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Activation of 3-Amino-1,2,4-benzotriazine 1,4-Dioxide Antitumor Agents to Oxidizing Species Following Their One-Electron Reduction

Robert F. Anderson,*,†,‡ Sujata S. Shinde,† Michael P. Hay,‡ Swarna A. Gamage,‡ and William A. Denny‡

Contribution from the Departments of Chemistry and Auckland Cancer Society Research Centre, The University of Auckland, Private Bag 92019, Auckland 1, New Zealand

Received July 8, 2002; E-mail: r.anderson@auckland.ac.nz.

Abstract: The mechanism by which a benzotriazine 1,4-dioxide class of anticancer drugs produce oxidizing radicals following their one-electron reduction has been investigated using tirapazamine (3-amino-1,2,4-benzotriazine 1,4-dioxide, **1**) and its 6-methoxy (**6**), 7-dimethylamino (**7**), and 8-methyl (**8**) analogues. By measuring the changes in absorption with pH, we found that the radical anions undergo protonation with radical pK_r values of 6.19 ± 0.05, 6.10 ± 0.03, 6.45 ± 0.04, and 6.60 ± 0.04, respectively. The one-electron reduced species underwent a first-order reaction, with increased rate constants from 112 ± 23 s^{-1} for **1** to 777 ± 12 s^{-1}(**6**), 1120 ± 29 s^{-1} (**7**), and 825 ± 89 s^{-1} (**8**) at pH 7. No overall change in conductance was observed following the one-electron reduction of **6**, and **8** at pH 4.5, consistent with the protonation of the radical anions, but a loss in conductance was seen for one-electron reduced **7** because of further protonation of the initially formed radical. This is assigned to the protonation of the dimethylamino group of the radical species, which has a pK_a of 8.8 ± 0.3. All conductance changes take place on a time-scale shorter than those of the above first-order reactions, which are not associated with the formation or loss of charged species. The absorption spectra present at the end of the unimolecular reactions were found to be similar to those formed immediately upon the one-electron oxidation of the respective substituted 3-amino-1,2,4-benzotriazine 1-oxides, and it is suggested that common benzotriazinyl radicals are formed by both routes. All these intermediate radicals underwent dismutation to produce final spectra matched by equal contributions of the parent compound and their respective substituted 3-amino-1,2,4-benzotriazine 1-oxides. By establishing redox equilibria between the intermediate radicals formed on the one-electron oxidation of the respective 3-amino-1,2,4-benzotriazine 1-oxides of the compounds and reference compounds, we found the one-electron reduction potential of the oxidizing radicals to range from 0.94 to 1.31 V. The benzotriazinyl radical of tirapazamine was found to oxidize dGMP and 2-deoxyribose with rate constants of (1.4 ± 0.2) × 10^8 M^{-1} s^{-1} and (3.7 ± 0.5) × 10^6 M^{-1} s^{-1}, respectively.

Introduction

The presence of hypoxic cells in certain tumors[1,2] has been linked with resistance of such tumors to radiotherapy treatment.[3] An approach to overcome this problem is the development of bioreductive drugs, which specifically kill hypoxic cells in tumors. Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-dioxide, TPZ, **1**) is the leading such drug[4] currently undergoing Phase III clinical trials, in combination with cisplatin, having shown promise in Phase II trials for the treatment of several cancers.[5−7] TPZ is reduced by one-electron reductases[8−12] to form a radical anion (**2**) which, in aerobic cells, is back oxidized by molecular oxygen[13] to generate the superoxide radical that mediates aerobic

† Department of Chemistry, The University of Auckland.
‡ Auckland Cancer Society Research Centre, The University of Auckland.
* Corresponding author, E-mail: r.anderson@auckland.ac.nz.

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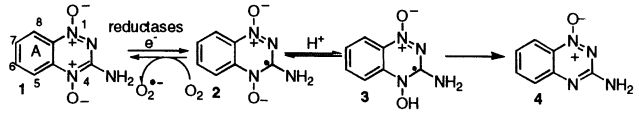
Scheme 1. Formation and Reoxidation of Radical Anion



toxicity. It is inferred that the one-electron reduced species, if not back oxidized, is cytotoxic to hypoxic cells as the two-electron reduced species, 3-amino-1,2,4-benzotriazine 1-oxide (**4**), is nontoxic.[14] Thus the one-electron reduced species is believed to be the key radical intermediate in the cytotoxic action of TPZ.

The cytotoxicity of TPZ increases with decreasing pH,[15] pointing to the protonated radical (**3**) (radical pK_a = 6.1[13]) as the cytotoxin, or as an obligate intermediate in forming a cytotoxin, Scheme 1.

However, the protonated radical, **3**, which may well be in the form of a nitroxide with the unpaired spin centered on the N-1 position,[16] is a reducing radical being readily oxidized by molecular oxygen.[13,16] It is difficult to understand how such a radical could also oxidize DNA and cause strand breaks, as has been suggested.[13,16,17] Another possibility, arising from work with radical scavengers,[18] is that the •OH radical is eliminated from **3**, although spin-trap EPR experiments are inconclusive.[19] In this paper, we examine the decay kinetics of **2**/**3** for tirapazamine and three analogues containing electron-donating substituents, (6-methoxy-,**6**, 7-(dimethylamino)-,**7**, and 8-methyl-, **8**), by varying the radiation dose (radical concentration) and using conductivity detection to detect if charged species are produced or lost in the process.

Results

Decay Kinetics of One-Electron Reduced 1, 6, 7, and 8. Pulse radiolysis experiments were conducted to rapidly reduce the compounds (50 μM) by the e^-_{aq} in N_2-saturated solutions containing 2-methylpropan-2-ol (0.1 M) and to observe the decay kinetics of the radicals. An earlier study[13] determined the second-order decay rates, k_2, of **2** (**2** + **3**) and reported another minor, but undetermined, decay route. We have examined the decay kinetics in detail with increasing radiation dose by determining the half-lives, $t_{1/2}$, of decay of the radicals at pH 7. For the competitive decay of a species of initial concentration C by both first-order (k_1) and a second-order (k_2) processes, with the first half-life $t_{1/2}$, it can be shown that $\exp(k_1 t_{1/2}) = (k_1 + k_2[C])/(2k_1 + k_2[C])$. When $[C] = 0$, that is, the intercept of a plot of $1/t_{1/2}$ versus $[C]$, $k_1 = \ln 0.5 \times 1/t_{1/2}$. We have measured $1/t_{1/2}$ values for a series of radical concentrations of **1** by monitoring the decay at 560 nm[13] for up to 20 Gy dose, Figure 1, from which a value for k_1 of 112 ± 23 s^{-1} is derived. This first-order reaction predominates at low radical concentration, which is the case in enzymatic reactions. To study this reaction under pulse radiolysis conditions (high initial radical concentration which favors bimolecular reaction), it is necessary to increase k_1 relative to k_2. We reasoned that derivatives of **1** containing electron-donating substituents would be appropriate and first determined the radical pK_a values of (**6**) 6.10 ± 0.03, (**7**) 6.45 ± 0.04, and (**8**) 6.60 ± 0.04 by plotting the change in absorbance at different pH values, Figure 2. On carrying out a similar kinetic study at 560 nm to that above, Figure 1, we found a substantial increase in k_1 values to 777 ± 12 s^{-1} (**6**), 1120 ± 29 s^{-1} (**7**), and 825 ± 89 s^{-1} (**8**), respectively.

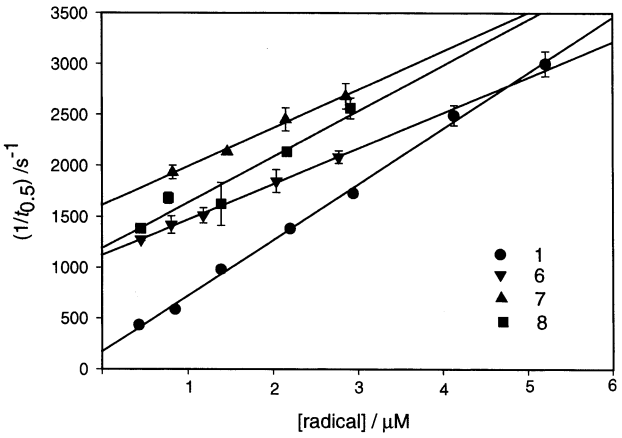


Figure 1. Dependence of the reciprocal of the first half-life of radicals on the initial radical concentration formed with increasing radiation dose. Radicals produced on scavenging the e^-_{aq} in N_2-saturated solutions containing 2-methylpropan-2-ol (0.2 M) at pH 7.0 (5 mM phosphate) and 150 μM concentrations of **1** (●), **6** (▼), **7** (▲), and **8** (■). Observations were made in triplicate at 560, 380, 360, and 440 nm, respectively.

Conductivity Studies. The possibility of charged species being formed or lost was investigated using conductivity detection. Control experiments, for comparative purposes, were carried out using the well-characterized system of dimethyl sulfoxide (DMSO, 1 mM) in N_2O-saturated solutions (to convert e^-_{aq} into •OH radicals) at both pH 4.5 and 10.0, Figure 3. The conductance changes (in μS) arise from the reaction of •OH radicals with DMSO producing sulfinic acid (pK_a 2.35).[20] The $H^+/CH_3SO_2^-$ ion pair gives rise to a conductance increase in acid solution (trace A) and a loss of conductance in basic solution through neutralization (trace B).

$$^{\bullet}OH + (CH_3)_2SO \rightarrow (CH_3)_2SO(OH)^{\bullet} \rightarrow {}^{\bullet}CH_3 + CH_3SO_2H \quad (1)$$

$$CH_3SO_2H + H_2O \rightleftharpoons CH_3SO_2^- + H_3O^+ \quad (2)$$

$$H_3O^+ + {}^-OH \rightarrow 2H_2O \quad (3)$$

Experiments with compounds **1**, **6**, **7**, and **8** were carried out in unbuffered N_2-saturated solutions at pH 4.5 containing the •OH radical scavenger, 2-methylpropan-2-ol (which forms an inert, nonconducting radical). An initial increase in conductance was seen following the electron pulse because of the formation of the radical anions on the scavenging of e^-_{aq} by the compounds and the proton produced by the pulse, followed by rapid protonation of the radical anions. While one-electron reduction did not result in a significant loss in the conductance of **1** (data on the long time scale not shown), **6** (trace D), and **8** (trace E), a loss in conductance was observed for **7** (trace C).

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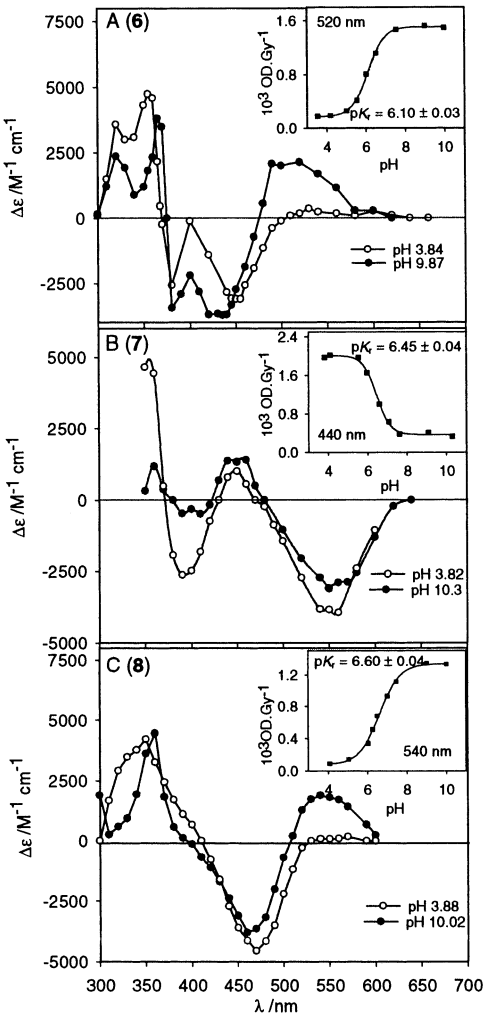

Figure 2. Absorption spectra of radicals, relative to parent compounds, following the one-electron reduction of (A) **6**, (B) **7**, and (C) **8** (100 μM) at high and low pH in N_2O-saturated solutions containing sodium formate (0.15 M). Inserts are the changes in absorbance with pH at given wavelengths from which the pK_a values of the radicals are determined.

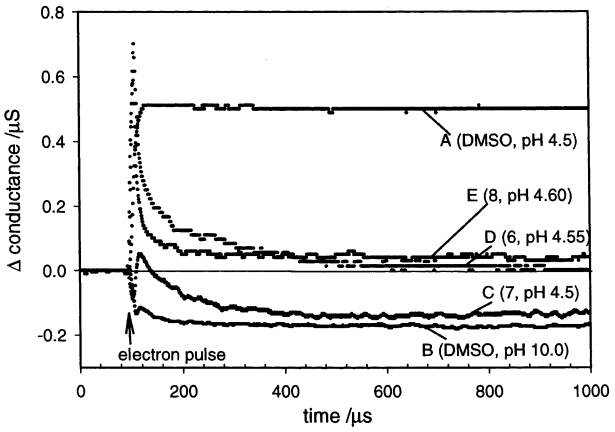

Figure 3. Changes in conductance following pulse radiolysis (2.5 Gy) of N_2-saturated solutions of the compounds (200 μM) and 2-methylpropan-2-ol (0.2 M), relative to that seen for dimethyl sulfoxide (1 mM) in N_2O-saturated solutions.

This loss in conductance is similar to the measured loss in conductance observed on the oxidation of DMSO in basic solution previously. The calculated change in conductivity in an N_2O-saturated basic solution of DMSO, due to the removal of OH^- by the produced sulfinic acid, is

$$\sum c_i|z_i|\Lambda_i = G_{e-aq}(\Lambda OH^- + \Lambda CH_3SO_2^{\bullet -} - \Lambda OH^-) + G_{\bullet OH}(\Lambda CH_3SO_2^{\bullet -} - \Lambda OH^-) + G_{H_3O^+}(-\Lambda OH^-) = -86\,\mu S\ cm^2\ M\ Gy^{-1}$$

The magnitude of this loss in conductance is equal to the loss of a H_3O^+ from solution and is consistent with an additional protonation of the radical of **7**, as

$$\sum c_i|z_i|\Lambda_i = G_{e-aq}(\Lambda R^{\bullet +} - \Lambda H_3O^+) = -83\,\mu S\ cm^2\ M\ Gy^{-1}$$

The above calculations are based on values of Λ for H_3O^+, OH^-, $CH_3SO_2^-$ and a singly charged benzotriazine 1,4-dioxide molecule being 331, 188, 35, and 35 S cm^2 mol^{-1}, respectively, at 21 °C.[21]

Production of Radical Spectra. A. One-Electron Oxidation of 3-Amino-7-dimethylamino-1,2,4-benzotriazine 1-Oxide, 10. The selenite radical ($SeO_3^{\bullet -}$) was found suitable for these experiments, as a rapid thermal reaction occurred between **10** and peroxodisulfate, preventing the sulfate radical ($SO_4^{\bullet -}$) being used. The $SeO_3^{\bullet -}$ radical reacted with **10** (0.2 mM) at pH 6 with a second-order rate constant of (2.0 ± 0.2) × 10^9 M^{-1} s^{-1}, resulting in an immediate spectrum having the known characteristic features of an aromatic dimethylamine, namely a narrow band of high absorbance near 350 nm and a broad band, of less absorbance, centered near 550 nm,[22−24] Figure 4A. The spectrum produced at pH 9.6 has a slightly different absorption characteristic above 500 nm from which a pK_a of 8.8 ± 0.3 was found from a plot of the absorption changes versus pH at 540 nm, Figure 4B insert. The species produced at pH 6.0 underwent a second-order reaction to form products whose final spectrum is matched by equal amounts of **10** and its benzotriazine 1,4-dioxide, **7**, Figure 4C.

B. One-Electron Reduction of 3-Amino-7-(dimethylamino)-1,2,4-benzotriazine 1,4-Dioxide, 7. The one-electron reduction spectrum of compound **7** (0.2 mM) at pH 6.0 was produced by electron transfer from the $CO_2^{\bullet -}$ species (k = 2.0 × 10^9 M^{-1} s^{-1}) in N_2O-saturated solutions containing formate ions (0.1 M). The initial radical spectrum, composed of a mixture of the radical anion and its protonated form **9**, exhibits a sharp absorbance band near 350 nm and a low absorbance tail (∼2000 M^{-1} cm^{-1}) to 650 nm, Figure 5A. This initial radical spectrum underwent a rapid transformation with first-order kinetics (k = (1.34 ± 0.3) × 10^3 s^{-1}) to form a new spectrum which has a higher absorbance than that of the initial spectrum at 350 nm and a broad band centered near 550 nm, Figure 5B. The features of the new transient spectrum match those of Figure 4B and provides strong support that the same radical species is formed, which is suggested to be the benzotriazinyl radical, **11**, Scheme 2. This transient species decayed to give a final spectrum which matches a composite spectrum of equal concentrations of **7** and **10**, Figure 5C.

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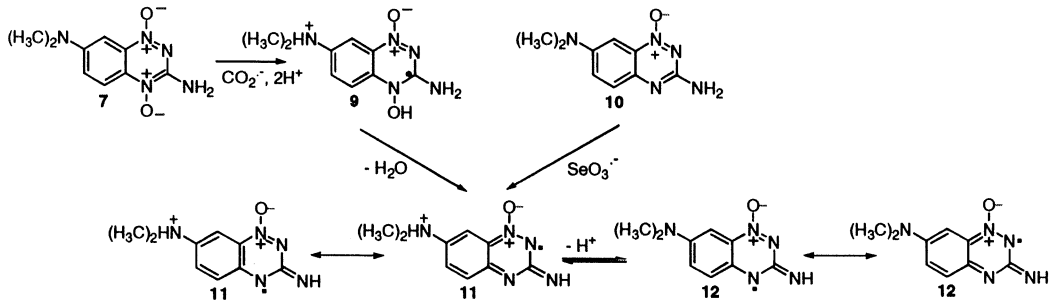
Scheme 2. Formation of Radical **11** upon One-Electron Reduction of **7** and Oxidation of **10**



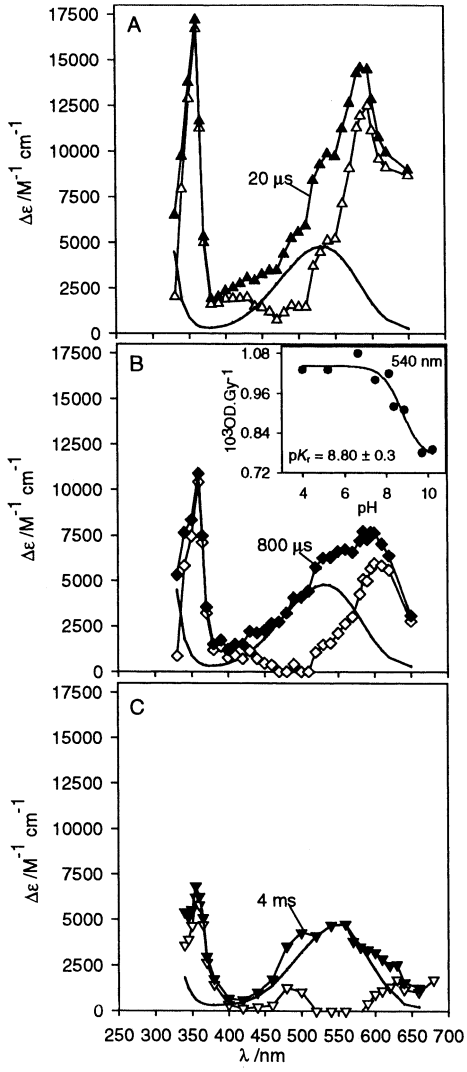


Figure 4. Time-resolved spectra following reaction of the selenite radical with **10**. The N_2-saturated solution contained **10** (100 μM), sodium selenate (50 mM), and 2-methylpropan-2-ol (0.2 M) at pH 6.0 (5 mM phosphate). Solid symbols are time points corrected for the absorption of compound **10** (solid line). Insert panel B displays the changes in absorption with pH of the initial radical formed.

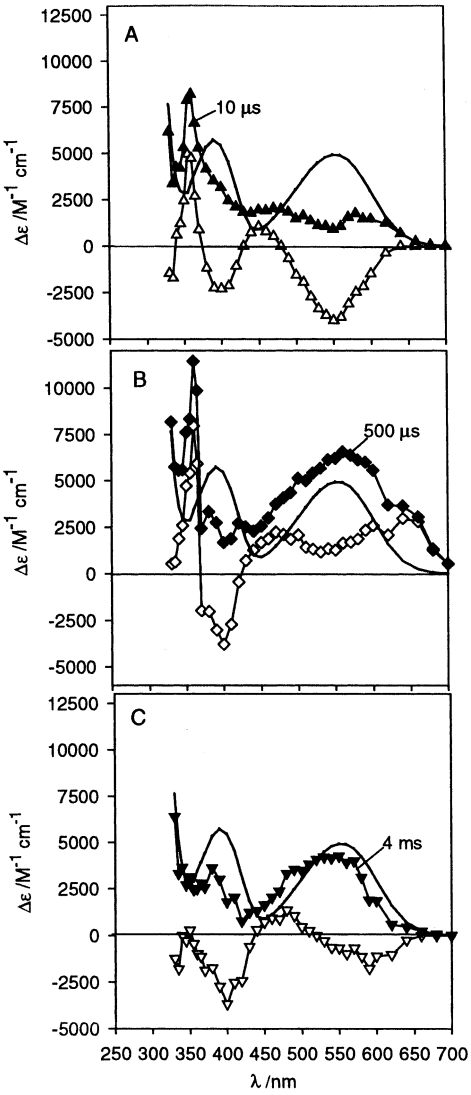
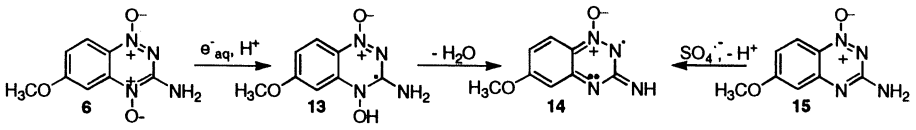


Figure 5. Time-resolved spectra following reaction of the $CO_2^{\bullet-}$ species with **7**. The N_2O-saturated solutions contained **7** (100 μM) and sodium formate (0.15 M) at pH 6.0 (5 mM phosphate). Solid symbols are time points corrected for the absorption of compound **7** (solid line).

C. One-Electron Reduction of 3-Amino-6-methoxy-1,2,4-benzotriazine 1,4-Dioxide, 6. The one-electron reduction of **6** (0.2 mM) by the $CO_2^{\bullet-}$ species was carried out at pH 5.0 to ensure formation of the protonated species **13** and to follow its decay. The initial radical spectrum, which has a peak near 350 nm with $\epsilon = 12\,000\ M^{-1}\ cm^{-1}$, Figure 6A, decayed with first-order kinetics to produce an intermediate spectrum, Figure 6B, which at 1 ms is composed of a small proportion of the initial spectrum and a new spectrum which exhibits a shoulder near 400 nm and a decreased peak at 350 nm. This composite spectrum decayed to a final spectrum which matches a spectrum of equal concentrations of **6** and 3-amino-6-methoxy-1,2,4-benzotriazine 1-oxide, **15**, Figure 6C. These observations, together with the conductivity data, indicate that neither a hydroxide nor $^{\bullet}OH$ radical is eliminated from the transient radical species, as there is no loss in conductance at pH 4.5 or quantitative formation of **15** by the first-order reaction.

Scheme 3. Radical Intermediates Formed on the One-Electron Oxidation of **15** and Intermediate Produced Following One-Electron Reduction of **6**



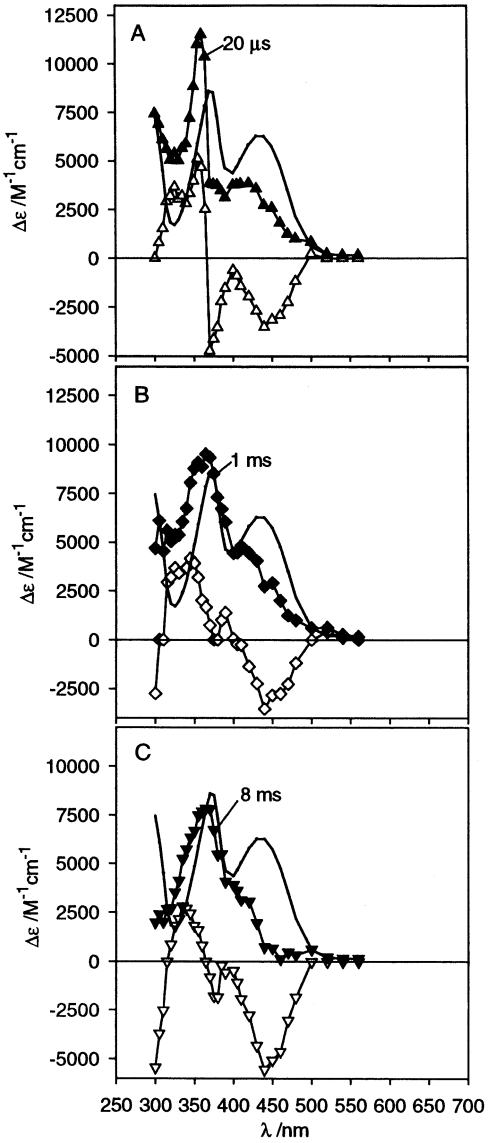


Figure 6. Time-resolved spectra following reaction of the $CO_2^{\bullet-}$ species with **6**. The N_2O-saturated solutions contained **6** (100 μM) and sodium formate (0.15 M) at pH 5.0 (5 mM phosphate). Solid symbols are time points corrected for the absorption of compound **6** (solid line).

D. One-Electron Oxidation of 3-Amino-6-methoxy-1,2,4-benzotriazine 1-Oxide, 15. The one-electron oxidation of **15** (0.2 mM) at pH 5.0 was carried out by reaction with the $SO_4^{\bullet-}$ radical. The initial radical spectrum, Figure 7, exhibits a small loss in absorption compared to that of the parent compound in the 350−400 nm region with an absorption tail above 500 nm. The radical spectrum decayed away to form a similar spectrum to that finally seen, following the one-electron reduction of **6** previously. It is seen that both the reduction of **6** and the oxidation of **15** produced a similar radical intermediate spectrum, suggested to be the benzotriazinyl radical **14**, when corrected for the absorption of the parent compounds and allowing for the incomplete decay of the initial radical due to the slow first-order rate constant measured earlier for **13**, Scheme 3.

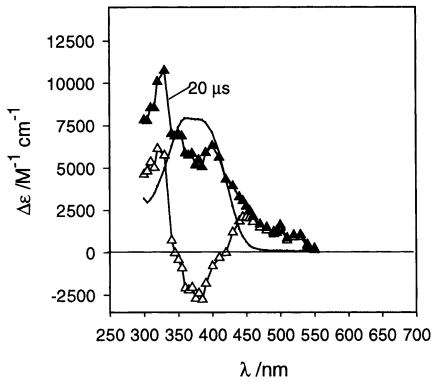


Figure 7. Radical spectrum produced upon reaction of the sulfate radical with **15**. The N_2-saturated solution contained **15** (100 μM), potassium peroxidodisulfate (25 mM), and 2-methylpropan-2-ol (0.2 M) at pH 5.0 (5 mM phosphate). Solid symbols are time points corrected for the absorption of compound **15** (solid line).

E. One-Electron Reduction of 3-Amino-8-methyl-1,2,4-benzotriazine 1,4-Dioxide, 8. The one-electron reduction of **8** (0.2 mM) by the $CO_2^{\bullet-}$ species was also carried out at pH 5.0 to ensure formation of the protonated species **16** and to follow its decay. The initial radical spectrum has a peak near 350 nm with $\epsilon = 4000$ M^{-1} cm^{-1}, Figure 8A, decayed with first-order kinetics to produce an intermediate spectra with a peak near 430 nm, Figure 8B, which in turn decayed to a final spectrum which matches a composite spectrum of **8** and 3-amino-8-methyl-1,2,4-benzotriazine 1-oxide, **18**, Figure 8C. The intermediate spectrum absorbs more strongly below 400 nm than that of the 1-oxide derivative, **18**, which indicates that a different species is formed at the end of the first-order reaction.

F. One-Electron Oxidation of 3-Amino-8-methyl-1,2,4-benzotriazine 1-Oxide, 18. The one-electron oxidation spectrum of compound **18** (0.2 mM) at pH 5.0 was produced upon reaction with the $SO_4^{\bullet-}$ radical, Figure 9. The initial radical spectrum exhibited a peak near 430 nm, similar to the above intermediate which is formed following the one-electron reduction of **8**, with $\epsilon = 4000$ M^{-1} cm^{-1}. It is suggested that the benzotriazinyl radical **17** is formed by both pathways, Scheme 4.

One-Electron Reduction Potential and Reactivity of Oxidizing Radicals. Pulse radiolysis can be used to determine the reduction potentials of radicals by measuring the equilibrium constants between the radicals formed upon one-electron oxidation of substrates and reference compounds.[25] The one-electron reduction potential of the benzotriazinyl radical **5**, E(**5**/**4**) (Scheme 5) of TPZ was measured by establishing a redox equilibrium between 3-amino-1,2,4-benzotriazine 1-oxide, **4**, and 1,4-dimethoxybenzene (DMB). Solutions contained sodium peroxodisulfate (50 mM), 2-methylpropan-2-ol (0.5 M) buffered at pH 7.0 (phosphate, 5 mM), and mixtures of **4** and DMB.

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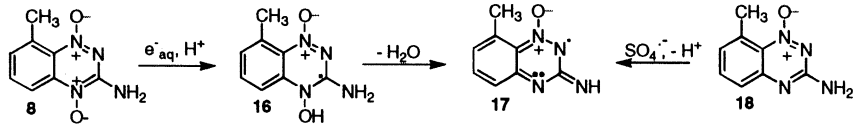
Scheme 4. Radical Intermediates Formed on the One Electron Oxidation of **18** and Intermediate Produced Following One-Electron Reduction of **8**



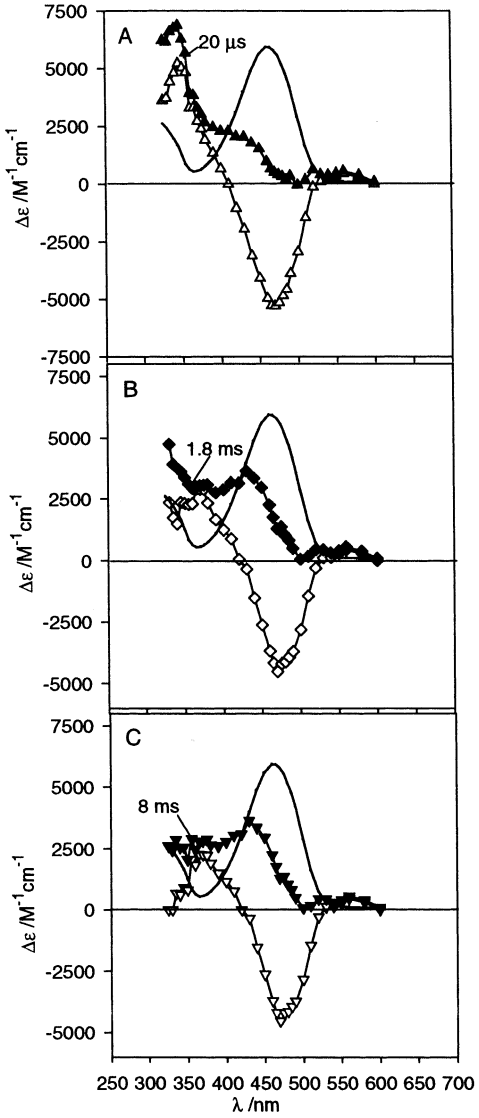


Figure 8. Time-resolved spectra following reaction of the $CO_2^{\bullet-}$ species with **8**. The N_2O-saturated solutions contained **8** (100 μM) and sodium formate (0.15 M) at pH 5.0 (5 mM phosphate). Solid symbols are time points corrected for the absorption of compound **8** (solid line).

The $SO_4^{\bullet-}$ radical produced oxidizes **4** and DMB in <2 μs, and the position of the subsequent equilibrium (4) was determined at 460 nm, where the radical cation of DMB ($DMB^{\bullet+}$) absorbs strongly.

$$\mathbf{4} + \mathrm{DMB}^{\bullet+} \rightleftharpoons \mathbf{5} + \mathrm{DMB} \qquad (4)$$

From three mixtures of **4** and DMB, $K_e = 0.59 \pm 0.02$. As $\Delta E = -(RT/F)\ln K_e = -13$ mV, and the value $E(\mathrm{DMB}^{+\bullet}/\mathrm{DMB}) = +1.30 \pm 0.01$ V,[26] a value $E(\mathbf{5}/\mathbf{4}) = +1.31 \pm 0.01$ V is determined. Similarly, the one-electron reduction potentials of the radicals formed by the one-electron oxidation of **10**, **15**, and **18** were determined as 0.94 ± 0.01 V, 1.30 ± 0.01 V, and 1.30 ± 0.01 V, respectively.

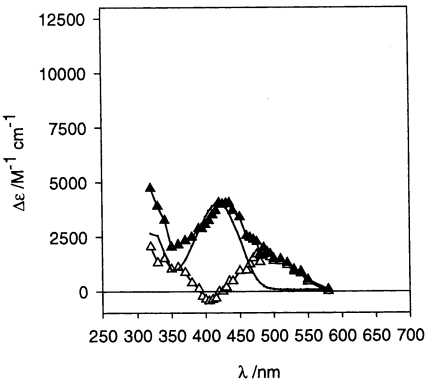


Figure 9. Radical spectrum produced upon reaction of the sulfate radical with **18**. The N_2-saturated solution contained **18** (100 μM), potassium peroxidodisulfate (25 mM), and 2-methylpropan-2-ol (0.2 M) at pH 5.0 (5 mM phosphate). Solid symbols are points corrected for the absorption of compound **18** (solid line).

Compound **4** was found to react with the $SO_4^{\bullet-}$ radical with a rate constant of $(4.8 \pm 0.3) \times 10^9$ M^{-1} s^{-1} by observing the rate of formation of the absorbance of radical **5** at 345 nm for four concentrations of **4** (20−100 μM) in N_2-saturated solutions containing 2-methylpropan-2-ol (0.5 M). In a series of experiments, **4** (0.75 mM) was reacted with the $SO_4^{\bullet-}$ radical to produce radical **5** in the presence of dGMP (0−0.15 mM). The initially formed absorption of **5** was observed to convert to the known, more highly absorbing, spectrum of the one-electron oxidized dGMP ($dGMP^{\bullet+}$) species,[27] reaction 6.

$$\mathrm{SO_4}^{\bullet-} + \mathbf{4} \rightarrow \mathbf{5} + \mathrm{SO_4}^{2-} \qquad (5)$$

$$\mathrm{dGMP} + \mathbf{5} \rightarrow \mathrm{dGMP}^{\bullet+} + \mathbf{4} \qquad (6)$$

The formation of $dGMP^{\bullet+}$ followed first-order kinetics, with the rate dependent on the concentration of the added dGMP, Figure 10, giving a second-order rate constant, k_6, of $(1.4 \pm 0.2) \times 10^8$ M^{-1} s^{-1}. In a similar set of experiments, no reaction was observed between the radical **12** and dGMP.

The decay kinetics of radical **5**, produced on the one-electron oxidation of **4** (1 mM) by the $SO_4^{\bullet-}$ radical, was also studied in the presence of 2-deoxyribose (dR) (0.1−0.4 mM) using the same kinetic treatment of the data as done earlier for the one-electron reduced compounds in Figure 1. The radical **5** is exclusively formed initially under these conditions, since the $SO_4^{\bullet-}$ radical reacts with dR 2 orders of magnitude slower at 3.8×10^7 M^{-1} s^{-1} [28] than its rate when reacting with **4**. Plots of $1/t_{1/2}$ against initial radical concentration for a series of radiation doses gave lines with intercepts dependent on the dR concentration, Figure 11. Plotting the first-order rate constants derived from the intercepts against the dR concentrations yields

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(27) Candeias, L. P.; Steenken, S. *J. Am. Chem. Soc.* **1993**, *115*, 2437−2440.

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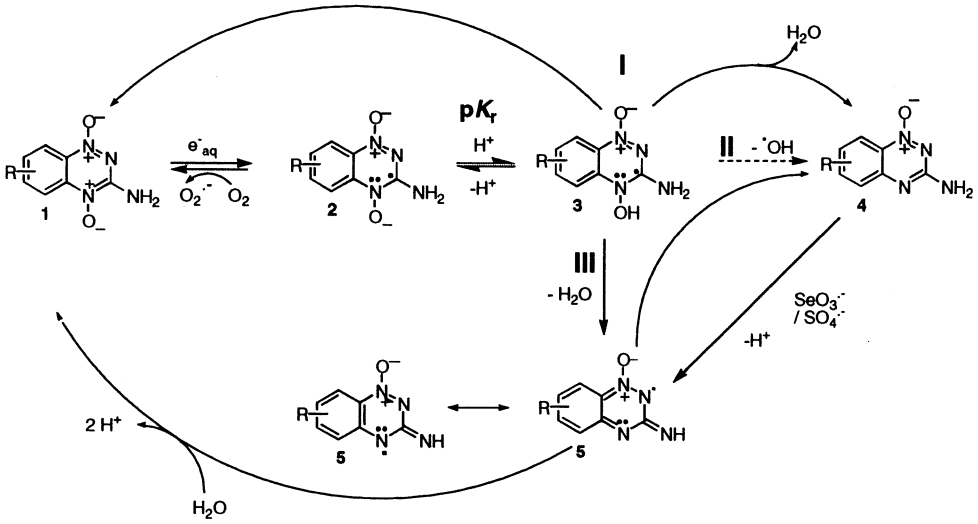
Scheme 5. Radical Pathways for the Formation of 1-Oxides Following One-Electron Reduction of Substituted 3-Amino-1,2,4-benzotriazine 1,4-Dioxides



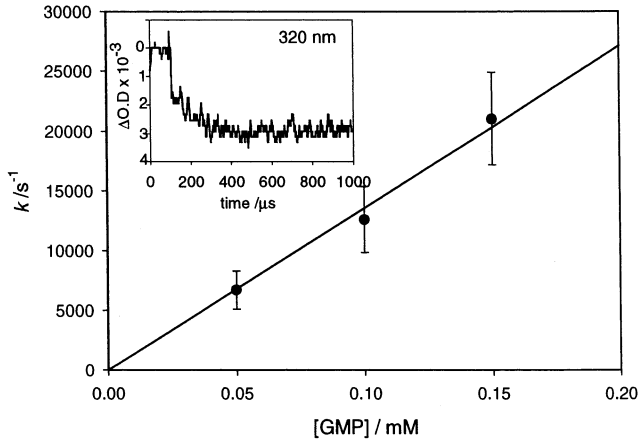


Figure 10. Dependence of the rate of oxidation of dGMP by **5** on dGMP concentration. The N_2-saturated solution contained **4** (500 μM), potassium peroxidodisulfate (25 mM), and 2-methylpropan-2-ol (0.2 M) at pH 7.0 (5 mM phosphate). Insert displays oscilloscope trace for solution containing 100 μM dGMP.

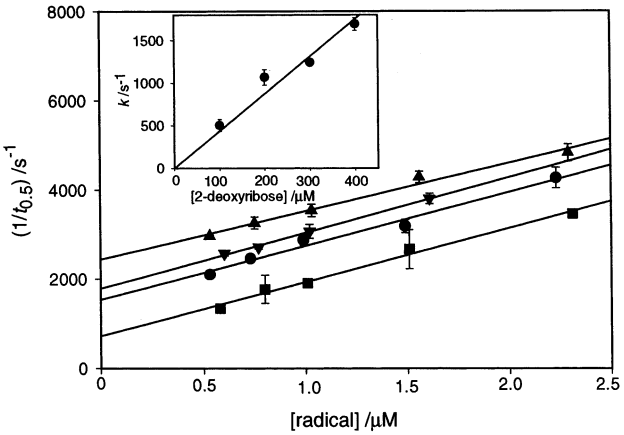


Figure 11. Dependence of the reciprocal of the first half-life of the **5** radical on the initial radical concentration formed with increasing radiation dose. Radicals produced on the oxidation of **4** (1 mM) by the sulfate radical produced in N_2-saturated solutions containing potassium peroxidodisulfate (25 mM) and 2-methylpropan-2-ol (0.1 M) at pH 7.0 (5 mM phosphate) in the presence of (■) 100 μM, (●) 200 μM, (▼) 300 μM, and (▲) 400 μM 2-deoxyribose. Observations were made in triplicate at 345 nm. Insert displays dependence of the first-order rate constants derived from the intercepts of the plots on the concentration of 2-deoxyribose.

the second-order rate constant for reaction 7 between **5** and dR as $k_7 = (3.74 \pm 0.5) \times 10^6\ M^{-1}\ s^{-1}$, Figure 11 insert.

$$\mathbf{5} + \mathrm{dR} \rightarrow \mathrm{dR}^{\bullet} + \mathbf{4} \qquad (7)$$

Discussion

It is known that the one-electron reduction of TPZ, followed by protonation of the radical anion, produces radical **3** as a key intermediate in effecting the hypoxia-selective cytotoxicity of TPZ. This study has shown that analogues of **3** undergo a first-order reaction to produce a radical intermediate that dismutates to the benzotriazine 1,4-dioxides, **1**, and respective benzotriazine 1-oxides, **4**. Possible pathways for the reactions of analogues of **3** in hypoxia are depicted in Scheme 5. Pathway I is only likely to be occurring under certain pulse radiolysis conditions which produce a high initial concentration of radicals favoring bimolecular reaction. Under conditions where **1** is reduced by enzymes or in steady-state radiolysis experiments where a low steady-state concentration of radicals is produced, the first-order reaction reported here will predominate. The appearance of a new intermediate spectrum that is different from that of the 1-oxide, **4**, followed by a final spectrum, which consists of equal amounts of **1** and **4**, suggests that the $^{\cdot}$OH radical is not eliminated by pathway II. The lack of charged species being formed or lost during the first-order reaction rules out the elimination of a hydroxide ion from **3**. As the redox nature of **3** is changed from a reductant to an oxidant by the first-order reaction, we suggest that a benzotriazinyl radical, **5**, is formed. Rearrangement of the initial protonated radical to a nitroxide or nitroxyl radical can be ruled out, as these are reducing species of low reduction potentials.[16,29] Nitrogen-centered radicals are oxidizing species, for example, the tryptophyl radical, where $E = +1.08$ V at pH 7.[30] Such a radical most likely arises from the elimination of water from **3**, pathway III, which was proposed earlier[13] without any supporting evidence.

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The reduction potentials of **5**, **14**, and **17** are all ~1.3 V. At this potential, radical damage on DNA, due to electron transfer from at least the purine bases, is likely as the reduction potentials of the guanine and adenosine radicals in neutral solution are 1.29 and 1.42 V, respectively.[31] We have shown previously that radical **5** does oxidize dGMP. The much lower reduction potential of 0.94 V measured here for the benzotriazinyl radical **11** means that it cannot effect similar radical damage to DNA. Compound **7** exhibits the poorest hypoxic and aerobic cytotoxicity to SCCVII cells of a series of benzotriazine 1,4-dioxide drugs covering a wide range of substituents,[32] and this may well be explained by the low reduction potential of its radical. However, the lethal lesion in the cytotoxic action of this class of drug is the production of double strand breaks in DNA. The initial event in this cytotoxic process is thought to be the abstraction of a hydrogen atom from the deoxyribose backbone of DNA, and we report in this paper the rate constant between **5** and dR in a model system. Whether benzotriazinyl radicals can induce double strand breaks per se or by effecting the initial damage, as modeled in this study, which is amplified by a subsequent reaction with a further benzotriazine 1,4-dioxide molecule,[33−35] warrants further study. It is known from steady-state radiolysis experiments that **1** does form an oxidizing radical following its one-electron reduction as hydrogen atoms are abstracted from hydrogen donor molecules, such as ribose and formate ions, to initiate short chain reactions.[13] Also the analysis of DNA damage produced following the one-electron reduction of **1** points to the formation of a strongly oxidizing species. The induction of double strand breaks by **1** may arise from clustered damage in which a benzotriazinyl radical oxidizes the sugar of DNA to form a reducing radical which in turn is oxidized by another **1** molecule to form a labile cation on the sugar leading to a strand break. The newly produced **3** species then undergoes elimination to form another benzotriazinyl radical local to the first lesion and by a similar series of reactions induces a strand break on the adjacent DNA strand to give a double strand break. In preliminary experiments, we have found short chain reactions do occur when **1** is reduced in the presence of excess 2-deoxyribose, similar to that reported for related compounds.[36] Our study gives evidence as to the exact nature of the oxidizing radical species and has determined the kinetic parameters relevant to the reactivity of the cytotoxic radical. Clearly, the radical's oxidizing power is affected by substituents on the A-ring, with strongly electron-donating substituents decreasing the one-electron reduction potential of the radical. For example, we have found no evidence for the further reduction of **7** by a short chain reaction in the presence of formate ions.

In summary, this work provides kinetic and spectral evidence that the oxidizing radical that is generated from TPZ under hypoxia, and which reacts with DNA as the initial event leading to strand breaks, is the benzotriazinyl radical **5**.

Experimental Section

Chemicals. All compounds used in this study were synthesized and characterized as described previously[32] except 3-amino-7-dimethylamino-1,2,4-benzotriazine 1-oxide, **10**. All reagents used were of analytical grade. Sodium formate, sodium hydroxide, perchloric acid, and phosphate buffers were obtained from Merck, and potassium thiocyanate was obtained from Riedel-de Haen. All other reagents were obtained from Aldrich Chemical Co. All solutions were prepared in water purified by the Millipore Milli-Q system. Solution pH values were adjusted using the phosphate salts (5 mM) and either NaOH or $HClO_4$ when necessary.

Synthesis. 7-Dimethylamino-1,2,4-benzotriazin-3-amine 1-Oxide (10). A solution of 7-fluoro-1,2,4-benzotriazine 1-oxide[32] (114 mg, 0.63 mM) and 40% aqueous Me_2NH (5 mol) in CH_3CN (15 mL) was stirred at 20 °C for 5 days. The solvent was evaporated, and the residue was partitioned between dilute ammonia (10 mL) and CH_2Cl_2 (15 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 15 mL), the combined organic extract dried, and the solvent evaporated. The residue was purified by chromatography, eluting with a gradient (0−3%) of MeOH/CH_2Cl_2 to give **10** (64 mg, 61%) as an orange powder: mp (CH_2Cl_2/hexane) 231−233 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.58 (dd, J = 9.4, 2.9 Hz, 1H), 7.45 (d, J = 9.4 Hz, 1H), 7.02 (d, J = 2.9 Hz, 1H), 6.97 (br s, 2H), 3.05 (s, 6H); ^{13}C NMR [$(CD_3)_2SO$] δ 158.3, 147.6, 130.3, 126.5, 125.6, 95.3, 40.0. Anal. Calcd for $C_9H_{11}N_5O$: C, 52.7; H, 5.4; N, 34.2. Found: C, 52.4; H, 5.3; N, 34.2.

Methods. Pulse radiolysis experiments at room temperature (22 ± 1 °C) were carried out using a 4 MeV linear accelerator, of variable pulse length (200 ns to 2 μs), to deliver a typical absorbed dose of 2.5 Gy for spectral studies and 2.5−20 Gy for kinetic studies.

Steady-state γ-radiolysis experiments were carried out using a ^{60}Co source delivering a dose rate of 18 Gy min^{-1}. Aqueous samples were evacuated and repurged with appropriate O_2-free gases in glass tubes for three cycles. The tubes were fitted with a sidearm incorporating a supracil spectrophotometer cell for UV−vis measurements.

Radiolysis of water produces three well-defined reactive radical species and molecular products (concentrations in μM per absorbed dose of 1 Gy (J kg^{-1}) given in parentheses).

$$H_2O \rightsquigarrow e^-_{aq}(0.28) + {}^\bullet OH(0.28) + H^\bullet(0.06) + H_2(0.04) + H_2O_2(0.07) + H_3O^+(0.28)$$

The one-electron reduction of substrates, A, can be carried out (i) by using the e^-_{aq} alone (forming 0.28 μM Gy^{-1}), while at the same time scavenging the oxidizing radicals with 2-methylpropan-2-ol to form an inert radical, or (ii) by electron transfer from the $CO_2^{\bullet-}$ species (E° $CO_2^{\bullet-}/CO_2 = -1.90$ V[37]) in N_2O-saturated solutions (to quantitatively convert the e^-_{aq} to $^\bullet$OH radicals) containing 0.15 M sodium formate. The radical yield of $A^{\bullet-}$ in this system is 0.68 μM Gy^{-1}.[38]

$$e^-_{aq} + A \rightarrow A^{\bullet-} \quad (8)$$

$$^\bullet OH(H^\bullet) + (CH_3)_3COH \rightarrow {}^\bullet CH_2(CH_3)_2COH + H_2O(H_2) \quad (9)$$

$$N_2O + e^-_{aq} \rightarrow {}^\bullet OH + OH^- + N_2 \quad (10)$$

$$^\bullet OH(H^\bullet) + HCOO^- \rightarrow H_2O(H_2) + CO_2^{\bullet-} \quad (11)$$

$$CO_2^{\bullet-} + A \rightarrow A^{\bullet-} + CO_2 \quad (12)$$

The rapid one-electron oxidation of the 3-amino-1,2,4-benzotriazine 1-oxide derivatives (B) was carried out using the selenite radical (E° $SeO_3^{\bullet-}/SeO_3^{2-} = +1.68$ V[39]) or the sulfate radical (E° $SO_4^{\bullet-}/SO_4^{2-}$ =

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$\sim$+2.5 V[40]) produced in N_2-saturated solution containing 0.2 M 2-methylpropan-2-ol and 20 mM selenate or peroxodisulfate. The radical yield (G value) of $B^{\bullet+}$ in these systems is 0.28 μM Gy^{-1}.

$$e^-_{aq} + SeO_4^{2-}/(S_2O_8^{2-}) + H_2O \rightarrow SeO_3^{\bullet-}/SO_4^{\bullet-} + 2OH^- + (SO_4^{2-}) \quad (13)$$

$$^{\bullet}OH(H^{\bullet}) + (CH_3)_3COH \rightarrow {}^{\bullet}CH_2(CH_3)_2COH + H_2O(H_2) \quad (9)$$

$$SeO_3^{\bullet-}/SO_4^{\bullet-} + B \rightarrow SeO_3^{2-}/SO_4^{2-} + B^{\bullet+} \quad (14)$$

The optical radical detection system and method of chemical dosimetry used for pulse radiolysis experiments have been described.[41] Conductivity measurements were carried out using a custom built AC system consisting of a balanced 250 kHz sine wave voltage source. This provides signals of $\pm$15 V (30 V) to Pt electrodes in a 2 cm suprasil cell. The current in the circuit is measured by a current transformer, which rejects the common mode electron pulse signal. The resulting signal is full wave rectified and is gated into a peak hold/follow circuit. This allows fast updates every 2 μs, which it holds until the next peak. A signal equal and opposite to the base conductance of the sample is generated and used to restore the output to zero; hence, small signals can be easily resolved.

The change in conductance, G_c, of a solution following pulse radiolysis is related to the conductivity, σ, by $\sigma = G_c k_c = 10^{-3} \sum_i c_i |z_i| \Lambda_i$, where c_i is the concentration of all species in mol L^{-1} of charge z, Λ is the equivalent conductivity in S cm^2 mol^{-1}, and k_c is the cell constant.[42]

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