Determination of Rate Constants of the Spin Trap 3,5-Dibromo-4-nitrosobenzenesulfonic Acid with Various Radicals by Pulse Radiolysis and Competition Kinetics

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The spin-trapping reactions of the aromatic nitroso compound 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS) with a number of inorganic [$^{\bullet}OH$, $^{\bullet}N_3$, $CO_2^{\bullet-}$, $O_2^{\bullet-}$, $(SCN)_2^{\bullet-}$] and organic radicals (methyl, hydroxymethyl, hydroxyethyl, 2-hydroxypropyl, 2-hydroxy-2-methylpropyl) have been studied by both pulse radiolysis and EPR spectroscopy. Direct kinetic as well as competition kinetic methods have been used to determine the respective rate constants. In the case of $CO_2^{\bullet-}$ and $O_2^{\bullet-}$, no corresponding spin adduct is observed, while with $^{\bullet}OH$ and $(SCN)_2^{\bullet-}$ only unspecific nine-line EPR signals occur. At neutral pH, only hyperfine splitting constants of spin adducts consistent with trapping of primary (β-hydroxyalkyl) radicals can be determined, while strongly acidic solutions are required to trap secondary and tertiary (α-hydroxyalkyl) radicals.

3,5-Dibromo-4-nitrosobenzenesulfonic acid (DBNBS) is an aromatic nitroso compound employed as a spin-trapping agent.[1–3] Its main advantage over other nitroso spin traps is its good solubility in water and minimal light sensitivity, as compared to 2-methyl-2-nitrosopropane,[1] making it potentially very useful for *in vivo* spin-trapping experiments.[4] Because of its limited use thus far in EPR/spin-trapping studies, hyperfine splitting constants (hfsc) of spin adducts are known only for a few alkyl radicals [1,5,6] and no trapping rate constants have yet been determined.

Using pulse radiolysis as a specific source of radicals, we have examined the trapping reactions with a variety of inorganic and organic radicals, establishing both the respective rate constants and the hfsc values of the spin adducts. Since only few of the radicals resulted in transient spectra with DBNBS, which could be kinetically resolved, we used competition experiments to obtain kinetic parameters. Thus, we were able to determine rate constants with practically all investigated radicals, whereas the corresponding spin adducts could not always be observed owing to their instability.

Experimental

DBNBS was synthesized from 3,5-dibromosulfanilic acid according to Kaur *et al.*[1] For the kinetic experiments, the competitors used were the porphyrins *meso*-tetra(*N*-methylpyridyl)porphine tetraiodide (TMPP) and *meso*-tetra(4-sulfonatophenyl)porphine tetrasodium (TSPP) from Strem and *p*-nitrosodimethylaniline (*p*-NDA) from Fluka. The iron–porphyrin complex iron(III) tetrakis(*N*-methylpyridyl)porphine (FTMP) was synthesized according to ref. 7. The inorganic radicals were generated from the respective anions by oxidation with hydroxyl radicals in N_2O-saturated aqueous solution.[8] Likewise, the organic alkyl radicals were obtained by oxidation with $^{\bullet}OH$, using dimethyl sulfoxide (DMSO) for $^{\bullet}CH_3$ and alcohols for various $^{\bullet}C(OH)R'R''$ radicals. All substances were of the highest available purity and used as purchased. Solutions were prepared with Milli-Q water and adjusted for pH values of 8.0–8.5 without the use of buffer. Pulse radiolysis experiments were performed with 100 ns pulses of 1.6 MeV electrons from a Febetron 705 source. Solute handling and data processing have been described before.[9] The EPR spectra of the spin adducts were recorded on a Bruker ESP 300 spectrometer, with a modulation amplitude of 0.1 G, a sweep rate of 2.8 G s^{-1} at a frequency of 9.75 GHz and a gain of 1×10^6.

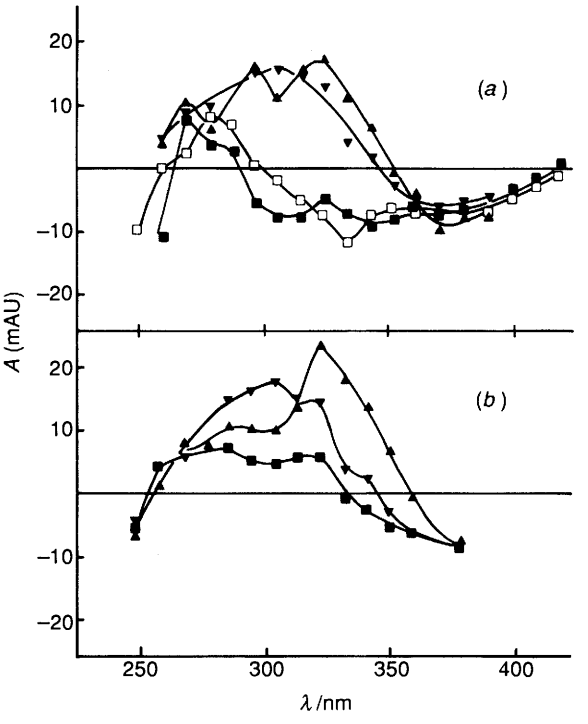

Fig. 1 Transient spectra of DBNBS after attack by different radicals (dose-normalized difference spectra at time of maximal absorption change, 300–450 μsec after the pulse). (*a*) Inorganic radicals: [DBNBS] = 0.13–0.15 mmol dm^{-3}, pH 8.8–8.9, (□) $^{\bullet}OH$, (■) $^{\bullet}N_3$, (▲) $CO_2^{\bullet-}$, (▼) $O_2^{\bullet-}$. (*b*) Alkyl radicals: [DBNBS] = 0.1 mmol dm^{-3}, pH 7.8–8.0) (■) $^{\bullet}CH_2OH$ (MeOH), (▲) $^{\bullet}CH(OH)CH_3$/$^{\bullet}CH_2CH_2OH$ (EtOH), (▼) $^{\bullet}C(OH)(CH_3)_2$/$^{\bullet}CH_2CH(OH)CH_3$ (Pr^iOH).

Results

Transient Spectra.—The transient spectra observed after attack of DBNBS by different radicals are depicted for the time of the maximum of the absorption change. Fig. 1(*a*) shows the inorganic radicals and 1(*b*) the organic hydroxyalkyl radicals. It is obvious, that the weak transient spectra do not allow a clear distinction of the individual radicals: (*i*) the highly electrophilic $^{\bullet}OH$ and $^{\bullet}N_3$ radicals (together with $^{\bullet}CH_2OH$) have the smallest absorption changes; (*ii*) $CO_2^{\bullet-}$, $O_2^{\bullet-}$ and $(CH_3)_2\dot{C}OH$, the radical derived from isopropanol, give very similar transient

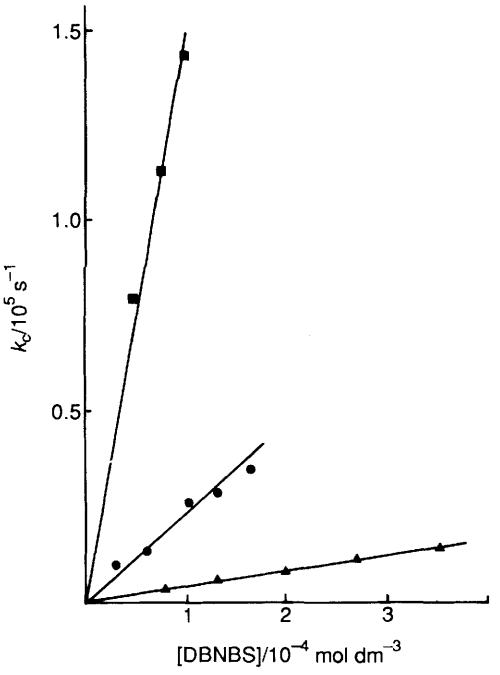

Fig. 2 Pseudo-first order reactions of DBNBS with inorganic radicals–direct kinetic evaluation (■) $CO_2^{\bullet -}$, (●) $^{\bullet}N_3$, (▲) $O_2^{\bullet -}$

Table 1 Rate constants of spin trapping reactions of DBNBS with inorganic radicals

Radical	Rate constant/ 10^8 dm^3 mol^{-1} s^{-1}	Method[a]	Comments
$^{\bullet}OH$	39.5 ± 0.2	c/k	*p*-NDA[b]
$CO_2^{\bullet -}$	15.0 ± 0.9	k/e	
$^{\bullet}N_3$	2.4 ± 0.4	k/e	
$O_2^{\bullet -}$	0.43 ± 0.04	k/e	
$(SCN)_2^{\bullet -}$	—	n/a	see text
$SO_3^{\bullet -}$	—	n/a	see text

[a] Abbreviations: c/k, competition kinetics; k/e, (direct) kinetic evaluation; n/a, not applicable (no rate constant). [b] Competitor *p*-NDA: $k(^{\bullet}OH) = 1.25 \times 10^{10}$ dm^3 mol^{-1} s^{-1}, ref. 12/#603.

Table 2 Rate constants of DBNBS and reference substance TSPP with organic radicals

Solute	Radical	Rate constant/10^8 dm^3 mol^{-1} s^{-1} DBNBS	TSPP
DMSO	$^{\bullet}CH_3$	15.5[a]	320
MeOH	$^{\bullet}CH_2OH$	2.55	1.05
EtOH	$^{\bullet}CH(OH)CH_3$/ $^{\bullet}CH_2CH_2OH$	0.63	0.52
Pr^iOH	$^{\bullet}C(OH)(CH_3)_2$/ $^{\bullet}CH_2C(OH)CH_3$	0.48	0.50

[a] A value of 28×10^8 dm^3 mol^{-1} s^{-1} was determined by competition with O_2; k ($^{\bullet}CH_3 + O_2$) = 47×10^8 dm^3 mol^{-1} s^{-1} (ref. 13, entry 3.1).

spectra, the transient with the ethanol-derived radical not being significantly different. There was even less difference between the observed transients at the final observation period of *ca.* 95 ms (data not shown).

No transient spectra were observed after $^{\bullet}OH$ attack on *tert*-butyl alcohol; however, distinct EPR signals were observed under these conditions (see below). With $(SCN)_2^{\bullet -}$ only its strong absorption (ε_{475} = 7600 dm^3 mol^{-1} cm^{-1}; ref. 10) is evident. The $NaHSO_3$ solution used to generate $SO_3^{\bullet -}$ radicals destroyed the native absorption of DBNBS prior to any PR or EPR experiments.

Rate Constants.—As can be imagined from the weak transient absorption changes, determination of rate constants from direct kinetic evaluation of the pseudo-first-order plots could be achieved only with some of the radicals, even though both absorption increases and decreases were checked. Only $^{\bullet}N_3$, $O_2^{\bullet -}$ and $CO_2^{\bullet -}$ gave reasonable plots with the rate constants taken as the slope of the concentration-dependent linear increases in absorption change. Fig. 2 shows the results after evaluating the bleaching reaction at 350 nm for $^{\bullet}N_3$ and 370 nm for $O_2^{\bullet -}$ and $CO_2^{\bullet -}$. To determine the rate constant for the reaction of $^{\bullet}OH$ radicals with DBNBS, we used *p*-NDA as competitor.[11] The results for the inorganic radicals studied are compiled in Table 1.

Unfortunately, *p*-NDA and several other compounds, despite exhibiting high rate constants with the hydroxyalkyl radicals, could not be employed as competitors against DBNBS in solutions containing 0.1 mol dm^{-3} of the alcohols because of chemical interactions prior to the generation of the radicals, or kinetic changes after the initiation of radical reactions. FTMP and its cationic iron-free salt TMPP were examined because of previous success with DMPO (Bors *et al.*, manuscript in preparation), but again were found to be incompatible. However, a similar compound, the anionic porphyrin salt TSPP, was discovered to be ideally suited.

Firstly, DBNBS did not interact with TSPP even at a more than 10-fold excess of the spin trap. Secondly, TSPP has reasonably high rate constants with the hydroxyalkyl radicals (even though the kinetic behaviour is quite complex and not completely resolved). Thirdly, it is the first substance we have encountered that reacts rapidly with $^{\bullet}CH_3$ radicals. We were thus able to determine the rate constants for four of the five alkyl radicals investigated, with $^{\bullet}CH_2(CH_3)_2COH$ derived from *tert*-butyl alcohol being the sole exception. The data are shown in Table 2, together with the pertinent reference rate constants of the competitor employed.

Included in Table 2 is a rate constant for DBNBS + $^{\bullet}CH_3$, which we determined by competition with oxygen, taking advantage of the fact that DBNBS does not seem to form adducts with peroxyl radicals (or that they are too unstable for EPR detection). This value agrees quite well with the one obtained from the competition with TSPP. Unfortunately, this was the only example where we could use this approach, owing to the exceptionally high rate constants and to the highly distinct EPR signal of the DBNBS–CH_3 adduct. The approach proved to be futile with Bu^tOH, for which no other spectroscopically observable reaction is presently available.

EPR Spectra.—Thus far, only a few spectra of DBNBS radical adducts have been published. Therefore, examples of EPR spectra of DBNBS spin adducts examined in this study are shown in Fig. 3. The corresponding hyperfine splitting constants are given in Table 3 for the inorganic radicals and in Table 4 for the alkyl radicals with the additional alkyl hydrogen splitting. Some of the radicals, *e.g.* $CO_2^{\bullet -}$ and $O_2^{\bullet -}$, do not produce spin adducts which are stable enough, so that after PR generation they cannot be observed by EPR spectroscopy. A surprising result was obtained with the hydroxyalkyl radicals derived from ethanol and isopropanol: in both cases only the β-hydroxyalkyl radicals were trapped at neutral pH, even though these are formed by $^{\bullet}OH$ to a much smaller extent than the respective α-hydroxyalkyl radicals.[14] It required strongly acidic solutions (pH < 2; ref. 6) to trap $^{\bullet}CH_2OH$ as well as the α- and β-hydroxyalkyl radicals of ethanol and isopropanol.

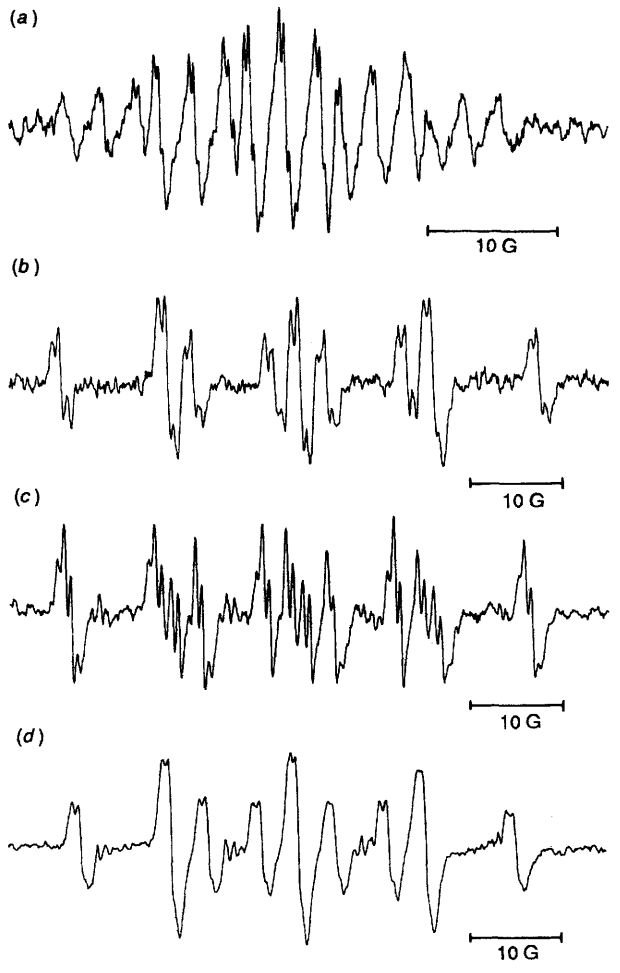

Fig. 3 EPR spectra of spin adducts of alkyl radicals with DBNBS. [DBNBS] = 0.5 mmol dm^{-3}, pH 7.5; for EPR spectrometer settings see Experimental section. (*a*) $^{\bullet}N_3$, (*b*) $^{\bullet}CH_2CH_2OH$, (*c*) $^{\bullet}CH_2C(OH)CH_3$, (*d*) $^{\bullet}CH_2C(OH)(CH_3)_2$.

Table 3 Hyperfine splitting constants of DBNBS spin adducts with inorganic radicals (all radicals generated by pulse radiolysis in aqueous solution)

		Hyperfine splitting constant[a]		
Solute/Gas	Radical	a_N	$a_{H(Ph)}$	Comment
H_2O/N_2O	$^{\bullet}OH$	[12.7	0.6/2]	*b*
N_3^-/N_2O	$^{\bullet}N_3$	2.6 + 6.9/2	0.4/2	*c*
$HCOO^-/N_2O$	$CO_2^{\bullet-}$	—	—	*d*
$HCOO^-/O_2$	$O_2^{\bullet-}$	—	—	*d*
SCN^-/N_2O	$(SCN)_2^{\bullet-}$	[12.7	0.65/2]	*b*

[a] a_N, nitrogen splitting; $a_{H(Ph)}$, splitting of the two equivalent hydrogen atoms in *m*-positions on the benzene ring. [b] Unspecific nine-line signal. [c] Strong signal. [d] No signal.

Discussion

We consider the knowledge of spin-trapping rate constants as essential to the interpretation of EPR spin trap spectra, whether these are obtained in *in vitro* or *in vivo* studies. First of all the rate constants allow an estimate of the probability of individual radicals reacting with the spin trap, which is certainly as important as the knowledge of the stability of the spin adducts. Secondly, they might help to improve the quantitation of signal intensity which is normally given only as a relative value (see also ref. 15).

In the present study we obtained the rate constants for the reactions of a number of radicals with the spin-trapping agent DBNBS, using pulse radiolysis as a specific source of radicals. While it would certainly be preferable to study such reactions with an *in situ* radiolysis/EPR technique, we are aware of only one study where this has been done to any extent.[16] Despite the drawbacks of separate PR and EPR facilities requiring transfer times of 35–45 s, we nevertheless believe that the PR approach to determine rate constants both by direct kinetic evaluation and, if this is impossible, by competition studies, is a valid one, at least as long as *in situ* measurements are unavailable.

Turning to the individual *k* values, it is clear that DBNBS is a highly effective spin trap, reacting with almost all investigated radicals at or near diffusion-controlled limits. The rate constants are quite similar to the corresponding ones found for MNP,[16] with the major exception of $^{\bullet}CH_3$, for which the rate constant with MNP is more than two orders of magnitude lower!

Owing to the different stabilities of the spin adducts, high reaction rate constants do not automatically translate into strong EPR signals. Furthermore, rate constants determined by kinetic spectroscopy of the depletion of a substrate and even more so by competition kinetics in pulse radiolysis do not allow any correlation with possible sites of attack. Only when the same radical forms an EPR-observable adduct, can we be sure that the reaction occurred at the nitroso group of DBNBS. Thus, the fast reactions with $CO_2^{\bullet-}$ and $O_2^{\bullet-}$ can lead either to unstable adducts or to EPR-silent products. While $^{\bullet}OH$ radicals are the only species which might also add to the aromatic ring, the similarity of the nine-line signal with that of $(SCN)_2^{\bullet-}$, and the signals reported by Ozawa and Hanaki[17–19] (see below), rather points to some degradation product. The major drawback of DBNBS is that it cannot be employed to trap oxygen-centred radicals, as was shown by the decrease of the alkyl radical EPR signal with increasing oxygen concentration. In fact this lack of reaction with peroxyl radicals (or the high instability of the peroxyl adducts) could be applied to determine the rate constant with $^{\bullet}CH_3$ by competition with O_2 (see Table 2).

Strong and very distinct EPR signals were observed for the adducts of all alkyl radicals, albeit for $^{\bullet}CH_2OH$ only in acidic solution. The fact that in neutral solution the far less prevalent β-hydroxyalkyl radical of ethanol[14] was trapped, in line with similar species from isopropanol and *tert*-butyl alcohol, points to steric hindrance in the trapping reaction. How this may be affected by the dissociation state of the sulfonic acid group of the DBNBS molecule is presently unclear, yet this is the only dissociable group in the molecule. Limiting the results to biologically more relevant neutral solutions, we lose only $^{\bullet}CH_2OH$ as a trapable species.

Looking at the EPR spectra after spin trapping with DBNBS, we have also to consider the existence of unspecific nine-line signals which we observed both with $(SCN)_2^{\bullet-}$, whose attack did not yield an appreciable rate constant, and after photolytic generation of $^{\bullet}OH$ radicals. Similar signals were reported after reaction of DBNBS with $O_2^{\bullet-}$,[17] with $SO_3^{\bullet-}$[18] and with $S_2O_3^{\bullet-}$.[19] With the $O_2^{\bullet-}$ signal discredited by both Mani and Crouch[20] and Nazhat *et al.*[21] and the $SO_3^{\bullet-}$ results dubious because of the apparent destruction of DBNBS by $NaHSO_3$ in our experiments, we consider this nine-line signal to be the result of some unspecific DBNBS degradation.

Nevertheless, DBNBS is definitely a welcome addition to the growing list of spin trapping agents because of its highly efficient trapping reactions, its distinct EPR signals with alkyl radicals and, last but not least, its good water solubility in comparison to other nitroso spin traps.

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Table 4 Hyperfine splitting constants of DBNBS spin adducts with organic radicals

Solute/Gas	Radical	Hyperfine splitting constant[a]				Comment
		a_N	a_H	$a_{H(Ph)}$	$a_{H\beta}$	
$DMSO/N_2O$	$^{\bullet}CH_3$	14.5	13.5/3	0.7/2	—	*b*
		14.5	13.5/3	0.7/2	—	*c*
$MeOH/N_2O$	$^{\bullet}CH_2OH$	13.6	9.0/2	0.6/2	—	*d*
		13.7	9.2/2	0.61/2	—	*c*
$EtOH/N_2O$	$^{\bullet}CH_2CH_2OH$	14.1	11.2/2	0.6/2	—	*b*
		14.05	11.3/2	—	—	*c*
	$^{\bullet}CH(OH)CH_3$	13.9	9.0	0.6/2	—	*d*
		14.0	9.2/1	0.58/2	—	*c*
Pr^iOH/N_2O	$^{\bullet}CH_2C(OH)CH_3$	14.0	9.7 + 11.3	0.7/2	0.7	*b*
		14.0	9.8 + 11.25	0.55/2	—	*c*
	$^{\bullet}C(OH)(CH_3)_2$	14.04	—	0.7/2	—	*c*
Bu^tOH/N_2O	$^{\bullet}CH_2C(OH)(CH_3)_2$	13.5	9.6/2	0.6/2	—	*e*

[a] a_N, nitrogen splitting; $a_H/a_{H\beta}$, splitting of the aliphatic hydrogen atoms; $a_{H(Ph)}$, splitting of the two equivalent aromatic hydrogen atoms. [b] Strong signal. [c] Average literature value (photolysis of H_2O_2, refs. 1, 5 and 6). [d] Signal only in acidic solution. [e] Weak signal.

References

1 H. Kaur, K. H. Leung and M. J. Perkins, *J. Chem. Soc., Chem. Commun.*, 1981, 142.
2 K. V. Ettinger, A. R. Forrester and C. H. Hunter *Can. J. Chem.*, 1982, **60**, 1549.
3 R. Konaka and S. Sakata, *Chem. Lett.*, 1982, 411.
4 A. Samuni, A. Samuni and H. Swartz, *Free Rad. Biol. Med.*, 1989, **7**, 37.
5 T. Ozawa and A. Hanaki, *Bull. Chem. Soc. Jpn.*, 1987, **60**, 2304.
6 P. Smith and J. S. Robertson, *Can. J. Chem.*, 1988, **66**, 1153.
7 R. F. Pasternack, H. Lee, P. Malek and C. Spencer, *J. Inorg. Nucl. Chem.*, 1977, **39**, 1865.
8 W. Bors, M. Saran, C. Michel and D. Tait, in *Advances on Oxygen Radicals and Radioprotectors*, eds. A. Breccia, C. L. Greenstock and M. Tamba, Lo Scarabeo, Bologna, 1984, p. 13.
9 M. Saran, G. Vetter, M. Erben-Russ, R. Winter, A. Kruse, C. Michel and W. Bors, *Rev. Sci. Instrum.*, 1987, **58**, 363.
10 G. L. Hug, *NSRDS-NBS Report*, 1981, **69**, Nat. Bur. Stand., US Dept Commerce, Washington, DC.
11 W. Bors, C. Michel and M. Saran, in *CRC Handbook of Methods for Oxygen Radical Research*, ed. R. A. Greenwald, CRC Press, Boca Raton, 1985, p. 181.
12 G. V. Buxton, C. L. Greenstock, W. P. Helman and A. B. Ross, *J. Phys. Chem. Ref. Data*, 1988, **17**, 513.
13 P. Neta, R. E. Huie and A. B. Ross, *J. Phys. Chem. Ref. Data*, 1990, **19**, 513.
14 K. D. Asmus, H. Möckel and A. Henglein, *J. Phys. Chem.*, 1973, **77**, 1218.
15 F. P. Sargent, *J. Phys. Chem.*, 1977, **81**, 89.
16 K. P. Madden and H. Taniguchi, *J. Am. Chem. Soc.*, 1991, **113**, 5541.
17 T. Ozawa and A. Hanaki, *Biochem. Biophys. Res. Commun.*, 1986, **136**, 657.
18 T. Ozawa and A. Hanaki, *Biochem. Biophys. Res. Commun.*, 1987, **142**, 410.
19 T. Ozawa and A. Hanaki, *Biochem. Int.*, 1990, **20**, 649.
20 V. Mani and R. K. Crouch, *J. Biochem. Biophys. Meth.*, 1989, **18**, 91.
21 N. B. Nazhat, G. Yang, R. E. Allen, D. R. Blake and P. Jones, *Biochem. Biophys. Res. Commun.*, 1990, **166**, 807.

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