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Rapid RNA Strand Scission Following C2′-Hydrogen Atom Abstraction

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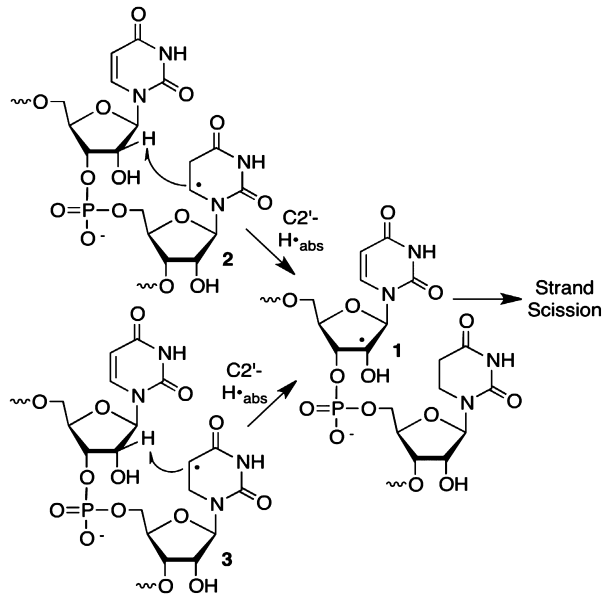
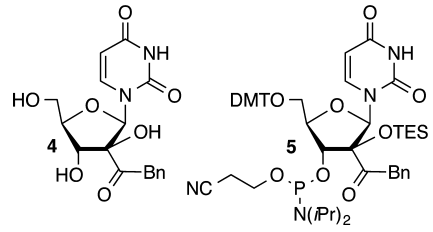
Supporting Information

ABSTRACT: C2′-Nucleotide radicals have been proposed as key intermediates in direct strand break formation in RNA exposed to ionizing radiation. Uridin-2′-yl radical (**1**) was independently generated in single- and double-stranded RNA via photolysis of a ketone precursor. Direct stand breaks result from heterolytic cleavage of the adjacent C3′-carbon−oxygen bond. Trapping of **1** by O_2 or β-mercaptoethanol (1 M) does not compete with strand scission, indicating that phosphate elimination is $>10^6$ s^{-1}. Uracil loss also does not compete with strand scission. When considered in conjunction with reports that nucleobase radicals produce **1**, this chemistry explains why RNA is significantly more susceptible to strand scission by ionizing radiation (hydroxyl radical) than is DNA.

Ionizing radiation is a common source of nucleic acid damage. Although radiation can cause cancer, it is used to treat a large percentage of human cancers, and DNA damage is the source of its cytotoxicity. Ionizing radiation induced nucleic acid damage is also a useful tool for probing nucleic acid structure, folding, and interactions with proteins in vitro and in vivo.[1−3] Radiation damages nucleic acids via direct ionization and following the excitation of water, which yields hydroxyl radical (OH•). The highly reactive hydroxyl radical, which is also produced by Fe·EDTA and related metal complexes, oxidatively damages nucleic acids and is more commonly used to characterize nucleic acids and their interactions than is γ-radiolysis.[4−6] Nucleobase addition is the favored pathway for OH• reaction with RNA and DNA, accounting for as much as 93% of the encounters.[7,8] Despite the similarity in the reactivity of the two families of nucleic acids with OH•, the efficiency for strand scission is significantly higher in RNA (∼40%) than in DNA (≤5%).[9,10] Strand cleavage requires oxidation of the carbohydrate backbone, and various mechanisms have been proposed for transferring spin from the nucleic acids' nucleobases to carbohydrates in RNA. The C2′-position is frequently suggested as the site from which a hydrogen is abstracted.[11] Recent studies on analogues of the OH•−uridine adducts (**2**, **3**) provided strong evidence in support of the intermediacy of uridin-2′-yl radical (**1**) en route to strand cleavage (Scheme 1).[12−14] However, questions remain concerning the reactivity of a C2′-ribonucleotide radical. Some of these questions are addressed herein by independently generating **1** at a specific site within RNA.

Scheme 1





Although intramolecular hydrogen bonding increases the solvent exposure of the C2′-hydrogen atom in some RNA structures, most such atoms in duplex RNA have negligible exposure to OH•.[15,16] However, its proximity to nucleobase radicals and its relatively weak bond strength make the C2′-hydrogen atom a good candidate for transferring spin to the carbohydrate component of RNA.[17] Monomer studies on independently generated C2′-ribonucleoside radicals and those putatively generated from reactions between nucleosides and OH• or $SO_4^{-\bullet}$ indicate that nucleobase loss is rapid.[18−20] Experiments in which reactive intermediates are randomly generated in biopolymers lead to proposals that strand scission from a C2′-radical is also rapid ($>10^4$ s^{-1}).[21,22] Product analysis and kinetic isotope effects support 5′-internucleotidyl C2′-

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hydrogen atom abstraction by **2** and **3**, as well as intra-nucleotidyl abstraction by the former.[12−14] Competitive kinetic experiments suggested that C2′-hydrogen atom abstraction was the rate-determining step, but an unequivocal answer to this question awaited independent generation of a C2′-nucleotide radical. Uridin-2′-yl radical (**1**) was recently independently generated from **4** upon photolysis with UV light.[19] We have utilized phosphoramidite **5** in solid phase oligonucleotide synthesis to prepare RNA oligonucleotides within which **1** is independently generated.[23,24]

5′-GAU CAG GC$_8$**4** U$_{10}$UG CCA UCG C
6

5′-GAU CAG GC**4** UUG CCA UCG C
3′-CUA GUC CGA AAC GGU AGC G
7

5′- $^{-2}$O$_3$PO-UUG CCA UCG C
8

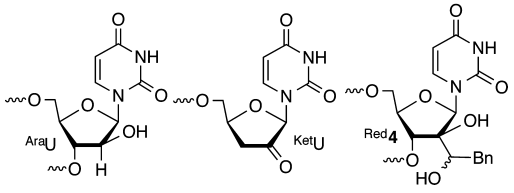
5′-GAU CAG GC^{Ket}U
9

5′-GAU CAG GC-OPO$_3^{-2}$
10

5′-GAU CAG GCU UUG CCA UCG C
11

5′-GAU CAG GC^{Ara}U UUG CCA UCG C
12

5′-GAU CAG GC^{Red}**4** UUG CCA UCG C
13



A single 3′-fragment is detected by denaturing polyacrylamide gel electrophoresis (PAGE) following photolysis of 3′-^{32}P-**6** or the respective duplex (3′-^{32}P-**7** in which the ketone containing strand is labeled) under either anaerobic or aerobic conditions (Figures S3, S4, Supporting Information).[23] Phosphatase treatment of the photolysate confirms that the radiolabeled fragment contained a 5′-phosphate terminus (**8**) (Figure S5).[23] Two major products (**9** and **10**) are observed by denaturing PAGE in the 5′-fragment following photolysis of 5′-^{32}P-**6** (or **7**) with or without O_2 in the presence of 5 mM β-mercaptoethanol (BME). Postphotolysis treatment with polynucleotide T4 kinase (PNK), which dephosphorylates 3′-phosphates, reveals that the major product under aerobic conditions is **10** (Figure 1B). The major product formed under anaerobic conditions reacts with isoniazid (**14**), which forms hydrazones with carbonyls in RNA, and is consistent with ketone **9**. Ketone **9** also forms preferentially over **10** when **1** is generated under aerobic conditions in single-stranded (5′-^{32}P-**6**) and double-stranded (5′-^{32}P-**7**) RNA at higher [BME] (100 mM, Figure 2). Although the 3′-phosphate (**10**) is favored over ketone **9** under aerobic conditions in the presence of ≤5 mM BME (Figures 1B, 2), more complicated mixtures are produced (Figures S1, S2).[23] The identities of the cleavage products (**8**−**10**) were corroborated by MALDI-TOF MS (Figure 3).

We could not estimate the true yield for RNA strand scission from **1** (identified as the yield of **8** from 3′-^{32}P-**6** and -**7**) without determining whether other products are formed in which the oligonucleotide remains intact. Although no such products were detected by denaturing PAGE, experiments were carried out to determine whether intact oligonucleotide products comigrate with **6** (Figures S6−S8).[23] Experiments

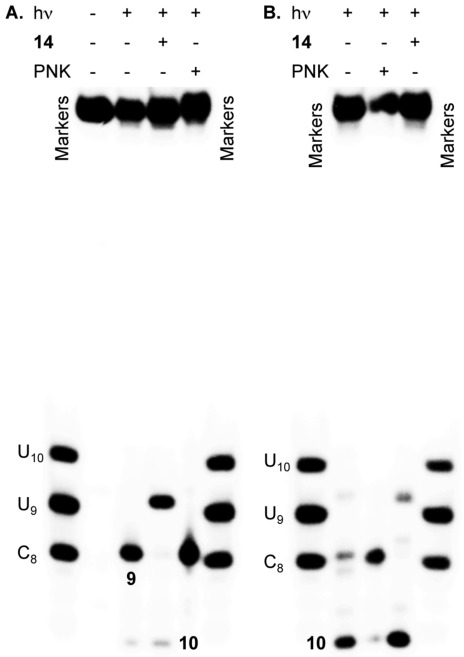


Figure 1. Autoradiogram of photolysis of 5′-^{32}P-**6** in the presence of 5 mM BME. (A) Anaerobic conditions. (B) Aerobic conditions. Markers contain 5′-phosphate and 3′-hydroxyl termini.

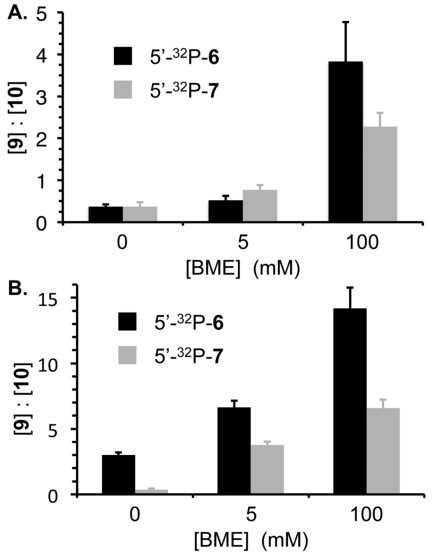


Figure 2. Product dependence on thiol (BME) concentration in single (5′-^{32}P-**6**) and double (5′-^{32}P-**7**) stranded RNA under aerobic (A) and anaerobic (B) conditions.

with independently synthesized oligonucleotides containing potential products in which the RNA remains intact (e.g., **11**), and treatment with $NaBH_4$ to reduce any ketone (**4**) that remains after photolysis indicated that the C2′-radical (**1**) does not produce any intact oligonucleotide products. Therefore, the percent strand scission detected by denaturing PAGE corresponds to the extent conversion of **4**, and cleavage from **1** is quantitative.

The amount of strand scission in single- (**6**, 59 ± 6%) and double-stranded (**7**, 25 ± 4%) RNA following photolysis under anaerobic conditions is independent of BME concentration (up to 1 M). Based upon the aforementioned product analysis, the differences in strand scission yield in single- (**6**) and double-

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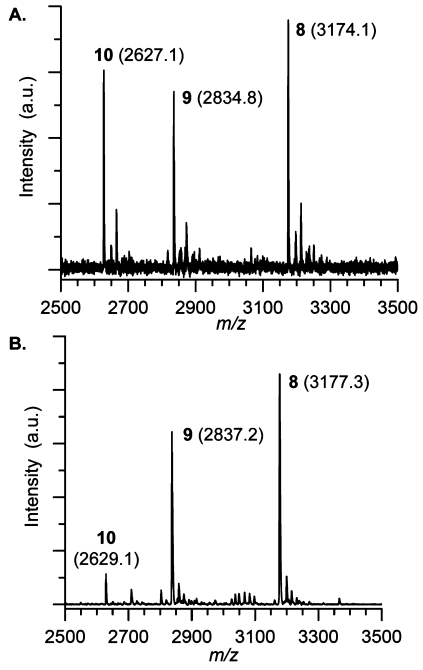
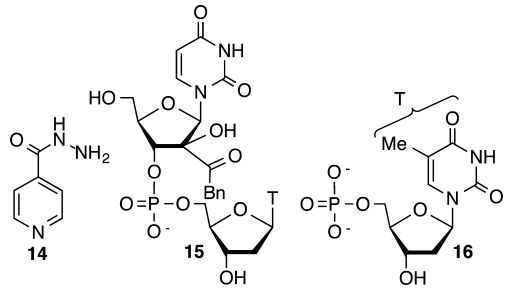


Figure 3. MALDI-TOF MS analysis of photolyzed **6** under (A) aerobic conditions or (B) anaerobic conditions in the presence of 5 mM BME. Calculated m/z: **8**, 3176.9; **9**, 2836.8; **10**, 2628.6. Observed m/z are in parentheses.



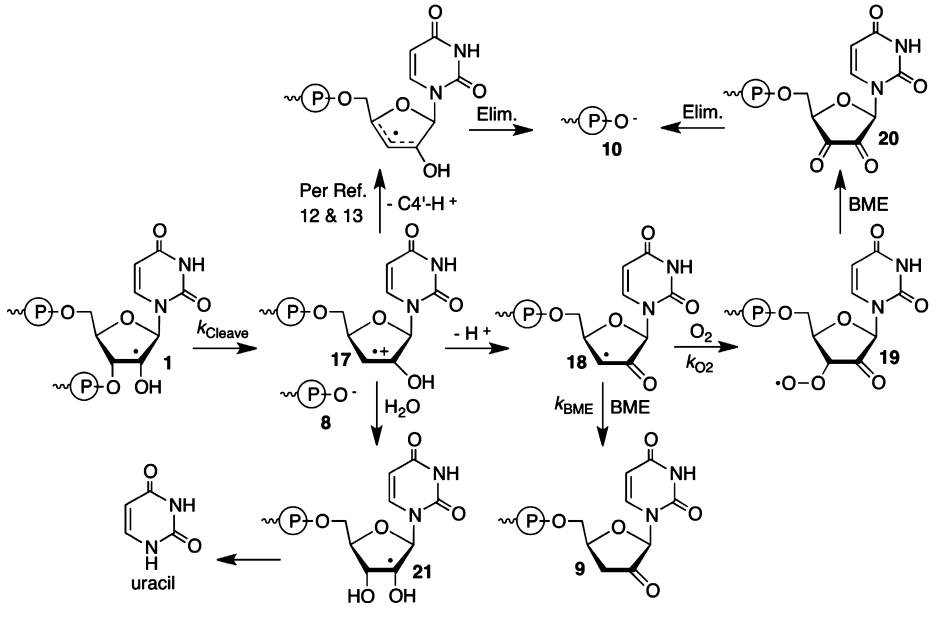
stranded (**7**) RNA are attributed to higher photochemical conversion of **4** in the former. A slight reduction in cleavage (38 ± 5% for **6** and 18 ± 2% for **7**) is observed under aerobic conditions, and is also attributed to lower conversion of **4** and not reaction of O_2 with **1**. Most importantly, these experiments indicate that reaction of neither O_2 or BME with **1** competes with strand scission from the radical.

These data are consistent with strand scission from **1** resulting from heterolytic cleavage of the 3′-phosphate (Scheme 2). The lack of an effect by O_2 (k_{O_2} = 2 × 10^9 M^{-1} s^{-1}, 0.2 mM) or 1 M BME (k_{BME} ∼ 1−10 × 10^6 M^{-1} s^{-1}) on strand scission yield suggests that the rate constant for cleavage (k_{Cleave}, Scheme 2) from **1** is >10^6 s^{-1}.[25] This estimate is based upon the anticipated rate constants for reaction between **1** and O_2 or BME (and the concentrations of these reagents), and the assumption that reduced strand scission would have been detected in the presence of either at the concentrations employed if k_{Cleave} was ≤10^6 s^{-1} in single-stranded or double-stranded RNA. This conservative estimate for strand scission from **1** is at least 1000-times faster than that from the C4′-radical in DNA where the 3′-phosphate cleaves heterolytically.[26]

The gel electrophoresis and mass spectrometry experiments do not address the possible loss of the nucleobase following formation of **1**, which was detected in photolyses of monomeric **4** and the analogous adenosine radical where the 3′-leaving group (hydroxyl) is much poorer.[18,19] Dinucleotide **15** was used to examine the competition between uracil release and phosphate cleavage by HPLC. As was the case in the oligonucleotides, the respective phosphate cleavage product (**16**) quantitatively accounts for the consumed **15**, and the yield of **16** was independent of O_2 or BME (1 M). In addition, uracil was formed in 35−40% yield relative to **16**, and was independent of O_2 or BME.

The steps proposed following strand scission are based upon product studies involving oligonucleotides **6** and **7**, and dinucleotide **15** (Scheme 2). Deprotonation of cation radical **17** yields the α-keto radical (**18**), which produces **9** upon

Scheme 2



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reduction. Oxygen trapping of **18** (**19**) ultimately yields the 3′-phosphate terminal product (**10**), possibly via one or more C3′-carbonyl intermediates (e.g., **20**). C4′-deprotonation of **17** may also lead to **10**.[12,13] The effect of BME concentration on the ratio of **9**:**10** indicates that the reactivity of **17** is similar in single-stranded (**6**) and double-stranded (**7**) RNA (Figure 2). The details for the formation of uracil from **6** and **7** are unknown at this time. However, the quantitative yield of **16** suggests that uracil cleavage occurs after strand scission from **1**. The lack of an effect of BME or O_2 on the yield of uracil from monomeric **1** in aqueous buffer indicates that this process is also significantly faster than bimolecular trapping by these reagents.[19] One possible mechanism involves competition between deprotonation from **17** and trapping by H_2O (Scheme 2). Reaction of **17** with H_2O regenerates an uridin-2′-yl radical (**21**) that is analogous to monomeric C2′-radical **1**, which rapidly loses uracil in protic solvent.[19] However, the mechanism is speculative, and further investigation is warranted.

Overall, the combination of recent studies on RNA cleavage resulting from nucleobase radical generation and the experiments described above solidify long-standing mechanistic proposals regarding the effects of ionizing radiation on nucleic acids and are relevant in other aspects of RNA chemistry.[7−9,11−14,16,20] These investigations affirm that RNA is more susceptible to strand scission than DNA because the major family of reactive intermediates formed at pyrimidine nucleotides, the nucleobase adducts of hydroxyl radical, induce direct strand scission via rate limiting C2′-hydrogen atom abstraction.[27] The ensuing C2′-radicals (e.g., **1**) rapidly eliminate the 3′-phosphate to yield strand breaks. Uracil cleavage is not competitive with strand scission, but occurs after cleavage from **1**. Direct strand scission in DNA is inefficient because C2′-hydrogen atom abstraction is significantly less favorable and the absence of a α-heteroatom substituent in the resulting C2′-radical significantly decreases the rate constant for stand scission via phosphate elimination. RNA's greater susceptibility to oxidative cleavage may also be relevant to its involvement in various human pathological conditions.[28,29] Finally, the data also support the proposal that strand scission emanating from OH• addition to nucleobases in RNA may be an additional source of structural information on the biopolymer in folding experiments.[3,12,13]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, representative autoradiograms, and mass spectrum of **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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