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Mechanistic Studies on RNA Strand Scission from a C2′-Radical

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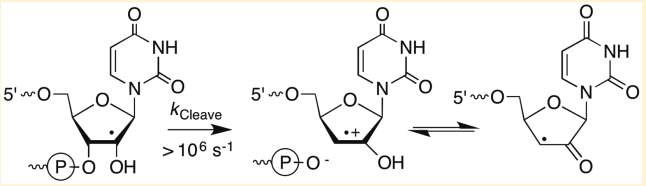
Supporting Information

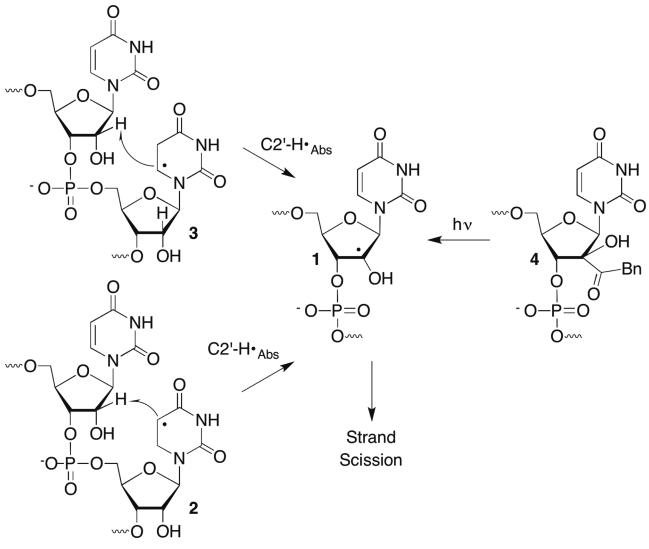
ABSTRACT: The C2′-carbon−hydrogen bond in ribonucleotides is significantly weaker than other carbohydrate carbon−hydrogen bonds in RNA or DNA. Independent generation of the C2′-uridine radical (**1**) in RNA oligonucleotides via Norrish type I photocleavage of a ketone-substituted nucleotide yields direct strand breaks via cleavage of the β-phosphate. The reactivity of **1** in different sequences and under a variety of conditions suggests that the rate constant for strand scission is significantly greater than 10^6 s^{-1} at pH 7.2. The initially formed C2′-radical (**1**) is not trapped under a variety of conditions, consistent with computational studies (*Chem.—Eur. J.* **2009**, *15*, 2394) that suggest that the barrier to strand scission is very low and that synchronous proton transfer from the 2′-hydroxyl to the departing phosphate group facilitates cleavage. The C2′-radical could be a significant contributor to RNA strand scission by the hydroxyl radical, particularly under anaerobic conditions where **1** can be produced from nucleobase radicals.



INTRODUCTION

Nucleic acid oxidation has profound biological effects and is also useful for probing RNA structure, RNA folding kinetics, and nucleic acid binding by small molecules and proteins.[1−6] Oxidation has even been used to examine nucleic acids in cells.[7,8] Ionizing radiation, such as γ-radiolysis, is a potent, common modality for inducing nucleic acid damage. Hydroxyl radical (HO•) is the primary reactive oxygen species generated from H_2O by ionizing radiation that reacts with nucleic acids. It is widely accepted that HO• adds to the π-bonds of nucleic acid nucleobases and abstracts hydrogen atoms from the (2′-deoxy)ribose backbone, but that its reactivity is dominated by the former.[9] However, oxidation of the carbohydrate moiety is required for HO• to induce strand scission, the chemical event most often used as a read-out of nucleic acid damage. One elegant study using isotopically labeled DNA substrates and Fe·EDTA to generate the reactive oxygen species led to the proposal that hydrogen atom abstraction from the C5′-carbon, followed by C4′-hydrogen atom abstraction, were the major pathways leading to strand scission by HO•.[10] Tullius' seminal publication on DNA damage is also cited when describing HO• cleavage of RNA, whose structure differs significantly from the former.[11,12] A large number of mechanistic studies have been carried out to understand how HO• produces strand breaks in DNA and RNA, in which the reactive oxygen species are generated by ionizing radiation. Under anaerobic conditions, hydrogen atom abstraction by a nucleobase radical from the ribose backbone is believed to be the major pathway for strand scission in poly(U).[13−15] However, investigations utilizing HO• are limited by the high reactivity and poor selectivity of this reactive species. Photochemical generation of reactive intermediates putatively produced in nucleic acids by HO• from stable radical precursors has also proven useful for understanding how nucleic acids are oxidatively damaged.[16−24] Recently, this approach was employed using **4** to generate the radical (**1**) resulting from C2′-hydrogen atom abstraction from uridine in RNA (Scheme 1).[25,26] Additional studies on the reactivity of uridin-2′-yl radical (**1**) are presented in this report.

Scheme 1



Direct strand breaks are formed ∼8-fold more efficiently in RNA than DNA exposed to HO•.[13,27] Approximately 40% of the reactions between HO• and RNA are believed to result in strand scission. Given that estimates for the contributions of HO• additions to nucleobases range from ∼80% to more than 90% of its reactions with nucleic acids, one or more pathways must exist for transferring spin from a nucleobase radical(s) to

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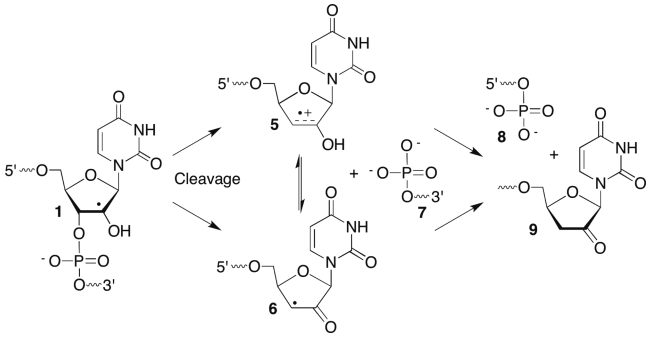
the carbohydrate moiety of RNA.[14,28] Independent generation of 5,6-dihydrouridin-5-yl (**2**) and 5,6-dihydrouridin-6-yl (**3**) radicals in RNA oligonucleotides identified pathways for direct strand scission from these nucleobase radical models of the respective HO• adducts via C2′-hydrogen atom abstraction (Scheme 1).[21−24] Both regioisomeric radicals abstract the C2′-hydrogen atom from the 5′-adjacent nucleotide, whereas **3** also induces intranucleotidyl hydrogen atom abstraction (not shown explicitly). Neither radical produces strand breaks at the 5′-adjacent nucleotide when a 2′-deoxyribonucleotide is present at that position. Furthermore, independent generation of the major hydroxyl radical adduct of thymidine within oligonucleotides also does not lead to direct strand scission at the 5′-adjacent nucleotide.[19] These observations suggest that the greater proclivity of RNA than DNA toward direct strand scission is due to initial nucleobase radical formation, followed by 5′-inter- and intranucleotidyl hydrogen atom abstraction.

The reactivity of a C2′-radical was examined by independently generating uridin-2′-yl radical (**1**) in a nucleoside and in RNA oligonucleotides (Scheme 2).[25,26] (Please note that for convenience, structures are referred to by the same number in the monomer or polymer.) The reactivity of **1** was characterized via product analysis and competitive kinetics. Uracil is rapidly lost from monomeric **1** (>10^5 s^{-1}), and thiol does not compete with this process in H_2O.[26] Similarly, strand scission via 3′-phosphate elimination is much faster (>10^6 s^{-1}) than β-mercaptoethanol (BME) trapping when **1** is generated in oligonucleotides.[25] The major products (**7**−**9**) obtained formed from **1** in oligonucleotides under aerobic and anaerobic conditions were determined, as well as their dependence upon thiol concentration. Additional questions concerning the reactivity of **1**, including how fast strand scission is and whether a discrete radical cation (**5**) forms, are addressed herein.

Scheme 2



RESULTS

Mass Spectral Analysis of Products. The major products (Scheme 2) previously characterized were ascribed to β-phosphate cleavage from **1**.[25] Additional information was sought by analyzing aerobic photolyses of trinucleotide **10** in the presence of 5 mM BME by LC/MS (Table 1 and Figure 1). The products (**11**−**13**, Table 1 and Scheme 3) observed from **10** were consistent with those formed from **17** that were characterized by gel electrophoresis (Scheme 2).

Ketone **13** (Figure 1) elutes as a mixture of the free carbonyl and the respective hydrate (**15**), with the former being present in greater amount. ^{18}O is not incorporated in **15** when the photolysis is carried out in $H_2{}^{18}O$ or $^{18}O_2$.

Table 1. Identification of Products from Aerobic Photolysis of 10 in the Presence of 5 mM BME by LC/MS

compound	calculated *m/z*	retention time (min)	observed *m/z*
11	307.0337	1.3	307.0316
12	321.0493	1.7	321.0469
13	529.0977	4.6	529.0963
15	547.1083	4.6	547.1074
14	563.1032	4.6	563.1002
^{18}O-14	565.1075	4.6	565.1086
10	955.1805	5.5	955.1792

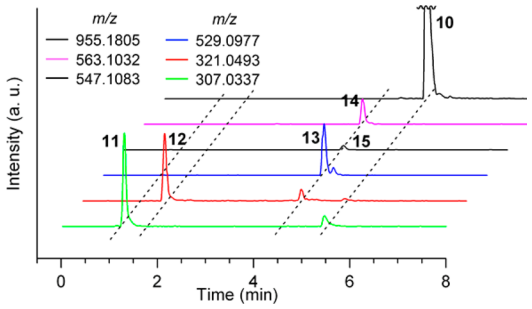
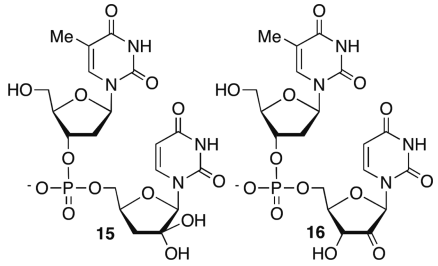


Figure 1. LC/MS analysis of photolysate of **10** (a total ion chromatogram is in the Supporting Information).

In addition, a product with *m/z* corresponding to **14** was observed (Scheme 3, Figure 1, and Table 1). Such a low concentration of BME (5 mM) is not expected to effectively compete with O_2 for alkyl radicals (e.g., **6**, Scheme 2) but should reduce peroxyl radicals and/or hydroperoxides. In contrast to **13**, complete hydration of **16** (**14**) is observed and is consistent with the perturbation of the carbonyl−hydrate equilibrium by an α-hydroxyl group.[29] Dioxygen was firmly established as the source of the α-hydroxyl group in **14** by carrying out aerobic photolysis of **10** in either $^{18}O_2$ or $H_2{}^{18}O$ (Figure 2). Isotopically enriched hydroxyl ketone hydrate (**^{18}O-14**) was present in the sample sparged with $^{18}O_2$ prior to photolysis (Figure 2) but not when $H_2{}^{18}O$ was the solvent.



5′-GAU CAG GC**4** UUG CCA UCG C
3′-CUA GUC CGA AAC GGU AGC G
17

5′-GAU CAG GG**4** UUG CCA UCG C
3′-CUA GUC CCA AAC GGU AGC G
18

5′-GAU CAG GC**4** GGG CAC UCG C
3′-CUA GUC CGA CCC GUG AGC G
19

5′-GAU CAG GC**4** UUG CCA UCG C
20

5′-GAU CAG GG**4** UUG CCA UCG C
21

5′-GAU CAG GC**4** GGG CAC UCG C
22

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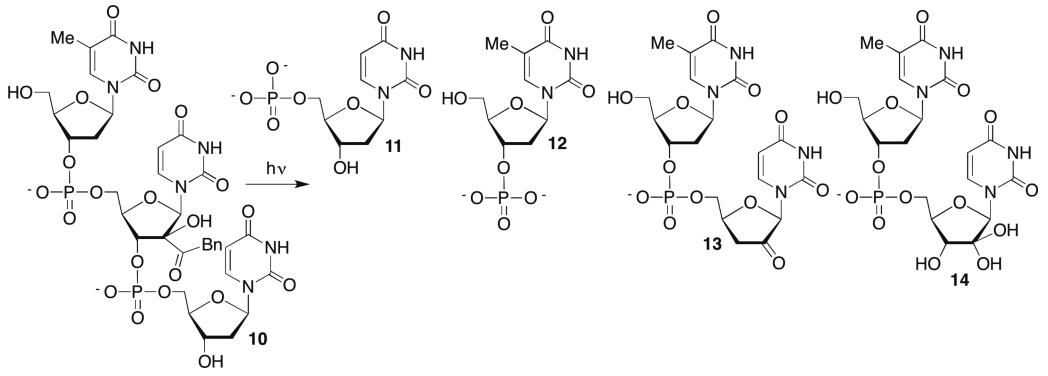
Scheme 3



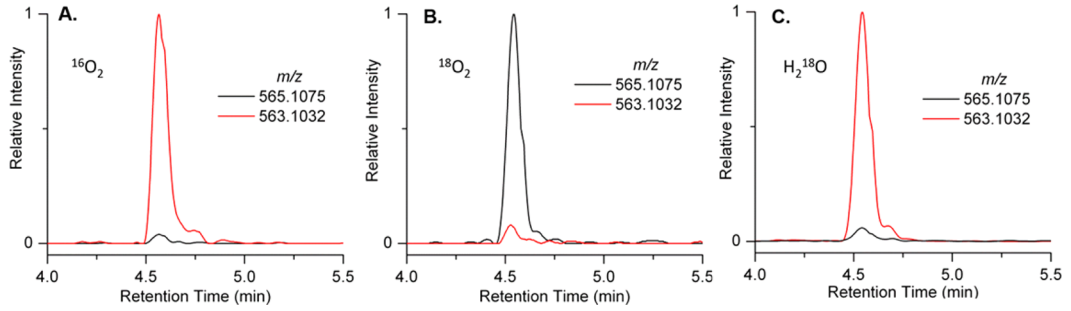


Figure 2. ^{18}O incorporation in **14** upon photolysis of **10** in (A) $H_2{}^{16}O/{}^{16}O_2$, (B) $H_2{}^{16}O/{}^{18}O_2$, and (C) $H_2{}^{18}O/{}^{16}O_2$.

RNA Sequence Effects on Product Distribution. The ratio of the two major 3′-terminal end group products (**9**/**8**) obtained from photolysis of **17** under aerobic conditions and 5 mM BME was slightly less than 1 (Table 2). The ratio of **9**/**8** was examined under the same photolysis conditions using substrates in which the radical precursor (**4**) was flanked by a GGG triplet on either the 5′- or the 3′-side. A proximal G-triplet should reduce the radical cation (**5**) more rapidly, which would result in a ratio of **9**/**8** being higher than that in **17**.[30] However, in both instances, the ratio of **9**/**8** was lower than that from **17** (Table 2). Photolysis of the corresponding single-stranded RNAs (**20**–**22**) produced similar ratios of **9**/**8** and were all <0.7 (Table 2), showing no evidence for G-triplet-mediated reduction of **5** in single- or double-stranded RNA. Finally, substituting D_2O for H_2O had no effect on the product distribution obtained from the photolysis of **18**.

Table 2. Ratio of 9/8 under Aerobic Conditions as a Function of Flanking Sequence

substrate	ratio of **9**/**8**
17	0.77 ± 0.11
18	0.28 ± 0.02
19	0.47 ± 0.14
20	0.52 ± 0.11
21	0.52 ± 0.18
22	0.66 ± 0.10

[a]Photolyses were carried out in the presence of 5 mM BME. All values are the average ± standard deviation of three independent experiments.

Environmental Effects on the Ratio of Ketone (9)/3′-Phosphate (8) Product. Investigations on strand scission from nucleobase radicals **2** and **3** in duplex RNA had shown that the **9**/**8** ratio under anaerobic conditions (no BME) increased with decreasing pH. In contrast, there is no pattern in the change of the **9**/**8** ratio as a function of sequence at higher pH (5.3 or 7.2) when **1** was produced directly from photolysis of **4** in **17**–**19** (Table 3). However, a significant increase in the ratio of **9**/**8** was observed upon reducing the pH from 5.3 to 3.6. The ratio of **9**/**8** varied from ∼4 to ∼7 among the three sequences examined. Although the ratio of **9**/**8** is certainly greater at pH 3.6 when **1** is flanked by GGG sequences, the observed change represents a small increase in the amount of **9** from ∼80% to less than 88%. In addition, anaerobic photolysis of single-stranded **17** (**20**) at pH 7.2 in the absence of BME produces a higher ratio of **9**/**8** (3.0 ± 0.2) compared to that of **17**.[23] Similar results were obtained from photolysis of **18** and its single-stranded variant **21** (3.5 ± 1.3) under these conditions.

Table 3. Ratio of 9/8 from 1 under Anaerobic Conditions as a Function of pH

	ratio of **9**/**8**		
substrate	pH 7.2	pH 5.3	pH 3.6
17	0.35 ± 0.11	0.17 ± 0.02	4.01 ± 0.57
18	0.12 ± 0.02	0.40 ± 0.11	7.02 ± 1.04
19	0.11 ± 0.04	0.20 ± 0.01	6.82 ± 1.32

[a]Photolyses were carried out in the absence of BME. All values are the average ± standard deviation of three independent experiments, unless otherwise noted.

Solvent Effects on the Rate of Phosphate Elimination. Strand scission from **1** in aqueous buffer (10 mM pH 7.2 phosphate, 5 mM $MgCl_2$, 100 mM NaCl) is too rapid for BME to compete. In attempts to reduce the cleavage rate constant, photolyses were carried out in the absence of salt and in acetonitrile cosolvent to reduce the polarity. Reducing the ionic strength by removing only $MgCl_2$ did not have a significant effect on the yield of RNA strand scission under anaerobic

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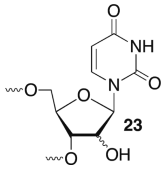
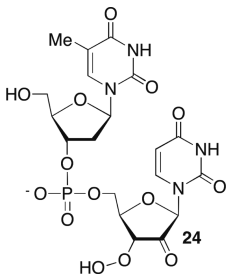
conditions in the presence of 0.5 M BME. Strand scission increased slightly from 25.3 ± 2.3% ([$MgCl_2$] = 5 mM) to 28.7 ± 3.6% upon removal of $MgCl_2$.

The effect of solvent polarity on the yield of strand scission was also investigated. Anaerobic photolysis in aqueous acetonitrile (50%) in the presence on 0.5 M BME without any salt again showed an increase in strand scission yield (30.5 ± 4.8%) compared with a similar photolysis (21.3 ± 2.4%) in aqueous buffer (10 mM pH 7.2 phosphate, 5 mM $MgCl_2$, 100 mM NaCl). In addition, we did not detect any thiol trapping products of **1** (e.g., **23**) by LC/MS in photolysates of trinucleotide **10** that were irradiated under degassed conditions in 50% aqueous acetonitrile and 0.5 M BME.[26] However, the previously described cleavage products (**11**−**13**, Scheme 3) were observed. Although rate constants for hydrogen atom transfer from thiols to alkyl radicals are modestly reduced in less polar solvents, these experiments suggest that the ratio of rate constants for thiol trapping of **1** and strand scission by the C2′-RNA radical are not increased significantly in the less polar conditions employed.[31]



DISCUSSION

Previous reports support the proposal that the greater susceptibility of RNA to cleavage by HO• is due to C2′-hydrogen atom abstraction, followed by rapid 3′-phosphate cleavage (Schemes 1 and 2).[14,21−23,25] The hydroxyl radical is not expected to frequently abstract the C2′-hydrogen atom in duplex RNA directly due to its low solvent exposure.[1] However, the C2′-hydrogen atom(s) is well positioned in the major groove to react with pyrimidine nucleobase (peroxyl) radicals, which are the major family of reactive intermediates formed by reaction of HO• with nucleic acids. In addition, radical cation formation via β-phosphate cleavage from α-heteroatom-stabilized alkyl radicals is well established.[32−36] Computational studies suggest that 3′-phosphate cleavage from a C2′-radical in RNA is facilitated by the 2′-hydroxyl proton and could be >10^9 s^{-1}.[37] Synchronous/concomitant proton transfer would formally bypass the radical cation and yield the α-keto radical (**6**, Scheme 2). We investigated RNA strand scission from the C2′-radical by independently generating this reactive intermediate via Norrish type I photocleavage of **4**. These initial studies yielded a conservative estimate that strand scission of **1** occurred with a rate constant >10^6 s^{-1} in phosphate-buffered saline (PBS) at 25 °C.[25] The product studies presented above were carried out to address how fast cleavage from **1** occurs, whether strand scission is sequence-dependent, whether the discrete radical cation (**5**) is produced, and whether the cleavage process serves as an initiation for hole transfer in the biopolymer.



Although the major products at the 3′-termini of the 5′-fragment were **8** and **9**, additional products were apparent by denaturing polyacrylamide gel electrophoresis following photolysis under aerobic conditions and low thiol concentration. One of these was characterized using trinucleotide **10**. Mass spectrometry established that the compound was the hydrate (**14**) of the α-hydroxy ketone (**16**) and not the hydroperoxide (**24**). Isotopic labeling established that O_2 was the source of the hydroxyl group, suggesting that the α-keto radical (**6**) was trapped and subsequently reduced by the thiol. The pseudo-first-order rate constant for H_2O trapping of a radical cation similar to **5** was estimated to be ∼1.1×10^8 s^{-1}.[38] If this is a good model, the absence of any evidence for oxygen incorporation in **14** (Scheme 2) from water suggests that deprotonation of radical cation (**5**) occurs significantly faster than 10^8 s^{-1}, and that the lifetime of diffusively free **5** if it is formed at all is <10 ns. This estimate is consistent with computational studies, which suggest that proton transfer from the C2′-hydroxyl group to the departing phosphate is barrierless and phosphate cleavage from **1** bypasses **5**.[37]

A GGG sequence was incorporated on either the 5′- (**18**, **21**) or 3′-side (**19**, **22**) of the radical precursor (**4**) to enhance the probability of reducing **5** if it is formed. Reduction of **5** would result in an increase in the ratio of products containing **9**/**8** at the 3′-termini of the 5′-cleavage fragments.[30] However, the ratio of **9**/**8** was within experimental error in three single-stranded RNAs of different sequence, two of which contained the GGG sequence (Table 2). Moreover, in the case of duplex RNAs, the ratio of **9**/**8** actually decreased slightly at neutral pH under aerobic and anaerobic conditions (Tables 2 and 3). Attempts to increase the lifetime of **5** by carrying out the reaction in D_2O, which would decrease the rate constant for deprotonation and formation of **6** also had no effect on the product ratio obtained from photolysis of **18**. Hence, if diffusively free **5** is formed, deprotonation is too fast for electron transfer within the duplex to compete. Based upon rate constants for hole transfer within duplex DNA (provided these are applicable to RNA), deprotonation would have to be faster than 10^9 s^{-1}.[39,40]

In contrast, Sugiyama showed that electron transfer within the RNA duplex competes with strand scission from **1**, albeit using the much stronger guanine radical cation oxidant.[41] We were unable to trap **1** using O_2 in aerated solutions or thiol in degassed solutions.[25] Previously, we modulated the rate constant for deglycosylation from monomeric **1** by reducing the solvent polarity.[26] Attempts to reduce the rate of strand cleavage from **1** so that BME could compete by reducing the ionic strength and solvent polarity were unsuccessful. (We cannot rule out that the reduced rate constant for phosphate cleavage is masked by a commensurate decrease in the rate constant for trapping of **1** by thiol in less polar solvent. However, the solvent effect on thiol trapping of radicals is <20-fold over a greater range of solvent polarity.[31]) The calculated barrier for deglycosylation of **1** is ≥8 kcal/mol higher than that

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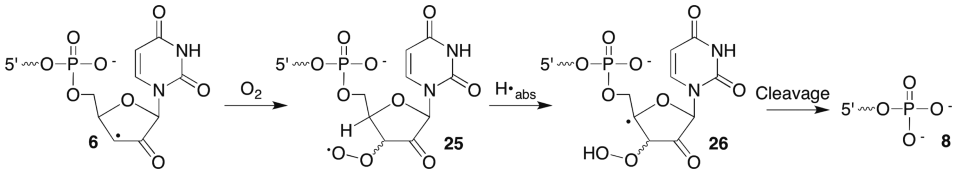
Scheme 4



for strand scission and could be more susceptible to moderation by changes in the reaction environment.[37] These experiments suggest that the calculated barrier for strand scission from **1** (≤4 kcal/mol) is accurate and that the rate constant at 298 K is significantly greater than the conservative value of 10^6 s^{-1} that our trapping experiments support.[25,37]

The above experiments and Eriksson's computational studies indicate that if **5** forms it rapidly deprotonates to **6**, which goes on to produce **8** and **9** (Scheme 2). In experiments in which nucleobase RNA radicals (e.g., Scheme 1) were produced under anaerobic conditions, the increased **9**/**8** ratio at lower pH was ascribed to a partitioning of **5**.[21−23] The same trend was observed when **1** was independently generated (Table 3) as previously observed for nucleobase radicals. However, in view of the above discussion concerning **5** and **6** (Scheme 2), the significant increase in the **9**/**8** ratio when the pH was reduced from 7.2 to 3.6 may also indicate that synchronous proton transfer from C2′-OH to form **6** becomes less important and that discrete formation of **5** contributes at lower pH. However, we cannot rule out changes in the relative stability of the final products and/or intermediates. The greater increase of the **9**/**8** ratio in duplexes containing G trinucleotide flanking sequences (**18**, **19**) at pH 3.6 compared to that in **17** is consistent with electron transfer from the GGG sequence to the radical cation at this pH (and not at pH 7.2 or 5.6). However, the overall change in the percent of **9** is modest, and we cannot definitively conclude that electron transfer contributes to the observed chemistry.

Previous reports help explain how synchronous cleavage (**1** to **6**, Scheme 2) and proton transfer (**5** to **6**) give rise to 3′-phosphate product (**8**) under aerobic conditions. Oxygen trapping of **6** yields **25**, which may ultimately yield **8** via a mechanism for which there is precedent in nucleic acid radical chemistry (Scheme 4). For instance, the peroxyl radical obtained from the O_2 trapping of the C3′-radical in DNA abstracts a hydrogen atom from the C4′-position.[42,43] The facility of the resulting C4′-radical (**26**) to yield strand scission via β-fragmentation is well established.[17,44,45] Formation of 3′-phosphate (**8**) from **6** under anaerobic conditions is difficult to explain. However, significant quantities of **8** are only formed under anaerobic conditions in the absence of thiol, and one cannot rule out trace amounts of O_2 that trap **6**, which is generated from <50 nM solutions of radical precursor. At such low substrate concentrations, O_2 would remain in excess even if 99.9% of it were removed. Similarly, the hydrogen atom source that yields **9** from **6** in the absence of thiol is also uncertain.

CONCLUSIONS

Although nucleobase radical formation by ionizing radiation (and HO•) is the major pathway for RNA and DNA damage, the former is significantly more susceptible to strand scission.[13,27] We previously showed that nucleobase radicals induce direct strand breaks in RNA by selectively abstracting C2′-hydrogen atoms.[21−23] We also showed that the C2′-RNA radical (e.g., **1**) rapidly eliminates phosphate (>10^6 s^{-1}) to produce a direct strand break.[25] Computational studies suggest that strand scission from **1** could be ≥10^9 s^{-1} at room temperature and that diffusively free radical cation **5** is avoided.[37] Additional experiments described above suggest that phosphate cleavage from **1** is significantly faster than 10^6 s^{-1}, and only at pH 3.6 is tentative evidence, electron transfer from a GGG trinucleotide, presented for discrete formation of **5**. When combined with the previously described preference for strand scission from nucleobase radicals in double-stranded compared to single-stranded RNA, these data suggest that strand scission efficiency from RNA nucleobase radicals is more likely to be affected by secondary structure that influences the rate of spin transfer to the sugar (Scheme 1) than from sequence or environmental effects on the cleavage reaction from C2′-radicals (Scheme 2).[21−23] Together, these observations reinforce the suggestion that additional structural information on RNA could be obtained by carrying out hydroxyl radical cleavage experiments under anaerobic conditions.

EXPERIMENTAL PROCEDURES

General Methods. Oligonucleotides were synthesized via standard automated oligonucleotide synthesis. RNA synthesis reagents were obtained from Glen Research. Oligonucleotides were purified by 20% denaturing gel electrophoresis and desalted using C18-Sep-Pak cartridges. Oligonucleotides were characterized by MALDI-TOF MS or ESI-MS. LC/MS was carried out using a quadrupole time-of-flight spectrometer. 5′-Radio-labeling was carried out using standard protocols (briefly described below) involving T4 polynucleotide kinase (PNK) and γ-^{32}P-ATP.[46] T4 PNK and γ-^{32}P-ATP were commercially available. Quantification of radio-labeled oligonucleotides was carried out using a phosphorimager. Radio-labeled samples were counted using a liquid scintillation counter. Photolyses were carried out using lamps with maximum output at 350 nm. BME solutions were freshly prepared. Anaerobic photolyses samples were degassed using standard freeze−pump−thaw degassing techniques (three cycles, 3 min each), sealed, and photolyzed in Pyrex tubes. Pyrex tubes were washed with dilute Absolve (sodium hydroxide) solution, rinsed with RNase-free water, and oven-dried to render them RNase-free.

Oligonucleotide Substrate Preparation. Oligonucleotides containing **4** were prepared via solid-phase oligonucleotide synthesis as previously described and purified by denaturing polyacrylamide gel electrophoresis.[25] Trinucleotide **10** was synthesized in the same manner but was purified by C_{18} reverse-phase HPLC. The resin was incubated in 80% aq AcOH (1 mL) for 2 h at room temperature, and the supernatant solution was decanted off. The resin was washed with 1:1 acetonitrile/H_2O (2 × 0.5 mL) and dried under vacuum. The trinucleotide was then cleaved from the resin by the treatment with 1:1 30% aq NH_3/40% aq $MeNH_2$ (0.8 mL) for 1 h at 65 °C. The resin was separated from the supernatant by centrifugation and washed with H_2O (2 × 0.25 mL). The supernatant and the washings were combined and concentrated, and the residue was purified by reverse-phase HPLC on a Phenomenex C18 column (250 × 4.6, 5 μm) using 0.1 M triethylammonium acetate (containing 5% acetonitrile, solvent A) and acetonitrile (containing 5% H_2O, solvent B) as mobile phases. A linear gradient of 0 to 40% B over 10 min was employed, followed

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by another gradient of 40 to 100% B over 6 min. The peak eluting at 12 min was collected and lyophilized: HRMS (ESI-TOF) m/z calcd for $C_{36}H_{41}N_6O_{21}P_2$ $(M - H)^-$ 955.1805, found 955.1792.

General Procedure for Oligonucleotide Photolysis. The strands of interest were labeled at their 5′-termini with γ-^{32}P-ATP using T4 PNK in T4 PNK buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 45 min, 37 °C). Radio-labeled oligonucleotides were separated from unincorporated ^{32}P-nucleotides by gel filtration using Sephadex G-25. Prior to photolysis, labeled strands were hybridized to the complementary strand(s) (1.5 equiv) in PBS (0.1 M NaCl, 10 mM sodium phosphate pH 7.2) by heating at 90 °C for 5 min and slowly cooling to room temperature. RNA was photolyzed (350 nm) for 7−8 h under aerobic/anaerobic conditions in Pyrex glass tubes (5 mm i.d.) in the presence of buffer (10 mM phosphate at pH 7.2, 10 mM citrate at pH 5.3 or pH 3.6), NaCl (0.1 M), $MgCl_2$ (5 mM), and the desired concentration of BME. The reaction mixtures were lyophilized, resuspended in formamide loading buffer, and analyzed by 20% denaturing PAGE.

Procedure for Trinucleotide (10) Photolysis. An aqueous solution (100 μL) of **10** (6.5 μM) containing phosphate (10 mM), NaCl (0.1 M), $MgCl_2$ (5 mM), and BME (5 mM) was photolyzed in Pyrex glass tubes (5 mm i.d.) under aerobic conditions for 9 h at room temperature. The reaction mixture was concentrated, resuspended in H_2O (60 μL), filtered (0.22 μm), and analyzed by LC/MS. For $^{18}O_2$ experiments, the reaction mixture was prepared without adding BME and bubbled with $^{18}O_2$ (gas) for 15 min at 0 °C. A previously degassed solution of BME was then added to a final concentration of 5 mM, and the mixture was photolyzed in the usual manner. For $H_2{}^{18}O$ experiments, the reaction mixtures were redissolved in $H_2{}^{16}O$ before LC/MS analysis. The samples were analyzed using a UPLC Q-ToF mass spectrometer with an Acquity UPLC HSS T3 C18 column (2.1 mm × 100 mm, 1.8 μm particle size) set at 35 °C following separation using 1% formic acid in water (solvent A) and acetonitrile (solvent B), 0.3 mL/min using the following linear gradient: 5% solvent B (2 min); 5 → 50% B (over 4 min); 50% → 97% B (over 1 min); 97% B (2 min); 97% → 5% B (over 1 min); 5% B (5 min). Mass spectra were acquired in negative ion mode with MSE using a capillary voltage of 2 kV, a sample cone voltage of 40 V, and an extraction cone voltage of 4 V. Desolvation temperature and source temperature were set to 500 and 130 °C, respectively. The acquisition range was m/z 100−3000. The LC/MS system was operated by MassLynx software v 4.1.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b01760.

Mass spectra of oligonucleotides (3) containing non-native nucleotides and the total ion chromatogram for the photolysis of **10** (PDF)

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Notes

The authors declare no competing financial interest.

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