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Bromopyridone Nucleotide Analogues, Anoxic Selective Radiosensitizing Agents That Are Incorporated in DNA by Polymerases

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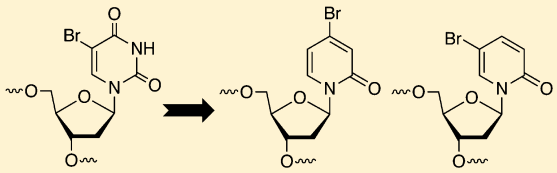
Supporting Information

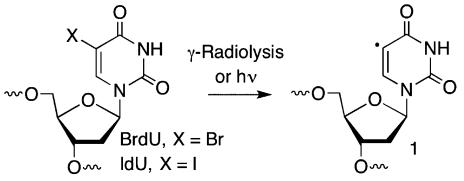
ABSTRACT: Ionizing radiation is frequently used to kill tumor cells. However, hypoxic solid tumor cells are more resistant to this treatment, providing the impetus to develop molecules that sensitize cells to ionizing radiation. 5-Bromo-2′-deoxyuridine (BrdU) has been investigated as a radiosensitizing agent in the lab and clinic for almost 5 decades. Recent reports that BrdU yields DNA interstrand cross-links (ICLs) in non-base-paired regions motivated us to develop radiosensitizing agents that generate cross-links in duplex DNA selectively under anoxic conditions. 4-Bromo- and 5-bromopyridone analogues of BrdU were synthesized and incorporated into oligonucleotides via solid-phase synthesis. Upon irradiation, these molecules yield DNA interstrand cross-links under anaerobic conditions. The respective nucleotide triphosphates are substrates for some DNA polymerases. ICLs are produced upon irradiation under anoxic conditions when the 4-bromopyridone is present in a PCR product. Because the nucleoside analogue is a poor phosphorylation substrate for human deoxycytidine kinase, a pronucleotide form of the 4-bromopyridone was used to incorporate this analogue into cellular DNA. Despite these efforts, the 4-bromopyridone nucleotide was not detected in cellular DNA. Although these molecules are improvements over previously reported nucleotide analogues designed to be hypoxic radiosensitizing agents, additional advances are needed to create molecules that function in cells.



INTRODUCTION

More than 50% of cancer patients receive radiation treatment, which kills tumor cells by damaging DNA. Radiation produces modified nucleotides, single strand breaks, as well as DNA−DNA interstrand cross-links (ICLs), DNA−protein cross-links, and double strand breaks. ICLs are typically more detrimental to cells than single strand breaks or modified nucleotides, but are formed in much lower yields. Solid tumors often possess deficient vasculature for delivering O_2 and other nutrients to cells. Ironically, the hypoxic character of solid tumor cells makes them less susceptible to the cytotoxic effects of γ-radiolysis due to the involvement of organic free radicals in the chemical processes and the need for O_2 to compete with thiols and "fix" DNA radicals produced upon irradiation. A long-standing problem in radiation mediated cell-killing concerns the development of molecules that help overcome O_2 deficiency in solid tumors.[1,2] One approach to developing such radiosensitizing agents involves incorporating nonnative nucleosides into DNA that enhance nucleic acid damage upon γ-radiolysis. Using halogenated pyrimidines, such as 5-bromo-2′-deoxyuridine (BrdU) and 5-iodo-2′-deoxyuridine (IdU) has achieved limited success in this regard, possibly in part due to the facile repair of the single strand break and alkali-labile lesions produced from these radiosensitizers (Scheme 1).[3] Despite this lack of effectiveness, these molecules are still under investigation after ∼50 years, and few if any other nucleotides have been developed as radiosensitizers.[4−7] We have designed molecules that sensitize DNA to ionizing radiation selectively under anaerobic conditions and produce DNA interstrand cross-links.

Scheme 1



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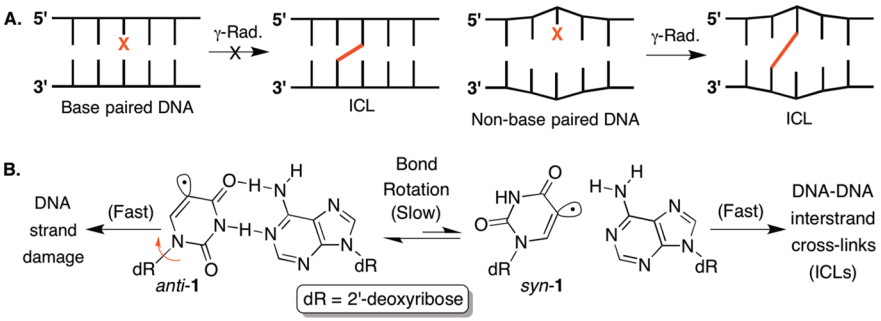
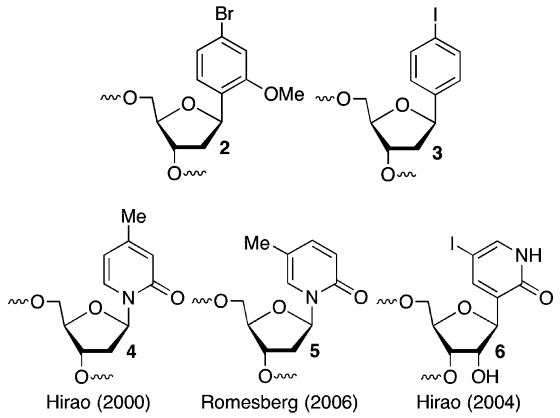


Figure 1. Irradiation of BrdU produces ICLs in nonbase paired DNA. (A.) Cartoon illustration of ICL dependence from BrdU (X) on DNA structure. (B.) ICL formation requires formation of *syn*-**1**, which in duplex DNA is slow compared to the reactivity of *anti*-**1**.

Desirable properties of nucleotide radiosensitizers include enhanced DNA damage upon irradiation selectively under O_2 deficient conditions and incorporation into cellular DNA by polymerases. The polymerases substitute BrdU and IdU for thymidine. BrdU and IdU sensitize DNA to radiation by scavenging solvated electrons, which are produced from the ionization of water and/or released from other portions of the DNA. The electron adduct releases the corresponding halide, yielding a highly reactive σ-radical (**1**, Scheme 1).[8,9] Radical **1** ultimately leads to strand breaks and alkali-labile lesions by abstracting hydrogen atoms from adjacent nucleotides.[10−15] The inspiration for our efforts is provided in part from recent research that describes ICL formation from BrdU in nonbase paired regions of DNA (Figure 1A).[16,17] ICLs are of particular interest because they are absolute blocks of transcription and replication, and are produced by extremely cytotoxic chemotherapeutics.[18−20] ICLs are generally regarded to be a more deleterious form of damage than the strand breaks and nucleotide modifications typically produced from irradiation of BrdU. As few as 20 ICLs can be lethal to a cell.[21] However, their formation only in nonbase paired DNA from BrdU is not useful for radiosensitizing cells. We believe that this limitation is attributable to the structure of BrdU, and that we can overcome this by using different halogenated nucleosides.[22]

Cross-linking requires that **1** adopt the *syn*-conformation (*syn*-**1**) in which the radical faces the opposing strand (Figure 1B). Our own research on ICL formation from DNA radicals, and the high reactivity of **1**, suggest that the barrier to rotation about the glycosidic bond prevents cross-linking in duplex DNA.[23,24] ICLs form via *syn*-**1** in nonbase paired regions of DNA, because Watson−Crick hydrogen bonds need not be broken in this environment.[17,23,25,26] Hence, adoption of *syn*-**1** is more facile. Our approach has been to design nucleotide analogues with reduced hydrogen bonding capacity that would encounter lower barriers for adopting conformations that can lead to interstrand cross-link formation. Our designs have been guided by the significant advances in the development of non-hydrogen bonding nucleotide analogues for probing polymerase mechanisms and expanding the genetic code.[27−36] Polymerase incorporation of nucleotide radiosensitizing agents is less challenging than expanding the genetic code because even low incorporation levels may be satisfactory since DNA interstrand cross-links are very deleterious to cells. In addition, nonselective polymerase incorporation opposite native nucleotides is satisfactory. This has led to the recent use of aryl halides (**2**, **3**) that yield ICLs.[22,35] The ICLs are produced by reaction with the opposing nucleotide and flanking nucleotides. The exact distribution of ICLs depends upon the identity of the nucleotide opposite the radical precursor. ICLs were produced selectively under anaerobic conditions when duplexes containing them were exposed to γ-radiolysis. Mechanistic studies are consistent with σ-radical formation via reaction with solvated electrons. The involvement of solvated electrons, and their diffusion controlled reaction with O_2 leads to the selectivity under hypoxic conditions. Although the halogenated aromatic nucleotide analogues produce ICLs, DNA polymerases did not accept the corresponding nucleotide triphosphates as substrates. Consequently, **1** and **2** could not be used in cells. Herein, we describe nucleotide analogues that selectively produce ICLs when DNA containing them is irradiated under anoxic conditions. Furthermore, the respective nucleotide triphosphates are accepted as substrates by DNA polymerase(s).



RESULTS AND DISCUSSION

The molecules described in this study were based on a combination of results mentioned above involving **2** and **3**, the expectation that molecules with compromised ability to form hydrogen bonds with nucleotides on the opposing strand will more readily adopt conformations conducive to ICL formation, and the importance of a hydrogen bond acceptor in the minor groove for acceptance by DNA polymerase(s).[22,35,37−39] The precedents for the pyridine-2-one moiety in nucleotides **7** and **8** are found in the work of Hirao, Romesberg, and Kuchta who employed nucleotide analogues of this variety in replication (**4**, **5**) and transcription experiments (**6**).[39−42] The iodine containing analogue (**6**) was used in photochemical cross-linking experiments.[40] Consequently, bromopyridones **7** and **8** were synthesized and evaluated as radiosensitizer candidates. (Please note that for simplicity the aryl halides are identified by

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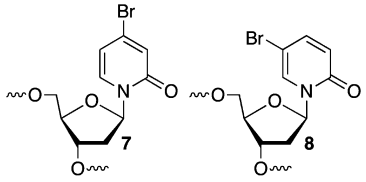
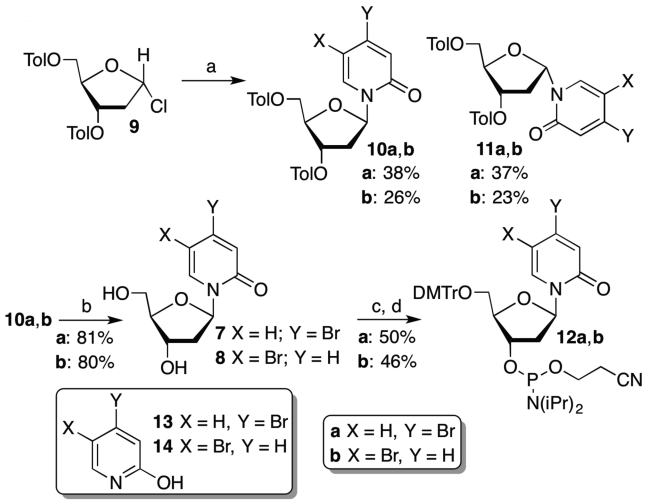
the same numerical descriptor whether they are present as the monomer or as a component within an oligonucleotide.)



Synthesis of the Nucleoside Analogues, Their Incorporation into Chemically Synthesized Oligonucleotides, Their Respective Triphosphates, and a Pro-Nucleotide. Various aspects of this investigation required the nucleoside analogues **7** and **8** or related molecules. Bromopyridones **7** and **8** were synthesized via a common strategy involving Lewis acid-mediated glycosylation of α-chloro sugar **9** (Scheme 2).[41−43]

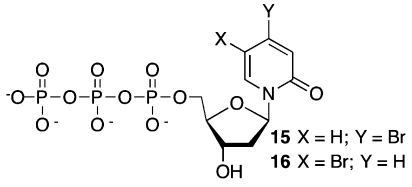
Scheme 2[a]



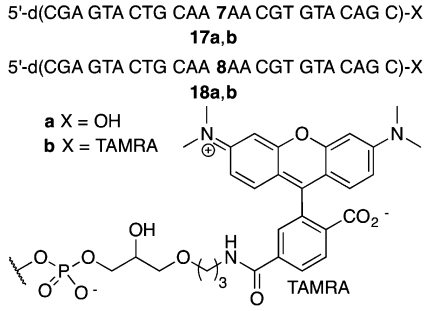
[a]Key: a. **13** or **14** and BSA, then **9**, $SnCl_4$, CH_2Cl_2 b. NaOMe, MeOH c. DMTrCl, pyridine d. 2-Cyanoethyl-*N*,*N*′-diisopropyl phosphoramidic chloride, diisopropylethylamine, CH_2Cl_2.

[a]Key: (a) **13** or **14** and BSA, then **9**, $SnCl_4$, CH_2Cl_2, (b) NaOMe, MeOH, (c) DMTrCl, pyridine, (d) 2-Cyanoethyl-*N*,*N*′-diisopropyl phosphoramidic chloride, diisopropylethylamine, CH_2Cl_2.

Silylation of 4-bromo- (**13**) or 5-bromo-2-hydroxypyridine (**14**),[44] followed by reaction with **9** yielded separable diastereomeric mixtures (**10**, **11**) of the glycosylation products. Methanolysis of **10a,b** provided the free nucleoside analogues **7** and **8**. The stereochemistry was evaluated by comparing the ^{1}H NMR spectra of **10a,b**, **7**, and **8** to the corresponding nonhalogenated compounds.[41,42] The nucleoside analogues were carried on to phosphoramidites **12a,b** and the respective nucleotide triphosphates (**15**, **16**) via standard methods.[45]

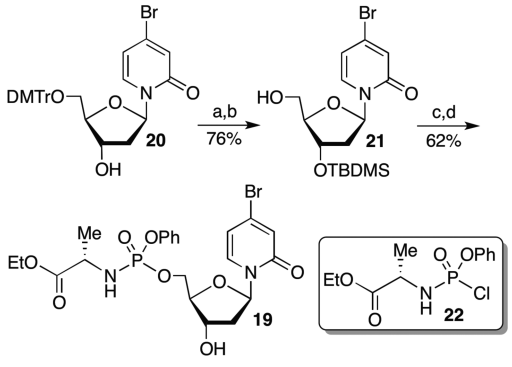


The phosphoramidites of the halogenated nucleotide analogues were incorporated into oligonucleotides (**17**, **18**) via automated solid phase synthesis. The coupling time for **12a,b** was extended to 15 min. Otherwise, standard procedures and reagents were employed. Oligonucleotides containing TAMRA (**17b**, **18b**) were prepared using commercially available "fast deprotecting" phosphoramidites and deprotected with 0.15 M K_2CO_3 in MeOH overnight. Otherwise, oligonucleotides were deprotected using 1:1 aqueous methylamine and concentrated NH_4OH at 65 °C for 90 min.[46] The oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis and characterized by ESI-MS.[47] No evidence for debromination was detected by MS.



Incorporation of **7** or **8** in cellular DNA requires that the respective nucleotide triphosphates (**15**, **16**) be substrates for DNA polymerase(s). However, the triphosphates are themselves poor candidates for passing through cell membranes, and must be synthesized within the cell. Phosphorylation of nucleosides to 5′-monophosphates is typically the rate limiting step in nucleotide triphosphate synthesis in cells. A pronucleotide (**19**) was synthesized because **7** is a poor substrate for the human deoxycytidine kinase (dCk), which phosphorylates 3 of the native nucleosides (see below).[47,48] Pronucleotides overcome the stringent selectivity of human kinases. Phosphoramidate **19** (Scheme 3) makes use of an

Scheme 3[a]



[a]Key: a. TBDMSCl, Imidazole, pyridine b. Trichloroacetic acid, CH_2Cl_2 c. **22**, *N*-methyl imidazole,THF d. TFA, H_2O.

[a]Key: (a) TBDMSCl, Imidazole, pyridine, (b) Trichloroacetic acid, CH_2Cl_2, (c) **22**, *N*-methyl imidazole, THF, (d) TFA, H_2O.

established approach for delivering masked nucleotides to cells that are chemoenzymatically transformed into the respective monophosphates.[49,50] The synthesis of **19** proceeded from **20** which was transformed into the appropriately protected substrate (**21**, Scheme 3) for introducing the phosphoramidate. Coupling of the phosphochloridate (**22**)[49] with **21** was induced by activating the electrophile with *N*-methyl imidazole instead of deprotonating the alcohol, which led to decomposition.[50]

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The 3′-silyl group was then removed under acidic conditions to provide pro-nucleotide **19**.

Interstrand Cross-Link Formation upon ^{137}Cs-Irradiation of Duplex DNA Containing Bromopyridones (7, 8). ^{137}Cs irradiation (500 Gy) of duplexes **23a−d** and **24a−d** containing **7** or **8** (prepared from **17b** or **18b**) under anaerobic conditions produced interstrand cross-links (Table 1). Cross-linking was not detected when a comparable duplex containing only native nucleotides was irradiated. There was no clear dependence of ICL yield on which bromopyridone was irradiated, or on the identity of the opposing nucleotide. Cross-linked products were identified based upon their migration in denaturing (20%) polyacrylamide gels, and the ICLs isolated in highest yield (Table 1) from **7** (**23a**) and **8** (**24d**) were characterized by ESI-MS.[47] In addition, cross-linking position(s) were determined in ICLs obtained from **23a** and **24d** using hydroxyl radical cleavage.[47,51,52] Both bromopyridones (**7** and **8**) yielded cleavage patterns indicative of cross-linking with more than one nucleotide on the opposing strand. This is consistent with previous studies on halogenated nucleotide analogues incapable of standard Watson−Crick base pairing, and is presumably due to local destabilization of the duplex.[35] However, the nucleotides at which the ICLs derived from **23a** and **24d** are distributed differ. Opposing dC_{13} was the major site of cross-linking from **8** in **24d**, whereas the preferred cross-link from **7** in **23a** was biased in the 5′-direction (T_{12}), as one might expect for a reactive species generated in the major groove of DNA.[53] This minor difference could be attributable to different opposing nucleotides and/or variations in the positioning of the radicals produced from **7** and **8**.

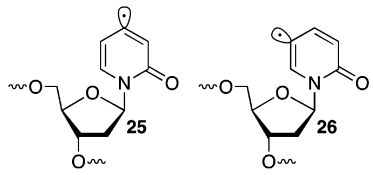
Table 1. Interstrand Cross-Link Yields Following γ-Radiolysis (500 Gy) of DNA Containing 7 or 8 under Anaerobic Conditions[a]

5′-d(CGA GTA CTG CA A **X** A A CGT GTA CAG C)-TAMRA
3′-d(GCT CAT GAC GTT$_{14}$ **Y**$_{13}$T$_{12}$T GCA CAT GTC G)

23a-d X = 7 a Y = A c Y = G
24a-d X = 8 b Y = T d Y = C

	ICL yields[a] (%)			
X	Y = A	Y = T	Y = G	Y = C
7	7.3 ± 0.9	3.8 ± 0.1	5.8 ± 3.0	6.7 ± 1.7
8	2.0 ± 0.5	4.7 ± 1.2	3.6 ± 2.2	5.3 ± 1.2

[a]Yields are the average ± std. dev. of 3 samples.



Experiments with O_2 and *t*-BuOH are consistent with formation of the respective σ-radicals (**25**, **26**) from **7** and **8** via reaction with solvated electrons that produce intermediate radical anions. Specifically, *t*-BuOH (10 mM) has no effect on ICL yields, consistent with the lack of involvement by hydroxyl radical. However, ICL formation is completely quenched in the presence of O_2 (~0.2 mM), which scavenges solvated electrons.[54] An alternative explanation for the O_2 effect on cross-linking is that **25** and **26** are trapped faster than they react with the opposing DNA strand, and that the resulting peroxyl radicals do not yield ICLs. These two mechanisms were distinguished by measuring the consumption of **7** in **27** following ^{137}Cs-irradiation (700 Gy). The depletion of **7** was measured by HPLC of digested DNA by comparing the relative areas of the nucleoside to an internal standard (2′-deoxyinosine, dI) added after irradiation but prior to enzyme digestion. The amount of **7** was significantly reduced in samples that were irradiated under anaerobic conditions. In contrast, the ratios of 7:dI in samples that were not irradiated and those irradiated under aerobic conditions (Table 2) were within experimental error of one another. We believe that this is consistent with the previously proposed mechanism that O_2 scavenges the solvated electron, preventing σ-radical formation (e.g., **25**, **26**).[3,22,35] However, the slightly lower average value in the irradiated samples may indicate a minor contribution by an alternative mechanism, such as O_2 trapping of σ-radicals **25** and/or **26**.

Table 2. Effect of O_2 on the Consumption of 7 in 27 upon ^{137}Cs-Irradiation[a]

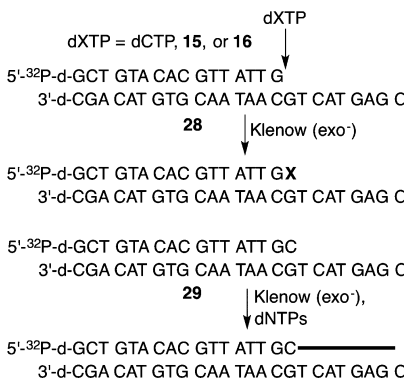
5′-d(CGA GTA CTG CAA **7**AA CGT GTA CAG C)
3′-d(GCT CAT GAC GTT ATT GCA CAT GTC G)
27

^{137}Cs Dose (Gy)	O_2	Area$_7$:Area$_{dI}$
0	+	0.51 ± 0.01
700	+	0.49 ± 0.01
700	−	0.42 ± 0.01

[a]Average ± std. dev. of 3 experiments.

Klenow (exo$^-$) Incorporation of 7 and 8 via Their Nucleotide Triphosphates (15, 16). The above experiments indicate that **7** and **8** can produce ICLs selectively under hypoxic conditions upon ^{137}Cs-irradiation. To be useful in cells the nucleotide triphosphates must be accepted as substrates by DNA polymerase(s). The discovery of nucleotide analogues that are efficiently incorporated into DNA by polymerases is challenging but several groups have made great strides in this endeavor.[28,31,36,55−58] Initial studies examined the ability of the Klenow fragment of *E. coli* DNA polymerase I (Klenow (exo$^-$)), a model polymerase, to accept **15** and **16** as substrates (Scheme 4). Single nucleotide incorporation opposite dG in **28** went to completion within 20 min using **15** or **16** (100 μM), or in the presence of dCTP (50 μM) as control.[47] However, extending the growing strand past the modified nucleotides, and incorporating more than one molecule of **7** or **8** in **28** was much less efficient, as is often the case when utilizing nonnative nucleotides in replication.[37,47,59] In addition, full-length product

Scheme 4



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was not observed when either **15** or **16** were substituted for dCTP and TTP in reactions with **28** or **29** (Scheme 4). The latter substrate provides a "running start" for modified nucleotide incorporation by requiring native dATP as the first position.[47,60]

Since the bromopyridone triphosphates were incompatible with producing full-length replication products using the model replicative polymerase, we also examined the incorporation of one of them (**7**) in **29**-**32** by DNA polymerase β (Pol β). Pol β is the primary polymerase utilized during base excision repair, and is more promiscuous than a replicative polymerase.[61−63] We rationalized that **7** might be more readily incorporated in genomic DNA if the cells were damaged under low dose conditions to instigate DNA repair during which the triphosphate of **7** (**15**) would be used as a substrate by Pol β. However, **7** was not incorporated by Pol β even when it was present at 800 μM (data not shown).

5′-^{32}P-d-GCT GTA CAC GTT ATT GC
3′-d-CGA CAT GTG CAA TAA CG**X** CAT GAG C
29 X = T; **30** X = A; **31** X = C; **32** X = G

Synthesis and ^{137}Cs Irradiation of Polymerase Chain Reaction (PCR) Products Containing Bromopyridones 7 or 8. To better model the random incorporation of **7** and **8** anticipated in cellular DNA, the respective triphosphates (**15**, **16**) were utilized in PCR using One Taq Hot Start polymerase. The PCR product formed consists of 916 bp of the p53 gene, and was prepared as previously described, except that polymerization was carried out in the presence of native deoxynucleotide triphosphates (100 μM) and **15** or **16** (200 μM).[64] Following isolation of the PCR product, 2 pmol were digested (DNase I, snake venom phosphodiesterase I, calf intestinal alkaline phosphatase) and the amount of **7** or **8** incorporated was quantified by LC−MS/MS using uridine as internal standard.[47] We were able to reliably quantify as little as 100 fmol of **7** or **8** by measuring the ions corresponding to the fragmented bromopyridone (m/z = 173.956). This assay indicated that **7** accounted for ∼0.7% of the nucleotides in the PCR product, and **8** ∼ 1.2%.

ICL formation in the PCR product upon ^{137}Cs irradiation was detected using alkaline agarose electrophoresis, and quantified using Sybr Gold. Although the synthetic duplexes containing **8** flanked by two dA's on its 5′- and 3′-sides (**24**, Table 1) produced ICLs, no cross-linking was detected when the PCR product was exposed to as much as 700 Gy. In contrast, dose dependent cross-linking (Figure 2) was observed when the PCR product containing **7** was irradiated under anaerobic conditions.[47] No cross-linking was detected in a comparable PCR product prepared using native nucleotide triphosphates only. It is unknown why **8** yields cross-links in **24** and not when randomly incorporated in the PCR product, but perhaps the local sequences that are conducive for incorporating **8** in DNA by One Taq Hot Start polymerase are ill-suited for producing ICLs.

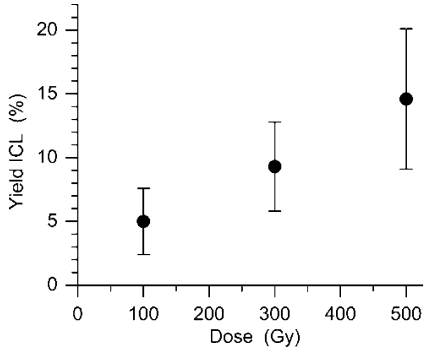

Figure 2. ^{137}Cs dose dependent ICL formation under anaerobic conditions from PCR product containing **7**.

Attempted Radiosensitization of Prostate Cancer Cells with 7. Although the triphosphate of **7** (**15**) was not a very good substrate for the model polymerase Klenow (exo$^-$), we examined its possible incorporation in prostate cancer cells (DU145). Experiments were carried out using pro-nucleotide **19**, because nucleoside **7** was a poor substrate for dCk, and nucleotide triphosphates do not pass through cell membranes readily. Under conditions where phosphorylation of dA (0.3 mM) is saturated, dCk (0.1 μM) reactivity of **7** is barely discernible.[47,65] Increasing the concentration of **7** to 1 mM still yields only slightly more efficient phosphorylation, and only at 2 mM **7** and 0.5 μM dCk is the respective monophosphate produced at a moderate rate (data not shown). An appropriate concentration of **19** to work with was established using a fluorescent assay in which a pro-fluorescent compound (resazurin) is converted into a fluorescent molecule (resorufin) in live cells. The viability of cells was measured in the presence of a range of concentrations of **19** (0.5−10 mM) over 2−4 days. Significant cell death was evident at 500 μM and above.[47] Subsequently, DU145 cells that were grown in the absence or presence of **19** (100, 300, and 500 μM) for 2 days were exposed to varying doses (0−10 Gy) of ^{137}Cs and cell survival was measured using a clonogenic assay. No difference in survival could be detected between cells that were treated with or without **19**.[47] Given the cytotoxicity of cross-linked DNA and the ability of **7** to produce such damage upon irradiation, the lack of an effect of **19** on the survival of irradiated cells suggested that the bromopyridone was not incorporated into the genomic material by replicative polymerases.

Consequently, we attempted to utilize the DNA repair pathway to incorporate **7** into the cellular DNA. Although **15** was not accepted as a substrate by Pol β in the sequence context present in **28**-**31**, we reasoned that significantly lower levels of incorporation in cellular DNA can be detected than in a gel assay (limit of detection ∼1%) and could still have an effect on sensitization. We reasoned that a low dose of irradiation would damage DNA and the ensuing base excision repair might stimulate incorporation of **7** via the pro-nucleotide (**19**). Hence, DU145 cells (10^7 cells) were preincubated in the presence of 300 μM **19** and subjected to 2 Gy of radiation. The cells were allowed to recover (37 °C, 4 h) after which the genomic DNA was isolated and digested to nucleosides in the presence of uridine (20 pmol) as internal standard to provide 48 μg of nucleosides (∼145 nmol). The digested sample was analyzed by LC−MS/MS as described above but **7** was not detected. Upon the basis of a 100 fmol detection limit of **7**, we believe that less than 1 molecule of the bromopyridone was incorporated per 10^6 nucleotides, or less than 10^{-4}%. The results of this experiment suggested that although **7** effectively produces cross-links upon irradiation under anoxic conditions, it is not a good candidate for studies in (prostate cancer) cells. From these experiments it is not possible to determine if the absence of **7** in genomic DNA is due to inefficient transformation of **19** into nucleotide triphosphate **15** and/or poor recognition of the latter by DNA polymerase(s).

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Summary. Bromopyridones **7** and **8** meet some of the requirements for hypoxic selective radiosensitizing agents that yield interstrand cross-links (ICLs) in DNA. The molecules are compatible with solid-phase oligonucleotide synthesis, and the respective nucleotide triphosphates are incorporated during PCR by One Taq Hot Start polymerase. LC−MS/MS provided more sensitive and quantitative detection of overall modified nucleotide incorporation in the DNA, and will be useful in future studies. The 4-bromopyridone (**7**) produced ICLs in a dose dependent manner, whereas **8** did not produce any cross-links in the same sequence, despite being incorporated in comparable amounts, as detected by LC−MS/MS. Although **7** was incorporated into DNA via its respective nucleotide triphosphate (**15**) during PCR, it was inefficiently incorporated by Klenow (exo$^-$) and not at all by DNA polymerase β. Nucleoside **7** was also not incorporated into genomic DNA in prostate cancer cells, although we do not know whether this is due to failure to transform the pro-nucleotide (**19**) into triphosphate **15**, and/or rejection of the latter by DNA polymerase(s), or even failure to enter cells.

EXPERIMENTAL SECTION

General Methods. Triethylamine, pyridine, tributylamine, *N*-methyl imidazole, MeOH, EtOAc, CH_3CN and CH_2Cl_2 were distilled from CaH_2 under Ar or under an appropriate vacuum. THF, Et_2O, and toluene were distilled from Na under Ar. $POCl_3$, PCl_3, $PO(OMe)_3$ were distilled from themselves under Ar. All other reagents were purchased from commercial sources and were used without further purification unless noted otherwise. 4-Bromo-2-hydroxypyridine was from Accela. All reactions were carried out under a positive pressure of argon atmosphere and monitored by TLC on Silica Gel G-25 UV_{254} (0.25 mm) unless stated otherwise. Spots were detected under UV light and/or by ethanolic solution *p*-anisaldehyde, aqueous solution of ammonium molybdate, ceric ammonium sulfate, or $KMnO_4$. Column flash chromatography was performed with Silicycle grade 70−230 mesh, 60−200 μm, 60 Å silica. The ratio between silica gel and crude product ranged from 100:1 to 20:1 (w/w). ^{1}H, ^{13}C, and ^{31}P NMR spectra were collected at 400, 101, and 162 MHz, respectively.

Oligonucleotides were synthesized via standard automated DNA synthesis on a commercial instrument. Commercially available oligonucleotide synthesis reagents were from Glen Research. The coupling time for the phosphoramidites of modified nucleotides 15 min. Oligonucleotides **17b** and **18b** were synthesized using pivalic anhydride, 2,6-lutidine, THF (1:1:8) as capping mix and fast deprotecting groups on phosphoramidites for dA (Pac) and dG (iPrPac). These oligonucleotides were deprotected using K_2CO_3-MeOH (450 μL, 0.15 M) overnight with occasional shaking and then neutralized by glacial acetic acid (450 μL, 0.15 M) before evaporating the solvents under a vacuum. All other oligonucleotides were prepared using standard phosphoramidites and deprotected using 1:1 methylamine (40% in water), concentrated NH_4OH at 65 °C for 75 min, or concentrated NH_4OH at 55 °C for 9 h (oligonucleotides containing native nucleotides only). Oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE). All oligonucleotides containing modified nucleotides were characterized by MALDI-TOF MS. Oligonucleotides were 5′-^{32}P-labeled by polynucleotide T4 kinase (New England Biolabs) and γ-^{32}P-ATP (PerkinElmer) using standard protocols.[66] One Taq Hot Start polymerase was from New England Biolabs. Radiolabeled oligonucleotides were hybridized with 1.5 equiv of complementary oligonucleotides in 10 mM potassium phosphate (pH 7.2) and 100 mM NaCl at 90 °C for 1 min and cooled to room temperature. All anaerobic reactions were carried out in sealed Pyrex tubes, which were degassed and sealed using freeze−pump−thaw (three cycles, 3 min each) degassing techniques. Experiments involving radiolabeled oligonucleotides were analyzed following PAGE using a Storm 840 phosphorimager or Typhoon 9410 variable mode imager. Roswell Park Memorial Institute Medium (RPMI) 1640 culture medium with L-glutamine and sodium bicarbonate (catalogue #R8758) (http://www.lifetechnologies.com/us/en/home/life-science/cell-culture/mammalian-cell-culture/classical-media/rpmi.html) and 10% fetal bovine serum (FBS, catalogue #F6178) were purchased from Sigma-Aldrich. Trypsin-EDTA solution (0.1 mM trypsin), and SYBR Gold nucleic acid stain were purchased from Life Technologies. Phosphate buffered saline (1 x, PBS, catalogue #21−040) was purchased from Corning Cellgro. DU 145 cells were obtained from the American Type Culture Collection (ATCC) and cultured as per the supplier's protocol (http://www.atcc.org/products/all/HTB-81.aspx#culturemethod).

Preparation of 10b. 5-Bromo-2-hydroxypyridine[44] (440 mg, 2.53 mmol) was dissolved in MeCN (10 mL), and bis(trimethylsilyl)-acetamide (625 μL, 2.53 mmol) was added dropwise. The mixture was stirred for 70 min at room temperature and then cooled on ice. Additional MeCN (10 mL) were added followed by 2-deoxy-3,5-di-*O*-(*p*-toluoyl)-α-D-*erythro*-pentofuranosyl chloride (1.5 g, 3.86 mmol). To this solution was added 1 M $SnCl_4$ in CH_2Cl_2 (1 M 1.5 mL, 1.5 mmol) dropwise. The reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was diluted with ethyl acetate (100 mL), washed with saturated $NaHCO_3$ (50 mL), and brine (50 mL). The organic layers were dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness in vacuo. The crude product was purified by silica gel column chromatography (EtOAc−Hexanes, 2:1) to afford the desired β-isomer **10b** (350 mg, 26%) as a white solid along with the α-isomer **11b** (300 mg, 23%) as a white foam. **10b**: ^{1}H NMR ($CDCl_3$) δ 7.97−7.89 (m, 4H), 7.84 (d, 1H J = 2.8 Hz), 7.33−7.22 (m, 5H), 6.53 (dd, 1H, J = 5.4, 8.2 Hz), 6.43 (d, 1H, J = 9.6 Hz), 5.62−5.58 (m, 1H), 4.75−4.73 (m, 2H), 4.64−4.61 (m, 1H), 2.99 (ddd, 1H, J = 1.6, 5.6, 14.5 Hz), 2.43 (s, 3H), 2.41 (s, 3H), 2.20 (ddd, 1H J = 14.5, 6.6, 4.2 Hz); ^{13}C NMR ($CDCl_3$) δ 166.1, 166.0, 160.4, 144.5, 144.3, 142.7, 131.9, 129.9, 129.7, 129.4, 129.3, 126.5, 126.4, 121.7, 98.5, 86.5, 83.6, 75.1, 64.0, 39.4, 21.73, 21.71; IR (NaCl plate) 3196, 2987, 1666, 1422, 1194 cm^{-1}; MALDI-TOF HRMS $C_{26}H_{24}BrNO_6Na$ (M + Na $^+$) calcd. 548.0679, obsd. 548.0679. **11b**: ^{1}H NMR ($CDCl_3$, δ) 7.95 (d, 2H, J = 8.0 Hz), 7.83 (d, 1H, J = 2.8 Hz), 7.71 (d, 2H, J = 8.0 Hz), 7.39 (dd, 1H, J = 2.8, 9.6 Hz), 7.29−7.20 (m, 4H), 6.47−6.43 (m, 2H), 5.66 (d, 1H, J = 5.6 Hz), 4.98 (dd, 1H, J = 4.0, 4.0 Hz), 4.59−4.50 (m, 2H), 3.01 (ddd, 1H, J = 6.6, 6.6, 15.4 Hz), 2.56 (ddd, 1H, J = 15.4, 15.4, 15.4 Hz), 2.42 (s, 3H), 2.40 (s, 3H); ^{13}C NMR ($CDCl_3$,) δ 166.1, 165.5, 160.6, 144.4, 144.3, 142.6, 132.5, 129.7, 129.6, 129.4, 129.3, 126.6, 126.1, 121.9, 97.9, 89.0, 85.8, 74.6, 64.1, 60.4, 39.3, 21.7. IR (NaCl plate) 3150, 3055, 2956, 1660, 1408, 1178 cm^{-1}; MALDI-TOF HRMS $C_{26}H_{24}BrNO_6Na$ (M + Na $^+$) calcd. 548.0679, obsd. 548.0683.

Preparation of 8. β-Isomer **10b** (320 mg, 0.61 mmol) was dissolved in 20 mL 1:1 MeOH/CH_2Cl_2. To this solution was added 2 mL 1 M NaOMe in CH_3OH. The reaction mixture was stirred for 2 h at room temperature. The organic solvent was removed in vacuo and the resulting residue was purified by silica gel flash chromatography (CH_2Cl_2−MeOH, 10:1) to give **8** (140 mg, 80% yield). ^{1}H NMR (CD_3OD) δ 8.37 (d, 1H, J = 2.8 Hz), 7.55 (dd, 1H, J = 2.4, 9.6 Hz), 6.42 (d, 1H, J = 9.6 Hz), 6.35 (dd, 1H, J = 6.2, 6.2 Hz), 4.39−4.35 (m, 1H), 3.99 (m, 1H), 3.85 (dd, 1H, J = 3.0, 12.2 Hz), 3.74 (dd, 1H, J = 3.4, 12.2 Hz), 2.47 (ddd, 1H, J = 4.4, 6.4, 13.6 Hz), 2.14−2.09 (m, 1H); ^{13}C NMR (CD_3OD) δ 162.7, 144.9, 135.1, 121.8, 100.2, 89.4, 87.8, 71.6, 62.3, 42.9. IR (NaCl plate) 3432, 3306, 2914, 1684, 1430, 1179 cm^{-1}; MALDI-TOF HRMS $C_{10}H_{12}BrNO_4Na$ (M + Na $^+$) calcd. 311.9842, obsd. 311.9841.

Preparation of Dimethoxytritylated 8. Nucleoside **8** (130 mg, 0.45 mmol) was azeotroped with pyridine (2 × 3 mL) and then dissolved in pyridine (3 mL). To this solution was added 4,4′-dimethoxytrityl chloride (198 mg, 0.58 mmol) and 4-dimethylaminopyridine (10 mg, 0.08 mmol). The reaction mixture was stirred at room temperature overnight, at which time methanol (3 mL) was added to quench the reaction. The organic solution was removed in vacuo and the residue was purified by flash chromatography (0−10% methanol in EtOAc) to afford dimethoxytritylated **8** (170 mg, 64%) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 8.06 (d, 1H, J = 2.4 Hz), 7.46−7.21

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(m, 10H), 6.85 (d, 4H, J = 8.8 Hz), 6.52−6.44 (m, 2H), 4.49 (dd, 1H, J = 2.8, 2.8 Hz), 4.21−4.19 (m, 1H), 3.81 (s, 6H), 3.47−3.36 (m, 2H), 2.80−2.76 (m, 1H), 2.24−2.16 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 160.8, 158.6, 144.5, 142.9, 135.64, 135.60, 132.7, 130.1, 128.04, 128.01, 126.9, 121.3, 113.3, 98.8, 86.9, 86.8, 86.6, 72.4, 63.5, 55.3, 42.3. IR (NaCl plate) 3380, 3153, 3055, 2986, 1655, 1464, 1062 cm^{-1}; MALDI-TOF HRMS $C_{31}H_{30}BrNO_6Na$ (M + Na$^+$) calcd. 614.1149, obsd. 614.1145.

Preparation of 12b. To a solution of dimethoxytritylated **8** (120 mg, 0.20 mmol) and diisopropylethylamine (71 μL, 0.40 mmol) in dichloromethane (5 mL) was added 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (59 μL, 0.26 mmol) at room temperature. After stirring for 2 h, the reaction mixture was diluted with dichloromethane (20 mL) and washed with saturated $NaHCO_3$ aq. (20 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness in vacuo. The crude product was purified by silica gel column chromatography (EtOAc−Hexanes, 2:1) to afford **12b** (115 mg, 72%) as a white foam. ^{1}H NMR ($CDCl_3$) δ 7.97−7.91 (m, 1H), 7.38−7.33 (m, 2H), 7.29−7.11 (m, 8H), 6.80−6.74 (m, 4H), 6.38−6.31 (m, 2H), 4.52−4.43 (m, 1H), 4.17−4.14 (m, 1H), 3.76−3.68 (m, 7H), 3.58−3.25 (m, 5H), 2.75−2.61 (m, 1H), 2.54 (t, 1H, J = 6.4 Hz), 2.36 (t, 1H, J = 6.4 Hz), 2.15−2.08 (m, 1H), 1.20−0.97 (m, 14H); ^{31}P NMR ($CDCl_3$) δ 149.1, 148.5; MALDI-TOF HRMS $C_{40}H_{47}BrN_3O_7PNa$ (M + Na$^+$) calcd. 814.2227, obsd. 814.2220.

Preparation of 10a. 4-Bromo-2-hydroxypyridine (440 mg, 2.53 mmol) was dissolved in MeCN (10 mL), and bis(trimethylsilyl)-acetamide (625 μL, 2.53 mmol) was added dropwise. The mixture was stirred for 70 min at room temperature and then cooled on ice. Additional MeCN (10 mL) was added, followed by 2-deoxy-3,5-di-*O*-(*p*-toluoyl)-α-D-*erythro*-pentofuranosyl chloride (1.5 g, 3.86 mmol). To this solution was added 1 M $SnCl_4$ in CH_2Cl_2 (1 M 1.5 mL, 1.5 mmol) dropwise. The reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was diluted with ethyl acetate (100 mL), washed with saturated $NaHCO_3$ (50 mL), and brine (50 mL). The organic layers were dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness in vacuo. The crude product was purified by silica gel column chromatography (EtOAc−Hexanes, 2:1) to afford desired β-isomer **10a** (514 mg, 38%) as a white solid along with α-isomer **11a** (500 mg, 37%) as a white solid. **10a**: ^{1}H NMR ($CDCl_3$) δ 7.96 (d, 2H, J = 6.4 Hz), 7.85 (d, 2H, J = 6.4 Hz), 7.58 (d, 1H, J = 7.6 Hz), 7.29−7.20 (m, 4H), 6.76 (d, 1H, J = 2.0 Hz), 6.48 (dd, 1H, J = 5.8, 8.2 Hz), 6.23 (dd, 1H, J = 2.2, 7.4 Hz), 5.61−5.58 (m, 1H), 4.75−4.58 (m, 3H), 3.01 (ddd, 1H, J = 2.0, 5.6, 14.5 Hz), 2.43 (s, 3H), 2.42 (s, 3H), 2.24 (ddd, 1H, J = 6.6, 8.0, 14.5 Hz); ^{13}C NMR ($CDCl_3$) δ 166.08, 166.06, 160.7, 144.5, 144.4, 136.1, 131.8, 129.8, 129.5, 129.4, 129.3, 126.5, 126.4, 122.5, 110.4, 86.6, 83.5, 75.0, 64.1, 39.4, 21.74, 21.72. IR (NaCl plate) 3065, 2964, 2913, 1654, 1472, 1126 cm^{-1}; MALDI-TOF HRMS $C_{26}H_{24}BrNO_6Na$ (M + Na$^+$) calcd. 548.0679, obsd. 548.0683. **α-isomer 11a**: ^{1}H NMR ($CDCl_3$) δ 7.96 (d, 2H, J = 8.4 Hz), 7.86 (d, 2H, J = 8.4 Hz), 7.54 (d, 1H, J = 7.6 Hz), 7.29 (d, 2H, J = 8.4 Hz), 7.21 (d, 2H, J = 8.0 Hz), 6.83 (s, 1H), 6.39 (d, 2H, J = 7.6 Hz), 5.61 (d, 1H, J = 5.6 Hz), 4.91 (s, 1H), 4.59 (d, 2H, J = 4.0 Hz), 3.03−2.95 (m, 1H), 2.68 (d, 1H, J = 15.6 Hz), 2.45 (s, 3H), 2.43 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 166.2, 165.6, 144.6, 144.3, 136.0, 132.3, 129.7, 129.6, 129.4, 129.2, 126.6, 126.1, 122.7, 109.7, 89.2, 86.0, 74.8, 64.1, 38.9, 21.7. IR (NaCl plate) 3055, 2984, 2923, 1685, 1455, 1377, 1178 cm^{-1}; MALDI-TOF HRMS $C_{26}H_{24}BrNO_6Na$ (M + Na$^+$) calcd. 548.0679, obsd. 548.0690.

Preparation of 7. β-Isomer **10a** (490 mg, 0.93 mmol) was dissolved in 20 mL 1:1 MeOH/CH_2Cl_2. To this solution was added 3 mL 1 M NaOMe in CH_3OH. The reaction mixture was stirred for 2 h at room temperature. The organic solvent was removed in vacuo and the resulting residue was purified by silica gel flash chromatography (CH_2Cl_2−MeOH, 10:1) to give **7** (220 mg, 81% yield). ^{1}H NMR (CD_3OD) δ 8.05 (d, 1H, J = 7.6 Hz), 6.75 (d, 1H, J = 2.0 Hz), 6.60−6.55 (m, 1H), 6.36 (t, 1H, J = 6.2 Hz), 4.40−4.37 (m, 1H), 4.03−4.00 (m, 1H), 3.84 (dd, 1H, J = 3.2, 12.0 Hz), 3.76 (dd, 1H, J = 3.6, 12.0 Hz), 2.51 (ddd, 1H, J = 4.0, 6.0, 13.6 Hz), 2.15−2.08 (m, 1H); ^{13}C NMR (CD_3OD) δ 162.9, 138.1, 135.2, 122.4, 112.1, 89.4, 87.7, 71.8, 62.6, 42.7. IR (NaCl plate) 3455, 3326, 2944, 1659, 1265, 1056 cm^{-1}; MALDI-TOF HRMS $C_{10}H_{13}BrNO_4$ (M + H$^+$) calcd. 290.0022, obsd. 290.0030.

Preparation of 20. Diol **7** (200 mg, 0.69 mmol) was azeotropically dried with pyridine (2 × 3 mL) and then dissolved in pyridine (5 mL). 4,4′-Dimethoxytrityl chloride (350 mg, 1.03 mmol) and 4,4-dimethylaminopyridine (10 mg, 0.08 mmol) were added to this solution. The reaction mixture was stirred at room temperature overnight, at which time methanol (3 mL) was added to quench the reaction. The organic solution was removed in vacuo and the residue was purified by flash chromatography (0−10% methanol in EtOAc) to afford **20** (280 mg, 69%) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 8.11 (d, 1H, J = 5.6 Hz), 7.88 (d, 1H, J = 7.2 Hz), 7.44−7.24 (m, 10H), 6.85 (d, 4H, J = 8.4 Hz), 6.74 (s, 1H, 6.48−6.40 (m, 2H), 6.10 (d, 1H, J = 7.2 Hz), 4.56−4.54 (m, 1H), 4.16 (s, 1H), 3.80 (s, 6H), 3.53−3.43 (m, 2H), 2.72−2.68 (m, 1H), 2.22−2.19 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 161.1, 158.6, 148.9, 144.5, 136.1, 135.6, 135.5, 133.3, 130.14, 130.12, 128.2, 128.0, 127.0, 122.0, 113.2, 110.3, 106.6, 86.8, 86.5, 86.2, 70.5, 62.8, 55.3, 42.2, 39.0. IR (NaCl plate) 3369, 3054, 2913, 1648, 1301, 1084 cm^{-1}; MALDI-TOF HRMS $C_{31}H_{30}BrNO_6Na$ (M + Na$^+$) calcd. 614.1149, obsd. 614.1153.

Preparation of 12a. To a solution of **20** (180 mg, 0.30 mmol) and diisopropylethylamine (106 μL, 0.60 mmol) in dichloromethane (10 mL) was added 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (88 μL, 0.39 mmol) at room temperature. After stirring for 2 h, the reaction mixture was diluted with dichloromethane (20 mL) and washed with saturated $NaHCO_3$ aq. (20 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness in vacuo. The crude product was purified by silica gel column chromatography (EtOAc−Hexane, 2:1) to afford **12a** (175 mg, 73%) as white foam. ^{1}H NMR ($CDCl_3$) δ 7.80−7.70 (m, 1H), 7.33−7.30 (m, 2H), 7.22−7.14 (m, 7H), 6.77−6.73 (m, 4H), 6.65 (s, 1H), 6.33−6.27 (m, 1H), 5.96−5.94 (m, 1H), 4.56−4.52 (m, 1H), 4.10−3.98 (m, 1H), 3.72−3.65 (m, 7H), 3.53−3.27 (m, 5H), 2.65−2.50 (m, 2H), 2.34 (t, 1H, J = 6.0 Hz), 2.16−2.10 (m, 1H), 1.22−0.96 (m, 14H); ^{31}P NMR ($CDCl_3$) δ 149.3, 148.7; MALDI-TOF HRMS $C_{40}H_{47}BrN_3O_7PNa$ (M + Na$^+$) calcd. 814.2227, obsd. 814.2237.

Preparation of 15. Nucleoside **7** (89 mg, 0.31 mmol) was dissolved in $(MeO)_3PO$ (3 mL) and proton sponge (197 mg, 0.92 mmol) was added to the solution. The reaction mixture was cooled to −15 °C. $POCl_3$ (34 μL, 0.37 mmol) was added and the mixture was stirred for 2 h at −15 °C. At that time a solution of bis-tributylammonium pyrophosphate (1.2 g, 2.14 mmol), tributylamine (1.0 mL, 4.29 mmol) in DMF (2 mL) was added dropwise via syringe to the reaction mixture over 5 min, and the reaction mixture was stirred for another 2 h. The reaction was quenched with TEAB buffer (5 mL, 1 M, pH 8) and stirred for another 45 min at 25 °C. The reaction mixture was lyophilized to dryness and dissolved in NaOAc (200 μL, 0.3 M, pH 5.6) precipitated with 3 volumes of ethanol at −78 °C. After centrifuging, the crude precipitate was dissolved in H_2O (300 μL) and subjected to FPLC purification on a mono Q 5/50 GL column (5 × 50 mm, 20 mM to 1 M TEAB buffer over 30 min, pH = 8.0). The fractions containing the triphosphate (t_R = 19 min) were collected, lyophilized and purified further by reverse-phase (C18) HPLC (0−50% CH_3CN in 0.1 M TEAB, pH 8). The fractions containing **15** (Ret. Time: 17 min) were collected, lyophilized and dissolved in H_2O (200 μL) to give a solution of **15** (10.6 mM, $\varepsilon_{300\ nm}$ = 8 × 10^3 M^{-1}cm^{-1}, based on nucleoside 7,5%): ^{1}H NMR (D_2O) δ 7.89 (d, J = 7.4 Hz, 1H), 6.82 (dd, J = 2.1, 0.6 Hz, 1H), 6.79 (ddd, J = 7.4, 2.1, 0.4 Hz, 1H), 6.36 (t, J = 6.4 Hz, 1H), 4.59−4.51 (m, 1H), 4.18 (dd, J = 5.0, 2.5 Hz, 3H), 2.47 (ddd, J = 13.9, 6.3, 4.0 Hz, 1H), 2.23 (dt, J = 13.3, 6.4 Hz, 1H); ^{31}P NMR (D_2O) δ −10.8 (d, J = 19.9 Hz), −11.5 (d, J = 19.9 Hz), −23.3 (t, J = 20.1 Hz). ESI-MS (m/z) [M + 3H$^+$] calcd for 524.9, found 527.8.

Preparation of 16. Nucleoside **8** (80 mg, 0.275 mmol) was dissolved in $(MeO)_3PO$ (3 mL) and proton sponge (197 mg, 0.92 mmol) was added to the solution. The reaction mixture was cooled to −15 °C. $POCl_3$ (34 μL, 0.368 mmol) was added and the mixture was stirred for 2 h at −15 °C. At that time a solution of bis-tributylammonium pyrophosphate (1.2 g, 2.14 mmol), tributylamine

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(1.0 mL, 4.29 mmol) in DMF (2 mL) was added via syringe dropwise to the reaction mixture over 5 min. The reaction mixture was stirred for another 2 h. The reaction was quenched with the addition of TEAB buffer (5 mL, 1 M, pH 8) and stirred for 45 min at 25 °C. The reaction mixture was lyophilized to dryness, dissolved in NaOAc (200 μL, 0.3 M, pH 5.6) and precipitated with 3 volumes of EtOH at −78 °C. After centrifuging, the crude precipitate was dissolved in H_2O (300 μL) and subjected to FPLC purification on a mono Q 5/50 GL column (5 × 50 mm, 20 mM to 1 M TEAB buffer over 30 min, pH = 8.0). The fractions containing **16** (t_R = 19 min) were collected, lyophilized and further purified by reverse-phase (C18) HPLC (0−50% CH_3CN in 0.1 M TEAB, pH 8). The fractions containing triphosphate (Ret. Time: 17 min) were collected, lyophilized and dissolved in H_2O (200 μL) to give a solution of triphosphate (8.3 mM, $\varepsilon_{300\ nm} = 8 \times 10^3\ M^{-1}cm^{-1}$, based on nucleoside, 3%). A portion of the triphosphate solution was cation exchanged for Na^+ salt by passing through a Dowex 50WX8−400 ion exchange column (0.5 × 5 cm) pretreated with MeOH (1 × 2 mL), 1 M NaOH (1 × 1 mL) and H_2O (until pH 7) for spectroscopic characterization and found to be 95% pure: ^{1}H NMR (D_2O) δ 8.03−7.87 (m, 1H), 6.51−6.36 (m, 1H), 6.25 (dd, *J* = 7.0, 2.2 Hz, 1H), 4.58−4.44 (m, 1H), 4.02 (dd, *J* = 6.5, 4.8 Hz, 2H), 2.95 (t, *J* = 7.3 Hz, 1H), 2.80 (dt, *J* = 14.6, 6.6 Hz, 1H), 2.05 (d, *J* = 14.8 Hz, 1H); ^{31}P NMR (D_2O) δ −6.44 (d, *J* = 21.3 Hz), −11.27, −22.70 (t, *J* = 20.6 Hz); ESI-MS (*m*/*z*) [M + 3H] calcd for 524.9, found 527.8.

Preparation of 21. Compound **20** (368 mg, 0.620 mmol) was azeotropically dried with pyridine (3 × 1 mL) under reduced pressure (0.1 Torr) and then dissolved in dry pyridine (6 mL). To this solution was added imidazole (88 mg, 1.29 mmol) and TBDMSCl (176 mg, 1.17 mmol). The mixture was then heated for 3 h at 50 °C, at which time the solvent was removed in vacuo and the crude mixture was purified by silica gel column chromatography (EtOAc/hexanes, 20−70% gradient). The fractions containing product were combined and concentrated under reduced pressure to give the silylated compound as a white foam: R_f = 0.8 (5% MeOH/CH_2Cl_2). This material was directly used in the next step without further purification. Dimethoxytritylated **21** (260 mg, 0.368 mmol) was dissolved in CH_2Cl_2 (2 mL). Trichloroacetic acid (120 mg, 0.74 mmol) was added in one portion. The reaction mixture turned bright red. After stirring for 10−15 min, the reaction was quenched by the addition of pyridine (1 mL). Solvents were removed in vacuo, and the crude reaction mixture was purified by silica gel chromatography, eluting with 10% EtOAc/hexanes (200 mL) and 20% EtOAc/hexanes (200 mL) to give **21** as a white solid (190 mg, 76%): R_f = 0.2 (15% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$) δ 7.67 (tt, *J* = 4.8, 1.6 Hz, 1H), 6.77 (dd, *J* = 2.1, 0.7 Hz, 1H), 6.37 (dd, *J* = 7.4, 2.1 Hz, 1H), 6.31−6.16 (m, 1H), 4.47 (dt, *J* = 6.6, 4.5 Hz, 1H), 4.00−3.96 (m, 1H), 3.94 (dd, *J* = 2.8, 0.7 Hz, 1H), 3.85−3.70 (m, 1H), 2.42 (ddd, *J* = 13.5, 6.6, 4.8 Hz, 2H), 0.89 (s, 9H), 0.07 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 161.2, 136.2, 133.8, 122.7, 110.6, 87.8, 71.0, 61.6, 41.5, 25.7, 17.9, −4.7, −4.9; IR (KBr plate) 3020, 2956, 2931, 2401, 2253, 1651, 1584, 1216 cm^{-1}; ESI HRMS (*m*/*z*) [M + Na^+] calcd for $C_{16}H_{26}BrNO_4SiNa$ 426.0712, found 426.0713.

Preparation of Silyl Protected 19. Alcohol **21** (20 mg, 0.049 mmol) was dissolved in THF (500 μL) and cooled to −78 °C. *N*-Methylimidazole (20 μL, 0.245 mmol) was added, followed by a solution of phosphorochloridate **22**[67] (43 mg, 0.148 mmol). The reaction was warmed to 25 °C over 12 h. The mixture was evaporated to dryness after pyridine (500 μL) was added. The crude reaction mixture was purified by a silica gel chromatography, eluting with 2% MeOH/CH_2Cl_2 (200 mL). The fractions containing product gave a (1:1) diastereomeric mixture of silylated **19** as a colorless oil (26 mg, 80%): R_f = 0.6 (2% MeOH/CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 7.60 (m, 2H), 7.37−7.28 (m, 4H), 7.24−7.13 (m, 6H), 6.74 (m, 2H), 6.39−6.22 (m, 4H), 4.57−3.86 (m, 14H), 3.66 (m, 2H), 2.52 (ddd, *J* = 13.6, 6.2, 4.0 Hz, 1H), 2.44 (ddd, *J* = 13.6, 6.3, 4.2 Hz, 1H), 1.89 (dt, *J* = 13.6, 6.4 Hz, 1H), 1.81 (dt, *J* = 13.7, 6.5 Hz, 1H), 1.37 (m, 6H), 1.25 (m, 6H), 0.88 (m, 18H), 0.14 (m, 12H); ^{13}C NMR ($CDCl_3$) δ 173.3, 173.3, 160.7, 150.5, 150.5, 135.9, 135.8, 132.4, 129.7, 129.7, 125.2, 125.1, 125.1, 122.3, 122.2, 120.0, 120.0, 119.9, 110.2, 110.1, 86.1, 85.8, 85.6, 85.5, 71.3, 71.1, 65.6, 65.3, 61.7, 61.6, 50.3, 50.2, 41.9, 25.6, 25.6, 21.1, 21.1, 21.0, 21.0, 17.8, 14.1, −4.6, −4.7, −4.9, −4.9; ^{31}P NMR ($CDCl_3$) δ 2.61, 2.51. IR (KBr plate) 3020, 2400, 1520, 1423, 1215 cm^{-1}; ESI HRMS (*m*/*z*) [M + Na^+] calcd for $C_{27}H_{40}BrN_2O_8PSiNa$ 681.1372, found 681.1370.

Preparation of 19. The silylated precursor (20 mg, 0.03 mmol) was dissolved in 90% TFA in H_2O (2 mL) and stirred for 2 h. Dry toluene (500 μL) was added and the solvents were coevaporated to dryness. The crude reaction mixture was purified by a silica gel column chromatography, eluting with 2% MeOH/CH_2Cl_2 (100 mL), 5% MeOH/CH_2Cl_2 (100 mL). The fractions containing product gave a (1:1) diastereomeric mixture of **19** as a white powder (12 mg, 77%): R_f = 0.12 (2% MeOH/CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 7.55 (d, *J* = 7.4 Hz, 2H), 7.40−7.27 (m, 4H), 7.24−7.10 (m, 6H), 6.76 (dd, *J* = 2.2, 0.5 Hz, 2H), 6.37 (t, *J* = 6.1 Hz, 2H), 6.33 (dd, *J* = 7.5, 2.1 Hz, 2H), 4.39 (dt, *J* = 7.3, 3.1 Hz, 6H), 4.24−4.10 (m, 6H), 4.10−3.94 (m, 4H), 3.77 (dd, *J* = 11.2, 9.5 Hz, 2H), 2.59 (ddd, *J* = 13.8, 6.5, 5.2 Hz, 2H), 1.94 (ddd, *J* = 13.9, 6.7, 5.8 Hz, 2H), 1.39 (dd, *J* = 7.1, 0.8 Hz, 6H), 1.27 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 142.5, 142.4, 132.5, 129.7, 129.6, 125.2, 124.8, 121.8, 121.7, 120.2, 120.2, 120.1, 98.2, 86.3, 86.0, 71.5, 71.3, 61.7, 61.5, 50.4, 42.0, 25.7, 21.1, 21.1, 17.9, 14.1, 14.1, −4.7, −4.7, −4.9, −4.9; ^{31}P NMR ($CDCl_3$) δ 3.47, 3.22; IR (KBr plate) 3053, 3020, 2986, 2254, 1656, 1580, 1265, 1216 cm^{-1}; ESI HRMS (*m*/*z*) [M + Na^+] calcd for $C_{21}H_{26}BrN_2O_8PNa$ 567.0508, found 567.0504.

γ-Radiolysis. γ-Radiolysis of DNA was carried out in Pyrex tubes in a J. L. Shepherd Mark I ^{137}Cs irradiator. After reaction, each sample (30 nM, 30 μL) was aliquoted to a 0.6 mL Eppendorf tube, lyophilized, and mixed with an equal volume of formamide loading buffer, and subjected to 20% denaturing PAGE analysis. Percent ICL yields were determined using the phosphorimage by dividing the volume of the ICL bands by the summation of all of the DNA in the lane (ICLs, intact DNA, cleaved DNA) and multiplying by 100.

Fe(II)−EDTA Digestion of Cross-Linked DNA after γ-Radiolysis. Unlabeled duplexes (**23a**, **24d**) were irradiated, as described above, followed by 5′-^{32}P-labeling. The radiolabeled ICLs were purified by 20% denaturing PAGE, isolated by the crush and soak method, desalted, and annealed by slowly cooling from 80 to 25 °C prior to treating with the restriction enzyme. ScaI-HF (1 μL, 20 U/μL) was added to a mixture of the isolated 5′-^{32}P-ICL sample (~200 pmol) in 10 × reaction buffer (5 μL, 500 mM KOAc, 200 mM Tris-acetate, 100 mM $Mg(OAc)_2$, 1 mg/mL BSA, pH 7.9 at 25 °C) and H_2O (44 μL). The reaction mixture was mixed thoroughly and then incubated for 1 h at 37 °C. After 1 h the reaction was stopped by heating at 80 °C for 2 min. The mixture was lyophilized, and suspended in formamide loading buffer (100 μL). The digested product was purified by 20% denaturing PAGE. *Note*: Prior to restriction enzyme digestion, 5′-^{32}P-ICL was annealed by slowing cooling in a heat block from 80 to 25 °C.

Fe(II)−EDTA cleavage reactions of 5′-^{32}P-ICL were carried out in 50 μM $Fe(NH_4)_2(SO_4)_2$, 0.1 mM EDTA, 1 mM sodium ascorbate, 5 mM H_2O_2, 100 mM NaCl, and 10 mM potassium phosphate (pH 7.2) for 5 min at 25 °C (total volume: 20 μL). The reactions were quenched with 100 mM thiourea (10 μL). Samples for ICL from **23a** were evaporated to dryness. Samples for ICL from **24d** were treated with 1 M piperidine (30 μL) at 70 °C for 5 min after lyophilization. After evaporating the cooled samples to dryness under a vacuum, residual piperidine was coevaporated with H_2O (50 μL × 3) from the samples by lyophilization. The dried samples were suspended in formamide loading buffer, and subjected to 20% PAGE analysis.

Determination of Consumption of 7 via γ-Radiolysis. Duplex **27** (3 nmol) was irradiated as described above under degassed and aerobic conditions, transferred to Eppendorf tubes, and concentrated to dryness under a vacuum. The material was redissolved in 30 μL H_2O, 10 × DNase I buffer (5 μL, 100 mM Tris·HCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.6 at 25 °C), and 2′-deoxyuridine (1.5 μL, 10 mM), 2′-deoxyinosine (1.5 μL, 1 mM) as internal standards were incubated with DNase I (5 μL, 2 U/μL), calf intestinal alkaline phosphatase (2 μL, 10 U/μL), and snake venom phosphodiesterase (5 μL, 1 μg/μL) for 24 h at 37 °C. The reaction mixture was filtered through a Nanosep 3K filter by centrifuging the mixture for 5−10 min at 16000*g*. The filter was washed once with H_2O (50 μL) and the combined

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filtrate was then transferred to a 0.2 mL tube. The solution was flash frozen in liquid N_2, lyophilized to dryness, and the residue dissolved in H_2O (30 μL). This solution (10 μL) was subjected to UPLC analysis (Ret. Time: 7, 19 min; dI, 11.9 min; dU, 11.1 min). UPLC analysis of the digestion products was carried out using a Microsorb-MV 100−5 C18 column (250 × 4.6 mm, 5 μM particle size) at 25 °C using the following separation conditions. Solvent A: Ammonium formate (10 mM), Solvent B: acetonitrile; 1.0 mL/min The following linear gradient was used: (time (min), % B) 0, 0; 5, 0; 16, 20; 19, 100; 24, 100; 27, 0, 42, 0.

Full-Length Extension Reactions. Klenow exo$^-$ (0.4 μL, 6.6 μM), 10 × reaction buffer (0.5 μL, 100 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM dithiothreitol, pH 7.9 at 25 °C) was added to H_2O (4.1 μL) to make a 500 nM enzyme solution in 1× reaction buffer. The above enzyme solution (1 μL) was added to a mixture of **15** or **16** (4.0 μL, 2.5 mM), dATP (0.5 μL, 10 mM), dGTP (0.5 μL, 10 mM), dTTP (0.5 μL, 10 mM), thermostable inorganic pyrophosphatase (1.0 μL, 2 U/μL), 10 × BSA (1 μL) and 10 × reaction buffer (1 μL, 100 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM dithiothreitol, pH 7.9 at 25 °C) and H_2O (0.5 μL) to make a 10 × enzyme/**15** (or **16**)/dATP/dGTP/dTTP cocktail. This 10 × solution (4 μL) was incubated for 10 min at room temperature and added to 36 μL of a solution containing 10 × BSA (4 μL), 10 × reaction buffer (4 μL, 100 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM dithiothreitol, pH 7.9 at 25 °C), DNA (4 μL, 500 nM), H_2O (24 μL) to create a final reaction solution containing 100 μM **15** or **16**, 50 μM of each of the native triphosphates, 5 nM Klenow exo$^-$ and 50 nM DNA in 1× reaction buffer. The reaction was carried out at 37 °C. Aliquots (5 μL) were removed at 10, 20, 30, 40, 50, and 60 min, quenched with 95% formamide/1 mM EDTA, heated to 90 °C for 2 min and immediately placed on ice. Prior to loading on a PAGE gel, the samples were denatured by heating for 2 min at 90 °C and placed back on ice. The reactions were loaded on a 20% denaturing PAGE and electrophoresed at 25 mA for 1.5 h. A similar reaction was carried out using native dNTPs (50 μM) as positive control.

PCR Experiments. A solution (50 μL) of primers (0.2 μM each), template DNA (0.25 pM), dNTPs (0.2 mM each), and One Taq hot start polymerase (1.25 U) in 1× One Taq standard reaction buffer (20 mM Tris-HCl, 22 mM NH_4Cl, 22 mM KCl, 1.8 mM $MgCl_2$, 0.06% IGEPAL CA-630, 0.05% Tween 20, pH 8.9 at 25 °C) with or without **15** or **16** (0.2 mM in case of 100% wrt native dNTPs) was partitioned into thin-walled PCR tube. The PCR cycle was performed as follows: (1) 94 °C for 2 min, (2) 94 °C for 30 s, (3) 58 °C for 30 s, (4) 68 °C for 1 min, (5) steps 2−4 repeated 29 times, (6) 68 °C for 5 min, and (7) held at 4 °C. The crude reaction mixture was purified by silica spin column chromatography (Qiagen PCR purification kit) following manufacturer's protocol. The purity and product length were confirmed on 1% agarose gel/1× TBE (90 mM Tris-borate, 2 mM EDTA, pH 8) precasted with ethidium bromide (0.5 mg/L). Typical yields were 150 nM in 30 uL solution, corresponding to ∼10^6 fold amplification.

The p53 PCR products were prepared using the forward primer 5′-d(GCA GTC AGA TCC TAG CGT CGA GC) and the reverse primer 5′-d(GGG CAG TGC TCG CTT AGT GC). A plasmid containing the p53 gene was the source of the template DNA.[68]

Alkaline Agarose Gel Electrophoresis. The products from γ-radiolysis of the PCR products bearing **15** were separated using alkaline agarose gel electrophoresis as follows. Low melting agarose powder (750 mg) was suspended in 75 mL buffer solution (30 mM NaCl, 2 mM EDTA, pH 7.5). The suspension was boiled in a microwave oven (at medium power for 2.5 min) until the agarose melted completely to give a clear solution. The solution was cooled to 65−70 °C and carefully poured on a clean casting plate (7 × 10 cm) with comb (0.5 cm wide lanes) and was allowed to solidify for 1 h. The gel along with the casting plate was immersed into the alkaline electrophoresis buffer (30 mM NaOH, 2 mM EDTA) at least 1 h prior to electrophoresis. Aliquots of DNA samples (10 μL each) were mixed with 6 × alkaline electrophoresis loading buffer (2 μL, 180 mM NaOH, 6 mM EDTA, 18% Ficoll 400, 0.05% bromocresol green). The samples were heated to 70 °C for 5 min and chilled on ice for 5 min and loaded. The gel was then electrophoresed at 3 V/cm under constant voltage conditions in alkaline electrophoresis buffer (30 mM NaOH, 2 mM EDTA) for approximately 7 h or until the dye runs approximately two-thirds of the way down the gel. A thin glass plate as a cover was placed on top of the gel after the dye has migrated ∼1 cm into the gel to lower the diffusion rate of bromocresol green out of the lanes. After electrophoresis, the gel along with the casting plate was immersed for 30 min in 300 mL neutralizing buffer (1 M Tris-HCl, 1.5 M NaCl, pH 7.5) and then for additional 45 min in 1× SYBR Gold solution in 1× TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8) prior to scanning.

Enzymatic Digestion of PCR Products to 2′-Deoxynucleosides for LC−MS/MS Analysis. PCR product (2 pmol) in 32 μL H_2O, 10 × DNase I buffer (5 μL, 100 mM Tris·HCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.6 at 25 °C) and uridine (1 μL, 20 μM) as internal standard were incubated with DNase I (5 μL, 2 U/μL), calf intestinal alkaline phosphatase (2 μL, 10 U/μL), and snake venom phosphodiesterase (5 μL, 1 μg/μL) for 24 h at 37 °C. The reaction mixture was filtered through a Nanosep 3K filter by centrifuging the mixture for 5−10 min at 16000*g*. The filter was washed once with 50 μL H_2O and the combined filtrate was then transferred to a 0.2 mL tube. The solution was flash frozen in liquid N_2, lyophilized to dryness, and the residue dissolved in H_2O (30 μL). This solution (5−10 μL) was subjected to LC−MS/MS analysis using the following conditions. Waters HSS T3 C18 column (2.1 mm × 100 mm, 1.8 μm particle size) at 35 °C, using 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) at 0.3 mL/min. The following linear gradient was employed: (time (min), % B) 0, 0; 4, 0; 9.5, 80; 10.4, 80; 10.5, 0; 15.0, 0. Mass spectra were acquired in positive ion mode with MS^E using a capillary voltage of 3 kV, a sample cone voltage of 30 V and an extraction cone voltage of 4 V. The cone gas flow was set to 30 L/h and desolvation gas flow was 800 L/h. Desolvation temperature and source temperature were set to 400 and 150 °C, respectively. The acquisition range was m/z 100−3000. The scan acquisition rate was 10 Hz.

Nucleoside Kinase Assay.[69] A solution (95 μL) containing ATP (1 mM), NADH (0.15 mM), phosphoenol pyruvate (1 mM), pyruvate kinase-lactate dehydrogenase (10 U/μL pyruvate kinase, 15 U/μL lactate dehydrogenase), reaction buffer (1×, 50 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT) and H_2O (56.6 μL) was transferred to a cuvette and placed in a Beckman Coulter DU 640 UV−vis spectrometer equipped with an autosampler and a temperature controller set at 37 °C. The UV absorption of NADH at 340 nm was monitored after the addition of human deoxycytidine kinase (2 μL of 5.3 μM, 0.1 μM final concentration). After 1 min, the reaction was started by the addition of **7** (3 μL of 10 mM, 0.3 mM final concentration) and the UV absorption of NADH at 340 nm was monitored for 30 min at 37 °C. A control reaction with dA (0.3 mM) and a background reaction without any nucleoside were conducted under the same conditions. Reaction velocities were determined during periods in which the background reaction rate did not exceed 0.05 A_{340}/min.

Cell Viability Assay with 19. A solution of **19** (50 mM) in cell culture grade DMSO was serial diluted in RPMI 1640 growth medium to prepare 5× solutions ranging from 10 mM to 0.5 μM. These 5× solutions of each concentration were then added to wells of a 96 well plate containing 4000 cells/well. Control experiments containing the same percentages of DMSO in the absence of **19** were carried out. Each set of conditions was carried out in triplicate. Separate cell plates were incubated for 2, 3, and 4 days, respectively at 37 °C in a humidified atmosphere of 5% CO_2. After the incubation period, CellTiter-blue (20 μL) were added to the wells and incubated for 4 h. The fluorescence was measured at 590 nm in a SpectraMax M5 microplate reader.

Clonogenic Assay with 19. DU145 cells (18 × 10^4) were seeded in 10 mm culture dishes (1 each for 100 μM, 300 μM, and 500 μM **19** for later treatment, 1 each for corresponding controls with same percentages of DMSO as that in solutions containing **19**, and 1 for blank) in RPMI 1640 growth medium supplemented with 10% FBS. After overnight seeding at 37 °C in a humidified atmosphere of 5% CO_2, cells were treated with **19** (1 mL/dish). After 2 days incubation

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with **19**, the growth medium was removed from each dish and the remaining cells were gently washed with PBS (2 × 1 mL). The cells were treated for 5−10 min with trypsin (0.1 mM, 0.3 mL/dish) to detach them from the plates and then diluted in RPMI 1640 medium (0.7 mL/dish). The single cell suspensions were collected in 1.6 mL tubes and counted. Stock solutions of single cell suspensions were made in two groups for each concentration with 5000 cells/mL and 500 cells/mL, respectively. The number of cells seeded from each group was adjusted to account for the effects of radiation on survival with the goal of obtaining comparable numbers of colonies after the growth period. The number of cells seeded was adjusted for varying radiation doses (0, 6, 8, 10 Gy) using 5000 cells/mL, and the amount of DMSO in controls (using the group with 500 cells/mL for 0 Gy) in growth medium. Higher radiation doses such as 10 Gy required a larger number of cells (10 000) to be seeded, while a smaller number of cells (1000) were used for lower doses (6 Gy). The DMSO controls were plated similarly. The plates were irradiated initially with 2 Gy and after 4 h of incubation, were exposed to additional radiation (total dose: 6, 8, 10 Gy). The plates were incubated in humidified atmosphere with 5% CO_2 for 14 d. After 14 d, the growth medium was discarded and the attached cells were treated with 0.2% crystal violet solution. After the excess dye was washed, the colonies (at least 50 cells) were counted under a stereomicroscope. Plating efficiencies (PE) and survival fractions (SF) were calculated as follows:

$$\mathrm{PE} = \text{number of colonies} \div \text{number of cells seeded}$$

$$\mathrm{SF} = \mathrm{PE} \div \mathrm{PE(control)}$$

Quantification of 7 in DU145 Genomic DNA Following Treatment with 19 and Low Dose Radiation. A monolayer of cells (2.8×10^6) was grown in the culture medium in a T75 flask (75 mL). After 3 days the number of cells reached 10×10^6. The cells were treated with **19** (300 μM) by adding sterile stock solution in DMSO (50 mM) directly to the growth media, irradiated (2 Gy), and incubated at 37 °C for 4 h. The cells were then harvested by removing the growth medium, and washing with PBS. The PBS was aspirated and replaced by 0.2% trypsin (2 mL) in each dish. After detaching the cells from the dish cells (3−5 min at 37 °C), they were collected in medium. Using a Cellometer to count cells 5×10^6 cells were transferred to a 1.5 mL tube for control experiments (no treatment with **19**) and 10×10^6 cells that were treated with **19** (300 μM) were transferred to two 1.5 mL tubes. The tubes were centrifuged for 5 min at 300*g*. The supernatant was removed and discarded being careful to not disturb the cell pellets, which were each resuspended in PBS (200 μL).

The genomic DNA was extracted by adding proteinase K (20 μL, 20 mAnsonU/mL) and lysis buffer AL (Qiagen) (200 μL) were added to each tube. The suspension was pulse-vortexed for 15 s and then incubated for 10 min at 56 °C. The tubes were then briefly centrifuged to bring the droplets from the sides to the bottom. EtOH (200 μL) was added to the samples and mixed by pulse-vortexing for 15 s. The tubes were again centrifuged to bring the droplets from the sides to the bottom. Each solution was (420 μL) loaded on a QIAamp Mini spin column (Qiagen) equipped with a 2 mL collection tube. The tubes were centrifuged at 6000*g* for 1 min. The filtrates were discarded and buffer AW1 (Qiagen) (500 μL) was added to each of the spin column, which was then centrifuged at 6000*g* for 1 min. Again, the filtrates were discarded. The spin columns were then placed in clean 2 mL collection tubes, buffer AW2 (Qiagen) (500 μL) was added to each column, followed by centrifugation at 20000*g* for 3 min. The columns were then placed in new collection tubes and centrifuged again for 1 min at 12000*g* to eliminate any possibility of carrying over buffer AW2. The filtrates were discarded. Finally, the DNA was eluted by placing the columns in clean collection tubes and incubating with buffer AE (Qiagen) (200 μL) at 25 °C for 1 min. The columns were centrifuged at 6000*g* for 1 min. This process was repeated again and filtrates containing the DNA were combined to yield a total volume of 400 μL. This solution was stored at −20 °C for future experiments.

Genomic DNA (48 μg) was digested to 2′-deoxynucleosides for LC−MS/MS analysis by incubating in H_2O (77 μL), 10 × DNase I buffer (15 μL), uridine (1 μL, 20 μM) as internal standard with DNase I (50 μL, 2 U/μL), calf intestinal alkaline phosphatase (2 μL, 10 U/μL), and snake venom phosphodiesterase (5 μL, 1 μg/μL) for 24 h at 37 °C. The reaction mixture was filtered through a Nanosep 3K filter by centrifuging the mixture for 5−10 min at 16000*g*. The filter was washed once with H_2O (100 μL) and the combined filtrate (250 μL) was transferred to a 0.6 mL tube. The solution was flash frozen in liquid N_2, lyophilized to dryness, and the residue was dissolved in H_2O (30 μL).

This solution (10 μL) was subjected to LC−MS/MS analysis as described above. The extracted ion chromatograms of the fragmented nucleobases were generated by monitoring transitions 252.109 → 136.063, 243.098 → 127.051, 268.097 → 152.057, 228.098 → 112.051, 245.069 → 113.034, 289.128 (or 291.128) → 173.956 (or 175.956), for dA, dT, dG, dC, U, and **7** and **8**, respectively. The extracted ion chromatograms were used for quantifying the amount of **7** by comparing the integrated areas with the internal standard U (20 pmol). Varying ionization abilities of the nucleobases were normalized by including factors that correspond to the ratios of the integrated areas with respect to 20 pmol uridine. The factors are 10.2, 2.5, 9.6, 4, 7.3, and 7.8 for dA, dT, dG, dC, **7**, and **8**, respectively.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b01833.

Representative hydroxyl radical cleavage autoradiogram for cross-link identification. Representative alkaline agarose gel autoradiogram showing interstrand cross-link from PCR substrate. ESI-MS for oligonucleotides containing **7** and **8**. ESI-MS/MS of interstrand cross-links obtained from irradiation of **23a** and **24d**. NMR spectra of new compounds. (PDF)

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Notes

The authors declare no competing financial interest.

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