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Electron-Mediated Aminyl and Iminyl Radicals from C5 Azido-Modified Pyrimidine Nucleosides Augment Radiation Damage to Cancer Cells

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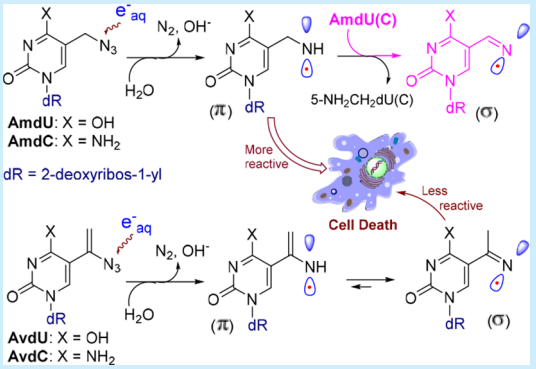
Supporting Information

ABSTRACT: Two classes of azido-modified pyrimidine nucleosides were synthesized as potential radiosensitizers; one class is 5-azidomethyl-2′-deoxyuridine (AmdU) and cytidine (AmdC), while the second class is 5-(1-azidovinyl)-2′-deoxyuridine (AvdU) and cytidine (AvdC). The addition of radiation-produced electrons to C5-azido nucleosides leads to the formation of π-aminyl radicals followed by facile conversion to σ-iminyl radicals either via a bimolecular reaction involving intermediate α-azidoalkyl radicals in AmdU/AmdC or by tautomerization in AvdU/AvdC. AmdU demonstrates effective radiosensitization in EMT6 tumor cells.



Pyrimidine nucleosides modified at C5 (e.g., 5-bromo-2′-deoxyuridine) are well-investigated as radiosensitizers in cancer therapy.[1] 5-(Phenylselenyl)methyl-2′-deoxyuridine and 5-thiocyanato-2′-deoxyuridine were shown to induce reactive benzyl-type[2] or uracil-C5-thiyl radical[3] for DNA interstrand cross-linking[2,3] and apoptosis in cancer cells.[4]

3′-Azido-3′-deoxythymidine (AZT) has shown a significant radiosensitization in irradiated human colon cancer, larynx squamous carcinoma, and malignant glioma cells.[5] Moreover, AZT has been employed as a radiation sensitizer in radiotherapy of tumors for HIV-positive patients.[6] We found that the radiation-produced prehydrated electrons[7] leads to the site-specific formation of a localized π-aminyl radical (RNH$^{\bullet}$) in azido-substituted nucleosides[8] and sugars.[9,10] Our ESR studies show that RNH$^{\bullet}$ formed from AZT undergoes bimolecular H atom abstraction from a C5-methyl group, generating a thymine allyl radical (dUCH$_2^{\bullet}$), or from a sugar moiety yielding C5′$^{\bullet}$.[8] Moreover, RNH$^{\bullet}$ and modified base radicals can produce sugar radicals which lead to DNA-strand breaks[1a] and eventually lead to induction of apoptosis.[1c] We hypothesize that incorporation of azido-modified nucleosides into genomic DNA would augment radiation-induced damage in cells owing to the reactions of aminyl radicals under hypoxic conditions and, therefore, act as potential radiosensitizers.

Owing to the poor incorporation of AZT in genomic DNA,[11] in this work, we have employed 5-azidomethyl-2′-deoxyuridine (AmdU, **4**), 5-azidomethyl-2′-deoxycytidine (AmdC, **6**), 5-(1-azidovinyl)-2′-deoxyuridine (AvdU, **12**), and 5-(1-azidovinyl)-2′-deoxycytidine (AvdC, **13**) analogues[12,13] (Schemes 1 and 2) to study the radiation-mediated formation of RNH$^{\bullet}$ and its subsequent reactions. Recently, AmdU has been metabolically incorporated into DNA in living cells for click labeling of DNA.[14] Moreover, its 5′-triphosphate was found to be the substrate for DNA polymerases and PCR amplification.[15,16] Herein, we report that π-aminyl RNH$^{\bullet}$ generated by radiation-produced electron addition to azido-nucleosides undergoes conversion to the thermodynamically more stable σ-iminyl radicals (R═N$^{\bullet}$). We also show incorporation of AmdU and AmdC phosphates into DNA fragments by polymerase-catalyzed reactions and present evidence that these azidonucleosides can act as radiosensitizers.

AmdU **4** was synthesized from thymidine **1** by sequential silylation, bromination with NBS, displacement of bromide with NaN_3, and desilylation (45% overall, Scheme 1).[14,17] Treatment of **3** with 2,4,6-triisopropylbenzenesufonyl chloride (TIPBSCl) in the presence of TEA/DMAP followed by displacement of the resulting aryl sulfate with NH_4OH and deprotection of **5** with TBAF provided AmdC **6** (57% overall).

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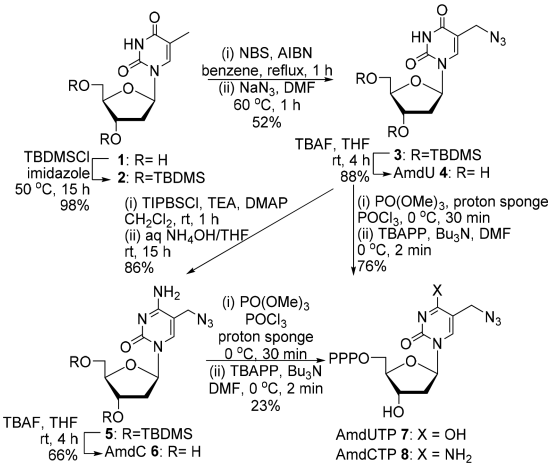
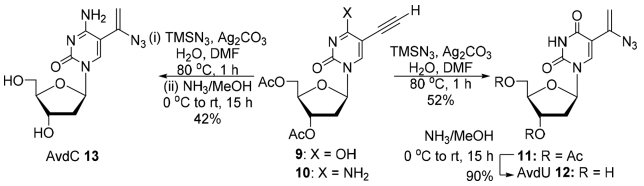
Scheme 1. Synthesis of 5-Azidomethyl Pyrimidine Nucleosides and Their 5′-Triphosphates



Scheme 2. Synthesis of 5-Azidovinyl Pyrimidine Nucleosides



AmdU and AmdC were triphosphorylated[18] to give AmdUTP **7** (76%) and AmdCTP **8** (23%).

Silver-catalyzed hydroazidation[19] of the protected 5-ethynyl-2′-deoxyuridine[20] **9** with $TMSN_3$ regioselectively produced *α*-azidovinyl **11** (52%; Scheme 2).[21] Deacetylation of **11** gave AvdU[13] **12** (90%). Analogous hydroazidation of the protected 5-ethynyl-2′-deoxycytidine[22] **10** followed by deacetylation provided AvdC **13** (42% overall). Conversation of uracil base in **11** to a cytosine counterpart by treatment[23] of **11** with TIPBSCl followed by ammonolysis also gave **13** (Supporting Information (SI)).

Radiation-produced electron addition to **4** allowed us to identify three radicals: (i) *π*-aminyl radical, *π*-U-5-CH_2−$ND^•$; (ii) *α*-azidoalkyl radical, U-5-$CH^•$−N_3; and (iii) *σ*-iminyl radical, U-5-CH=$N^•$ (Figures 1 and S1, Scheme 3). The center of the spectrum in Figure 1A (blue) does not show the doublet[24] expected for $U^{•-}$, thereby indicating that the azide moiety of **4** scavenged nearly all radiation-produced electrons to form the *π*-aminyl $RNH^•$ via a dissociative electron attachment. The initial electron attachment is followed by rapid protonation of the incipient nitrene anion radical (Scheme 3). The spectrum extends over 178.5 G, and its hyperfine structure shows line components from the hyperfine coupling (HFCC) due to single axially symmetric anisotropic nitrogen [$A_{||}$ = ∼42.5 G, $A_{\perp}$ = ∼0] with $g_{||}$ and $g_{\perp}$ values that are typical of aminyl radical nitrogen (Table S1). In U-5-CH_2−$ND^•$, the radical-site *p*-orbital strongly couples with two *β*-CH_2^- protons, generating the wide doublet (ca. 93.5 G). The simulated spectrum of U-5-CH_2−$ND^•$ (Figure 1A, red; see the SI for simulation parameters) matches the line components of the experimental blue spectrum in Figure 1A.

On subsequent stepwise annealing at 140, 160, 165, and 170 K, new line components appear which are assigned to two new species (Figure 1B−E, blue). The first species initially observed

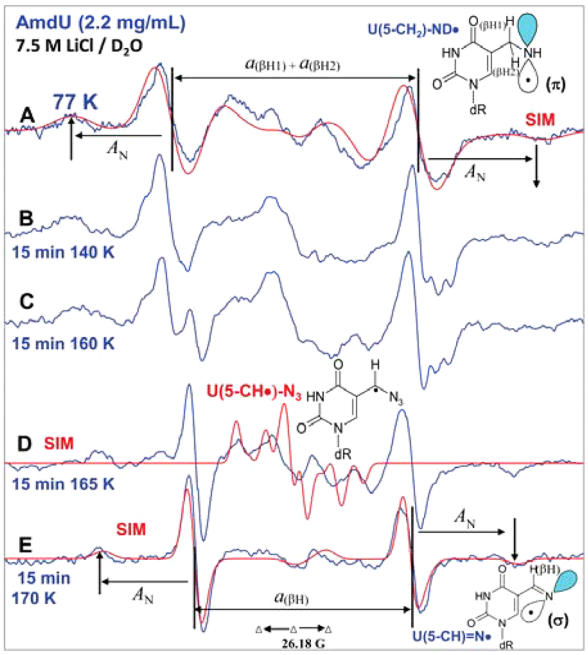
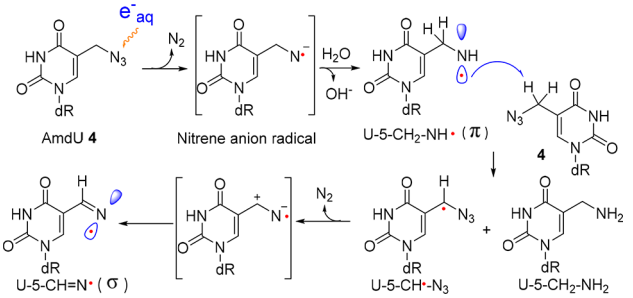


Figure 1. (A) ESR spectrum (blue) after radiation-produced one-electron addition to AmdU **4** (2.2 mg/mL) at 77 K (*γ*-irradiation, 500 Gy) in 7.5 M LiCl/D_2O in dark. Spectra B−E were obtained via stepwise annealing of the sample for 15 min at 140, 160, 165, and 170 K in the dark. All spectra were recorded at 77 K. The radiation produced background $Cl_2^{•-}$ spectrum was subtracted from spectra A and B for clarity. Three reference triangles show Fremy's salt resonances with central marker at g = 2.0056.

Scheme 3. Formation of *π*-Aminyl Radical from AmdU and Its Bimolecular Conversion to the *σ*-Iminyl Radical



in the center of spectrum 1C and resolved as a multiplet in spectrum 1D is assigned to a C-centered *α*-azidoalkyl radical (U-5-$CH^•$−N_3) formed via H-abstraction by $RNH^•$ from a proximate parent **4** (Scheme 3). In the spectrum in Figure 1C, the second species begins to form. The total hyperfine splitting decreases by ca. 8 G from that found in Figure 1A,B (i.e., the wings line components move in), and this new species becomes the only radical found in Figure 1E. The spectrum in Figure 1E is assigned to the *σ*-iminyl radical, U-5-CH=$N^•$ resulting from couplings of one anisotropic nitrogen and one *β*-proton. For assignments of radicals contributing to the spectra in Figure 1D,E and HFCC, see the SI (pages S6 and S7, Table S1, and Figures S1 and S2). These results show that the reactive *π*-U-5-CH_2−$ND^•$ abstracts an H-atom from the parent **4** to form the azidoalkyl radical intermediate, U-5-$CH^•$−N_3. Subsequently, U-5-$CH^•$−N_3 promptly undergoes a unimolecular *β*-N_2 elimination from the azide group to

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produce the thermodynamically more stable σ-U-5-CH=N• (Scheme 3).

The π-C-5-CH_2−ND• formation and its subsequent conversion to the σ-iminyl radical, C-5-CH=N•, has also been observed upon radiation-produced electron attachment to cytosine counterpart AmdC **6** (Scheme S1, Figure S2, and Table S1). The α-azidoalkyl radicals generated from photolysis of α-azidoacetophenones[25] or γ-azidobutyrophenone[26] as well as α-azido o-iodoanilides under tin-mediated radical reactions[27] are known to undergo similar conversion to the σ-iminyl radicals.

A radiation-produced electron addition to the vinyl azide **12** also generates π-RNH•, which undergoes facile tautomerization to thermodynamically more stable σ-iminyl radical (Figure 2 and Scheme 4). The 77 K ESR spectrum (black, Figure 2A)

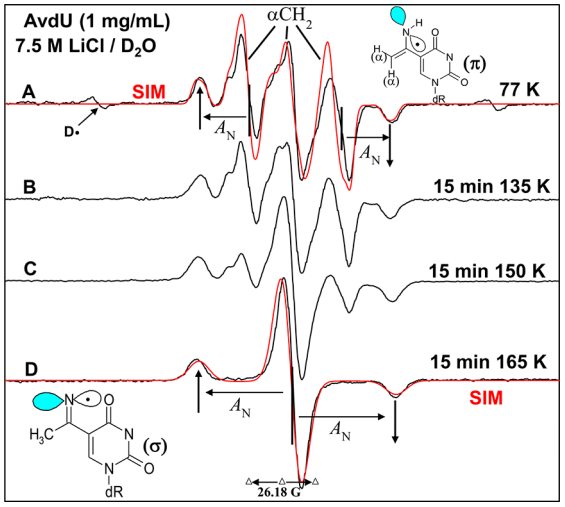
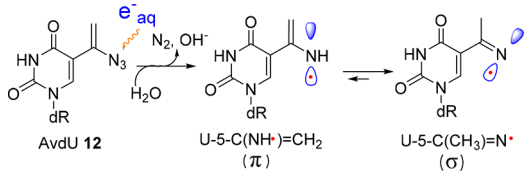


Figure 2. (A) ESR spectrum (black) after radiation-produced one-electron addition to **12** (1 mg/mL) at 77 K (γ-irradiation, 500 Gy) in 7.5 M LiCl/D_2O in dark. (B−D) Spectra after stepwise annealing at 135, 150, and 165 K recorded at 77 K. The red spectra in (A) and (D) are the simulated spectra. The background $Cl_2^{\bullet-}$ spectrum has been subtracted from spectra A and B for clarity.

Scheme 4. Tautomerization of π Aminyl Radical, Generated from AvdU, to σ-Iminyl Radical



shows line components from single axially symmetric anisotropic nitrogen HFCCs and from two anisotropic protons of the $=CH_2$ group in **12** (Table S1). We assign these to the π-aminyl radical, U-5-C(ND•)=CH_2 (Scheme 4). Formation of U-5-C(ND•)=CH_2 results from a dissociative electron attachment pathway similar to that shown in Scheme 3. The simulated red spectrum (see Table S1 for simulation parameters) matches line components of the black spectrum and supports our assignment (Figure 2A). HFCC values for the anisotropic nitrogen and CH_2 α-protons in U-5-C(ND•)=CH_2 and its optimized geometry are reported in Table S1 and Figure S5.

ESR spectra obtained upon progressive annealing (Figure 2B−D, black) show that height of the singlet at the center increases with concomitant decrease of the line components from the two anisotropic α $=CH_2$ protons. The black spectrum (Figure 2D) is due solely to an axially symmetric anisotropic nitrogen (for HFCCs see Table S1) and is assigned to the σ-iminyl radical, U-5-C(CH_3)=N•. The simulated spectrum (Figure 2D, red; Table S1) matches the line components of the black spectrum.[28] Nearly identical spectra were obtained from one-electron attachment to AvdC, proving that formation of the π-aminyl radical (C-5-C(ND•)=CH_2) and its facile tautomerization to the σ-iminyl radical (C-5-C(CH_3)=N•) has a general character and occurs independent of the nucleobase.

Increasing the concentration (1 to 5 mg/mL) of **12** has no effect on the extent of this conversion as observed from spectra recorded under the same microwave power, modulation, and gain. From these results, we conclude that the conversion of π-RNH• to σ-R=N• observed in **12** and **13** is by tautomerization; it occurs via facile intramolecular proton transfer from the aminyl group to the double-bonded CH_2 group in the π-RNH•. Conversion of π-RNH• to σ-R=N• has also been observed in one-electron oxidized 1-methylcytosine and its derivatives.[28]

In contrast to our previous results in which π-RNH• produced from one-electron reduction of AZT and azidopentoses[8,9] undergoes H atom abstraction reactions, we find that π-RNH• from **4**, **6**, **12**, and **13** undergoes conversion to σ-R=N•. For **4** and **6**, the π-RNH• to σ-R=N• conversion is bimolecular which, at first, involves H-abstraction to form an α-azidoalkyl radical followed by a unimolecular decomposition to σ R=N• (Scheme 3). However, **12** and **13** undergo tautomerization of π-RNH• to σ-R=N• (Scheme 4). Owing to the high free-radical scavenger concentrations in cells,[1a] the bimolecular conversion of π-RNH• to σ-R=N• from **4** and **6** is unlikely to take place. However, the tautomerization of π-RNH• to σ-R=N• from **12** and **13** should occur even in cells. Further, reactivity of σ-R=N• from **12** and **13** is far less than that of a π-RNH•. Therefore, it is expected that the π-RNH• from **4** and **6** could augment radiation damage more effectively than the σ-R=N• from **12** and **13**.

Incorporation of nucleosides into DNA is important for them to exhibit radiosensitization.[1b−d,29] Thus, AmdUTP was successfully incorporated by the *E. coli* Klenow fragment of DNA polymerase I (pol I) and human repair DNA polymerase β (pol β) during DNA leading and lagging strand synthesis and BER (base excision repair) using an open template, one-nucleotide gap substrates, and one-nucleotide substrate containing a 5′-THF (a tetrahydrofuran ring which mimics a sugar residue; Figure S6, Table S2; see the SI for more details). Incorporation of AmdUTP in the presence of dATP, dGTP, and dCTP showed that both polymerases readily inserted AmdUTP into dsDNA and extended the nucleotide during DNA replication and BER (Figure S7). AmdU incorporation can be ligated into duplex DNA during DNA replication and BER in the presence of LIG I (Figure S8). These results are consistent with a recent finding that AmdU was efficiently incorporated into newly synthesized DNA in human cancer cells.[14] The AmdCTP **8** was also incorporated into DNA by pol β during DNA replication and BER (Table S2, template 2; Figure S9).

To test our hypothesis that azidonucleosides incorporated into DNA can act as radiosensitizers, we investigated the

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radiation response of EMT6 breast cancer cells to the presence of 100 μM azido-modified nucleosides in both aerobic and hypoxic environments in terms of survival fraction (SF). For survival fraction measurements in aerobic cells, the cultures were treated with a 100 μM azido compound or vehicle for 48 h. For the measurements in hypoxic cells, hypoxic conditions were applied for 4 h after a 44 h aerobic incubation with 100 μM azido compounds or vehicles. To investigate the radiosensitizing effect, cells were irradiated with 7.5 Gy X-ray during the final few minutes of the 48 h incubation (Figure 3 and Table S3).

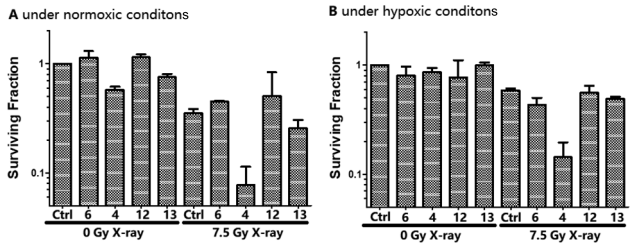

Figure 3. Radiosensitizing effect of 100 μM azido-modified nucleosides on EMT6 cells: (A) normoxic and (B) hypoxic conditions.

AmdU showed radiosensitization under both normoxic and hypoxic environments with sensitization enhancement ratios (SER) at 7.5 Gy X-ray ($SER_{SF7.5}$) of 4.57 and 4.10, respectively (Figure 3, Table S3). These results show that aminyl radicals generated in AmdU augments radiation damage to cells. In a hypoxic microenvironment, $RNH^{\bullet}$ formed from electron addition to AmdU can be involved in the H atom abstraction reactions[1a,8,9] leading to lesions that can induce apoptosis of cancer cells.[1c] On the other hand, in the aerobic cells, the aminyl radical generated from AmdU can react with oxygen to generate aminylperoxyl radical $RNHOO^{\bullet}$ and eventually lead to aminoxyl (nitroxyl) radicals $RNO^{\bullet}$,[30] which also can lead to DNA damage.[31] Other azido nucleosides showed a lower radiosensitizing effect with $SER_{SF7.5}$ index of 1.35 for AmdC under hypoxic cells and 1.37 for AvdC under aerobic cells.

Reasons for the differences in radiosensitization between AmdU **4** and AmdC **6** are unclear. Possibilities include differences in drug uptake into cells, metabolic phosphorylation,[32] and/or reactivity of the aminyl radical generated at the uracil and cytosine base. The higher radiosensitizing effect of AmdU compared with that of AvdU **12** and AvdC **13** could be explained by the higher reactivity of π-$RNH^{\bullet}$ from AmdU compared to that of σ-$R{=}N^{\bullet}$ from AvdU and AvdC.

In conclusion, the pyrimidine nucleosides modified at the C5 position with azidomethyl or azidovinyl moieties have been designed as potential radiosensitizers. The 5′-phosphates of 5-azidomethyl analogues were incorporated into DNA fragments by polymerase-catalyzed reactions. The π-aminyl radicals generated from **4**, **6**, **12**, and **13** undergo facile conversion to more stable σ-iminyl radicals, either bimolecularly involving an α-azidoalkyl radical intermediate or by tautomerization. AmdU may act as effective radiosensitizer in EMT6 cancer cells in the presence or absence of oxygen. Since AmdU **4** has been used for DNA labeling in cells,[14,33] **4** can serve a dual purpose of labeling tumor cells prior to, during, or after radiotherapy and may radiosensitize the tumor during radiotherapy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b03035.

Experimental procedures, mechanistic and theoretical studies, and compound characterization data for all new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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