EPR spin-trapping studies of the reaction of hydroxyl and other electrophilic radicals with uridine and related compounds. Isotopic substitution to refine analyses and aid quantification †

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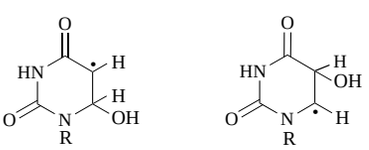
EPR spin-trapping experiments using MNP (2-methyl-2-nitrosopropane) have been employed to study radicals formed by reaction of $HO^{\bullet}$ (generated from reaction of H_2O_2 with Fe^{2+}) with uridine as a model nucleoside; studies with [5-^{2}H]uridine, [5,6-$^2H_2$]uridine and [1,3-$^{15}N_2$] have allowed us to obtain a more detailed analysis of the spectra following $HO^{\bullet}$ attack on uridine, for which EPR spectra cannot at present be unambiguously assigned. These studies provide detailed information concerning the analysis of hyperfine splittings and hence the quantification of the different amounts of the C(5)- and C(6)-hydroxyl-radical adducts formed following $HO^{\bullet}$ attack, which are comparable to results obtained from pulse radiolysis studies. Studies have also been extended to investigate the reactions of both $SO_4^{\bullet -}$ and $Bu^tO^{\bullet}$ with uridine.

Introduction

Hydroxyl-radical reactions with nucleic acids and their constituents have been the subject of extensive studies,[1,2] and a variety of techniques, including pulse radiolysis with optical and conductivity detection,[3,4] EPR spectroscopy with continuous-flow and spin-trapping[5–10] and product studies[11,12] have been employed to investigate the selectivity and mechanisms of these reactions. The technique of EPR spin-trapping has been applied with considerable success to identifying the sites of damage in nucleobases and nucleosides. For example, previous studies have established that reaction of $HO^{\bullet}$ with uridine can result in the formation of both the C(5)- and C(6)-OH radicals adducts;[1,2,6–8,10] EPR studies give spectra [see Fig. 1(*a*)] in which two major doublets are observed. The dominant 'inside' doublet [a(N) = 1.51 and a(H) = 0.35 mT] has been assigned by us previously to the adduct of the C(6)-OH, C(5)-yl-radical (**1**), and the 'outside' doublet [a(N) = 1.50 and a(H) = 0.68 mT] to the adduct of the C(5)-OH, C(6)-yl-radical **2**;[6,7] however, the latter signals are not completely resolved and possible extra hyperfine splittings from the C(5)-OH, C(6)-yl radical may be obscured by overlapping signals from the C(6)-OH, C(5)-yl radical. Studies by Kuwabara and co-workers, using HPLC investigations in conjunction with EPR spin-trapping experiments, led to the suggestion that additional nitrogen splittings arising from N(1) on the pyrimidine ring are observed from one of the adducts, assigned to the C(6)-yl species from uridine[8] and cytidine[9] following $HO^{\bullet}$ attack. In order to provide unambiguous assignment, we have carried out a detailed study of the reactions of $HO^{\bullet}$ (generated from reaction of H_2O_2 with Fe^{2+}) with [5-^{2}H]uridine, [5,6-$^2H_2$]uridine and [1,3-$^{15}N_2$]uridine by use of EPR spin-trapping using the spin-trap 2-methyl-2-nitrosopropane (Me_3CNO, MNP). These studies were then extended to investigate the reactions of other electrophilic radicals such as $SO_4^{\bullet -}$ and $Bu^tO^{\bullet}$.



1

C(6)-OH

2

C(5)-OH

Results and discussion

Hydroxyl-radical reactions with uridine, [5-^{2}H]uridine, [5,6-$^2H_2$]-uridine and [1,3-$^{15}N_2$]uridine

The hydroxyl radical was generated from reaction of H_2O_2 with Fe^{2+} (see Experimental) and its reaction with the substrate was then examined in the presence of the spin-trap MNP in the pH range 1–7.4. EPR spectra were recorded shortly after mixing the solutions which were deoxygenated by purging with nitrogen. Concentrations (after mixing) were typically; Fe^{2+}–EDTA (ethylenediaminetetracetic acid) (5×10^{-3} mol dm^{-3}), H_2O_2 (10^{-2} mol dm^{-3}), substrate (10^{-2} mol dm^{-3}) and MNP (5×10^{-3} mol dm^{-3}).

The spectrum obtained throughout the pH range is typified by that shown in Fig. 1(*a*), in which two triplets of doublets can be clearly seen. Similar reactions with [5-^{2}H]uridine **3** gave the spectrum shown in Fig. 1(*b*); comparison with the spectrum from unlabelled uridine shows that the 'inside' doublet assigned to the C(6)-OH, C(5)-yl-adduct **4** has collapsed to the expected singlet [a(N) = 1.51 mT] and the outside lines of the wider 'doublet' attributed to the C(5)-OH, C(6)-yl-adduct **5**, are unaltered (see Table 1). However, further additional resonances are now revealed following the collapse of the 'inside' doublet, and these can be interpreted in several ways. For example, these signals may represent a further triplet of *doublets* [a(N) = 1.50 and a(H) = 0.37 mT], *e.g.* a trapped sugar radical. Alternatively, they may be part of the outside signals [due to the C(5)-OH adduct], which can be analysed in three ways: either a triplet of doublets further split into doublets [a(N) = 1.50, a(β-H) = 0.51 and a(γ-H) = 0.17 mT], where the additional doublets are due to an extra hydrogen splitting, or a triplet of doublets, further split into *triplets* by the nitrogen atom adjacent to the C(6) atom in the pyrimidine ring [a(N) = 1.50, a(H) = 0.16 and a(β-N) = 0.26 mT], or with the hydrogen and nitrogen splittings

† Presented at the 30th International Meeting of the Electron Spin Resonance Group of the RSC, University of Lancaster, 6–10th April 1997.

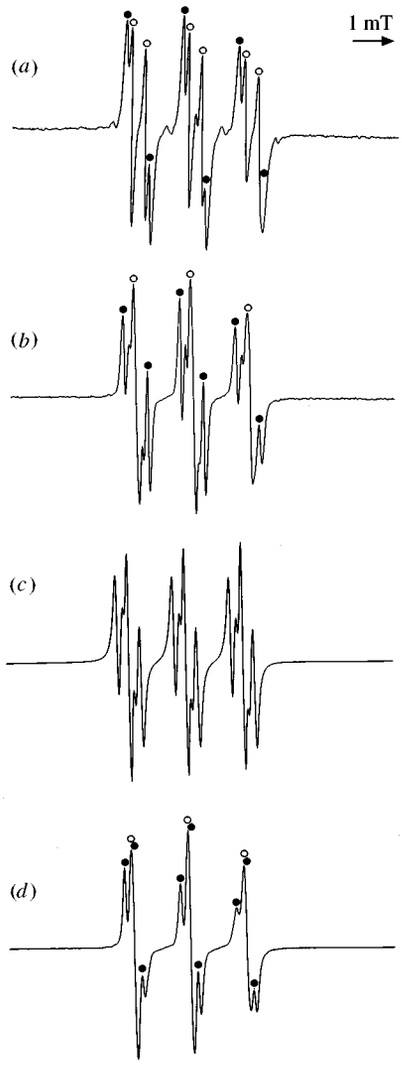


Fig. 1 (*a*) EPR spectra of spin adducts **1** (○) and **2** (●) formed from the addition of $HO^{\bullet}$ (generated from the reaction between Fe^{2+}–EDTA and H_2O_2) to uridine (1×10^{-2} mol dm^{-3}) in the presence of MNP at pH 7.4, only the outside lines of adduct **2** are indicated; (*b*) as in (*a*), except with [5-^{2}H]uridine, signals are assigned to spin adducts **4** (○) and **5** (●) with computer simulation (*c*) (see Table 1); (*d*) as in (*a*), except with [5,6-$^2H_2$]uridine, signals are assigned to spin adducts **7** (○) and **8** (●)

reversed in order [*e.g.* a(H) = 0.36 and a(β-N) = 0.16 mT]. Computer simulations of all the possibilities give spectra with similar characteristics [though in the case when a(H) > a(β-N), it was not possible to obtain a good simulation]. The spectrum obtained with [5,6-$^2H_2$]uridine provides further evidence for the presence and the magnitude of the additional nitrogen splitting [see Fig. 1(*d*)], in which the 'inside' doublet assigned to the C(6)-OH, C(5)-yl-radical **7** has collapsed to the expected singlet [a(N) = 1.51 mT] and the outside lines of the wider 'doublet' assigned to the C(5)-OH, C(6)-yl-radical **8** have also collapsed to a triplet a(N) = 1.50 mT with a second triplet splitting a(β-N) = 0.26 mT. Kuwabara and co-workers have suggested that an additional nitrogen splitting is indeed observed in one of the separated adducts, but with a(N) < a(H) [a(N) = 1.52, a(H) = 0.31 and a(β-N) = 0.14 mT].[8] We also note that patterns of hydrogen and nitrogen splittings [with a(β-N) larger than a(H) as suggested here] are characteristic of the C(5)-OH, C(6)-yl radical from nucleobases such as uracil[6] and cytosine.[9]

This analysis is confirmed by ^{15}N substitution; thus the reaction of $HO^{\bullet}$ with [1,3-$^{15}N_2$]uridine **9** in the presence of MNP gave a spectrum [see Fig. 2(*a*)] again attributed to a mixture of two species. The first has a triplet of doublets [a(N) = 1.51 and

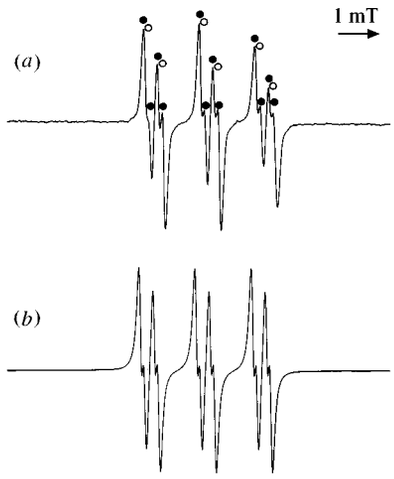


Fig. 2 (*a*) EPR spectra of spin adducts **10** (○) and **11** (●) formed from the addition of $HO^{\bullet}$ to [1,3-$^{15}N_2$]uridine in the presence of MNP at pH 7.4; (*b*) Computer simulation of (*a*) (see Table 1)

a(H) = 0.35 mT] which as expected, is the same as the doublet observed in the spectrum obtained following $HO^{\bullet}$ attack on uridine [see Fig. 1(*a*)] and therefore assigned on the C(6)-OH, C(5)-yl-radical **10**. The second, dominant signal must contain a second nitrogen splitting as indicated by the decrease in the width of the overall spectrum on going from ^{14}N (see Fig. 1) to ^{15}N. This spectrum can be interpreted as a triplet of doublets further split into doublets [a(N) = 1.50, a(H) = 0.16 and a(β-^{15}N) = 0.37 mT] (see Table 1). This is attributed to the C(5)-OH, C(6)-yl-radical **11**, for which an additional nitrogen splitting is observed (from the ^{15}N atom, with a doublet splitting approximately 1.4 times larger than the ^{14}N splitting). This verifies the analysis of the ^{14}N spectrum as a triplet of doublets, further split into triplets [a(N) = 1.50, a(H) = 0.16 and a(β-N) = 0.26 mT], notably with a(β-N) larger than a(H) as suggested earlier (and in contrast to that suggested by Kuwabara[8,9]).

Detailed quantitative analysis of the spectra from computer simulations of uridine and its isotopically substituted analogues reveals that $HO^{\bullet}$ attacks predominantly at the C(5) position, to generate the C(6)-yl radical; ratios of 83:17 [C(5):C(6)-adducts] give the most accurate simulations of the original spectra. The implied selectivity of attack in these reactions is in close agreement with related pulse radiolysis work, for which it has been shown that the C(5)-OH, C(6)-yl radical is the predominant species formed following $HO^{\bullet}$ attack on uracil and poly (U), with ratios of 82:18 and 70:23, respectively, for C(5):C(6).[2,3] This suggests that spin-trapping can indeed be used in this way to obtain quantitative information.

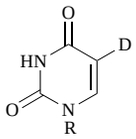
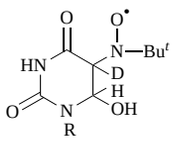
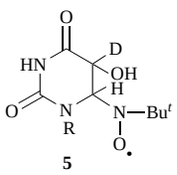
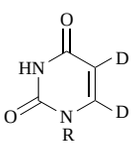
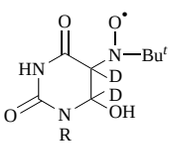
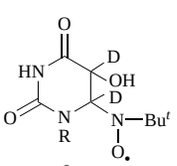
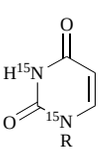
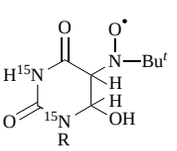
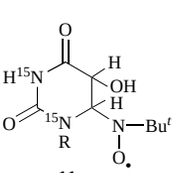
The reaction of *tert*-butoxyl with uridine and its isotopically labelled counterparts

The *tert*-butoxyl radical was generated from the reaction of Bu^{t}OOH with Fe^{2+}–EDTA and its reaction with uridine was examined in the presence of the spin-trap MNP (see Experimental). Reaction of Bu$^tO^{\bullet}$ with uridine, [5-^{2}H]uridine, [5,6-$^2H_2$]uridine and [1,3-$^{15}N_2$]uridine in the presence of MNP gave spectra that were very similar but not identical to those obtained from their reactions with $HO^{\bullet}$ respectively; the spectra differed only in minor details (see Table 2). The signals obtained in these studies are attributed to the C(5)- and C(6)-OBu^t adducts, with ratios of *ca.* 88:12 [C(5):C(6)], indicating that the selectivity of Bu$^tO^{\bullet}$ is almost identical to that of $HO^{\bullet}$ itself, as suggested previously.[13]

The reactions of the sulfate radical-anion with uridine and its isotopically labelled counterparts

The reaction of the sulfate radical-anion ($SO_4^{\bullet -}$) with *N*-substituted pyrimidines (for example, methylated uracil compounds) has been the subject of previous detailed studies

Table 1 EPR parameters of radicals derived from hydroxyl radical attack on [5-^{2}H]uridine, [5,6-$^2H_2$]uridine and [1,3-$^{15}N_2$]uridine throughout the pH range 1–7.4 in the presence of the spin trap MNP

Substrate	Radicals	a(N)[a]	a(β-H)[a]	a(other)[a]
Uridine	**1**	1.50	0.35	—
	2	1.50	0.16	0.26 (β-N)
 **3**	 **4**	1.51	—	—
	 **5**	1.50	0.16	0.26 (β-N)
 **6**	 **7**	1.51	—	—
	 **8**	1.50	—	0.26 (β-N)
 **9**	 **10**	1.51	0.35	—
	 **11**	1.50	0.16	0.37 (β-^{15}N)

[a] Typically ±0.005 mT, except where indicated otherwise; g = 2.0059 ± 0.0001.

using pulse radiolysis,[3,14] continuous-flow EPR spectroscopy[15,16] and spin-trapping.[17] EPR investigations in the range pH 1–9 establish that both C(5)- and C(6)-hydroxyl radical adducts can be detected (directly from continuous-flow investigations[15,16] and indirectly from spin-trapping studies[17]); ratios of adducts were found to depend on the concentrations of persulfate (used to generate $SO_4^{\bullet -}$) employed.[16] This has led to suggestions that one-electron oxidation of these substrates by $SO_4^{\bullet -}$ results in the formation of base radical-cations, which undergo rapid reaction with water to form the appropriate hydroxyl-radical adducts.[14–17] It is also possible that such adducts may also be formed from direct hydrolysis of the sulfate-adducts (for example *via* an S_N2 reaction).[16] We have used the isotopically substituted uridine compounds, in an attempt to obtain further information on this point. The sulfate radical-anion ($SO_4^{\bullet -}$) was generated from the reaction of either oxone (potassium peroxymonosulfate $2KHSO_5$, $KHSO_4$, K_2SO_4) or sodium persulfate with Fe^{2+}–EDTA 1 : 1 complex in the pH range 1–7.4.

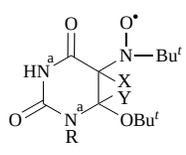
Reaction of $SO_4^{\bullet -}$ with uridine, [5-^{2}H]uridine, [5,6-$^2H_2$]uridine and [1,3-$^{15}N_2$]uridine at pH 7.4 gave spectra which showed the presence of two radicals. These are clearly identified as the hydroxyl adducts [for example see Figs. 1, 2 and 3(*a*)], for which ratios of *ca.* 85 : 15 [C(5) : C(6)] were obtained; similar results were observed in the pH range 2.5–7.4.

In contrast, studies at pH 2 with typical oxone and sodium persulfate concentrations of 3×10^{-2} and 3×10^{-3} mol dm^{-3}, respectively, resulted in a different spectrum [see *e.g.* Fig. 3(*b*)]. This appears to show the presence of a dominant radical, which for uridine itself is analysed in terms of a triplet of doublets of triplets [a(N) = 1.44, a(H) = 0.17 and a(β-N) = 0.17 mT], which is clearly not due to either or both the hydroxyl-adducts but is nevertheless typical of a C(5)-adduct, C(6)-yl radical. Reactions with [5-^{2}H]uridine gave a similar spectrum [Fig. 3(*c*)] together with an additional triplet [a(N) = 1.44 mT], consistent with the trapping of a C(6)-adduct, C(5)-yl radical (not clearly revealed in the undeuterated spectrum). Similar studies with [1,3-$^{15}N_2$]-uridine gave a spectrum [Fig. 3(*d*)] which showed the presence

Table 2 EPR parameters of radicals derived from sulfate radical-anion (in the range pH 2.5–7.4) and *tert*-butoxyl radical (at pH 7.4) attack on uridine, [5-$^2H_2$]uridine and [1,3-$^{15}N_2$]uridine in the presence of the spin trap MNP

Attacking species	Substrate	Radicals	a(N)[a]	a(β-H)[a]	a(other)[a]
$Bu^tO^{\bullet}$	Uridine	**12**	1.51	0.35	—
		13	1.50	0.16	0.26 (β-N)
	[5-^{2}H]Uridine **3**	**14**	1.51	—	—
		15	1.50	0.16	0.26 (β-N)
	[5,6-$^2H_2$]Uridine **6**	**16**	1.51	—	—
		17	1.50	—	0.26 (β-N)
	[1,3-$^{15}N_2$]Uridine **9**	**18**	1.51	0.35	—
		19	1.50	0.16	0.37 (β-^{15}N)
$SO_4^{\bullet -}$	(*a*) pH 2.5–7.4[c]				
	Uridine	**1**	1.51	0.35	—
		2	1.50	0.16	0.26 (β-N)
	(*b*) pH 2				
	Uridine	**22**	1.44	0.17	0.17 (β-N)
	[5-^{2}H]Uridine **3**	**23**[b]	1.44	—	—
		24	1.44	0.17	0.17 (β-N)
	[5,6-$^2H_2$]Uridine **6**	**25**[b]	1.44	—	—
		26	1.44	0.17	0.17 (β-N)
	[1,3-$^{15}N_2$]Uridine **9**	**27**	1.44	0.17	0.24 (β-^{15}N)

[a] Typically ±0.005 mT, except where indicated otherwise; $g = 2.0059 \pm 0.0001$. [b] Observed when low concentrations of either oxone or persulfate are employed (see text for further details). [c] See Table 1 for hyperfine splittings obtained with other isotopically substituted uridine compounds.



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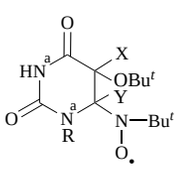
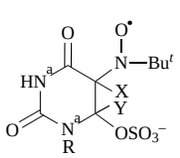


13 X = Y = H
14 X = D, Y = H
16 X = Y = D
18 X = Y = H, N^a = ^{15}N



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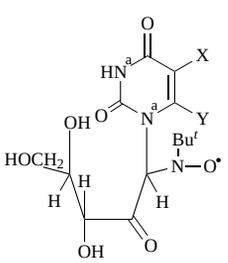


22 X = Y = H
24 X = D, Y = H
26 X = Y = D
27 X = Y = H, N^a = ^{15}N

of a dominant radical, attributed to a triplet of doublets of doublets [a(N) = 1.44, a(H) = 0.17 and a(β-N) = 0.24 mT], as expected, and again consistent with the trapping of a C(5)-adduct, C(6)-yl radical (see Table 2). Unexpectedly, reactions with [5,6-$^2H_2$]uridine [Fig. 3(*e*)] gave signals [a(N) = 1.44, a(H) = 0.17 and a(β-N) = 0.17 mT] and [a(N) = 1.44 mT] which were similar to those observed with uridine itself; this demonstrates that the adduct detected at low pH is not attributed to a simple C(5)-adduct, C(6)-yl-radical on account of the β-hydrogen (^{1}H) splitting. The possibility of hydrogen atom exchange with water was also ruled out as experiments conducted in D_2O showed no changes; therefore, the findings suggest the trapping of a radical with both a(H) and a(β-N) splittings, evidently arising from the formation of a sugar-derived radical.

When concentrations of oxone and persulfate were raised to *ca.* 8×10^{-2} and 3×10^{-2} mol dm^{-3}, respectively, studies with [5-^{2}H]uridine and [5,6-$^2H_2$]uridine gave spectra which showed the presence of the same C(5)-adduct, C(6)-yl-radical [a(N) = 1.44, a(H) = 0.17 and a(β-N) = 0.17 mT]. The additional triplet has now been removed (we believe it to originate from a precursor radical that is readily oxidised by excess oxone or persulfate[17]).

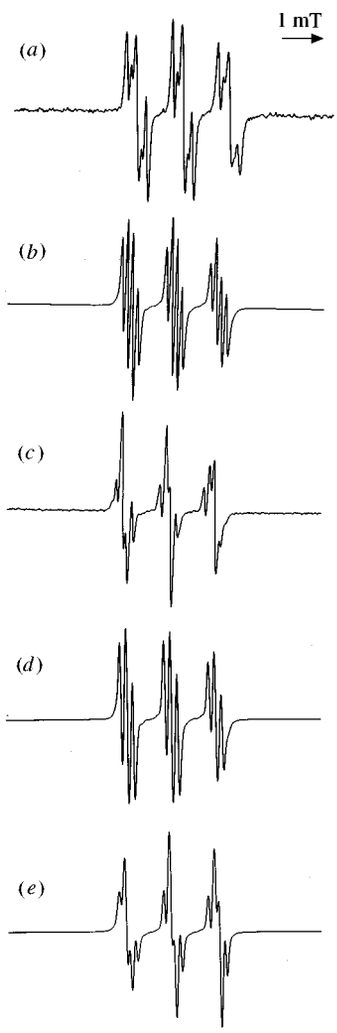
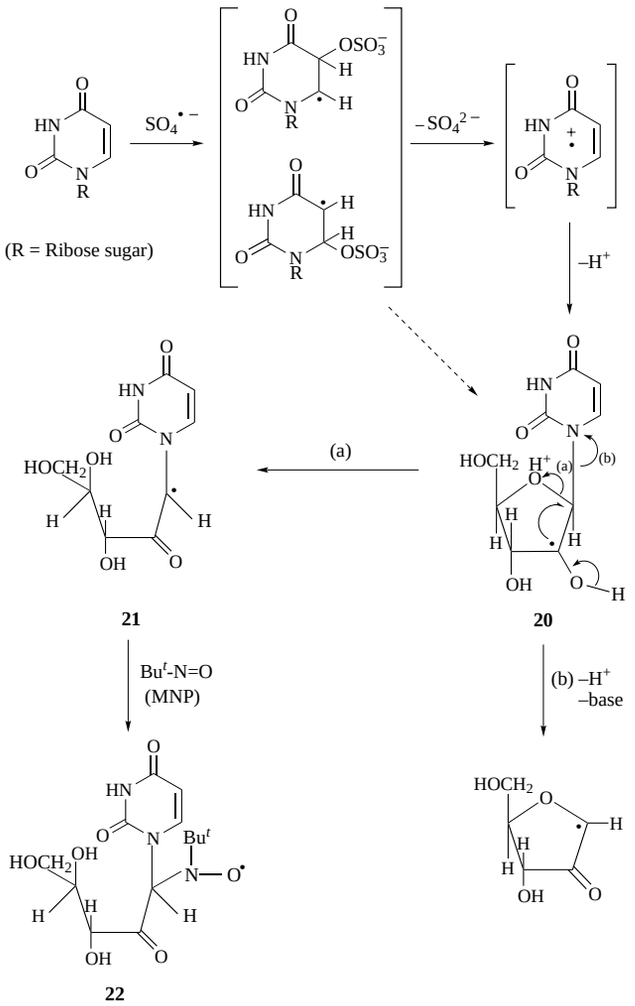


Fig. 3 EPR spectra of spin adducts formed from the addition of the $SO_4^{\bullet -}$ (from the reaction between Fe^{2+}–EDTA and sodium persulfate) in the presence of MNP to (*a*) [5-^{2}H]uridine at pH 7.4 [*cf.* adducts (**4**) and (**5**) in Fig. 1(*b*)]; (*b*) uridine at pH 2; (*c*) [5-^{2}H]uridine at pH 2; (*d*) [1,3-$^{15}N_2$]uridine at pH 2; (*e*) [5,6-$^2H_2$]uridine at pH 2

Our results strongly suggest that at pH < 2, a radical from the sugar moiety is formed. The species detected can best be assigned to an adduct with the radical centre located at the C(1) position in the sugar ring, so that the adjacent proton and nitrogen atom (from the base moiety) give the 1 : 2 : 2 : 1 pattern

observed. We believe that this can occur as a result of ring-opening by cleavage of the C(1)–oxygen bond in the C(2) radical **20** to give radical **21** which is trapped to give **22** (see Scheme 1).



Scheme 1

1). Previous studies (involving the reactions of $SO_4^{\bullet -}$ and $HO^\bullet$ with nucleosides at low pH) have shown that radicals formed initially at the C(2)-atom of the sugar (generated *via* radical-cations obtained initially from the pyrimidine), result in base loss;[10] however, it is also likely that the C(2) radical can undergo an analogous acid-catalysed cleavage of the C(1)–oxygen bond in a related reaction, to give the adduct identified here. When similar experiments were carried out with 2-deoxyuridine, in which hydrogen abstraction at C(2) atom in the sugar moiety would not be expected to occur (due to the absence of the radical-stabilising hydroxyl function), no evidence for this 1 : 2 : 2 : 1 signal was obtained; instead, hydroxyl-radical adducts (Fig. 1) were observed, these presumably being formed as a result of hydrolysis of the base radical-cation as suggested previously. This observation also lends support to the conclusion that the radical observed with uridine is indeed the C(1) sugar radical, formed by rearrangement of an initial C(2) sugar radical.

Conclusions

The reaction of $HO^\bullet$ with [5-^{2}H]uridine, [5,6-$^2H_2$]uridine and [1,3-$^{15}N_2$]uridine in the presence of MNP reveals unambiguously the presence of an additional nitrogen splitting from the C(5)-OH, C(6)-yl-radical. In addition, simulations also show that the C(5)-OH, C(6)-yl-radical is the predominant product of reaction [83 : 17 for C(5) : C(6)-adducts] and that these results are in good agreement with results obtained from pulse radiolysis.[2,3,10]

Related studies with $Bu'O^\bullet$ also indicate the formation of C(5)- and C(6)-adducts, in which the presence of the additional nitrogen splitting is evident, and predominance of attack is again at the C(5)-position. Investigations with $SO_4^{\bullet -}$ at pH 7.4 show that hydroxyl adducts are generated, probably *via* attack of water on the appropriate radical-cation or sulfate adduct (with a stereoselectivity close to that observed for direct $HO^\bullet$ and $Bu'O^\bullet$). In contrast, studies at pH < 2 indicate the formation of a sugar radical; it is believed that this may be formed as a result of ring-opening of the sugar following hydrogen-atom abstraction from C(2) at the sugar moiety (by the base radical-cation).

Experimental

EPR experiments were carried out on either Bruker ESP300 or JEOL JES RE1X EPR spectrometers, using an aqueous solution sample cell. Samples were prepared by mixing aqueous solutions of the substrate (1×10^{-2} mol dm^{-3}), with Fe^{2+}–EDTA (5×10^{-3} mol dm^{-3}), MNP (5×10^{-3} mol dm^{-3}) and hydrogen peroxide, H_2O_2, (1×10^{-2} mol dm^{-3}) which was used to generate $HO^\bullet$. Oxone [potassium peroxymonosulfate $2KHSO_5$, $KHSO_4$, K_2SO_4, ($3–8 \times 10^{-2}$ mol dm^{-3})] or sodium persulfate (3×10^{-3}–3×10^{-2} mol dm^{-3}) were employed to generate $SO_4^{\bullet -}$; we used $Bu'OOH$ (5×10^{-3} mol dm^{-3}) to generate $Bu'O^\bullet$. The Fe^{2+}–EDTA solution was deoxygenated by purging with nitrogen and the MNP solution contained 3–17% v/v acetonitrile to aid dissolution to the trap. Control of pH was achieved by use of 5×10^{-2} mol dm^{-3} phosphate buffer for experiments carried out at pH 7.4, pH variation was obtained by addition of H_2SO_4.

All chemicals were commercial samples of high purity and used without further purification. The deuterium-labelled samples were obtained from CDN Isotopes (91.9 and 98.3% atom of D for [5-^{2}H]uridine and [5,6-$^2H_2$]uridine, respectively); [1,3-$^{15}N_2$]uridine (99% pure) was obtained from Cambridge Isotope Laboratories. The other chemicals were supplied by either Sigma and/or Aldrich.

Computer simulations of spectra were carried out using a program written by Dr M. F. Chiu and adapted by Dr A. C. Whitwood (University of York) to run on a PC (IBM-compatible).

Acknowledgements

The authors would like to thank the Association for International Cancer Research for support (studentship and equipment) and Dr G. S. Timmins for helpful discussions.

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Paper 7/02491F
Received 11*th April* 1997
Accepted 20*th June* 1997