A Novel Mechanism for the Formation of Direct Strand Breaks upon Anaerobic Photolysis of Duplex DNA Containing 5-Bromodeoxyuridine

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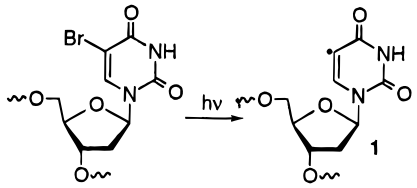
Abstract: The mechanism of UV-induced cleavage of duplex DNA containing 5-bromodeoxyuridine under anaerobic conditions was investigated via product analysis and kinetic isotope effect measurements using gel electrophoresis. KIEs strongly suggest that strand breaks result from abstraction of the C2′ hydrogen atom from the 5′-adjacent nucleotide by deoxyuridin-5-yl (**1**). It is proposed that under anaerobic conditions the C2′ radical is subsequently oxidized by the cation radical of adenine. The resulting carbocation undergoes 1,2-hydride migrations from C1′ and C3′ competitively, giving rise to cleavage products containing 3′-phosphate and the novel, labile 3′-ketone (**4**). The identity of the latter product is supported by electrospray mass spectrometric analysis of the crude photolysate. The results also suggest that the formation of alkaline labile lesions proceed via hydrogen atom abstraction from the C2′ position by **1**.

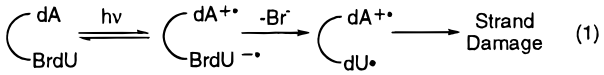
Replacement of thymidine by isosteric 5-bromodeoxyuridine (BrdU) in DNA creates biopolymers which are sensitized to damage induced by γ-radiolysis and UV photolysis.[1,2] This property of BrdU has been exploited in clinical studies and more recently has been used in concert with other DNA-damaging agents for increasing double strand cleavage efficiency.[3] The ability of 5-bromodeoxyuridine and other 5-halopyrimidine nucleosides to form protein-nucleic acid cross-links has also been exploited for mapping protein−nucleic acid interactions.[1b,4] Both biologically relevant processes are of mechanistic interest. However, we have focused our efforts on addressing the mechanism(s) by which strand damage is induced. Product studies and kinetic isotope effects allow us to put forth a mechanism for DNA damage produced via 5-bromodeoxyuridine that unifies two discordant proposals.

Irradiation of duplex DNA containing BrdU produces direct strand breaks and alkaline labile lesions at nucleotides attached to the 5′-phosphate of BrdU.[1,2,5] As such, BrdU is one of a few examples of nucleic acid damage processes in which spin is transferred from a nucleobase to the sugar moiety of an adjacent nucleotide.[6] Inspection of models of duplex DNA led to the suggestion that deoxyuridin-5-yl (**1**), formed from BrdU by bond homolysis, initiates strand scission via abstraction of a hydrogen atom from the C2′ position of the adjacent 5′-nucleotide.[1a] Hydrogen atom abstraction from the C2′ position of deoxyribonucleotides in DNA damage processes is



uncommon, being limited to ionizing radiation.[6,7] The C2′ position is disfavored on the basis of the relatively high bond strength of the respective C−H bond.[8]

The role of the sequence of the nucleic acid surrounding the BrdU was not considered in the original proposal concerning strand damage. In contrast, alkaline labile lesions formed in duplex DNA containing BrdU were reported to show a significant nucleic acid sequence dependence. This observation was proposed to result from a selective photoinduced single electron transfer (PSET) originating from a deoxyadenosine moiety linked to the 5′-phosphate of the BrdU (eq 1).[5] This



(1)

observation was intriguing, because deoxyguanosine is typically thought of as the electron source in DNA.[9] More surprising to us was the proposal that alkaline labile lesions resulted from **1** (formed following loss of bromide from the radical anion of BrdU) via hydrogen atom abstraction from C1′ of the adjacent deoxyadenosine.[5] This proposal was revised recently to include hydrogen atom abstraction at both C1′ and C2′.[10] The abstraction of the C1′ hydrogen atom by **1** was surprising, because these reactive entities are situated in different grooves of the duplex. Inspection of molecular models does suggest that the C1′ hydrogen atom is not readily accessible to **1**. We now report

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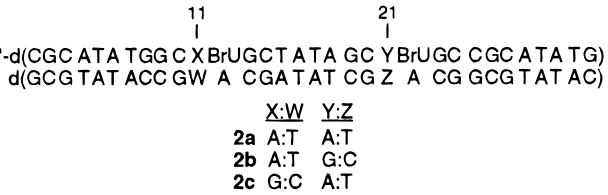
results which indicate that direct strand breaks resulting from photolysis of duplex DNA containing BrdU under anaerobic conditions are a consequence of C2′ hydrogen atom abstraction by **1**. Furthermore, our observations lead us to hypothesize that alkaline labile lesions produced from **1**, under anaerobic conditions, are the result of the same initial hydrogen atom abstraction that leads to direct strand breaks.

Results and Discussion

The recent proposals put forth for the production of strand damage upon irradiation of oligonucleotides containing BrdU were based upon observations made using DNA duplexes containing less than one full helical turn.[5,10] While the structure of duplex DNA is dynamical, we were concerned that a hexanucleotide would not be representative of B-form DNA. Consequently, with the exception of the electrospray mass spectrometry (ESMS) experiments, studies reported here were carried out using duplexes containing three turns of an α-helix. In these instances, the BrdU nucleotides were imbedded approximately one helical turn from the ends of the duplexes. Furthermore, the oligonucleotide duplexes employed in the sequence specificity analysis and kinetic isotope effect experiments contained two BrdU sites spaced approximately one helical turn apart. Utilizing DNA duplexes containing two BrdU sites enabled us to treat one cleavage site as an internal standard. This is described in more detail for the individual experiments.

Sequence Selectivity for Direct Strand Breaks. Prior studies of the photochemistry of 5-bromouracil revealed the presence of two closely excited states that exhibited distinct chemical reactivity.[11] The lower energy excited state was ascribed to an n,π^*-triplet. This excited state, which was populated using an excimer laser, participated in electron transfer chemistry. A higher lying singlet excited state (λ_{max} = 276 nm) was shown to give rise to C−Br bond homolysis, in competition with intersystem crossing. We did not have access to a monochromatic light source. Hence, wavelength effects were investigated using a 270 nm (±5 nm) interference filter in conjunction with quartz photolysis tubes to selectively populate the higher energy excited state. The Pyrex-filtered output of a transilluminator (λ_{max} = 302 nm) was utilized to populate the n,π^*-excited state of BrdU. This is also the irradiation source employed during studies in which electron transfer was implicated in the formation of **1** within duplex DNA.[5]

Photolysis of duplex DNA containing BrdU under anaerobic conditions produced direct strand breaks at the nucleotide attached to the 5′-phosphate of BrdU (Figure 1). Irradiation of **2a** for between 0.5 and 2.0 min revealed a continuous increase



	X:W	Y:Z
2a	A:T	A:T
2b	A:T	G:C
2c	G:C	A:T

in strand scission adjacent to both BrdU sites. The ratio of cleavage at the two sites was approximately 1.0, indicating that the extent of conversion of **2a** was sufficiently small so that each molecule of the biopolymer underwent only one photochemical reaction (single hit range). Irradiation of **2a** and **2b** showed that direct strand breaks were formed approximately 8 times more efficiently at a 5′-dABrdU sequence than at a 5′-dGBrdU site, regardless of the position of BrdU in the duplex.

(11) Dietz, T. M.; von Trebra, R. J.; Swanson, B. J.; Koch, T. H. *J. Am. Chem. Soc.* **1987**, *109*, 1793.

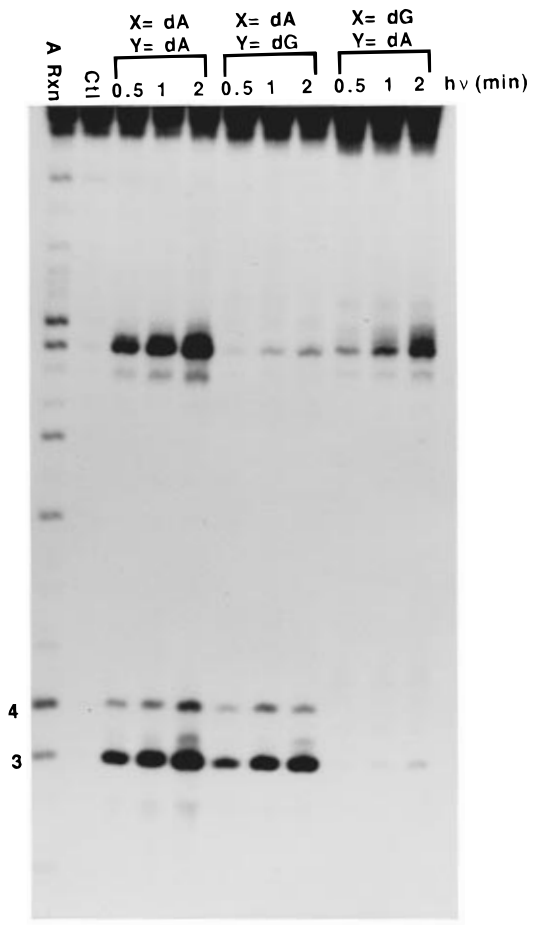


Figure 1. Anaerobic photolysis of **2a**−**c**. Photolyses were carried out in quartz tubes for the times shown using the interference filtered (270 ± 5 nm) output of a Hg/Xe high pressure lamp (400 W). The lane marked Ctl corresponds to unphotolyzed DNA.

Utilizing duplexes that contain 5′-dGBrdU and 5′-dABrdU sites within the same polymer (**2b** and **2c**) enabled us to compare the selectivity of cleavage directly, independent of the extent of conversion in a given sample. Furthermore, photolysis of **2a**−**c** showed that the preference for strand scission at 5′-dABrdU was identical upon irradiation at 270 or 302 nm. These results suggest that, under the above irradiation conditions, a single excited state is responsible for strand scission at nucleotides adjacent to the 5′-phosphate BrdU, consistent with the proposed PSET generation of **1** (eq 1).[5] This explanation for the observed sequence effect is preferred over one involving energy transfer, because recent studies have shown that the triplet states of dA and dG are too close in energy (<1 kcal/mol) to lead one to suggest preferential energy transfer to BrdU.[12] The PSET mechanism is also consistent with the product studies and KIE experiments discussed below.

The observed sequence effect does not explain why the proximity of the more facile (thermodynamically) electron donor, dG, to BrdU, is less efficient at generating **1**. We believe that the observed sequence specificity is a consequence of slower back electron transfer from BrdU to dA (eq 1).[5] This is based upon the assumption that loss of bromide ion to form **1** is the strand damage fixing event and that the quantum yield for the formation of the two respective radical ion pairs are approximately equal. Slower back electron transfer in the dA BrdU radical ion pair enables loss of bromide from the radical

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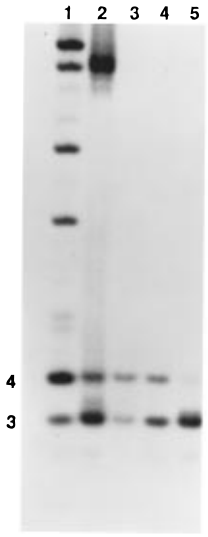

Figure 2. Alkaline lability of **4** obtained via photolysis of **2a**: lane 1, dA sequencing reaction; lane 2, crude anaerobic photolysate; lane 3, isolated **3** and **4**; lane 4, 0.1 N NaOH, 95 °C, 60 min; lane 5, 1.0 N NaOH, 95 °C, 60 min.

anion of BrdU to compete with strand repair by back electron transfer. This explanation is consistent with the reported absence of a sequence effect in 5-iododeoxyuridine (IdU) containing oligonucleotides.[13] The lack of a sequence effect had been ascribed to direct irradiation of the IdU moiety. However, it may not be necessary to suggest such a vastly different mechanism for DNA damage induced by two different 5-halopyrimidines. Loss of iodide from the radical anion of IdU is faster than the respective process from the radical anion of BrdU.[14] Hence, the lack of any observed sequence specificity for IdU-containing oligonucleotides could be due to the fact that formation of **1** competes more effectively with back electron transfer.

Product Analysis. Gel electrophoresis revealed that irradiation of [3′-^{32}P]-**2a** produced direct strand break products containing a single type of end group. Enzymatic end group analysis showed that the radiolabeled fragments produced contained termini consisting exclusively of 5′-phosphates.[15] However, fragments containing two distinct 3′-end groups were observed when 5′-^{32}P-labeled oligonucleotides were photolyzed (Figure 1). Using T4 kinase, the faster moving of these fragments was shown to contain a 3′-terminal phosphate (**3**).[15] The second cleavage product migrates more slowly. Its migration is similar to the 3′-phosphate product that one would obtain upon piperidine-induced cleavage at the BrdU site in **2a**. However, kinase treatment of this slower moving cleavage fragment reveals that it is not a 3′-terminal phosphate. Due to the similar electrophoretic migratory aptitude of this unknown product to the 3′-phosphate product formed via cleavage at BrdU and the lability of BrdU to piperidine, the unknown product was excised from the gel prior to establishing its own alkaline lability. The unknown product was partially converted to **3** during isolation and completely so upon treatment with base (Figure 2). These observations and those described below led us to propose that the slower moving, alkaline labile product obtained from **2a** was **4**. DNA cleavage resulting from formal oxidation of the C3′ position has been invoked for major groove binding oxidants.[16] However, to our knowledge, this is the first time that an alkaline labile lesion such as **4** has been directly observed.

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(14) Rivera, E.; Schuler, R. H. *J. Phys. Chem.* **1983**, *87*, 3966.
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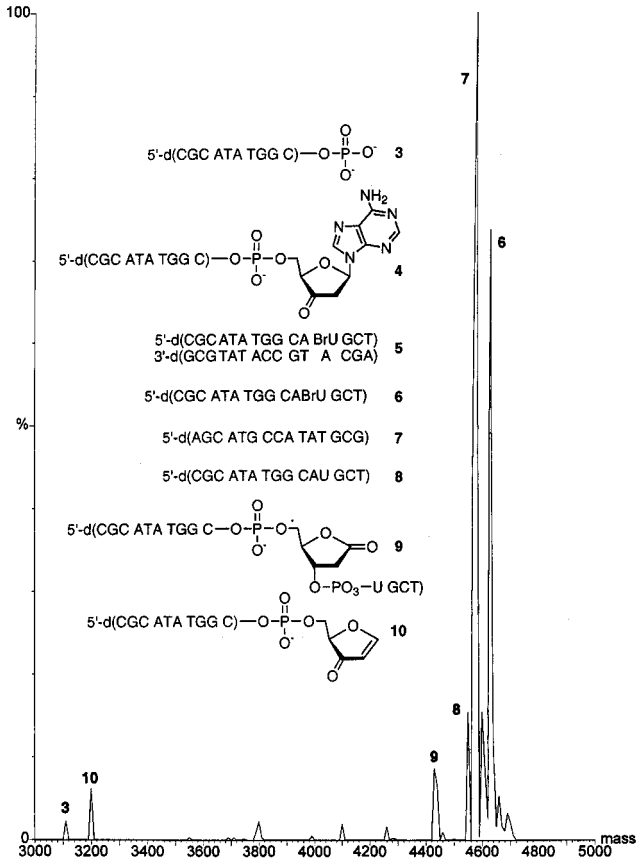
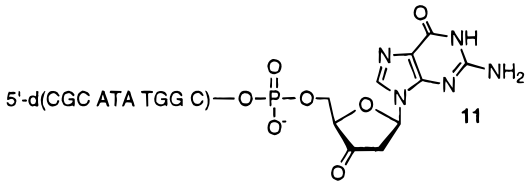


Figure 3. Electrospray mass spectrum obtained from the crude photolysate of **5**. The observed (calculated) masses are as follows: **3**, 3110.0 (3107.0); **6**, 4630.0 (4632.9); **7**, 4580.0 (4577.0); **8**, 4550.0 (4554.0); **9**, 4430.0 (4432.9); **10**, 3200.0 (3203.1).

Photolysis products derived from BrdU were characterized further via electrospray mass spectrometry (ESMS) analysis of the crude anaerobic photolysate of **5** (Figure 3). Duplex **5**, containing a single 5′-dABrdU site, was used in ESMS experiments in order to reduce the number of possible products. In order to minimize the structural differences between the DNA used in the gel electrophoresis and ESMS experiments, **5** was designed to have the exact structure of the first 15 nucleotides of **2a**. Following irradiation of **5** with a transilluminator, molecular ions were observed for the individual oligonucleotides that make up **5** (**6** and **7**), as well as photoreduction product **8**. The previously reported alkaline labile lesion product resulting from formal C1′ oxidation of the dA adjacent to BrdU (**9**) was also observed.[5] The ESMS experiment was also consistent with the observation via gel electrophoresis of two products containing differing 3′-termini. These products were identified as 3′-phosphate cleavage product (**3**) and the novel ketone-containing fragmentation product (**10**). The unsaturated ketone (**10**) is believed to arise from **4** via elimination of adenine upon ionization within the mass spectrometer. This hypothesis is based upon the observation that the analogous product obtained



from **2c** (**11**) migrated slightly faster than that obtained from photolysis of **2a** (**4**). This indicates that the products observed

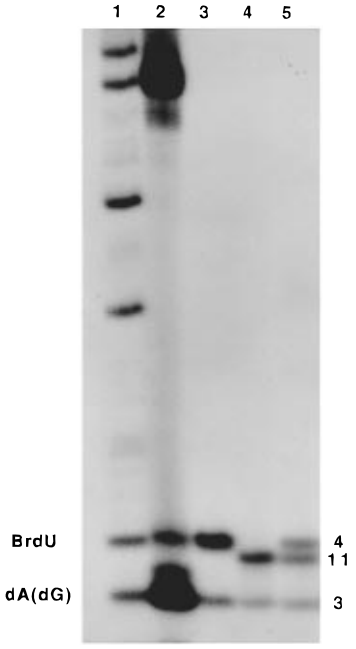
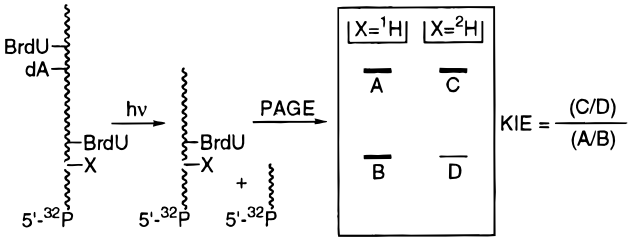


Figure 4. Comparison of 3′-ketonucleotide-containing DNA fragments obtained upon irradiation of **2a** and **2c**: lane 1, dA sequencing reaction of **2a**; lane 2, crude anaerobic photolysis of **2a**; lane 3, isolated **3** and **4** from **2a**; lane 4, isolated **3** and **11** from **2c**; lane 5, coloading of isolated **3**, **4**, and **11** from **2a** and **2c**.

Scheme 1



via gel electrophoresis contained the respective purine moieties (Figure 4).

Kinetic Isotope Effect Experiments. Insight into the mechanism for formation of cleavage products **3** and **4** was obtained from deuterium kinetic isotope effect (KIE) experiments (Scheme 1). KIEs have been useful in the study of a number of nucleic acid damage processes.[17,18] In the current study, duplexes containing two 5′-dABrdU sites with identical local sequence were used as substrates (**2a**).[17] In the control duplex, neither dA adjacent to BrdU was deuterated. KIEs were determined by measuring the ratio of strand scission at the two sites in the all protio duplex (control), relative to the same ratio in which either dA_{11} or dA_{21} was deuterated at C1′ or dideuterated at C2′. The requisite deuterated nucleosides were prepared via known methods.[19,20] For an oligonucleotide that is deuterated at dA_{11} and labeled with ^{32}P at its 5′-terminus,

(17) Greenberg, M. M.; Dervan, P. B., unpublished results, California Institute of Technology.

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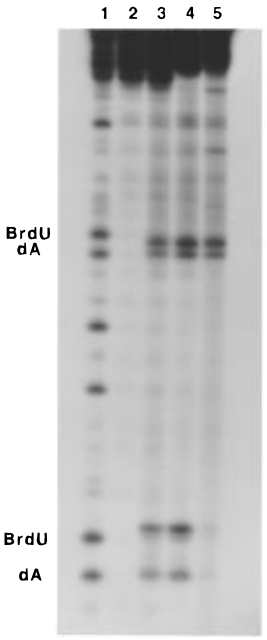


Figure 5. Demonstration of kinetic isotope effect measurement using **2a**. Lane: 1, dA sequencing reaction; 2, unphotolyzed DNA; 3, cleavage of **2a**; 4, cleavage of C1′-^{2}H-dA_{11}; 5, cleavage of C2′-^{2}H-dA_{11}.

Table 1. Kinetic Isotope Effects for Direct Strand Scission Produced via Irradiation at 302 nm[a]

11 21

5′-d(CG C A T A TGG C ABrU G C T A TA GC ABrU GC CGC A TA TG)
d(GC G T A T ACC G T A CGA T AT CG T A CG GCG T AT AC)

deoxyribose position isotopically substituted	isotopically labeled at dA_{11}	isotopically labeled at dA_{21}
C1′	0.91 ± 0.01	0.89 ± 0.06
C2′	4.43 ± 0.19	2.75 ± 0.20

[a] All oligonucleotides were labeled with ^{32}P at their 5′-termini.

the KIE is determined from the ratio of cleavage at the four sites, as shown in Scheme 1. Measuring the ratio of strand scission at two sites in two different oligonucleotides (only one of which is isotopically substituted) simplifies the determination of the KIE by removing the extent of conversion as a variable. A sample of primary data is shown in Figure 5. In a typical experiment, five samples of each oligonucleotide were irradiated, and the cleavage product(s) at each site were quantitated. The KIE was determined from the average cleavage ratio in each oligonucleotide. The standard deviations presented above represent the deviation from the average KIE for a series of experiments. Individual KIE experiments deviated from the reported values by less than 25% at the 95% confidence limit.[15]

Dideuteration of C2′ of deoxyadenosine (dA_{11} or dA_{21}) in **2a** resulted in significant observed KIEs on strand scission (Table 1). The KIEs reported are based upon total cleavage observed at each site (products **3** + **4**). The magnitude of the observed KIEs at each site are independent of the irradiation wavelength (Table 2). At first glance, the KIEs for dideuteration of the C2′ position appear to be different for isotopic substitution at dA_{11} and dA_{21} (Table 1). However, independently radiolabeling either the 5′- or 3′-terminus of the BrdU-containing oligonucleotide in the duplex reveals that this is an artifact of the position in the oligonucleotide with respect to the radiolabel where cleavage occurs (Table 3). A smaller effect on strand scission results from deuteration of the C2′ position at the site further away from the radiolabeled terminus; the product of which migrates more slowly in the gel. Regardless, the KIEs

Table 2. Kinetic Isotope Effects for Direct Strand Scission as a Function of Irradiation Wavelength[a,b]

11 21
5'-d(CGCATATGGCABrUGCTATAGCABrUGCCGCATATG)
d(GCGTATACCGTACGATATCGTACGGCGTATAC)

deoxyribose position isotopically substituted	270 nm	302 nm
C1′	0.95 ± 0.14	0.91 ± 0.01
C2′	4.75 ± 0.30	4.43 ± 0.19

[a] All oligonucleotides were labeled with ^{32}P at their 5′-termini. [b] All measurements were carried out on oligonucleotides in which dA_{11} was isotopically substituted.

Table 3. Kinetic Isotope Effects for Direct Strand Scission as a Function of Isotopic Labeling and Radiolabeling Positions[a,b]

11 21
5'-d(CGCATATGGCABrUGCTATAGCABrUGCCGCATATG)
d(GCGTATACCGTACGATATCGTACGGCGTATAC)

oligonucleotide isotopically labeled	terminus radiolabeled	deoxyribose isotopically substituted at C1′	deoxyribose isotopically substituted at C2′
dA_{11}	5′	0.91 ± 0.01	4.43 ± 0.19
dA_{21}	5′	0.89 ± 0.06	2.75 ± 0.20
dA_{21}	3′	0.93 ± 0.05	3.89 ± 0.18

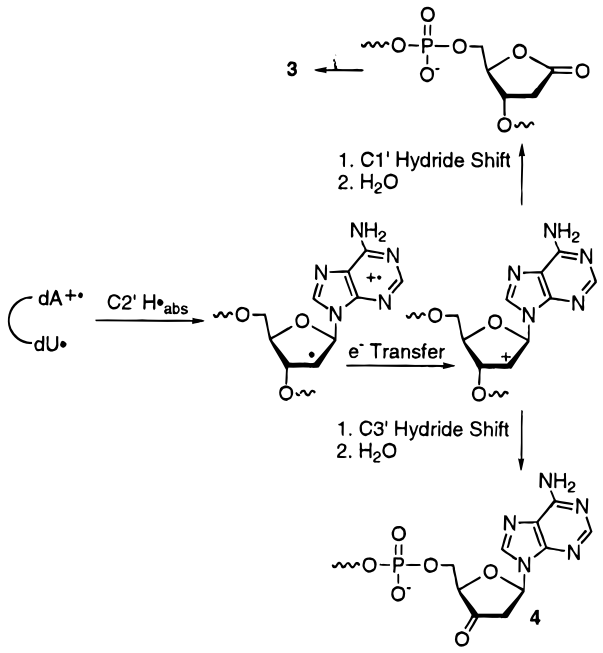
[a] All measurements were carried out on oligonucleotides in which dA_{21} was isotopically substituted. [b] All irradiations were carried out at 302 nm.

observed at both sites clearly imply that direct strand breaks involve abstraction of the C2′ hydrogen from the dA bound to the 5′-phosphate of BrdU. Furthermore, the magnitudes of the observed KIEs are similar to those measured for σ radicals reacting with organic substrates, strongly supporting proposals that direct strand scission results from abstraction of the C2′ hydrogen by **1**.[1a,21]

In contrast, deuteration of the C1′ position of either dA_{11} or dA_{21} gave rise to small overall KIEs, suggesting that a tiny fraction (if any) of direct strand breaks were derived from hydrogen atom abstraction from this position by **1** (Table 1−3). However, examination of the C1′ KIE at dA_{11} for **3** and **4**, individually, reveals a small normal effect for the formation of **3** (1.08 ± 0.03), but a significant inverse effect (0.79 ± 0.01) on the formation of **4**. The inverse KIE is attributed to the partitioning of a common intermediate which gives rise to **3** and **4**. Deuteration of the C1′ position shifts the partition toward formation of **4**.

A mechanism that incorporates the above observations involves oxidation of the C2′ radical on dA that results from hydrogen atom abstraction by **1** (Scheme 2). The oxidant is the cation radical of the adenine which was formed in the initial PSET process. Literature values for the reduction potentials of the dA cation radical and secondary alkyl cations as models for the C2′ deoxyribosyl radical suggest that this will be a thermodynamically favorable process.[22] The resulting carbocation subsequently undergoes 1,2-hydride migrations, as recently

Scheme 2



observed in the photolysis of 2′-deoxy-2′-iodouridine.[23] Hydride migration from C3′, followed by solvation and hydrolysis, leads to direct strand scission and formation of the oligonucleotide fragment containing a 2′-deoxy-3′-ketonucleotide (e.g. **4**, **11**). Hydride migration from C1′ forms the deoxyribonolactone, which ultimately results in strand breaks containing 3′-phosphate termini (e.g. **3**).[5,7,24] Deuteration of C1′ retards the rate of hydride migration from this center, resulting in an inverse KIE for formation of **4**.

Summary. The above isotopic labeling experiments show that C2′ hydrogen atom abstraction plays an important role in the formation of direct strand breaks in UV-irradiated duplex DNA containing BrdU under anaerobic conditions.[1a,13,25] ESMS indicates that the product (**9**) whose formation served as the impetus for proposing that the C1′ hydrogen is abstracted by **1** is produced in these experiments. However, KIE experiments do not support C1′ hydrogen atom abstraction by **1**. Mechanistic economy suggests that invocation of geometrically unfavorable hydrogen atom abstraction from C1′ and/or C3′ of the adjacent nucleotide by **1** may be unnecessary, as products resulting from formal oxidation at these positions can be attributed to subsequent reactivity of the radical at C2′.[26] Investigations into these mechanistic aspects of DNA damage are in progress.

Experimental Section

General Procedures. Oligonucleotides were synthesized on an Applied Biosystems Inc. 380B DNA synthesizer using standard protocols. All H_2O used was obtained from a Nanopure Barnstead still. Commercially available DNA synthesis reagents were obtained from Glen Research Inc. Deuterated phosphoramidites were prepared via known procedures.[19,20,27] Oligonucleotides containing BrdU were deprotected in 28% NH_4OH at room temperature for 48 h. All other oligonucleotides were deprotected at 55 °C overnight. Oligonucleotides

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(22) E_{red} for deoxyguanosine cation radical in DNA at pH 7 is 1.04 V (see a). Reduction potentials for adenine cation radicals are typically ≈0.1 V higher than for guanine cation radicals (see b). E_{red} for 2-propyl and cyclohexyl cations are 0.78 and 0.71 V, respectively (see c). All reduction potentials are reported relative to NHE. Values for nucleotides are in H_2O. Values for alkyl cations are in CH_3CN. (a) Jovanovic, S. V.; Simic, M. G. *Biochimica et Biophysica Acta* **1989**, *1008*, 39. (b) Jovanovic, S. V.; Simic, M. G. *J. Phys. Chem.* **1986**, *90*, 974. (c) Wayner, D. D. M, unpublished results, NRCC, Ottawa, Canada.

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were purified and analyzed via 20% denaturing polyacrylamide gel electrophoresis (PAGE). Oligonucleotides were radiolabeled and subsequently handled using standard protocols.[28] Enzymatic end group analysis was carried out using standard procedures.[28] Oligonucleotides were sequenced using a reaction specific for adenine.[29] Radionuclides were obtained from Amersham. T4 polynucleotide kinase and calf intestine alkaline phosphatase were obtained from New England Biolabs. Terminal deoxynucleotidyl transferase was obtained from United States Biochemical. DNA samples were dried in a Savant Speed-Vac following precipitation and removal of the supernatants. Radioactive samples were counted via Cerenkov counting, using a Packard Tri-Carb 1500 scintillation counter. Electroelution was carried out using a S & S Elutrap electro separation system.

Electrospray mass spectrometry was carried out on a VG Fisons Quattro. Samples were prepared by precipitating from NH_4OAc prior to photolysis at 302 nm.[30] Photolyses at 270 nm were carried out in quartz tubes using the filtered output of a 1000 W Hg/Xe high pressure lamp, set to 400 W. The interference filter ($\lambda_{max} = 270$ nm) was from Oriel. Photolysis tubes were positioned 24 cm from the filter. Photolyses at 302 nm were carried out in Pyrex tubes using a UV Photoproducts dual wavelength transilluminator containing four 8 W lamps. Photolysis tubes were positioned 8 cm from the source. Phosophorimaging analysis was carried out using a Molecular Dynamics Phosphorimager equipped with Imagequant software (version 3.3). Densitometry was carried out using a CCD camera in conjunction with a commercial software package from Technology Resources Inc.

Standard Photolysis Conditions. Photolyses were carried out in Pyrex or quartz tubes containing 50 μL of a 10 mM phosphate (pH 7.0), 10 mM NaCl buffered solution of DNA. All photolyses were carried out in sealed tubes that were degassed by three freeze, pump, thaw cycles, prior to flame sealing under dynamic vacuum. The photolysate was transferred to autoclaved Eppendorf tubes (0.6 mL) using a plastic draw bulb pipet. Each photolysis tube was washed with H_2O (50 μL). The photolysate was precipitated by adding 3 N NaOAc (pH 5.2, 20 μL), calf thymus DNA (1.3 mM in base pairs, 5 μL), and EtOH (400 μL). The samples were frozen in a dry ice−EtOH bath for 12 min, followed by centrifugation at 14 000 rpm for 12 min. The supernatant was carefully removed by pipet, and the remaining DNA pellet was dried in a Speed-Vac. The dried DNA pellet was resuspended in formamide loading buffer (8 μL) by heating at 55 °C for 20 min. The resuspended DNA was quantitated by scintillation counting. Denaturing 20% PAGE analysis was carried out using 30 000 cpm from each photolysis.

Isolation of Photocleavage Product 4. Method A. Anaerobic photolyses were performed as described. The crude photolysate was purified by 20% denaturing PAGE. An autoradiogram was used to locate **4** on the acrylamide gel. Product **4** was excised from the gel using a scalpel, and the gel slice was placed in a sterile test tube (15 mL). The gel slice was crushed with a sterile wooden applicator stick until it had the consistency of a powder, and 5 mL of sterile elution buffer (0.2 M NaCl, 1 mM EDTA) was added. The mixture was incubated at room temperature for 4 h with periodic vortexing. The elution buffer solution was filtered through a Quick Sep filter. The filtrate was concentrated by butanol extraction to a volume of 200−400 μL. The concentrate was transferred to a sterile Eppendorf tube (1.5 mL) and precipitated as described above. The dried pellet was resuspended in H_2O (400 μL) and filtered through an Amicon 3 filter. The retentate from the Amicon 3 contained **4**, which was washed off the filter with H_2O.

Method B. The ketone-containing DNA fragment (**4**) was purified by PAGE and excised from the gel as described in method A. The acrylamide gel slice was placed in the electroelution apparatus containing TBE buffer.[27] Electroelution was performed at 150 V for 1.5 h, after which no radiation was detectable in the acrylamide gel slice by a survey meter. The elution buffer was filtered through an Amicon 3 as described in method A.

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Supporting Information Available: Autoradiograms for enzymatic end group analyses and statistical treatment of individual kinetic isotope effect experiments (5 pages). See any current masthead page for ordering information and Internet access instructions.

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