The effects of secondary structure and O_2 on the formation of direct strand breaks upon UV irradiation of 5-bromodeoxyuridine-containing oligonucleotides

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Background: 5-Bromodeoxyuridine is a radiosensitizing agent that is currently being evaluated in clinical trials as an adjuvant in the treatment of a variety of cancers. γ-Radiolysis and UV irradiation of oligonucleotides containing 5-bromodeoxyuridine result in the formation of direct strand breaks at the 5′-adjacent nucleotide by oxidation of the respective deoxyribose. We investigated the effects of DNA secondary structure and O_2 on the induction of direct strand breaks in 5-bromodeoxyuridine-containing oligonucleotides.

Results: The efficiency of direct strand break formation in duplex DNA is dependent upon O_2 and results in fragments containing 3′-phosphate and the labile 3′-ketodeoxyadenosine termini. The ratio of the 3′-termini is also dependent upon O_2 and structure. Deuterium product isotope effects and tritium-transfer studies indicate that hydrogen-atom abstraction from the C1′- and C2′-positions occurs in an O_2- and structure-dependent manner.

Conclusions: The reaction mechanisms by which DNA containing 5-bromodeoxyuridine is sensitized to damage by UV irradiation are dependent upon whether the substrate is hybridized and upon the presence or absence of O_2. Oxygen reduces the efficiency of direct strand break formation in duplex DNA, but does not affect the overall strand damage. It is proposed that the σ radical abstracts hydrogen atoms from the C1′- and C2′-positions of the 5′-adjacent deoxyribose moiety, whereas the nucleobase peroxyl radical selectively abstracts the C1′-hydrogen atom from this site. This is the second example of DNA damage amplification by a nucleobase peroxyl radical, and might be indicative of a general reaction pattern for this family of reactive intermediates.

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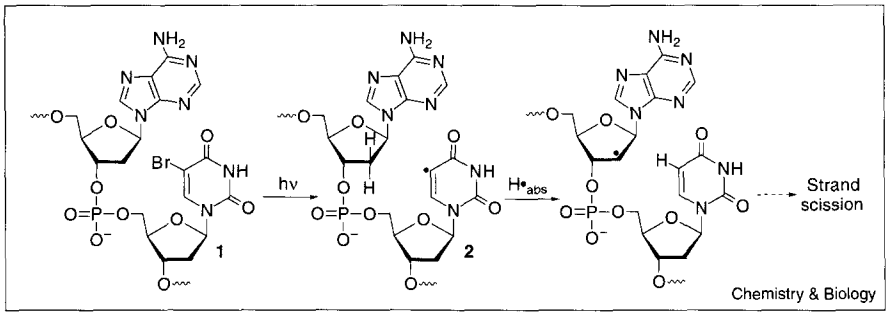
Introduction

5-Bromodeoxyuridine (BrdU, **1**) is a useful tool for analyzing biopolymer structure and interactions, and is potentially useful therapeutically. This pyrimidine continues to be explored as an adjuvant to radiation and chemotherapy in the treatment of a variety of malignancies. The clinical utility of **1** is attributed to its ability to sensitize DNA, in which it has been substituted for thymidine, to damage by γ-radiolysis [1–3]. 5-Bromodeoxyuridine also sensitizes DNA to UV irradiation, resulting in increased amounts of strand damage. The reaction mechanism(s) by which **1** enhances the formation of DNA lesions upon irradiation has been discussed for more than 30 years [4]. Significant advances have been made more recently in understanding the chemistry of DNA damage that is initiated by irradiation of **1** [5–11]. 2′-Deoxyuridin-5-yl (**2**), which is formally derived from the halopyrimidine by homolytic bond scission of the weak carbon–bromine bond, is believed to be the critical reactive intermediate in strand scission.

Initial inspection of models of duplex DNA and consideration of thermodynamics led to the proposal that the s radical (**2**) that is produced upon homolysis of the carbon–bromine bond induces direct strand scission at the 5′-adjacent nucleotide by abstracting one of this nucleotide's C2′-hydrogen atoms (Figure 1) [4]. Subsequent investigations have revealed that the mechanism of direct strand break formation induced by UV irradiation is more complicated than originally proposed. Deoxyuridin-5-yl (**2**) itself might result from two different pathways, one of which involves selective photoinduced single-electron transfer (PSET) from a 5′-adjacent deoxyadenosine in duplex DNA [5,6,10]. In addition, it has been suggested that **2** abstracts hydrogen atoms from the C1′- and C2′-positions of adjacent nucleotides (Figure 2) [5,8,10]. Recently, we put forth a proposal to explain the formation of direct strand breaks upon irradiation of 5-bromodeoxyuridine-containing duplex DNA that unifies previously proposed mechanisms (Figure 3) [5]. The key element in this proposal is that products resulting from formal oxidation of the C3′- (**3**) and C1′- (**4**) positions might result in part from initial C2′-hydrogen atom abstraction, followed by further intranucleotidyl rearrangements. We now wish to report an extension of these studies in an attempt to

Figure 1



The proposed mechanism for strand damage produced upon irradiation of duplex DNA containing 5-bromodeoxyuridine (**1**) [4]. $H^{\bullet}_{abs}$ denotes hydrogen atom abstraction.

explain the effects of O_2 and DNA hybridization on the formation of direct strand breaks emanating from irradiation of biopolymers containing **1**. Our results provide support for a rare example of oxygen-dependent DNA damage amplification and point out another limitation of using product isotope effects [12].

Results

The effects of oligonucleotide hybridization and O_2 on product formation

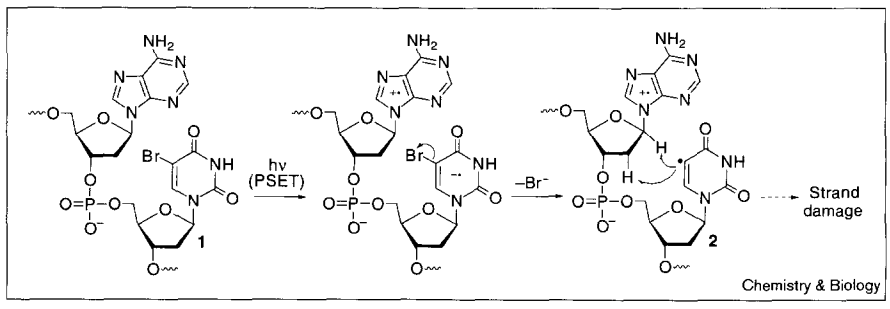
Previously, a significant preference for the formation of direct strand breaks was reported at 5′-dABrdU versus 5′-dGBrdU sequences upon irradiation of duplexes (**5d**, Figure 4) under anaerobic conditions. The selectivity was attributed to a contra-thermodynamic PSET process that is modestly affected by O_2 (Table 1). The formation of direct strand breaks in single-stranded DNA was between 2.5 and 4.0 times less efficient than in the comparable duplex. In addition, selectivity for cleavage at deoxyadenosine was not observed in single-stranded DNA (**5s**) when irradiations were carried out under either aerobic or anaerobic conditions. Also, O_2 did not affect the overall efficiency of direct strand scission in **6s**, whereas approximately twice the amount of such lesions were observed in the corresponding duplex (**6d**) under anaerobic conditions than in the presence of O_2. The total amount of alkaline labile lesions and direct strand breaks formed upon irradiation of **6d**, however, were independent of O_2.

The distribution of fragmentation products resulting from oxidation of the 5′-adjacent nucleotide also proved to be highly dependent upon O_2 and the secondary structure of the biopolymer. The ratio of labile 3′-ketodeoxyadenosine (**3**) : 3′-phosphate (**4**) is reduced in **6d** from ~1.0 under anaerobic conditions to ~0.2 when O_2 is present. In single-stranded DNA (**6s**), the ratio of **3**:**4** formed under anaerobic conditions is somewhat variable, but is reduced to between 0.25 and 0.10 from ~1.0 in duplex DNA. Furthermore, **3** is not observed and is estimated to be < 10%, when single-stranded DNA (**6s**) is irradiated under aerobic conditions. In contrast, the identity of the 5′-terminal end groups of the other oligonucleotide fragment that accompanies the formation of **3** and **4** is unaffected by hybridization or O_2 and consists exclusively of phosphate monoesters.

Deuterium kinetic isotope effects

Deuteration of particular positions in the deoxyribose ring of a nucleotide at which oxidative strand damage occurs has been employed for directly or indirectly determining sites of hydrogen atom abstraction by DNA damaging agents [13–15]. Product kinetic isotope effect (KIE) studies have been particularly useful for indirectly determining sites of hydrogen atom abstraction induced in a number of DNA-damaging systems [16–20]. Previously, we reported on a method by which observed KIEs were measured using oligonucleotides containing two cleavage sites with identical local sequences as substrates (Figure 5) [5]. Control experiments in which neither dA

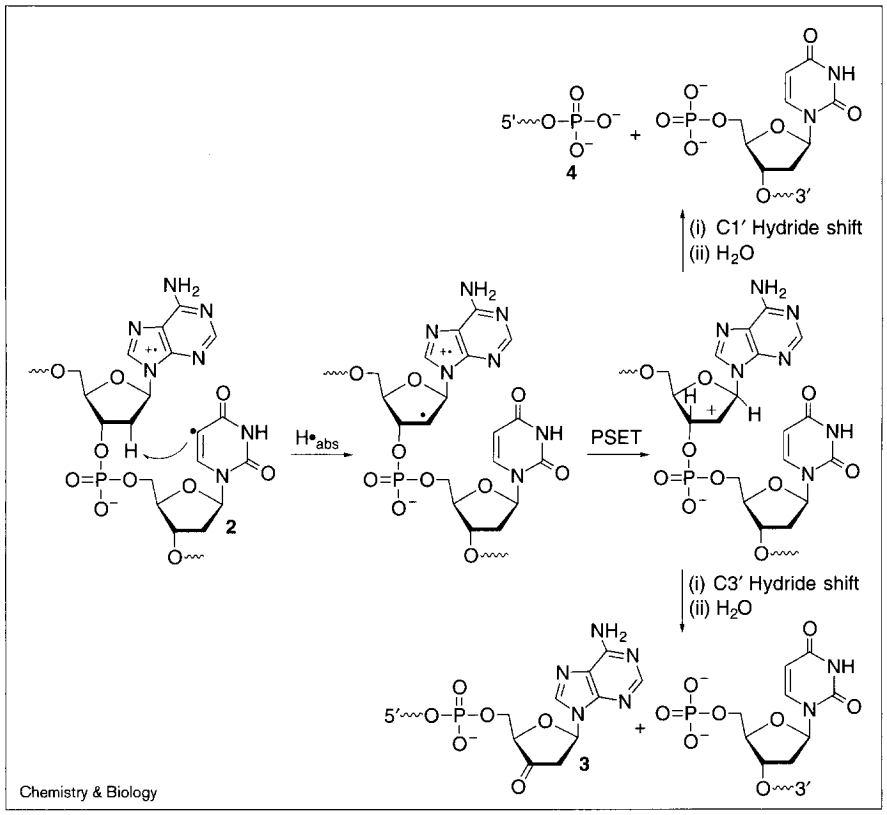
Figure 2



The proposed mechanism for strand damage produced upon irradiation of duplex DNA containing 5-bromodeoxyuridine (**1**) involving PSET-mediated generation of deoxyuridin-5-yl (**2**) [10].

Figure 3

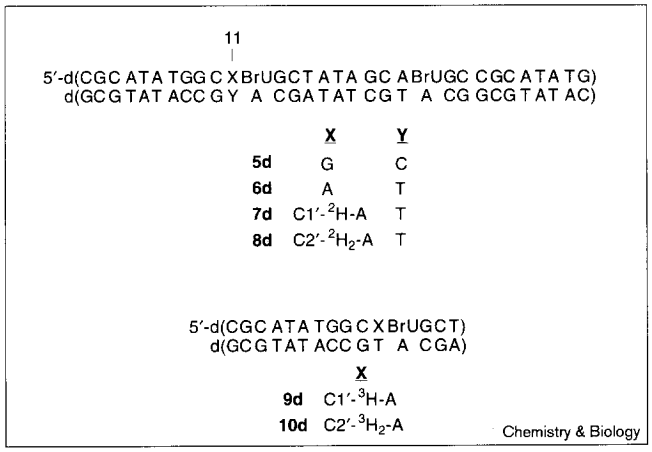
The proposed mechanism for the formation of direct strand breaks upon irradiation of duplex DNA containing 5-bromodeoxyuridine (**1**) involving PSET-mediated generation of deoxyuridin-5-yl (**2**) and selective C2′ hydrogen atom abstraction [5].



adjacent to **1** was deuterated were run side by side. KIEs were determined by measuring the ratio of strand scission at the two sites in the protonated duplex (control, **6d**), relative to the same ratio in which dA_{11} was selectively deuterated at the C1′- (**7d**) or dideuterated at the C2′-positions (**8d**). In this way, the exact percentage of cleavage in the biopolymer was removed as a variable in the experiment. In a typical experiment, five samples of each oligonucleotide were irradiated and the cleavage product(s) at each site were quantitated using a phosphorimager. The KIE was determined from the average cleavage ratio in each oligonucleotide. The standard deviations presented (Table 2) represent the deviation from the average KIE for a series of experiments carried out as described above. All of the KIEs reported herein were determined for 5′-^{32}P-labeled oligonucleotides deuterated at dA_{11} that were photolyzed at 302 nm.

C1′-deuteration and C2′-dideuteration were previously shown to differ significantly in their effects on the formation of the 3′-phosphate (**4**) and 3′-ketodeoxyadenosine (**3**) products in double-stranded oligonucleotides irradiated under anaerobic conditions. The observations were rationalized in terms of the proposed mechanism involving C2′-radical oxidation, followed by competing 1,2-hydride rearrangements (Figure 3) [5]. The magnitude of the observed KIEs upon deuteration of these positions are affected by O_2 and whether the oligonucleotide is complexed with its complement. In duplex DNA, the

Figure 4



Sequences of 5-bromodeoxyuridine-containing oligonucleotides. The respective single-strand substrates are composed of the 5-bromodeoxyuridine-containing oligonucleotides (top strand of duplexes shown) and are designated in the text using the same numbers with the suffix s (**6s–10s**).

Table 1

Effect of O_2 and hybridization on sequence selectivity of direct strand scission.

Secondary structure of oligonucleotide	Cleavage ratio (dA:dG)*	
	Aerobic	Anaerobic
ssDNA†	0.8 ± 0.2	0.8 ± 0.1
dsDNA‡	8.0 ± 0.7	13.8 ± 0.7

*Cleavage ratio is an average of a minimum of two experiments. Each experiment consists of at least four samples. †Oligonucleotide **5s** was used. ‡Oligonucleotide **5d** was used.

observed KIEs upon deuteration of the C1′- (**7d**) and C2′-positions (**8d**) under aerobic conditions are intermediate between those measured under anaerobic conditions. Measurable KIEs are also observed upon deuteration of these positions when the single-stranded substrate is irradiated under anaerobic conditions. Photolysis of single-stranded deuterated oligonucleotides under aerobic conditions yields an observed KIE only when the C1′-position (**7s**) is deuterated.

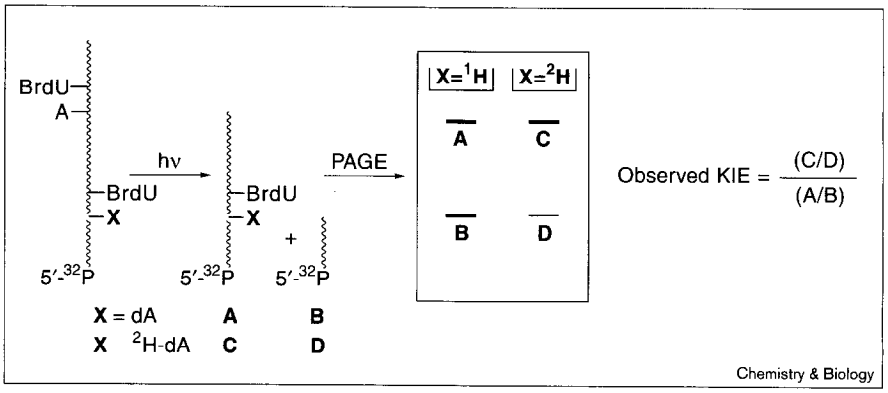
Tritium-transfer experiments

The deuterium kinetic isotope effects are not intrinsic but are derived from the effects of isotopic substitution on strand break (product) formation. By their nature, they are indirect measurements and might reflect a step(s) in a reaction process other than, or in addition to, hydrogen atom abstraction. Furthermore, the observed kinetic isotope effects are modulated by the fraction of the product (a direct strand break) that is produced by the pathway(s) involving the isotopically substituted site [12,20]. Direct nonquantitative evidence for the transfer of atoms under anaerobic conditions from specific positions of the sugar to the uracil ring was obtained using tritiated biopolymers (**9–10**, **d**,**s**). Oligonucleotides **9** and **10** contain a single 5′-dABrdU site and were chemically synthesized using the same methods described for their deuterated analogs [5]. The specific activity of **9s** and **10s** were 1.1 and 2.4 (1.2 per atom mol) Ci/mol, respectively. Tritium transfer was not examined under aerobic conditions because of the anticipated lability of the tritium abstracted by the putative peroxyl radical. Tritium transfer involved in direct strand break formation was determined using liquid scintillation counting of the amount of tritium in deoxyuridine following enzymatic digestion of the direct strand scission product (isolated using anion exchange high-performance liquid chromatography (HPLC)) produced under anaerobic photolysis. The amount of tritium transferred from each respective position (C1′ and C2′) was consistent with the more efficient formation of direct strand breaks in double- stranded DNA than in single-stranded DNA. When comparable amounts of **9s**,**d** and **10s**,**d** were irradiated for equal amounts of time, the amount of tritiated deoxyuridine formed from the double-stranded substrate was approximately 3.4 (**9d**:**9s**) and 3.3 (**10d**:**10s**) times greater than from the single-stranded biopolymer, which is consistent with the greater overall photolability of the double- stranded substrate. Moreover, tritium transfer from C1′ was favored relative to that from the C2′-position in single-stranded (4.6) and double-stranded (4.3) substrates.

Discussion

The previous mechanistic proposals regarding the UV-induced strand scission of oligonucleotides containing **1** must be expanded in light of the observations described above. The effects of oligonucleotide hybridization and O_2 are evident from examination of the efficiency of direct strand break formation, the distribution of 3′-phosphate (**4**) and 3′-ketodeoxyadenosine (**3**) products (Figure 3), and the product deuterium isotope effects (Table 2). The 3′-ketodeoxyadenosine product is indicative of the generation of **2** by PSET. Moiety **4**, however, can be produced by initial PSET or direct carbon–bromine bond homolysis (see below). The decrease in the ratio of fragments containing termini consisting of **3** to 3′-phosphate in single-stranded

Figure 5



A schematic design of deuterium kinetic isotope effect (KIE) experiment. The polyacrylamide gel is represented within the box and A–D correspond to bands associated with specific cleavage products. A and B are products from the protonated control oligonucleotide; C and D are products from the deuterated oligonucleotide. A and C correspond to the BrdU site that is closer to the 3′ terminus of the oligonucleotide. B and D correspond to the BrdU site that is closer to the 5′ terminus of the oligonucleotide.

Table 2

Observed deuterium kinetic isotope effects at dA_{11} as a function of hybridization and O_2.

Secondary structure of oligonucleotide	O_2	KIE*	
		C1′-^{2}H-dA_{11}	C2′-$^2H_2$-dA_{11}
ssDNA†	+	1.70 ± 0.21	0.91 ± 0.09
ssDNA†	−	1.68 ± 0.13	2.18 ± 0.29
dsDNA‡	+	1.66 ± 0.07	2.04 ± 0.07
dsDNA‡§	−	0.91 ± 0.01	4.43 ± 0.19

*Values reported are the average of at least three experiments. Each experiment consists of five samples. †Oligonucleotides **7s** and **8s** were used. ‡Oligonucleotides **7d** and **8d** were used. §Previously reported in [5].

DNA irradiated in the absence of O_2 relative to the respective duplex suggests that the contribution of the PSET pathway to the overall UV-irradiation-induced formation of direct strand breaks is greater in the double-stranded substrate. This is attributable to the decrease in π-stacking in single- stranded oligonucleotides. In addition, the observed decrease in overall strand break formation in single-stranded oligonucleotides also suggests that the PSET pathway gives rise to direct strand scission more efficiently.

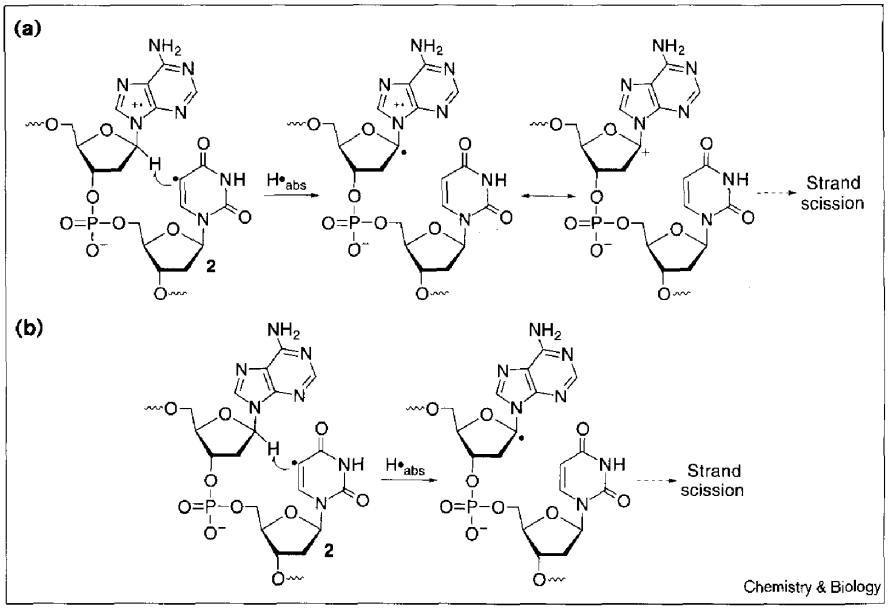
The pronounced effect of O_2 on the formation of 3′-ketodeoxyadenosine (**3**) and overall efficiency of direct strand break formation in double-stranded DNA might be attributed to quenching of the PSET process. This explanation is consistent with the observation that O_2 reduces the observed sequence selectivity for direct strand scission attributed to the PSET generation of **2** (Table 1), but is eliminated from consideration on the basis of the observation that O_2 does not diminish the overall formation of lesions (direct strand breaks and alkaline labile lesions) in the duplex substrate. Instead, we ascribe the effects of O_2 on direct strand break formation to competitive trapping of one or more of the radicals formed following deoxyuridin-5-yl (**2**) generation en route to the fragmented products, which alters the product distribution and the efficiency of their formation. For instance, the pathway to **3** can be diverted by trapping **2** with O_2. This explanation can also be reconciled with the observed effect of O_2 on sequence selectivity in **5d**. Given that cleavage at 5′-dGBrdU is presumably caused by direct carbon–bromine bond homolysis, and cleavage at 5′-dABrdU is caused by this pathway and PSET, more efficient divergence of the latter pathway from direct strand scission by O_2 would result in the observed effect of O_2 on sequence selectivity.

The observed deuterium KIE's are consistent with the above effects of hybridization and O_2 on product formation. The large KIEs observed upon dideuteration of C2′-A_{11} in duplex DNA under anaerobic conditions have been ascribed to hydrogen atom abstraction from this site [5]. In single-stranded DNA, substantial KIEs are observed under anaerobic conditions upon deuteration of both the C1′- (**7s**) and C2′-positions (**8s**). The reduced magnitude of the KIE observed at C2′ and increased KIE upon deuteration of C1′ in A_{11} suggest decreased and increased extents of hydrogen atom abstraction, respectively, from these centers compared with irradiation of duplexed DNA under anaerobic conditions. This hypothesis is consistent with the greater conformational freedom in the single-stranded substrate, providing easier access to the C1′-hydrogen atom. One cannot discount different proportionation of direct strand breaks through the C1′-and C2′-radical manifolds in single- stranded DNA and/or changes in the intrinsic KIEs for hydrogen atom abstraction (particularly from C1′), however, because of a decrease in the fraction of strand scission produced by the PSET pathway (Figure 6).

These issues also warrant consideration when reconciling the deuterium KIEs with the results of tritium transfer studies. Upon photolysis of duplex DNA substrates containing approximately equal specific activity (on a per atom basis), direct strand breaks containing tritium-transferred from the C1′-position are formed more efficiently than those containing tritium transferred from the C2′-position. There are several points to consider when explaining this apparent discrepancy. The intrinsic effect of isotopic substitution of the C2′-position (which is an aggregate effect of a primary and a secondary KIE, and will be even larger for tritium transfer) might induce a greater proportion of abstraction of hydrogen from another position(s). Such a shuttling of reactive positions has been seen in other DNA-damaging systems [18,19]. In addition, the intrinsic KIE in duplex DNA involving abstraction at C1′ might be significantly smaller than that encountered upon C2′-hydrogen atom abstraction. Assuming that in duplex DNA the deoxyuridin-5-yl (**2**) is formed by PSET, the C1′-carbon hydrogen bond would be analogous to the β carbon hydrogen bond of an aminium ion radical (Figure 6). This bond is very weak, and hydrogen atom abstraction from it should be extremely rapid [21–23]. Bimolecular rate constants for hydrogen atom abstraction from one such ion radical are extremely rapid, even by relatively unreactive radicals (e.g. *t*-butyl thiyl, $k = 3.2 \times 10^9\ M^{-1}s^{-1}$) [21]. Consequently, by analogy with the hydroxyl radical, hydrogen atom abstraction from an ion radical by a highly reactive s radical, such as **2**, might result in very small KIEs [20,24–26]. Finally, it should be noted that hydrogen atom abstraction from C1′ of the radical cation gives rise to the carbocation from which direct strand breaks in the form of **4** have been suggested, but remains to be proved [27–29]. This is significant, because less efficient strand scission will reduce the observed KIE.

The apparent increased contribution (according to deuterium KIEs) of direct strand breaks proceeding through

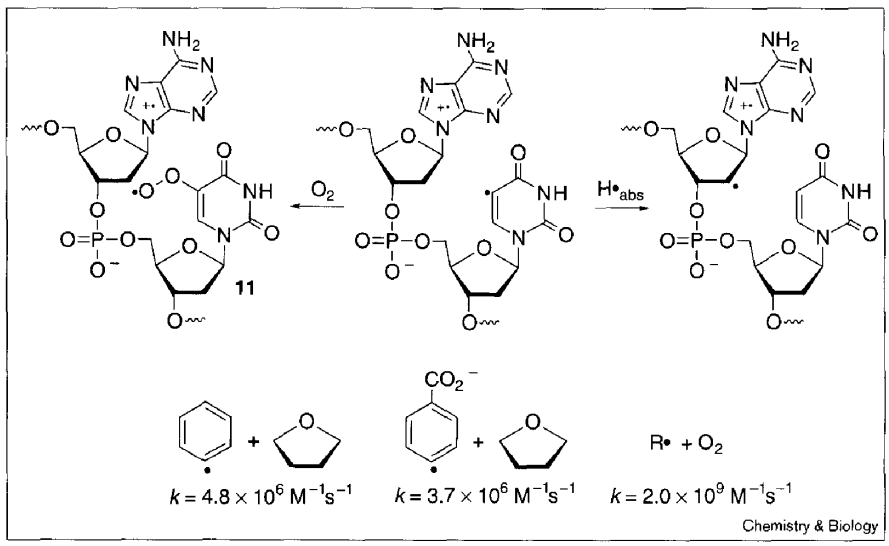
Figure 6



The effect of the mechanism of generation of deoxyuridin-5-yl (**2**) on hydrogen atom abstraction ($H^{\bullet}_{abs}$) from C1′ of the 5′-adjacent deoxyadenosine. **(a)** PSET; **(b)** direct photolysis.

C1′-hydrogen atom abstraction in the single-stranded oligonucleotide substrate compared with duplexed material might be reconciled with the fact that formation of deoxyuridin-5-yl (**2**) by the PSET process is at most a minor pathway in the unhybridized material. Consequently, C1′-hydrogen atom abstraction might face an intrinsically larger KIE than in a double-stranded substrate (Figure 6). It is also possible that C1′-hydrogen atom abstraction produces direct strand breaks more efficiently when the σ radical is formed by direct carbon–bromine bond homolysis compared with the process discussed above following PSET. Independent generation of deoxyuridin-1′-yl at a defined site in an oligonucleotide has shown that direct strand breaks make up a small fraction of the total strand damage resulting from C1′-hydrogen atom abstraction [30]. In contrast, as mentioned above, it is unclear whether or not C1′-nucleotide carbocations produce direct strand breaks. The uncertainty in the fate of nucleotide radicals in biopolymers also clouds any arguments that might be put forth to explain the decrease in the observed KIE upon C2′-dideuteration in single-stranded DNA. Very little is known a priori about DNA damage resulting from C2′-hydrogen atom abstraction, and it is possible that strand scission is less efficient by this

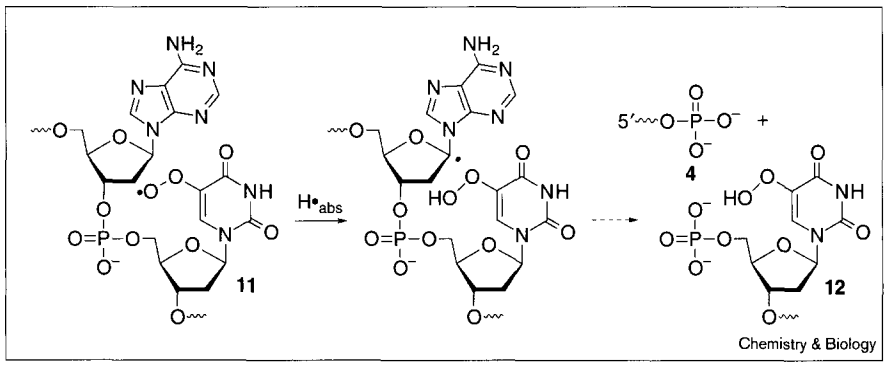
Figure 7



The partitioning of deoxyuridin-5-yl (**2**) in DNA between trapping by O_2 and hydrogen atom abstraction ($H^{\bullet}_{abs}$) from C2′ of the 5′-adjacent deoxyadenosine.

Figure 8

The proposed mechanism for the formation of two contiguous DNA damage sites by **11**.



pathway in the absence of prior electron donation by the appended adenine [31].

Similarly, irradiation of **8s** under aerobic conditions might fail to exhibit a substantial KIE at C2′ due to inefficient strand scission following hydrogen atom abstraction at this position. Prior experiments involving the generation of a nucleobase radical in single-stranded DNA under aerobic conditions, however, suggest that an alternative explanation is more likely [32]. Given that PSET-initiated strand scission is believed to be at most a minor pathway in single-stranded DNA, the aforementioned product studies and product KIE are more easily explained in terms of formation of a highly-selective nucleobase peroxyl radical. On the basis of the previously reported selectivity of the peroxyl radical derived from O_2 trapping of 5,6-dihydrothymidin-5-yl, the observed KIEs in single-stranded DNA (Table 2) are believed to be due to exclusive C1′-hydrogen atom abstraction by **11** (Figure 7), which is formed faster than hydrogen atom abstraction by deoxyuridin-5-yl [32]. Attempts at characterizing the expected hydroperoxide-containing fragmentation product (**12**) resulting from hydrogen atom abstraction by electrospray or matrix-associated laser desorption ionisation–time of flight (MALDI–TOF) mass spectrometry were unsuccessful, and are attributed to the expected instability of the vinyl hydroperoxide (Figure 8). Nevertheless, this proposal is also consistent with recently reported gel electrophoretic studies carried out on an irradiated single-stranded oligonucleotide containing 5-bromodeoxyuridine [33]. It should also be noted that the involvement of **11** in the formation of lesions results in damage at two contiguous nucleotides and represents another example of intrastrand DNA damage amplification [32].

Finally, the most economical explanation that is consistent with the observation of significant KIEs at C1′ and C2′ under aerobic conditions in duplex DNA includes hydrogen atom abstraction by different radical intermediates derived from 5-bromodeoxyuridine. In contrast to single-stranded DNA, we suggest that the conformational constraints of the duplex increase the effective molarity of the adjacent sugar, enabling hydrogen atom abstraction by **2** to compete with O_2 trapping. Extrapolation of known rate constants for hydrogen atom abstraction from tetrahydrofuran by aryl radicals suggest that the aforementioned processes will be competitive with one another provided the effective molarity of the sugar component of A_{11} is on the order of 0.1 M (Figure 7) [34,35]. Consequently, the isotope effect observed upon deuteration of C2′ is interpreted to mean that hydrogen atom abstraction by the deoxyuridin-5-yl σ radical (**2**) occurs from this site, as observed under anaerobic conditions. The reduced magnitude of the C2′ KIE compared with that observed under anaerobic conditions, is attributed to the partitioning of direct strand break formation between the σ radical and the peroxyl species. The peroxyl radical, as it does in single-stranded DNA, selectively abstracts a hydrogen atom from the C1′ position.

Significance

5-Bromodeoxyuridine (1) sensitizes DNA to UV irradiation and γ-irradiation. In this study UV-induced direct strand break formation in oligonucleotides containing 5-bromodeoxyuridine was shown to occur more efficiently in a double-stranded oligonucleotide than in the respective unhybridized material. This is attributed to the higher proportion of cleavage events resulting from initial photoinduced single-electron transfer (PSET) between a 5′-deoxyadenosine and 1 in the double-stranded substrate.

The kinetic isotope effects (KIEs) suggest that the s radical (2) abstracts hydrogen atoms from the C1′- and C2′-positions in single-stranded DNA, but that a greater proportion of C2′-hydrogen atom abstraction occurs in double-stranded DNA. Tritium-transfer experiments indicate preferential transfer from the C1′-position independent of secondary structure. These experiments reveal the limitation of the use of deuterium product KIEs, as well as qualitative isotope-transfer experiments. Together, the isotopic labeling experiments indicate that

both deoxyribose positions are accessible to the σ radical and that further studies of the inherent reactivity of the sugar radicals are necessary in order to fully elucidate the details of these processes.

The peroxyl radical (**11**), obtained from O_2 trapping of **2**, selectively abstracts the C1′-hydrogen atom from the 5′-adjacent nucleotide, as was observed previously for the respective peroxyl radical of 5,6-dihydrothymidin-5-yl [32]. In double-stranded DNA this results in a decreased efficiency for the formation of direct strand breaks, albeit presumably via a rare example of a pathway that produces two contiguous damaged nucleotides in a single oligonucleotide. One should note that the total amount of direct-strand breaks and alkaline labile lesions are independent of O_2 in double-stranded DNA. Moreover, the enhanced efficiency of **1** in sensitizing duplex DNA towards direct strand breaks under anaerobic conditions is a desirable property given the application of this agent in the treatment of cancerous cells that are often hypoxic.

Materials and methods

General methods

All H_2O used was obtained from a Nanopure® Barnstead still. Commercially available DNA synthesis reagents were obtained from Glen Research Inc. Radionuclides and NaB_3H_4 were obtained from Amersham. The T4 polynucleotide kinase and calf intestine alkaline phosphatase were obtained from New England Biolabs. Oligonucleotides were synthesized on an Applied Biosystems Inc. 380B DNA synthesizer using standard protocols. Deuterated and tritiated oligonucleotides were prepared as described previously [5]. Oligonucleotides containing **1** were deprotected in 28% NH_4OH at room temperature for 48 h. All other oligonucleotides were deprotected in 28% NH_4OH at 55°C overnight. DNA manipulations were carried out using standard procedures [36]. Oligonucleotides were sequenced using a reaction selective for adenine [37]. Preparative and analytical oligonucleotide separations were carried out on 20% polyacrylamide denaturing gels (5% crosslink, 45% urea (by weight)). Radioactive samples were counted by Cerenkov counting, using a Packard Tri-Carb 1500 scintillation counter. Photolyses were carried out at 302 nm in Pyrex tubes using a UV Photoproducts dual wavelength transilluminator containing eight 8 W lamps. Photolysis tubes were positioned 8 cm from the source. Anaerobic photolyses were carried out in tubes that were flame sealed under vacuum, following freeze-pump-thaw degassing (three cycles). Phosphorimaging 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.

Tritium atom transfer experiments

Photolyses of freeze-pump-thaw degassed samples were carried out for 4 h. The direct strand scission product(s) from the crude photolysate were separated from intact and alkaline labile containing oligonucleotides using anion exchange chromatography. The direct strand break products (tetramers) eluted from the column between 5.1 and 6.6 min, and were collected in sterile 15 μl polypropylene tubes and concentrated to dryness in a vacuum. The intact and alkaline labile lesion containing oligonucleotides co-eluted at 16.2 min. The oligonucleotide samples were resuspended in H_2O (81 μl), and reaction buffer (15 mM $MgCl_2$, 0.1 mM Tris–acetate pH 8.8, 10 μl). Snake venom phosphodiesterase (SVP, 3 μl) was added and the reaction incubated at 37°C for 2 h. The SVP digest was heat denatured and a second portion of SVP (3 μl) added, followed by incubation at 37°C for an additional 2 h. Calf alkaline phosphatase (3 μl) was added directly to the digest, and the reaction was incubated for 12 h at 37°C, after which the reactions were precipitated from EtOH. The dried pellet was resuspended in H_2O and concentrated to dryness (2 × 50 μl). The oligonucleotide pellet was then resuspended in H_2O (50 μl) and analyzed directly using C_{18} reverse phase HPLC. The free nucleoside digestion products were collected in 20 ml scintillation vials, mixed with 10 ml of Picofluor scintillation cocktail, and allowed to stand for 6 h. The tritium content of each fraction was measured by liquid scintillation counting and converted to dpm using a standard tritium quench curve.

Anion exchange HPLC conditions

A Vydac 301 VHP575 column was held at 50°C with an Eppendorf TC-45 temperature controller and a CH-30 Eppendorf column heater. Solvent A, 10 mM Tris–HCl pH 8.0; Solvent B, 10 mM Tris–HCl pH 8.0, 1.0 M NaCl. Flow conditions: initial to 10 min, 10% B (1.0 μl/min); linearly to 100% B and 2 μl/min over 5 min; 100% B (2 μl/min) for 10 in.

Reverse-phase HPLC conditions

A Microsorb-MV, 100 Å, 5 mm C18 reverse phase column was used at ambient temperature. Solvent A, 10 mM KH_2PO_4, 2.5% MeOH, pH 7.0; Solvent B, 10 mM KH_2PO_4, 20% MeOH, pH 7.0. Flow conditions (1.0 μl/min): Initial 100% A; linearly to 100% B over 15 min; 100% B for 15 min. Retention times: dC, 10.5 min; dU, 14.3 min; dG , 20.2 min; T, 21.0 min; dA, 26.0 min.

Standard photolysis conditions of ^{32}P-labeled samples

Photolyses were carried out in phosphate-buffered saline solution (50 μl, 10 mM phosphate (pH 7.0), 10 mM NaCl) of DNA. Photolyses were carried out for between 0.5 and 2 min. The photolysate was transferred to autoclaved Eppendorf tubes. Each photolysis tube was washed with H_2O (50 μl). The photolysate was precipitated from EtOH. Denaturing 20% PAGE analysis was carried out using 30,000 cpm from each photolysis.

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References

1. Prados, M.D., *et al.*, & Phillips, T.L. (1998). Influence of bromodeoxyuridine radiosensitization on malignant glioma patient survival: a retrospective comparison of survival data from the Northern California Oncology Group (NCOG) and radiation therapy oncology group trials (RTOG) for glioblastoma multiform and anaplastic astrocytoma. *Int. J. Rad. Oncol. Biol. Phys.* **40**, 653-659.
2. Limoli, C.L., Wu, C.C.L., Milligan, J.R. & Ward, J.F. (1997). Photochemical production of uracil quantified in bromodeoxyuridine-substituted SV40 DNA by uracil DNA glycosylase and a lysyl–tyrosyl–lysine tripeptide. *Mutagenesis* **12**, 443-447.
3. Chelladurai, M., *et al.*, & McLaughlin, P.W. (1997). Bromodeoxyuridine improves the cytotoxic effect of cisplatin. A comparison with 5-fluorouracil. *Cancer Chemother. Pharmacol.* **40**, 463-468.
4. Hutchinson, F. (1973). The lesions produced by ultraviolet light in DNA containing 5-bromouracil. *Quart. Rev. Biophys.* **6**, 201-246.
5. Cook, G.P. & Greenberg, M.M. (1996). A novel mechanism for the formation of direct strand breaks upon anaerobic photolysis of duplex DNA containing 5-bromodeoxyuridine. *J. Am. Chem. Soc.* **118**, 10025-10030.
6. Fujimoto, K., Sugiyama, H. & Saito, I. (1998). Sequence dependent photoreduction of 5-bromouracil-containing oligonucleotides via electron transfer. *Tetrahedron Lett.* **39**, 2137-2140.
7. Sugiyama, H., Fujimoto, K. & Saito, I. (1995). Stereospecific 1,2-hydride shift in ribonolactone formation in the photoreaction of 2′-iododeoxyuridine. *J. Am. Chem. Soc.* **117**, 2945-2946.
8. Sugiyama, H., Fujimoto, K. & Saito, I. (1996). Evidence for intrastrand C2′ hydrogen abstraction in photoirradiation of 5-halouracil-containing oligonucleotides by using stereospecifically C2′-deuterated deoxyadenosine. *Tetrahedron Lett.* **37**, 1805-1808.

9. Sugiyama, H., Tsutsumi, Y., Fujimoto, K. & Saito, I. (1993). Photoinduced deoxyribose C2′ oxidation in DNA. Alkali-dependent cleavage of erythrose-containing sites via a retroaldol reaction. *J. Am. Chem. Soc.* **115**, 4443-4448.
10. Sugiyama, H., Tsutsumi, Y. & Saito, I. (1990) Highly sequence selective photoreaction of 5-bromouracil-containing deoxyhexanucleotides. *J. Am. Chem. Soc.* **112**, 6720-6721.
11. Dietz, T.M., von Trebra, R.J., Swanson, B.J. & Koch, T.H. (1987). Photochemical coupling of 5-bromouracil (BU) to a peptide linkage. A model for BU-DNA protein photocrosslinking. *J. Am. Chem. Soc.* **109**, 1793-1797.
12. Goto, Y., Watanabe, Y., Fukuzumi, S., Jones, J.P. & Dinnocenzo, J.P. (1998) Mechanism of N-demethylations catalyzed by high-valent species of heme enzymes: novel use of isotope effects and direct observation of intermediates. *J. Am. Chem. Soc.* **120**, 10762-10763.
13. Meschwitz, S.M., Schultz, R.G., Ashley, G.W. & Goldberg, I.H. (1992). Selective abstraction of ^2H from C-1′ of the C residue in AGC–ICT by the radical center at C-2 of activated neocarzinostatin chromophore: structure of the drug/DNA complex responsible for bistranded lesion formation. *Biochemistry* **31**, 9117-9121.
14. Hangeland, J.J., *et al.*, & Ellestead, G.A. (1992). Specific abstraction of the 5′(S)- and 4′-deoxyribosyl hydrogen atoms from DNA by calicheamicin γ_1^I. *J. Am. Chem. Soc.* **114**, 9200-9202.
15. De Voss, J.J., *et al.*, & Schreiber, S.L. (1990). Site-specific atom transfer from DNA to a bound ligand defines the geometry of a DNA-calicheamicin g1I complex. *J. Am. Chem. Soc.* **112**, 9669-9670.
16. Kozarich, J.W., Worth, L., Frank, B.L., Christner, D.F., Vanderwall, D.E. & Stubbe, J. (1989). Sequence-specific isotope effects on the cleavage of DNA by bleomycin. *Science* **245**, 1396-1399.
17. Kappen, L.S., Goldberg, I.H., Wu, S.H., Stubbe, J., Worth, L., Jr. & Kozarich, J.W. (1990). Isotope effects on the sequence-specific cleavage of dC in d(AGC) sequences by neocarzinostatin: elucidation of chemistry of minor lesions. *J. Am. Chem. Soc.* **112**, 2797-2798.
18. Kappen, L.S., *et al.*, & Stubbe, J. (1991). Neocarzinostatin-induced hydrogen atom abstraction from C-4′ and C-5′ of the T residue at a d(GT) step in oligonucleotides: shuttling between deoxyribose attack sites based on isotope selection effects. *Biochemistry* **30**, 2034-2042.
19. Xu, Y., Xi, Z., Zhen, Y. & Goldberg, I.H. (1995). A single binding mode of activated enediyne C1027 generates two types of double-strand DNA lesions: deuterium isotope-induced shuttling between adjacent nucleotide target sites. *Biochemistry* **34**, 12451-12460.
20. Balasubramanian, B., Pogozelski, W.K. & Tullius, T.D. (1998). DNA strand breaking by the hydroxyl radical is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone. *Proc. Natl Acad. Sci. USA* **95**, 9738-9743.
21. Alder, R.W., Bonifacic, M. & Asmus, K.-D. (1986). Reaction of a stable N–N bonded radical cation with free radicals generated by pulse radiolysis: exceedingly rapid hydrogen abstraction from C–H bonds. *J. Chem. Soc. Perkin Trans. II* 277-284.
22. Zhang, X.-M. (1998). Homolytic bond dissociation enthalpies of the C–H bonds adjacent to radical centers. *J. Org. Chem.* **63**, 1872-1877.
23. Dinnocenzo, J.P., Farid, S., Goodman, J.L., Gould, I.R., Todd, W.P. & Mattes, S.L. (1989). Nucleophile-assisted cleavage of silane cation radicals. *J. Am. Chem. Soc.* **111**, 8973-8975.
24. Anbar, M., Meyerstein, D. & Neta, P. (1966). Reactivity of aliphatic compounds toward hydroxyl radicals. *J. Chem. Soc. B.* 742-747.
25. Walling, C. & Kato, S. (1971). The oxidation of alcohols by Fenton's reagent. The effect of copper ion. *J. Am. Chem. Soc.* **93**, 4275-4281.
26. Snook, M.E. & Hamilton, G.A. (1974). Oxidation and fragmentation of some phenyl-substituted alcohols and ethers by peroxydisulfate and Fenton's reagent. *J. Am. Chem. Soc.* **96**, 860-869.
27. Meijler, M.M., Zelenke, O. & Sigman, D.S. (1997). Chemical mechanism of DNA scission by (1,10-phenanthroline)copper. Carbonyl oxygen of 5-methylenefuranone is derived from water. *J. Am. Chem. Soc.* **120**, 1135-1136.
28. Cheng, C.-C., Goll, J.G., Neyhart, G.A., Welch, T.W., Singh, P. & Thorp, H.H. (1995). Relative rates and potentials of competing redox processes during DNA cleavage: oxidation mechanisms and sequence-specific catalysis of the self-inactivation of oxometal oxidants by DNA. *J. Am. Chem. Soc.* **117**, 2970-2980.
29. Chen, T. & Greenberg, M.M. (1998). Model studies indicate that copper phenanthroline induces direct strand breaks via β-elimination of the 2′-deoxyribonolactone intermediate observed in enediyne mediated DNA damage. *J. Am. Chem. Soc.* **120**, 3815-3816.
30. Tronche, C., Goodman, B.K. & Greenberg, M.M. (1998). DNA damage induced via independent generation of the radical resulting from formal hydrogen atom abstraction from the C1′-position of a nucleotide. *Chem. Biol.* **5**, 263-271.
31. Hissung, A., Isildar, M. & von Sonntag, C. (1981). Radiolysis of aqueous solutions of nucleosides halogenated at the sugar moiety. *Int. J. Radiat. Biol.* **39**, 185-193.
32. Greenberg, *et al.*, & Venkatesan, H. (1997). DNA damage induced via 5,6-dihydrothymid-5-yl in single-stranded oligonucleotides. *J. Am. Chem. Soc.* **119**, 1828-1839.
33. Doddridge, Z.A., Warner, J.L., Cullis, P.M. & Jones, G.D.D. (1998). UV-Induced strand break damage in single stranded bromodeoxyuridine-containing DNA oligonucleotides. *J. Chem. Soc. Chem. Commun.* 1997-1998.
34. Garden, S.J., Avila, D.V., Beckwith, A.L.J., Bowry, V.W., Ingold, K.U. & Lusztyk, J. (1996). Absolute rate constant for the reaction of aryl radicals with tri-n-butyltin hydride. *J. Org. Chem.* **61**, 805-809.
35. Scaiano, J.C. & Stewart, L.C. (1983). Phenyl radical kinetics. *J. Am. Chem. Soc.* **105**, 3609-3614.
36. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* (2nd Ed.), Cold Spring Harbor Laboratory Press, New York, USA.
37. Iverson, B.L. & Dervan, P.B. (1987). Adenine specific DNA chemical sequencing reaction. *Nucleic Acids Res.* **15**, 7823-7830.

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