical completes the process. This mechanism is supported by previous work by Epling and Wang involving the cleavage of dithioacetals with the use of photoinduced electron transfer.[120]

Diazonium salts have also been studied as photocleavage agents. Behr reported DNA photocleavage by a benzenediazonium chromophore that was covalently linked to spermine (**54**).[121] This compound nicked supercoiled plasmids at concentrations as low as 10 nM, apparently because of the high affinity of spermidine for DNA. Photocleavage patterns on radiolabeled restriction fragments were found to be shifted by 2−3 bp toward the 5′-end on opposite strands, as expected for binding of the spermine in the minor groove.[122] This feature was exploited in the photofootprinting of the minor groove binding drug distamycin.

The authors proposed that irradiation of **54** led to expulsion of N_2 and that the resulting aryl cation attacked the phosphates rather than either the sugars or nucleobases. Unfortunately, mechanistic information was not provided to support this intrigu-

ing hypothesis. Recent work by Gasper, Devadoss and Schuster demonstrated that diazonium salts irradiated in solution produce aryl cations which could be trapped either by addition of nucleophiles or by hydrogen atom abstraction, depending on the spin state of the aryl cation, a feature which is sensitive to the electron-donating ability of substituents on the aryl ring.[123] Thus, whether the phenyl cation produced by irradiation of **54** adds to phosphates or abstracts hydrogen atoms from deoxyribose is indeterminate. Substitution of single phosphate linkages with methylphosphonates or phosphorothioates could shed light on the cleavage mechanism for the diazonium salts.

D. Sequence-Selective Photocleavage by Oligonucleotide-Linked Cleavage Agents

A growing number of reports describe DNA damage by oligonucleotide-linked photocleavage agents. Oligonucleotides can recognize single- and double-stranded nucleic acid targets by forming Watson−Crick base-paired duplexes or Hoogsteen base-paired triplexes, respectively. There are two significant advantages to tethering a photocleaver to an oligonucleotide: (i) depending on the cleavage mechanism, the opportunity exists for sequence-specific cleavage of the nucleic acid target and (ii) restricting the photocleavage agent to one (or only a few) binding sites can greatly facilitate elucidation of the cleavage mechanism, as described above for the PNA-linked anthraquinone.

1. Porphyrins

The G-selective photocleavage exhibited by porphyrins has been exploited by Hélène and co-workers in their study of porphyrin−oligonucleotide conjugates.[124] DNA oligomers consisting of seven consecutive thymine residues and a porphyrin photosensitizer located on either the 3′- or 5′-end were synthesized by solid-phase methods. Hybridization of the oligomers with a DNA 27mer single strand containing an A_7 recognition site and irradiation with visible light led to extensive cross-linking of the two strands.[125] Piperidine treatment led to a significant decrease in the amount of cross-linked material and the appearance of cleavage bands at guanine sites. The strongest cleavage bands were found in the direction where the porphyrin was expected to be positioned (i.e., 5′-end for the 3′-linked porphyrin and vice versa) and the intensity of cleavage was greatest at the G closest to the porphyrin. The directionality and base selectivity of the cleavage are consistent with production of singlet oxygen by the excited porphyrin and then reaction of the singlet oxygen within the vicinity of the porphyrin. Performing the experiment under conditions where the two strands were dissociated led to uniform cleavage at all G sites and no cross-linking. Similar results have since been reported for numerous other conjugates between DNA oligomers and porphyrin derivatives.[126]

The extended porphyrin photocleavage agents reported by Sessler and co-workers have also been linked to oligonucleotides in order to enhance their cleavage selectivity. Compound **55** was linked to the 5′-end of an RNA sequence and shown to initiate selective, piperidine-dependent cleavage of G residues in a DNA target having a complementary sequence to the RNA.[78] (Damage on the photocleaver-bearing RNA strand was not reported.) In subsequent work, Sessler, Iverson, and co-workers covalently linked sapphyrin **56** to the 5′-end of a DNA T_{12} sequence and observed selective photocleavage at guanine sites (after piperidine treatment) in a single-stranded DNA target containing an A_{12} sequence.[127] In addition to the piperidine-dependent cleavage, photo-cross-linking of the two strands was observed.

2. C_{60}

There are also reports that the fullerene C_{60} cleaves DNA selectively at G by singlet oxygen production.[128] However, a recent study by Foote and co-workers questions this proposal.[129] In their system, C_{60} was covalently linked to a DNA oligonucleotide that was then hybridized with a complementary strand. While photocleavage indeed occurred at guanine residues, neither enhancement in D_2O nor inhibition by azide, a singlet oxygen quencher, was observed. A similar conjugate between the singlet oxygen generator eosin and the oligonucleotide was also synthesized. G-selective cleavage again occurred, but in that case cleavage was enhanced by D_2O and inhibited by azide, as expected for a singlet oxygen mechanism. It seems unlikely that the D_2O and the azide would have such different effects if singlet oxygen were involved in cleavage for both C_{60} and eosin. In addition, photocleavage by the fullerenes occurs at all sites within the DNA; cleavage at guanines is simply enhanced above this background. These results indicate that C_{60} does not photocleave DNA exclusively by a singlet oxygen mechanism, although that could account for part of the cleavage.

3. Azides

Hélène and co-workers synthesized an azidoproflavine derivative (**57**) linked to a DNA T_8 sequence in which the α-anomer of deoxyribose was present at each position[130] (as opposed to the naturally occurring β-anomers[131]). The azidoproflavine−DNA conjugate binds to a DNA oligomer containing an A_8 sequence in a 2:1 stoichiometry, giving a triple-stranded structure. Irradiation of the complex leads to photo-cross-linking and piperidine-dependent strand breaks at both ends of the target region, indicating that the two T_8 strands bind in opposite orientations. Addition of the same DNA to a duplex DNA containing an $A_8{\cdot}T_8$ sequence and irradiation led to piperidine dependent cleavage on both strands but only at one end of the recognition site. This result indicated that only one T_8 strand was binding to the duplex target and that one orientation was preferred, namely parallel alignment of the third strand with the A_8 strand. Similar photo-cross-linking and cleavage results were obtained for an [α]-T_8 substituted with an azidophenacyl derivative (**58a**).[132]

Levina and co-workers also reported the photocleavage of a complementary DNA using the perfluoroazidophenacyl derivative **58b**.[133] Photo-cross-linking and piperidine-dependent cleavage of the

DNA 8mer target 5′-TGTTTGGC-3′ was observed with cleavage occurring preferentially at G_6 and G_7. Approximately 75% of the total DNA target was photo-cross-linked in this reaction, indicative of an efficient photochemical reaction by the azido reagent.

4. Additional DNA–Photocleaver Conjugates

Tanaka and co-workers recently reported the synthesis and DNA cleavage properties of the diazapyrenium−DNA conjugate **59**.[134] Hybridization with a single-stranded DNA containing an A_7 target and irradiation led to cross-linking and piperidine-dependent cleavage within the target region. Neither mechanistic information nor a quantum yield were reported for this interesting system.

Schuster and co-workers reported the photocleavage of a duplex DNA target by a PNA-linked AQI derivative.[135] Homopyrimidine PNAs are known to bind to duplex DNA by strand invasion, wherein one PNA strand locally displaces the DNA pyrimidine strand in order to hybridize with the DNA purine strand in a Watson−Crick sense. A second PNA strand then binds to the PNA−DNA hybrid to form a local PNA_2−DNA triplex.[136] The affinity of the PNA for the DNA target is raised substantially by covalently linking the two PNA strands, forming a bisPNA. Conjugation of anthraquinone-2,3-anhydride (**60**) to the PNA N-terminus transforms the strand-invading bisPNA into a sequence-selective photocleavage agent. Irradiation of the bisPNA−AQI conjugate leads to cleavage of a DNA restriction fragment with high selectivity at an A_5 target site. (No damage is observed at an A_4 site also present in the target.) The damage is only observed in the displaced strand and is localized to the last three T residues at the 3′-end. While spontaneous cleavage is observed, piperidine treatment significantly enhances the yield, casting some doubt onto the mechanism. Further studies will be required to determine if damage arises from oxidation of the base or sugar in this system. Nevertheless, this represents the shortest sequence (5 bp) of duplex DNA targeted by a strand-invading PNA.

Finally, Hélène et al. synthesized the ellipticine−DNA conjugate **61** and studied its ability to initiate photocleavage of duplex DNA.[137] The homopyrimidine sequence of **61** permits it to bind to an appropriate sequence of duplex DNA in the major groove, forming a triplex. Irradiation led to a small amount of cross-linking and a higher yield of cleavage occurring on both strands at one end of the target site. Significantly, the cleavage occurred spontaneously and was observed at A, T, and C as well as G. These observations suggest that the excited ellipticine reacted directly with deoxyribose moieties rather than with the nucleobases. Photocleavage of DNA by free ellipticine led to doubling of the bands at some cleavage sites, suggestive of H-abstraction from C-1′ or C-4′ of the sugars. While the observation of cleavage on both strands does not mean that a single ellipticine makes a double-stranded cleavage, this remains the only example of an oligonucleotide-linked photocleavage agent that initiates spontaneous scission of a DNA target with little dependence on the base at the cleavage site.

V. Other DNA Photocleavage Agents

This section briefly reviews additional photocleavage agents for which little mechanistic information is available. First, metal complexes of Co(III)[138] and Re(I)[139] containing the dipyridophenazine (dppz) ligand (**62**) have recently been shown to photonick supercoiled DNA. This is an interesting observation given the reports of Rh(II) and Os(II) dppz complexes functioning as "molecular light switches" having very low fluorescent quantum yields in solution but greatly enhanced yields upon binding to DNA.[140] The dppz ligand in those cases is believed to intercalate into the DNA.

The pentacycle **63** is an intriguing structure, combining a viologen-like moiety (rotationally locked by the dimethylene bridge) and an intercalating phenanthridinium group.[141] The fluorescence of **63** is quenched in the presence of DNA, and irradiation leads to photonicking of supercoiled DNA. Singlet oxygen and hydroxyl radical scavengers had no effect on the cleavage, suggesting an electron-transfer mechanism. It will be interesting to see if GG selective cleavage is observed for **63**.

Along very different lines, Epling and co-workers covalently linked rose bengal (**64**) to T7 RNA polymerase (RNAP) in order to initiate sequence-specific cleavage of DNA (on the basis of specific binding of the polymerase to a promoter sequence in the DNA).[142] An active ester form of rose bengal was linked to reactive side chains on the RNAP giving a distribution of products differing in their number and sites of substitution. For proteins derivatized with 1−4 chromophores, there was no loss in enzymatic activity, but also no photocleavage. However, substitution with 5−8 chromophores yielded a protein that was still capable of RNA synthesis *and* capable of photonicking of supercoiled DNA. This indicates that the first four rose bengal chromophores are located at positions that do not permit photocleavage, perhaps on the side of the protein that faces away from the DNA. The photocleavage efficiency increased in the absence of oxygen, ruling out a singlet oxygen-based cleavage mechanism. The sequence selectivity of cleavage has not been reported, but could provide insight into not only the cleavage mechanism, but also the derivatization site(s) that lead to photocleavage.

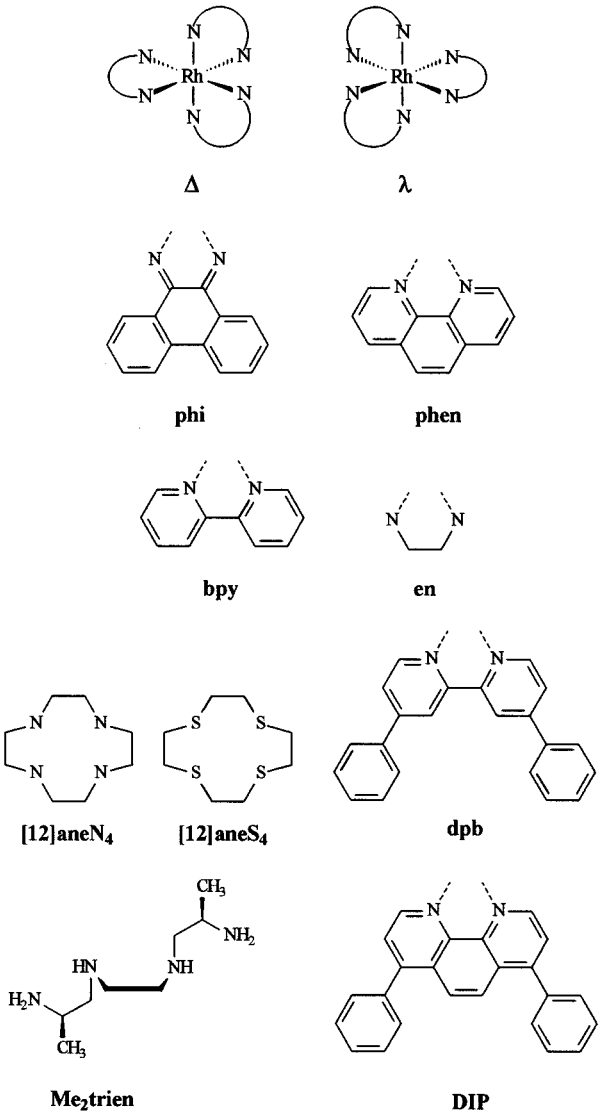
VI. Photocleavage of RNA

The relatively few reports of RNA photocleavage are mainly focused on structure probing rather than on mechanistic studies. Thus, very little information regarding cleavage products, is available and simply assuming that the compounds react with RNA by the same mechanisms as they do with DNA may not necessarily be valid. Nevertheless, a few general themes are beginning to emerge.

A. $[Rh(Phen)_2Phi]^{3+}$ and $[Rh(DIP)_3]^{3+}$

Barton and co-workers studied photocleavage of yeast $tRNA^{Phe}$ by the rhodium complexes $[Rh(phen)_2$-

Chart 2



phi]$^{3+}$ and [Rh(DIP)$_3$]$^{3+}$ (see Chart 2).[143] Cleavage of the RNA occurred at a few select sites without aniline treatment. Free bases were detected by HPLC, indicating that the rhodium complexes oxidize the ribose sugar residues rather than the nucleobases. Cleavage sites were observed for each of the four nucleotides but there was considerable sequence selectivity that differed for the two rhodium complexes. The [Rh(phen)$_2$phi]$^{3+}$ complex cleaved the tRNA in the vicinity of tertiary interactions, particularly adjacent to base triples, where a base from one domain of the RNA associates with a duplex domain by hydrogen bonding in the major groove. The Rh(DIP)$_3^{3+}$ complex cleaved at C-70, which is located on the 3′-side of a U which is involved in a G−U "wobble" base pair, and at the modified nucleotide Ψ55.

A subsequent report from the same group elaborated on the photocleavage of tRNA by [Rh(phen)$_2$phi]$^{3+}$.[144] This complex fails to cleave the RNA in standard A-form stem regions or in unstructured loops. [Rh(phen)$_2$phi]$^{3+}$ binds to B-form DNA by intercalation of the phi ligand from the major groove, so the narrow and deep major groove in A-RNA apparently prevents binding of the complex to stem regions. Similarly, a lack of structure in the uncleaved loop regions could inhibit binding of the complex if a suitable arrangement of the nucleobases in the loop cannot be induced by the complex.

Cleavage by [Rh(phen)$_2$phi]$^{3+}$ occurs at stem−loop junctions where there should be considerable fraying of the last base pair of the stem. Cleavage at the base triple sites can also be rationalized on a structural basis by considering that the third base provides a larger surface on which to stack the phi ligand and stabilize binding of the complex. Several mutants which contained various alterations involving the base triples were examined and it was found that conservative mutations (i.e., where changes in both the helix base pair and the third base preserved the triple) were cleaved effectively by the complex but an RNA where the third base was removed by depurination was cleaved at the triple site very weakly. Finally, cleavage was observed mainly in those loop regions that exhibited some structure, such as hydrogen bonding between bases within the loop. The stacking surface generated by the base pairing is apparently large enough to promote binding of the complex to the loop.

[Rh(phen)$_2$phi]$^{3+}$ has also been used to probe the structure of two RNAs for which high-resolution X-ray diffraction or NMR structures are not available: 5S rRNA and the TAR RNA sequence from HIV-1. The types of cleavage sites in 5S rRNA mirror those observed in tRNA: stem−loop junctions and potentially structured loops are targeted (based on secondary structure prediction).[145] Stem regions are also cleaved at sites where mismatched base pairs are formed; the lack of (or diminished) hydrogen bonding at these sites will likely widen the major groove, allowing binding of the rhodium complex. The authors note that the transcription factor protein TFIIIA recognizes many similar structural features as [Rh(phen)$_2$phi]$^{3+}$. Thus, [Rh(phen)$_2$phi]$^{3+}$ could be a useful probe for identifying potential protein binding sites on RNA.

A recent study by Neenhold and Rana further strengthens the apparent correlation between [Rh(phen)$_2$phi]$^{3+}$ cleavage sites, RNA major groove width and protein binding sites.[146] The TAR RNA sequence from HIV-1 is a 59-base stem−loop structure present at the 5′-end of all mRNAs. There is also a three-base bulge in the stem relatively close to the loop. The Tat protein binds to this sequence in vivo and regulates transcription. [Rh(phen)$_2$phi]$^{3+}$ was able to cleave at each position in the loop and bulge in the TAR RNA sequence. Binding of a truncated version of the Tat protein to the RNA inhibited cleavage. Mutant RNAs that either lacked the bulge entirely or had a single-base bulge were only cleaved in the loop by the rhodium complex. However, a two-base bulge (5′-UC-3′; wild type = 5′-UCU-3′) sequence was cleaved. Intriguingly, the Tat protein does not bind to the bulgeless or single-base bulge TAR RNA mutants, but will bind to a two-base bulge (5′-UU-3′). The fact that a protein α-helix is too large to be accommodated in an A-form major groove but that it could fit into a widened major groove has been

noted by Weeks and Crothers, who proposed that widening of the major groove in the vicinity of the three-base bulge leads to selective binding of the Tat protein to the TAR RNA sequence.[147] Again, the structural distortion introduced into the RNA stem by the bulge promotes both protein binding as well as $[Rh(phen)_2phi]^{3+}$ photocleavage.

As mentioned above, the $[Rh(DIP)_3]^{3+}$ complex cleaves $tRNA^{Phe}$ at the modified nucleotide Ψ55. If the position is unmodified, as in tRNA obtained by in vitro transcription, cleavage does not occur. Likewise, Ψ-modification at other positions are not cleaved, indicating that a sequence-specific binding is involved in the photocleavage. A deeper understanding of this cleavage site must await more binding and mechanistic data.

The second cleavage site within $tRNA^{Phe}$ targeted by $[Rh(DIP)_3]^{3+}$ was adjacent to a G−U base pair. $[Rh(DIP)_3]^{3+}$ cleaves 5S rRNA at several sites which have the same pattern: the damaged nucleotide is always on the 3′-side of a U at a G−U pair.[148] Any base can be present at the cleavage site, although some variation in cleavage efficiency is observed. Some G−U pairs do not trigger photocleavage, but these sites are often found adjacent to loops, with the position 3′ to the U being the first nucleotide of the loop. This indicates that the wobble pair must be embedded within a base-paired duplex in order for this cleavage chemistry to occur.

B. Flavins

Burgstaller and Famulok recently reported the photocleavage of several RNA substrates by three flavin derivatives: riboflavin (**38**), lumiflavin (**65**), and flavin mononucleotide (FMN, **66**).[149] The first RNA substrate was a sequence that evolved under in vitro selection and amplification to bind selectively to FMN. Intriguingly, each flavin initiated spontaneous photocleavage at two sites which were on the 3′-side of a U in a G−U wobble pair, precisely the same selectivity as for $Rh(DIP)_3^{3+}$. Further investigation with a series of RNA sequences which lacked preselected flavin binding sites but did contain G−U wobble pairs led to the same cleavage selectivity, demonstrating that this cleavage process is not the result of specific recognition of the flavin by the RNA.

The fact that the cleavage occurs spontaneously would seem to disfavor a process which targets the nucleobase, particularly since the reaction occurs regardless of the identity of the base at the cleavage site. Rather, sugar oxidation is likely at the heart of this type of RNA photocleavage. One possible mechanism involves direct H-abstraction by the flavin excited state. Famulok and co-workers recently prepared a computer model illustrating the binding of FMN at a G−U site that demonstrates the feasibility of direct H-abstraction by the flavin from either C-1′ or C-4′ of the ribose residue on the 3′-side of the uracil.[150] The geometric requirements are rather strict for this process and would require that both the flavin and $Rh(DIP)_3^{3+}$ bind adjacent to the wobble base pair in such an orientation as to allow H-abstraction to occur. While the ring nitrogens and carbonyl groups of the flavin can be envisioned as potential H-acceptors, it is not readily apparent which position on $Rh(DIP)_3^{3+}$ would serve as the H-acceptor.

An alternative possibility mentioned by Famulok and co-workers is that the flavin oxidizes the RNA by electron transfer rather than by H-abstraction. This mechanism would not require the photocleavers to bind at the G−U site in a specific orientation; in fact, it would not require them to bind at all to the wobble pair, if the oxidation can migrate through the RNA in a manner similar to its migration in DNA. At G−U sites, a base radical could conceivably abstract a hydrogen directly from the sugar on the 3′-side of the U. (Spontaneous strand breaks are often observed in experiments where the nucleobases are oxidized and are attributed to this type of process, which effectively transfers the radical from the base to the sugar.[151]) In this sense, the cleavage adjacent to G−U wobble pairs could actually be the RNA analogue to the GG cleavage observed in DNA, albeit with significantly different paths followed after the initial electron transfer and migration of damage through the helix. As more photocleavers are tested on RNA structures, it will be interesting to see if more examples of this type of cleavage selectivity are observed.

C. Other RNA Photocleavage Agents

Three other examples of photocleavers directed toward RNA substrates have been reported. The aryldiazonium salts studied initially by Behr as DNA cleavage agents have also been applied to study $tRNA^{Asp}$ in isolation and as its complex with aspartyl-tRNA synthetase.[152] The RNA is cleaved by benzenediazonium with some preference for loops versus stems. When the cognate synthetase is present, one set of cleavage sites, corresponding to the surface of the RNA to which the protein binds, are protected from cleavage while the sites which remain exposed to solvent are still cleaved, providing a footprint of the bound protein.

The putrescine− and spermine−diazonium conjugates prefer to cleave the tRNA in regions where phosphates approach one another. The polyamines seem to selectively bind in these regions of high negative charge density, leading to the observed cleavage selectivity. Thus, these reagents could have some utility as probes of tertiary interactions in folded RNA molecules, although care will have to be taken to ensure that the binding of the polyamine does not actually alter the structure of the RNA.

Celander and Nussbaum have studied the photocleavage of $tRNA^{Phe}$ and the RRE sequence of HIV-1 by several tetracationic porphyrin derivatives (**34**).[153] Under low-salt conditions, the porphyrins cleave the tRNA with high selectivity at G65, which is located in a hinge region between two helices. Cleavage at this site is inhibited under high-salt conditions, however, and new cleavage bands appear at guanines in loop regions. The added salt serves to stabilize the structure of the RNA, in particular promoting coaxial stacking of the helices bordering the hinge region which is the preferred cleavage site at low salt. This inhibition of cleavage is taken as an indicator

of coaxial stacking of helices, which inhibits binding of the porphyrin, forcing it out into solution or into the loop regions. The mechanism for the photocleavage was not studied; while it is likely that singlet oxygen contributes to the cleavage, since guanines are targeted and aniline is required to reveal the damage, two uracils are also cleaved under low-salt conditions, so an additional pathway might be available.

In the RRE RNA from HIV-1, photocleavage at guanines in mismatches, bulges, internal loops and at the ends of helices is observed under low-salt conditions. Cleavage at the ends of helices is inhibited when the salt concentration is raised, indicating a structural transition in the RNA that is attributed to coaxial stacking of the helices and protection of the guanines at the internal ends.

It should be noted that these are the same porphyrins investigated by Leontis and co-workers in probing DNA three way junctions. The results from the two studies indicate that these compounds can be useful probes for nonduplex secondary structures as preferential binding of the porphyrins appears to occur at these sites. The strong cleavage selectivity for guanines does introduce a limitation though, since nonduplex structures which lack an adjacent G−C base pair or a G in the single-stranded region will be invisible.

Finally, uranyl ion has found application in probing RNA structures. It cleaves at all positions in yeast $tRNA^{Phe}$, with some preference for loop regions.[154] This likely arises because the phosphate backbone has sufficient flexibility in nonduplex regions to adapt to the uranyl structure and permit simultaneous coordination of two phosphates by the UO_2^{2+}. Uranyl has also been used to probe the hammerhead ribozyme[155] and RNA-protein interactions.[156]

VII. Future Directions

After reviewing the wealth of DNA/RNA photocleavage agents reported in the past decade, it is certainly appropriate to ask: What now? It should be clear from the previous sections that analyzing cleavage events with radiolabeled nucleic acids and sequencing gels provides much greater insight into cleavage mechanisms than do experiments involving supercoiled DNA targets and agarose gels. The next logical step in studying cleavage mechanisms involves structural analysis of the lesions, particularly those that can only be visualized by PAGE after piperidine or aniline treatment. One technique which could be readily applied in this field is matrix-assisted laser desorption ionization−time-of-flight mass spectrometry (MALDI-TOF MS). For example, adduct formation by azido reagents with nucleobases would lead to an easily detected increase in mass for the nucleic acid. Moreover, enzymatic digestion of the damaged DNA and subsequent MS analysis could pinpoint not only the presence of an adduct but also its location in the DNA. The small quantities of material required for MS, the rapid data acquisition, and growing availability of the instrumentation should contribute to a real growth in the use of MS to analyze nucleic acid cleavage mechanisms.

It is also valid to wonder whether there is a need to develop and study new photocleavers. There are at least four areas of study that can be reasonably expected to bear intellectual fruit. First, more sequence-selective photocleavage agents should be synthesized. While there are numerous examples of electron transfer or singlet oxygen generating conjugates of this type, new ground in this field will be broken by photocleavage agents which function by a hydrogen abstraction mechanism. This will lead to localized cleavage events determined largely by the recognition sequence and the tether linking the cleavage and recognition moieties.

Second, photocleavage agents that selectively cleave non-B-form DNA would have great utility. The ability to detect transiently unwound DNA, H-form (i.e., intramolecular triplex) DNA, and intramolecular hairpins which could form as the result of triplet repeat mutations are all worthwhile goals for photocleavers, particularly since they offer the best hope for in vivo use. The report by Leontis and co-workers regarding detection of DNA three-way junctions by porphyrin photocleavage is noteworthy in this respect. Along these lines, an agent that could cleave DNA at any single-stranded region, regardless of the sequence, is highly desirable. An advance of this sort will probably require that the compound bind selectively to nonduplex regions, but react with the sugar residues (or phosphates) rather than with the bases.

A third area for future application of photocleavers is in the mapping of RNA structure. As described in section VI, there have been a few reports of work in this area and the results are certainly encouraging. The broad spectrum of RNA structure and function, combined with the difficulty in obtaining high-resolution structural information indicates that introduction of new probes for distinguishing among the different types of RNA secondary and tertiary structure would be welcome. There should be a high priority placed on reagents which cleave RNA spontaneously, since RNA will not tolerate piperidine treatment and it is not clear how effective the currently used aniline treatment is for visualizing all types of modification sites.

Finally, a photocleavage agent that cleaves nucleic acids by a hydrolytic mechanism would be a significant advance in the field. In addition to yielding exclusively spontaneous cleavage, no sequence information would be lost in the process of cleavage by such a reagent, unlike the current compounds which degrade the nucleotide at which they react. Furthermore, the cleavage products could be religatable, leading to their use in the synthesis of recombinant DNA. This is perhaps the final frontier in cleavage of B-DNA but it is certainly well worth exploring.

VIII. Abbreviations

PAGE	polyacrylamide gel electrophoresis
EDTA	ethylenediaminetetraacetic acid
G	guanine
A	adenine
C	cytosine
T	thymine
U	uracil

Ψ	pseudouracil
dG	2′-deoxyguanosine
8-oxo-G	7,8-dihydro-8-oxoguanine
7-deazaG	7-deazaguanine
poly(dA)-poly(dT)	polydeoxyadenylic acid-polythymidylic acid
BLM	bleomycin
Tris	tris[(hydroxymethyl)amino]methane
DMPO	5,5-dimethylpyrroline *N*-oxide
TEMPO	2,2,6,6-tetramethylpiperidine *N*-oxide
FMN	flavin mononucleotide (riboflavin 5′-phosphate)
PNA	peptide nucleic acid
tRNA	transfer RNA
mRNA	messenger RNA
rRNA	ribosomal RNA
TAR	transactivation response element
RRE	Rev response element

IX. Acknowledgments

I thank Professor Gary B. Schuster, not only for the suggestion to write this review, but also for his past and continuing support and guidance. Much of my interest in this field was stimulated by a postdoctoral appointment in Professor Schuster's group, first at the University of Illinois and, subsequently, at Georgia Tech. Sincere thanks also go to the many current and former Schuster group members with whom I learned about the diverse mechanisms by which one can cleave nucleic acids. I am grateful to Professor C. S. Foote for a preprint of ref 97. Finally, I thank the reviewers of the original version of this manuscript for their many thoughtful suggestions.

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CR960428+