Investigation of Reactions of Radiation-Produced Electrons with Azido-Nucleosides: Formation and Identification of Radical Intermediates

Submitted by

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Chemistry

To

The Honors College

Oakland University

In partial fulfillment of the

requirement to graduate from

The Honors College

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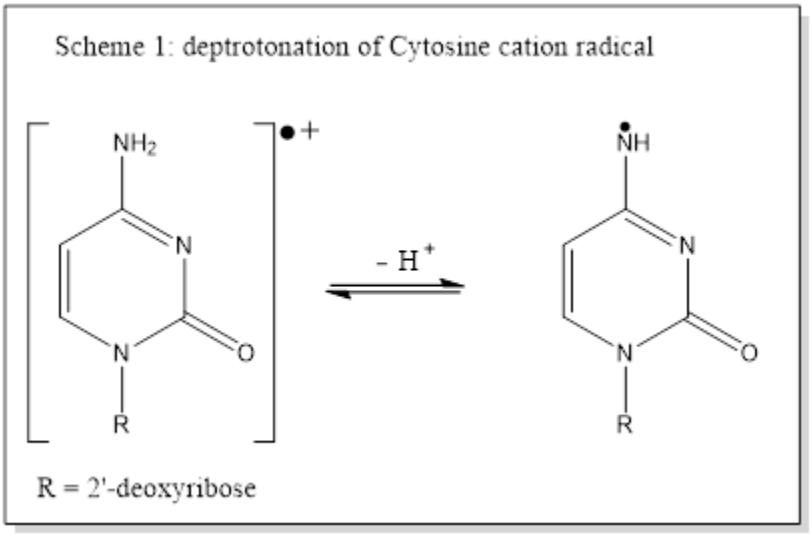
December 4, 2020

Abstract

This project elucidates the mechanisms involved in the formation of site-specific π-type neutral aminyl radicals (π-RNH•) via the reaction of radiation-produced electrons with certain azido-DNA model systems including azidonucleosides in the absence of oxygen. Subsequent reactions of these oxidizing π-RNH• that were produced reductively, were investigated. Experimental investigations on formation of these π-RNH• and their subsequent reactions were carried out using Electron Spin Resonance (ESR) spectroscopy. The hyperfine coupling constant (HFCC) values were calculated employing Density Functional Theory (DFT). Combination of ESR spectral studies and DFT calculations led to radical assignments and interpretations of ESR spectra. Reactions of these π-RNH• would reveal whether these azido-DNA model systems can be applied as potential radiosensitizers (compounds which augment radiation damage), used for radio-chemotherapy of tumors.

Background

Aminyl radicals (RNH•) are a vital component in the chemistry of biomolecules such as nucleic acids and proteins [3]. Formation of these radicals can occur in a number of ways: (a) RNH• can be produced via deprotonation of aminyl cation radicals and is also formed from oxidation of primary amines through the reductive quenching cycle that occurs during photo-redox catalysis. For example, deprotonation of either a purine cation radical or pyrimidine cation radical leads to RNH• formation (scheme 1) [9].



(b) It has also been discovered that radiation-produced electrons help form localized π-RNH• in azido-substituted nucleoside compounds that had the azido substitution in the sugar [2] and in the base [4]. (c) Additionally, it has been recently observed that in azido-substituted pentofuranoses with different configurations, the site and pathway of formation of the localized π-RNH• are strongly influenced by the sugar ring conformation [3].

The mechanism of formation of RNH• from azido-nucleosides is illustrated as follows: the RNH• forms through a particular pathway at 77 K [1-5], for example, when the compound "3′-

Azido-3′-deoxythymidine" (3′-AZT)" is irradiated. The 3′-AZT anion radical, $RN_3\bullet^-$, forms through addition of radiation-produced electrons to 3′-AZT (scheme 2, reaction 1). This azide anion radical, $RN_3\bullet^-$, then undergoes a quick loss of N_2 to form a nitrene anion radical, $RN\bullet^-$, (scheme 2, reaction 2) which then forms the π-type neutral aminyl radical, RNH•, through rapid protonation from the solvent, H_2O (scheme 2, reaction 3). Lastly, the RNH• species leads to the amine (RNH_2) product through hydrogen abstraction (scheme 2, reaction 4). Only the RNH• shown in these reactions have been detected by ESR spectra for AZT and other specific azide containing molecules [2-5]. In addition, aminyl cation radicals offer increased reactivity and selectivity in chemical reactions [14-16], making them useful for studying electron-mediated site-specific formation of RNH• and their reactions in azido-nucleosides.

Scheme 2

Azide anion radical formation [1]

$$RN_3 + e^- \longrightarrow RN_3\bullet^-$$

Nitrene anion radical formation [2]

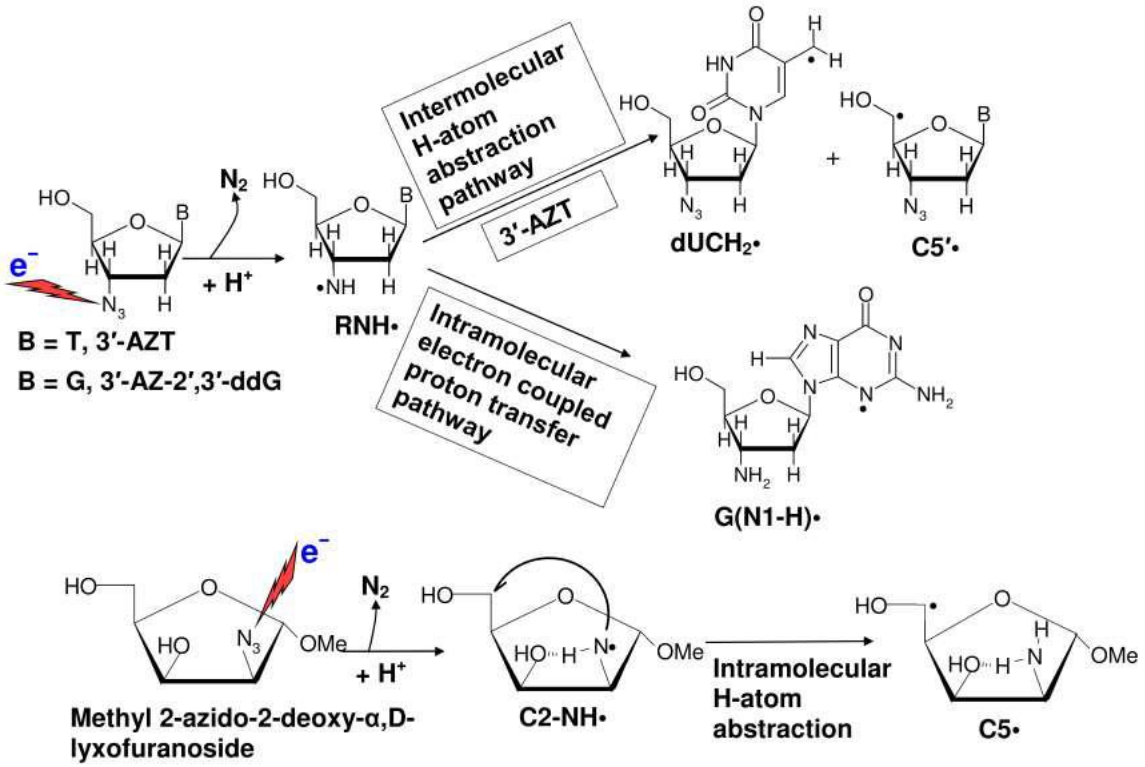
$$RN_3\bullet^- \longrightarrow RN\bullet^- + N_2$$

Aminyl radical formation [3]

$$RN\bullet^- + H_2O \longrightarrow RNH\bullet$$

Amine formation by H-atom abstraction [4]

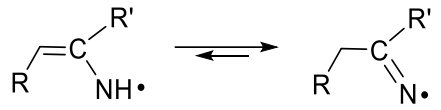
$$RNH\bullet + R'-H \longrightarrow RNH_2 + R'\bullet$$



Scheme 3. p-RNH• Formation and its reactions [1].

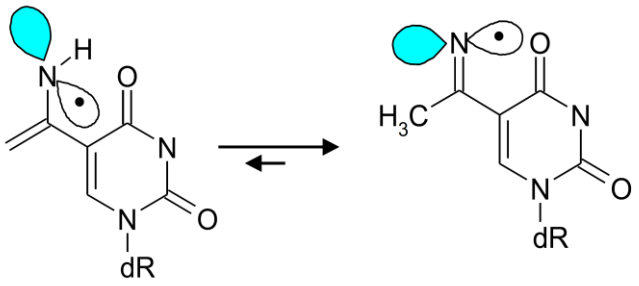
In 3′-AZT, RNH• undergoes a subsequent bimolecular H-atom abstraction both from the methyl group at C5 in the thymine base to give the allylic $dUCH_2$• and from the C5′-atom to give C5′• of a proximate 3′-AZT (scheme 3) [1,2]. Our lab established that sugar ring configuration influences the pathway and site of the H-atom abstraction reactions for RNH• formed from azido-substituted pentofuranoses [3]. ESR studies have presented evidence of facile and intramolecular 1,5 H-shift in RNH• in lyxofuranoside leading to the formation of C5• (scheme 3) [3,5]. Furthermore, ESR studies of 3′-azido-2′,3′-dideoxyguanosine (3′-AZ-2′,3′-ddG) present evidence of an intramolecular electron transfer process from the guanine base to the radiation-produced 3′-aminyl radical leading to the formation of one-electron oxidized guanine base G(N1-H)• (scheme 3) [2], which is identical to the radical produced via a radiation-mediated direct ionization process

[1,2] as well as via radiation-produced •OH addition to the C4=C5 bond of guanine base followed by water elimination from the adduct radical [1,2]. π-Type RNH• is shown to undergo tautomerization to the σ-type R=N•:



π-Type RNH• σ-type R=N•

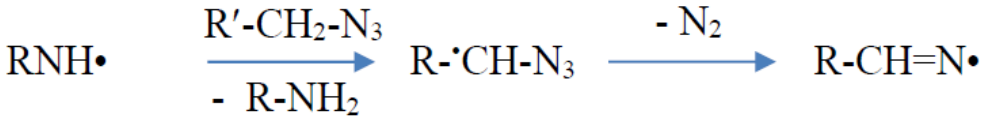
For example, electron spin resonance (ESR) studies have established that π-type aminyl radical (U-(5-C=CH_2)-NH•) formed from 5-(1-azidovinyl) modified 2'-deoxyuridine, facilely tautomerizes to the σ-type iminyl radical, U-(5-C-CH_3)=N• [4]:



U-(5-C=CH_2)-ND• (π) U-(5-C-CH_3)=N• (σ)

Scheme 4. Tautomerization of π-R-NH• to σ-R=N•

In addition, intermolecular H-atom abstraction by RNH• from a proximate compound (e.g., 5-azidomethyl-2′-deoxyuridine) having an α-azidoalkyl moiety leads to the formation of an α-azidoalkyl radical which undergoes a facile conversion to the σ-type R′=N• [4,5]:



Very recently, our lab investigated electron-induced site-specific formation of neutral π-type aminyl radicals (RNH•) and their reactions with pyrimidine nucleoside analogs azido-labeled at various positions in the sugar moiety, e.g., at 2′-, 3′-, 4′-, and 5′- sites along with a model compound 3-azido-1-propanol (3AZPrOH) [5]. These experiments have established that RNH• at a primary carbon site (5′-azido-2′,5′-dideoxyuridine, 3AZPrOH) facilely converted to a σ-type iminyl radical (R=N•) via a bimolecular H-atom abstraction forming an α-azidoalkyl radical. RNH• when at a secondary carbon site (e.g., 2′-azido-2′-deoxyuridine) underwent bimolecular electrophilic addition to the C5=C6 double bond of a proximate pyrimidine base. Finally, RNH• at tertiary alkyl carbon (4′-azidocytidine) underwent little reaction. These results show the influence of stereochemical and electronic environment on RNH• reactivity [5].

Methods

First, Spartan was used to virtually build the molecules that were studied in silico, then, Gaussian was employed for high level computational quantum chemistry calculations. The molecules in question were azido-substituted nucleosides and free radicals generated by electron addition to the azido-substituted molecules. The azidonucleosides were kindly provided by Prof. Wnuk and his group [6]. Following the ongoing work of azidonucleosides in our lab [1-5], Gaussian was used to optimize the structure of the compounds being studied and revealed important properties of compounds such as hyperfine coupling, spin density, and dipole moments. Afterwards, quantum chemical calculations were done using DFT. Then, the substances that were simulated were obtained and studied practically in the lab. The samples were prepared in the lab by mixing 2 to 10 mg of the azide compounds in 1 mL of 7.5 M LiCl in

H_2O or in D_2O and bubbling with nitrogen gas to eliminate oxygen gas from the sample. The sample was then drawn into a 4 mm suprasil quartz tube and frozen in liquid nitrogen to form a glass at 77K. The low temperature of 77 K slowed down potential reactions. Finally, the samples were irradiated, creating Aminyl radicals. After the samples were prepared and irradiated, photoexcitation, thermal annealing, and finally ESR spectroscopy were performed to produce spectra that were interpreted to compare with the theoretical results.

Results and Discussion

ESR spectral and theoretical investigations of one-electron reduced "tetrazole compound" and of "protected 4-azidocytidine":

Following the reactions (1-3) shown earlier in scheme 2, we expect the aminyl radical formation from the "protected 4-azidocytidine". Also, we checked the possibility of electron-mediated aminyl radical formation in the tetrazole compound (see Figure 1 for the structures of these compounds). EPR spectra of the radicals formed from matched γ-irradiated (absorbed dose = 600 Gy at 77 K) homogeneous glassy (or, supercooled homogeneous 7.5 M $LiCl/D_2O$ solutions) samples (concentration = 2-3 mg/mL) of "protected 4-azido-2'-deoxyuridine" and "tetrazole compound" were acquired. ESR spectra were recorded at 77 K just after irradiation and subsequently by gradually warming these samples up to 145 K (Figure 1, Panels A and B). It is evident from the ESR spectra that the center of the spectra, lineshape, and overall hyperfine splitting (i.e., total spectral width) did not change before (i.e., at 77 K) and after warming (i.e., at 145 K) in the dark. The spectra in panels A and B in Figure 1 are matched with a simulated spectrum (Figure 1, Panel B, black) that has been obtained employing a mixed

(Lorentzian/Gaussian=10) lineshape, two axially symmetric anisotropic ^{14}N (nuclear spin = 1) HFCC (hyperfine coupling constant) values of (22.0, 0, 0) G and (20, 0, 0) G, g_{zz}, g_{xx}, g_{yy} (2.0017, 2.0041, 2.0041) along with an isotropic Gaussian line-width of 6 G. Even the 77 K ESR spectrum obtained after subsequent photoexcitation of sample protected 4-azido-2'-deoxyuridine at 145 K for 45 min in the dark remains unchanged. These results establish that the same radical is formed via reaction of radiation-produced prehydrated electron with both compounds in the homogeneous glassy system.

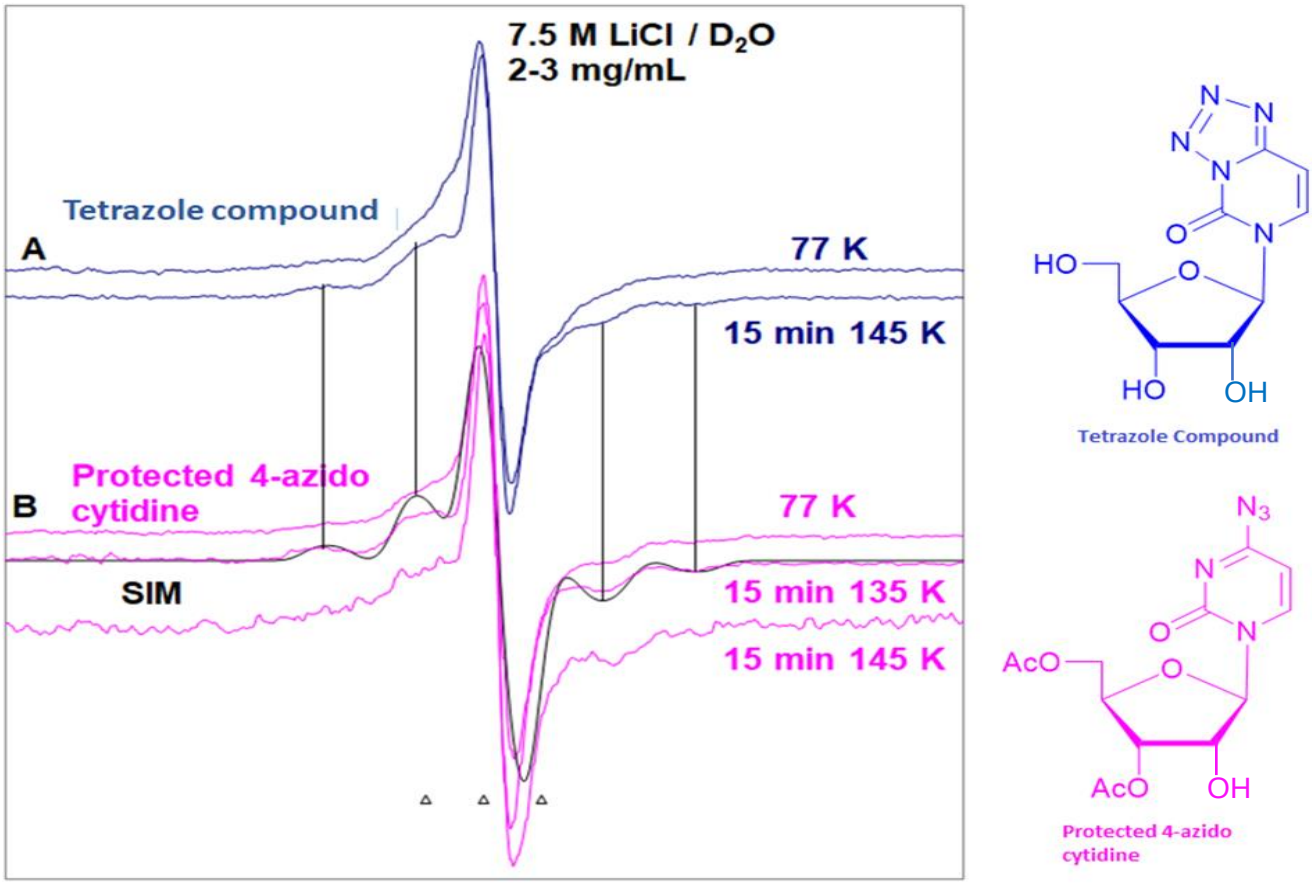
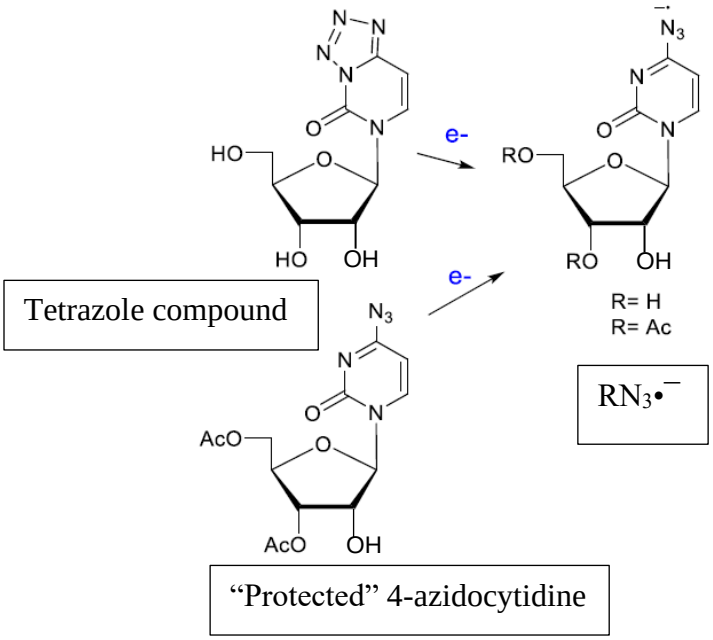


Figure 1. ESR spectra obtained from matched homogeneous glassy samples (2-3 mg/ml in 7.5 M $LiCl/D_2O$ (pH/pD ca. 5)) of tetrazole (blue) and of protected 4-azidocytidine (pink). The first

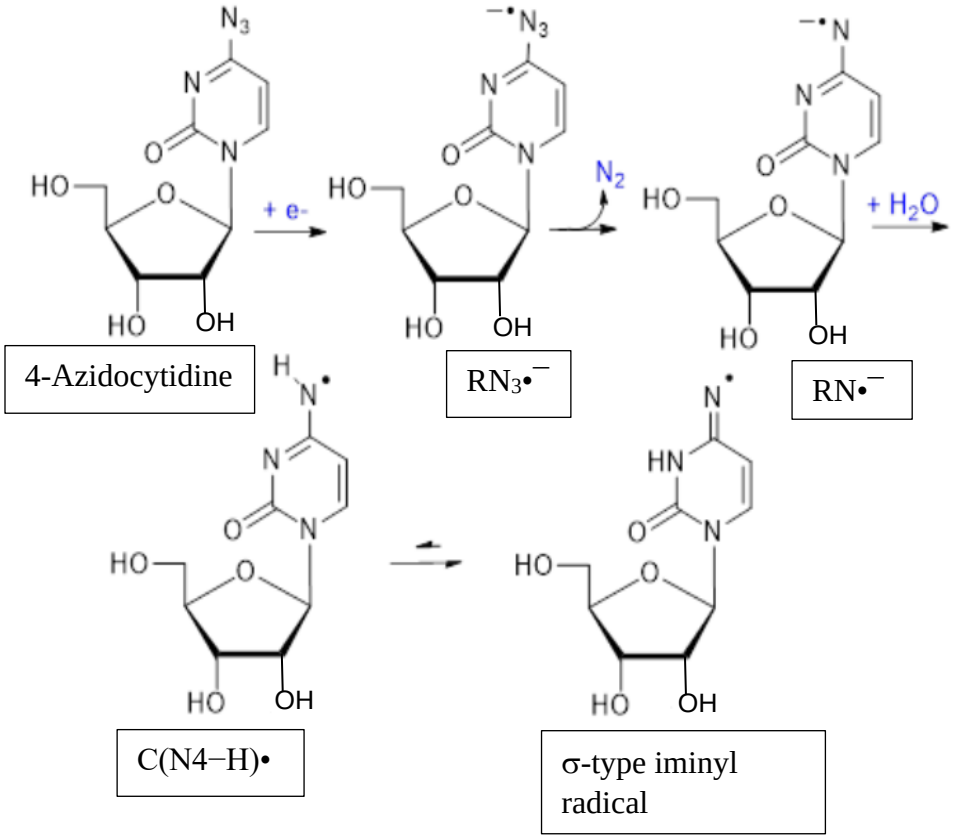
spectrum in panel (A) and in panel (B) shows $RN_3^{\bullet -}$ formation via radiation-produced (absorbed dose = 600 Gy) prehydrated electron attachment at 77 K in the dark. Other Spectra in panel (A) and in panel (B) were obtained after warming or annealing the samples for 15 min in the dark at 135 K and at 145 K. The simulated spectrum (black) is shown in panel (B) and for simulation parameters, consult text. Similarities of the $\boldsymbol{A_{zz}}$ HFCC in the spectra of both compounds were shown by vertical solid lines. All spectra were recorded at 77 K.

These spectra are different from the reported spectrum of π-type neutral aminyl radical (C(N4−H)•) from 1-methylcytosine [7]. Thus, we assign these spectra to the azide anion radical ($RN_3^{\bullet -}$); these ESR spectral results further show that (a) in tetrazole, $RN_3^{\bullet -}$formation occurs via dissociative electron attachment—the radiation-produced electron attaches to the tetrazole ring at 77 K and this is followed by breakage of the cytosine ring N3-azidoN bond in the anion radical also at 77 K (Scheme 4).



Scheme 4. Electron-mediated $RN_3^{\bullet -}$ formation from the tetrazole compound and "protected 4-azidocytidine".

(b) under the experimental conditions of ESR spectroscopic investigations (homogeneous glassy solutions at low temperatures (77 K to 145 K)) $RN_3^{\bullet -}$ from these compounds do not get converted to the expected neutral aminyl radical, C(N4−H)• (scheme 5), in the ground state as expected from earlier studies with azido-DNA-models [1-5].



Scheme 5. Plausible formation of σ-type iminyl radical via tautomerism of C(N4−H)• under reductive environment from "4-azidocytidine". Note that formation of σ-type iminyl radical via tautomerism of C(N4−H)•, when C(N4−H)• production happened via deprotonation of $C^{\bullet+}$ (cytosine cation radical), has been reported in ref. 7.

Since these radicals are base radicals and not sugar radicals, theoretical calculations were performed by employing 4-azido-1-methyl cytosine to check the influence of N3-proton on the hyperfine coupling constants. These theoretical calculations (Figures 2 and 3) propose a particular conformation of the azide anion radical ($RN_3^{\bullet-}$) which give rise to two high

anisotropic N-couplings ($\boldsymbol{A_{zz}}$ = ca. 27 G and 34 G respectively, $\boldsymbol{A_{xx}} = \boldsymbol{A_{yy}} = 0$) compared to the experimentally observed $\boldsymbol{A_{zz}}$ values of aniosotropic N-couplings ($\boldsymbol{A_{zz}}$ = 22 G, and 20 G, $\boldsymbol{A_{xx}} = \boldsymbol{A_{yy}} = 0$, vide supra) for the "protected" 4-azidocytidine. Similar results were obtained for the "tetrazole compound". These outcomes strongly supported the results shown in Figure 1 and in scheme 4 and helped in the radical assignments.

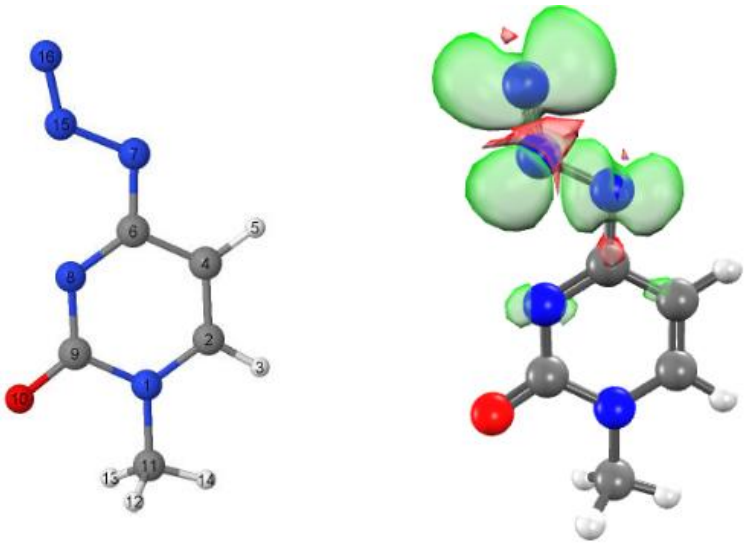


Figure 2. The azide anion radical ($RN_3\bullet^-$) in N3-deprotonated 4-azido-1-methylcytosine was geometry-optimized employing DFT/B3LYP/6-31G** method. Spin density distributions and hyperfine coupling constants were calculated employing the geometry-optimized structure of the $RN_3\bullet^-$using DFT/B3LYP/6-31G** method, taking into account full solvation (IEF-PCM) under Gaussian'09 suit of program.

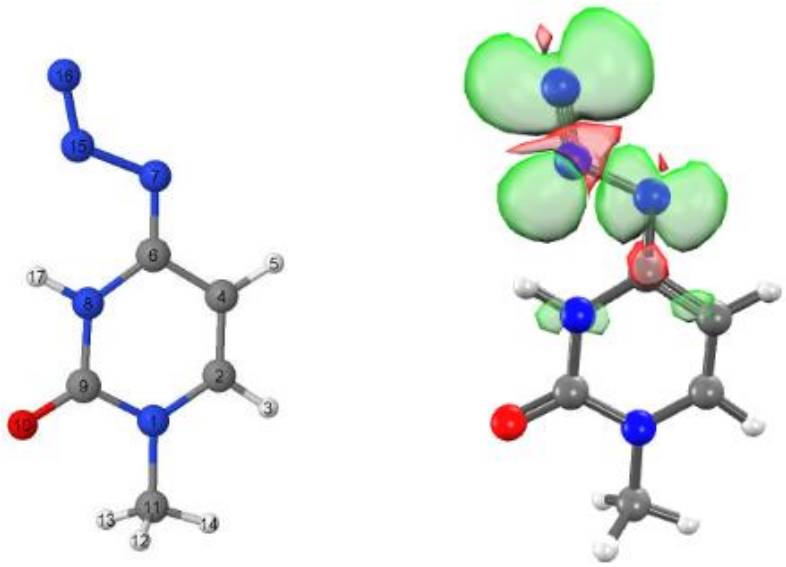


Figure 3. The azide anion radical ($RN_3^{\bullet -}$) in N3-protonated 1-methylcytosine was geometry-optimized employing DFT/B3LYP/6-31G** method. Spin density distributions and hyperfine coupling constants were calculated employing the geometry-optimized structure of the azide anion radical using DFT/B3LYP/6-31G** method taking into account of full solvation (IEF-PCM) under Gaussian'09 suit of program.

ESR spectral studies of one-electron addition to "5-azidouridine":

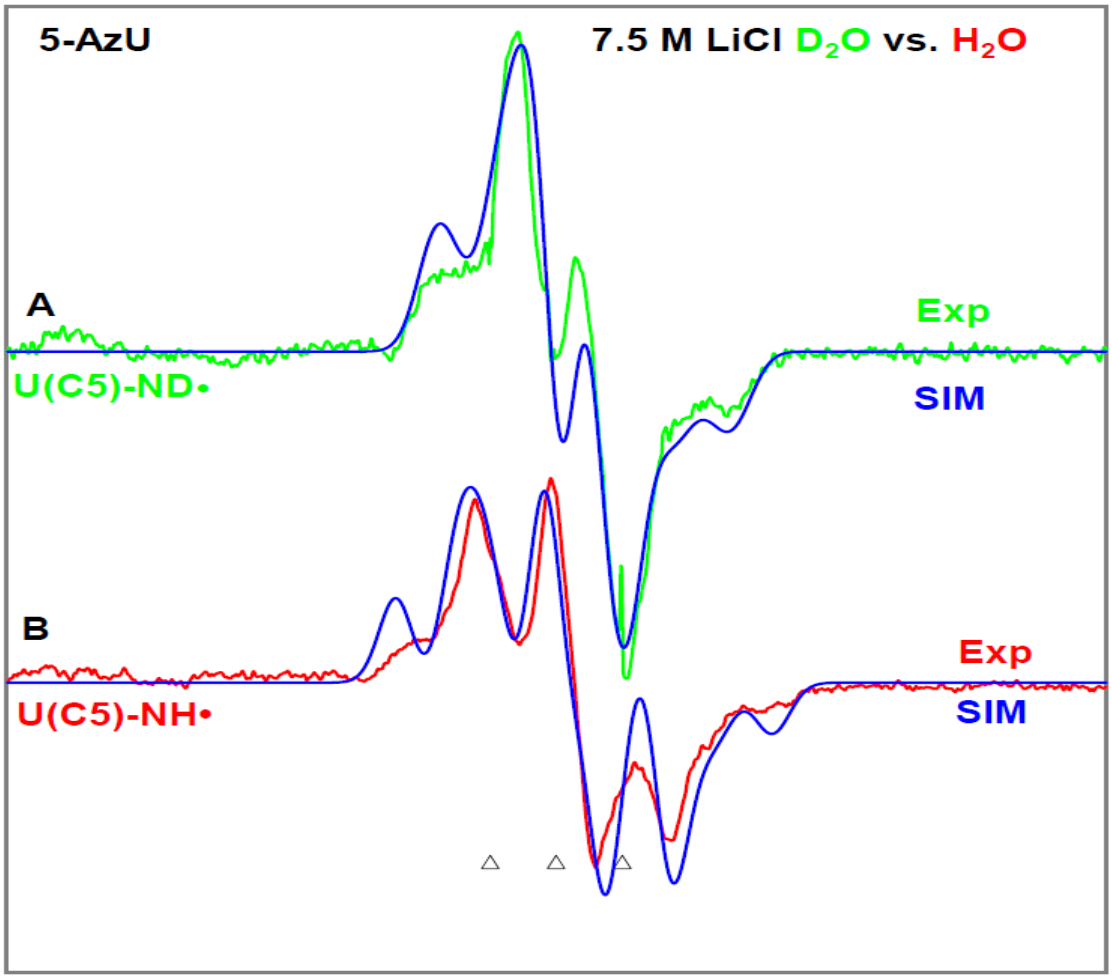


Figure 4. 77 K ESR spectrum of the aminyl radical (U(C5)-ND•/(U(C5)-NH•)) formed via radiation-produced one-electron attachment by γ-irradiation (absorbed dose = 600 Gray at 77 K) of 5-AzU (A) (U(C5)-ND•) (green color) in 7.5 M LiCl (D_2O), (B) (U(C5)-NH•) (red color) in 7.5 M LiCl (H_2O). The simulated spectra (blue) (for simulation parameters, see text below) are superimposed on the top of the experimentally recorded spectrum.

Comparison of the experimentally recorded spectra with those obtained via simulation clearly show that the $\boldsymbol{A_{zz}}$ component ca. 17 G due to the a-N-H proton of the aminyl radical in H_2O (U(C5)-NH•) (red color) is lost in the D_2O glasses (green color). All spectra were recorded at 77 K and the line components due to $Cl_2^{\bullet-}$ are subtracted from these recorded spectra. Following the identification of the aminyl radical (T(C3')-NH•) in 3′-AZT (schemes 1 and 3), we then ran samples from commercially available 5-azidouridine (5-AzU) to check whether formation of azide anion radical would occur in D_2O and in H_2O upon radiation-produced electron addition to 5-AzU. The ESR spectral results are presented in Figure 4. In Figure 4A, following the identification of the aminyl radical (T(C3')-ND•/T(C3')-NH•) in 3′-AZT [2], the ESR spectrum (green color) of the aminyl radical (U(C5)-ND•) from 5-AzU in D_2O (7.5 M LiCl), recorded at 77 K and then by subtraction of the line components due to $Cl_2^{\bullet-}$, is shown. $Cl_2^{\bullet-}$ is produced by scavenging of radiation produced holes in the matrix (7.5 M LiCl)). In Figure 4B, ESR spectrum of the aminyl radical (U(C5)-NH•) (red color) is obtained under identical conditions (γ-irradiated at 77 K in the dark, recorded at 77 K, the line components due to $Cl_2^{\bullet-}$ are subtracted from these recorded spectra) from 5-AzU in H_2O (7.5 M LiCl). Comparison of widths of these two spectra clearly shows that an additional 17 G proton hyperfine coupling in the aminyl radical in H_2O (U(C5)-NH•) (red color) is present in the H_2O glasses and is missing in the D_2O glasses (green color). These results show that the source of the proton in the formation of the aminyl radical (U(C5)-NH•) via protonation of the nitrene anion radical (U(C5)-N•$^-$)), is the solvent (H_2O). This additional hyperfine coupling of 17 G is the $\boldsymbol{A_{zz}}$ component of the N-H (α-H) coupling that contributes to the outer line components and this component adds to the $\boldsymbol{A_{zz}}$ component of the anisotropic hyperfine coupling of the N-atom of

U(C5)-NH•. Thus, from the experimentally recorded spectrum (red) of U(C5)-NH• (Figure 4B), only the $\boldsymbol{A_{zz}}$ component of the N–H (α-H) hyperfine coupling could be determined by experiment. The spectrum of U(C5)-ND• in Figure 4A and that of the U(C5)-NH• in Figure 2B have been simulated (blue color) by employing the following parameters. For U(C5)-ND•, the simulation parameters are: C6-H HFCC (14.5, 6.0, 11.0) G, radical site anisotropic axially symmetric N HFCC (0, 0, 23 G) G, N-D (for the exchangeable α-H = 4.0, 1.0, 2.5) G, $\boldsymbol{g_{xx}}$, $\boldsymbol{g_{yy}}$, $\boldsymbol{g_{zz}}$ =(2.0043, 2.0043, 2.0020), a mixed (Lorentzian/Gaussian=1.0) lineshape, and an isotropic linewidth of 7 G. For U(C5)-NH•, the simulation parameters are: C6-H HFCC (17.0, 6.0, 13.6) G, radical site anisotropic axially symmetric N HFCC (0, 0, 20 G) G, N-H (for the exchangeable α-H = 25.0, 6.0, 17.0) G, $\boldsymbol{g_{xx}}$, $\boldsymbol{g_{yy}}$, $\boldsymbol{g_{zz}}$ =(2.0043, 2.0043, 2.0020), a mixed (Lorentzian/Gaussian=1.0) lineshape, and an isotropic linewidth of 9 G. The simulated spectra match quite well with that of the experimentally observed spectra.

ESR spectral investigations of 2-azido-2'-deoxyinosine (2-Az-dGuo):

After finding the formation of azide anion radical in the protected 4-azidocytidine and in the tetrazole compound, we tested the guanyl aminyl radical (G(N2-H)•) formation in 2-azido-2'-deoxyinosine (2-Az-dGuo) [8-13]. The ESR spectrum obtained from 2-Az-dGuo sample is compared with the ESR spectrum found from a matched sample of the tetrazole compound and also to the authentic spectrum of G(N2-H)• that was obtained via one-electron oxidation of 1-methyl-2′-deoxyguanosine (1-methyl-dGuo) and in DNA-oligomers [8-13]. These results are presented in Figure 5.

The spectra in panel A of Figure 5 show that the 77 K ESR spectrum obtained from radiation-produced electron addition at 77 K to 2-Az-dGuo followed by annealing to 135 K for 15 min is very similar to the corresponding 77 K spectrum found from radiation-produced electron addition at 77 K and subsequently annealed to 145 K for 15 min from a matched sample of the tetrazole compound. This result concludes that addition of radiation-produced electron to 2-Az-dGuo and to the tetrazole compound (for its structure, see Figure 1) led to $RN_3^{\bullet -}$ formation and HFCC values of the two axially symmetric anisotropic N of $RN_3^{\bullet -}$ from 2-Az-dGuo are very close to those for $RN_3^{\bullet -}$ from the tetrazole compound.

Comparison of the authentic spectrum of G(N2-H)• that was obtained via one-electron oxidation of 1-methyl-dGuo (black spectrum in panel B, Figure 5) with the ESR spectrum obtained from radiation-produced electron addition to 2-Az-dGuo (panel A) clearly shows that the two spectra are not identical in terms of lineshape, overall hyperfine splitting, and the center of the spectra. Thus, electron addition to 2-Az-dGuo does not lead to the formation of G(N2-H)• unlike the reaction of radiation-produced electron with azides that are directly linked to the sugar moiety or [1,2,3,5] or with azides at C5 of the pyrimidine ring [4] including 5-AzU (Figure 4).

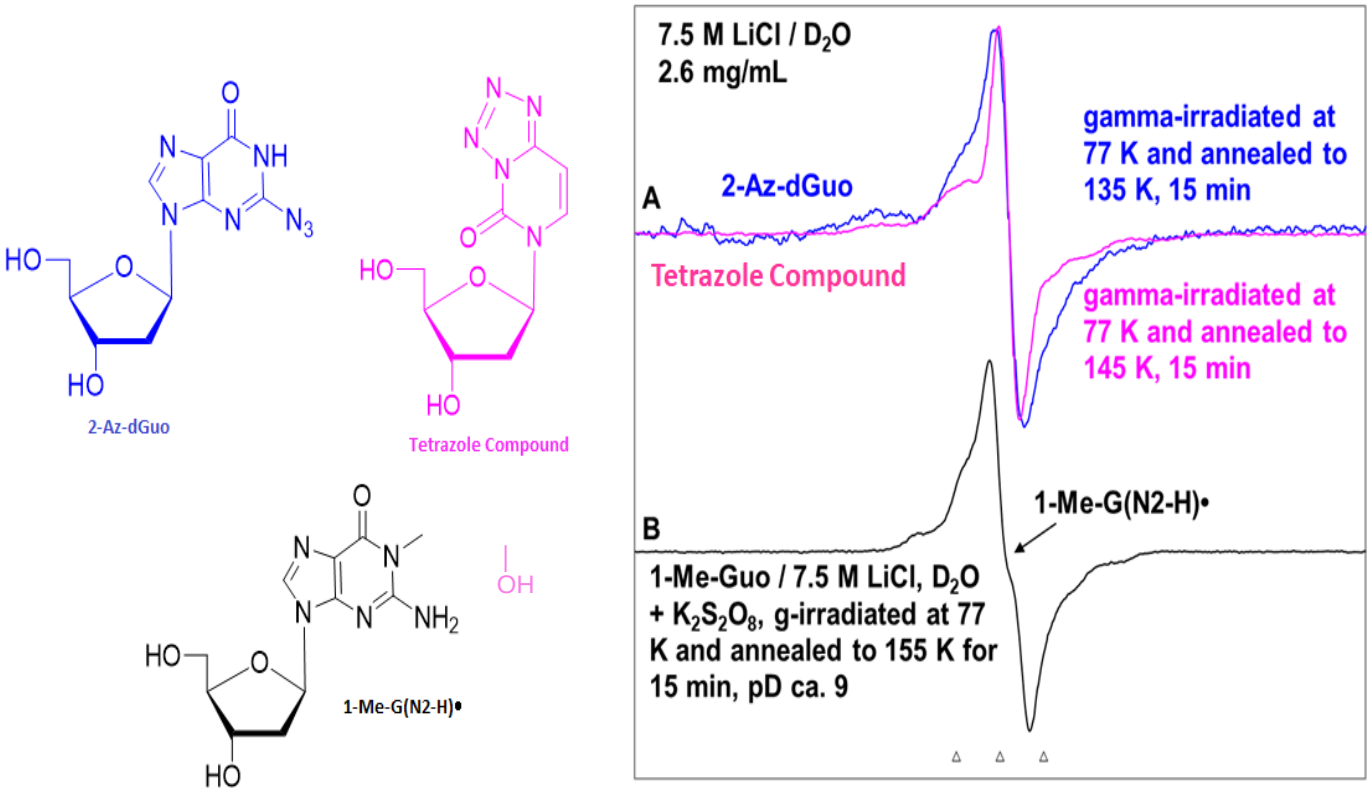


Figure 5. Comparison of EPR spectra acquired for 2-azido-2'-deoxyinosine and 4-tetrazolo-2'-deoxyuridine. Panel (A) ESR spectrum obtained from radiation-produced (absorbed dose of γ-radiation = 600 Gy in the dark) electron addition at 77 K to matched samples of 2-Az-dGuo (2-3 mg/mL, blue) followed by annealing to 145 K for 15 min in the dark and of tetrazole compound (2-3 mg/mL, pink) followed by annealing to 135 K for 15 min in the dark. The line components due to $Cl_2^{\bullet-}$ are subtracted from these recorded spectra. Panel (B) authentic ESR spectrum of the G(N2-H)• from 1-methyl-dGuo (1-Me- G(N2-H)•) (γ-irradiation absorbed dose at 77 K = 2.5 kGy) via one-electron oxidation by $Cl_2^{\bullet-}$ due to annealing at ca. 155 K for 15 min in the dark at pD ca. 9 in homogeneous glass (7.5 M LiCl in D_2O) in the presence of the electron scavenger $K_2S_2O_8$ (6-8 mg/mL) [9].

Conclusions

In 5-AzU, the azido group is at meta position with respect to the N1 ring nitrogen; on the other hand, in the tetrazole as well as in 4-azidocytidine, the azido group is at para position with respect to the N1 ring nitrogen (compare structures shown in Figures 1 and 2). Based on these ESR studies and theoretical results of HFCC and spin density distribution calculations, the azido group at C4 in the pyrimidine base makes the azide anion radical more delocalized and hence more stable in comparison to other azido-modified nucleosides previously studied [1-5] and to the azide anion radical in 5AzU.

In 2-Az-dGuo, the azido moiety is at the ortho position with respect to either N1 or N3 of the guanine ring (see structures shown in Figure 5). Therefore, formation of the azide anion radical as found in the tetrazole compound, in 4-azidocytidine, and is expected from 2-Az-dGuo samples rather than the neutral aminyl radical.

Therefore, our results propose that 5AzU could be an effective radiosensitizer. On the other hand, 8-azidopurine forms a very stable radical that does not progress to the next step of the reaction, making it promising for use in click chemistry, which is used to label tumors [17]. Thus, we can apply a combination of 5AzU and 8-azidopurine and this combination can serve a dual purpose of labeling tumor cells prior to, during, or after radiotherapy and may radio-sensitize the tumor during radiotherapy. We will test this hypothesis in tumor models.

Citations

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