EPR studies of the structure of transient radicals formed in photolytic reactions of some 2-nitrobenzyl compounds. Characterisation of aryl alkoxy aminoxyls and nitroaromatic radical-anions in the photolysis of caged ATP and related compounds

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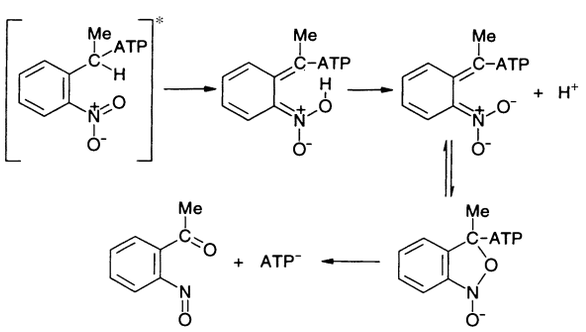
Radical species generated on photolysis of 2-nitrobenzyl compounds, including derivatives of 1-(2-nitrophenyl)ethyl phosphate (caged ATP **1** and caged monomethyl phosphate **3**) have been studied by EPR spectroscopy and their identity confirmed by independent chemical generation of the appropriate nitrogen-centred radicals. The reactions which occur include photo-isomerisation, photo-fragmentation, photo-assisted electron-transfer, intramolecular addition and spin-trapping reactions of the nitroso compounds produced *via* molecular rearrangement. In particular, a mechanistic scheme is advanced to explain the co-formation of a radical-anion and a cyclic aryl alkoxy aminoxyl (**2** and **30**, respectively, from caged ATP). Which of these species is observed in the EPR spectrum depends upon the particular experimental conditions.

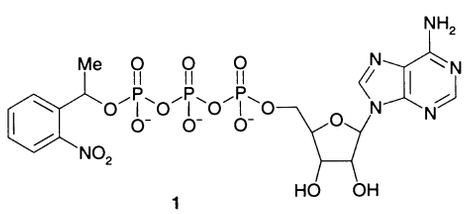
Introduction

Biological compounds that have their activity blocked by attachment of a photolabile protecting group, colloquially termed "caged" compounds, are useful in studies of fast biological processes because photolysis with a brief (ns–ms) pulse of near-UV light allows rapid release of a biologically-active species at its site of action.[1] For example, the P^3-1-(2-nitrophenyl)ethyl ester of adenosine triphosphate (caged ATP) **1** has been used to study the events associated with the contraction of muscle fibres.[2] The photolysis of caged ATP has been investigated in detail[3,4] and the accepted mechanism for fragmentation following photo-excitation is shown in Scheme 1; note

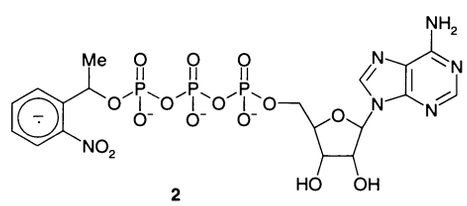


Scheme 1

that no role is postulated for free radicals. Nevertheless, a transient free-radical (half life ~ 1 s at 1.5 °C, pH 8.5) has been detected by EPR spectroscopy during 351 nm laser flash photolysis (LFP) of solutions of **1** and 10 mM dithiothreitol (DTT) in aqueous buffers over the pH range 6–9.[5] This species was estimated to represent *ca.* 10% of the reaction flux under the particular conditions of the laser flash experiments and was provisionally assigned as the radical-anion **2** formed by transfer of one electron, possibly from tertiary amines present as buffer salts, to an excited state of the starting material.[5] There are several other instances in which free-radical mechanisms have been postulated during photolysis of 2-nitrobenzyl derivatives,[6a] although in the best documented example the lifetime was only ~500 ps.[6b]

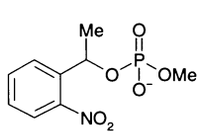


1



2

In order to characterise more clearly the nature of radicals formed during photolysis of **1** and related compounds, and the relevance of these findings to the formation of radicals and radical-anions of 2-nitrobenzyl compounds in a wider context, we have employed EPR spectroscopy in conjunction with continuous photolysis ($\lambda > 250$ nm) of caged ATP **1** and the analogous caged monomethyl phosphate (caged MeP) **3**. In separate experiments, we have generated chemically a range of 2-substituted nitrobenzene radical-anions and aryl alkoxy aminoxyls and recorded their EPR spectra to aid characterisation of the transient species generated by photolysis.



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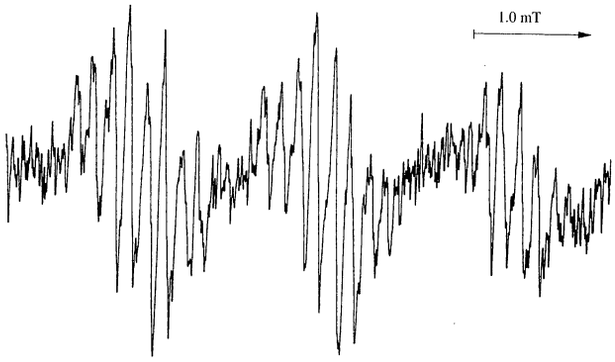


Fig. 1 EPR spectrum obtained on initial photolysis of caged ATP (50 mM) in the presence of DTT (100 mM) and EPPS buffer (200 mM, pH 8.5). The signals are assigned to the caged ATP radical-anion **2**.

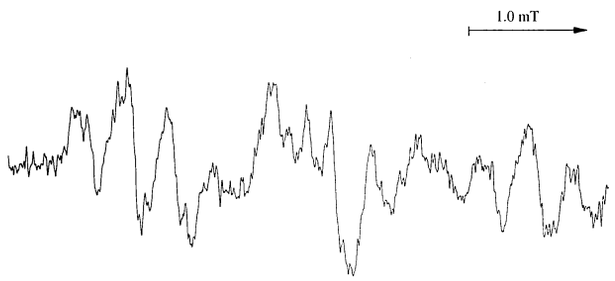


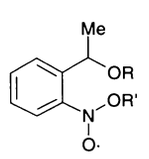
Fig. 2 EPR spectrum obtained on initial photolysis of caged ATP (50 mM) in EPPS buffer (200 mM, pH 8.5). The signals are assigned to the cyclic aryl alkoxy aminoxyl **30**.

Results and discussion

(i) Continuous photolysis of aqueous solutions of caged ATP 1

Initial experiments involved broad band (250–400 nm) irradiation of aqueous solutions of **1** (50 mM) in pH 8.5 EPPS buffer [4-(2-hydroxyethyl)piperazine-1-propanesulfonate, 200 mM], in the presence of dithiothreitol (DTT, 100 mM). The solution conditions were similar to those used in the previous LFP investigation.[5] The clearly resolved EPR spectrum (Fig. 1) had the following parameters: a_N 1.55, a_{2H} 0.29, a_{2H} 0.11 and a_H 0.17 mT, g 2.0047. The experiments described below indicate that the spectrum is that of the radical-anion **2**, whose splittings are typical of a species in which the unpaired electron is delocalised over the nitro group and the aromatic ring [with splittings from the *ortho* and *para* protons (0.29 mT), the α-CH (0.17 mT) and the *meta* protons (0.11 mT)]. When the irradiation ceased, the signals decayed over 1–2 min. There was no evidence of the species observed under LFP conditions (see below).

When the experiments were repeated in the same buffer but without DTT, the spectrum detected at the start of irradiation (see Fig. 2) was different, but similar to that previously seen with LFP (a_N 1.66, a_{2H} 0.33 mT, g 2.0046; improved resolution allowed an extra splitting, a_{2H} 0.10 mT, to be observed). The spectrum is characteristic of another delocalised species in which significant spin density is present on both nitrogen and in the *ortho* and *para* positions of the aromatic ring (a_{2H} 0.33 mT) with a small amount in the *meta* position (a_{2H} 0.10 mT). As shown below, this is assigned to an aryl alkoxy aminoxyl of the general structure **4**; when the irradiation ceased, the signals decayed over 1–2 min. After prolonged irradiation, a third species was detected, with hyperfine splittings a_N 1.16, a_{2H} 0.28 mT, g 2.0056; this is assigned an aminoxyl structure **5**, evidently resulting from a spin-trapping reaction of 2-nitrosoacetophenone (a by-product of photolysis, see Scheme 1). No EPR signals were observed on continuous irradiation of a solution of caged ATP **1**, in the absence of DTT and EPPS buffer.



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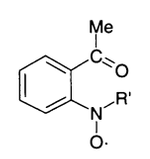
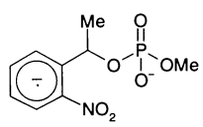


5

(ii) Continuous photolysis of aqueous solutions of caged MeP 3

Experiments similar to those described above were carried out using the model compound **3**. Photolysis of **3** (50 mM in 100 mM EPPS, pH 8.5) in the presence of DTT (100 mM) gave signals similar to those observed with caged ATP (a_N 1.55, a_{2H} 0.29, a_{2H} 0.11, a_H 0.17 mT, g 2.0047) and the spectrum is again assigned to a radical-anion, **6**. When irradiation ceased, the EPR signals decayed over a period of 1–2 min. The behaviour on irradiation in the absence of DTT, but in the presence of EPPS buffer was similar to that observed for caged ATP **1**: a spectrum with a_N 1.66, a_{2H} 0.33, a_{2H} 0.10 mT, g 2.0046 was initially observed and prolonged irradiation gave an aminoxyl radical with a_N 1.16, a_{2H} 0.28 mT, g 2.0056. No EPR signals were observed on irradiation of a solution of caged MeP **3**, in the absence of DTT and EPPS buffer.



6

(iii) Chemical generation of some 2-substituted nitrobenzene radical-anions

As the radical-anions of the caged compounds are possible candidates for the species observed in the photolysis experiments described above, we sought to generate these species independently. EPR spectra of many *para*-substituted nitroarene radical-anions have been studied and the splitting patterns are well established;[7,8] however, relatively few examples of *ortho*-substituted species have been reported.[9] One method of generation, appropriate for aqueous solutions, involves reduction of the parent compounds by sodium dithionite in strongly alkaline solution (pH ~ 13).[7] We employed a static method of radical generation (rather than the continuous-flow system used previously)[7] to minimise the amount of material required. This method gave strong signals from the appropriate radical-anion; the spectra had extremely narrow line-widths (typically < 0.01 mT), largely because oxygen (which can cause line-broadening) is scavenged by excess dithionite. The radical-anions generated in this way typically persisted for 5 to 15 min. A typical EPR spectrum of the nitroaromatic radical-anions is shown in Fig. 3 and parameters of the radical-anions of substrates **1**, **3** and **7**–**20** are in Table 1. The spectra have a large nitrogen splitting (1.4–1.6 mT), splittings in the range 0.28–0.34 mT from the *ortho* and *para* protons and smaller splittings from the *meta* hydrogens (*ca.* 0.1 mT) on the aromatic ring. These splittings characterise species with significant unpaired electron density on the aromatic ring. Additional small splittings (0.12–0.27 mT) are detected from the α-protons of the *ortho*-substituent; in some cases, other magnetic nuclei and more remote protons on the *ortho*-substituent give additional splittings. These findings confirm that continuous photolysis of the caged compounds **1** and **3** in the presence of both DTT and EPPS buffer gives the appropriate radical-anions.

Several features of the EPR spectra of this diverse group of *ortho*-substituted nitrobenzene radical-anions are worthy of comment. A number showed signs of anisotropy of the nitrogen hyperfine splitting as a distinctive broadening of the higher field multiplet. This was most pronounced for the larger molecules such as the caged ATP radical-anion **2**, as expected for the slower tumbling rates of the larger molecules. There is also a

Table 1 EPR splittings (mT)[a] for nitrobenzyl radical anions (in 4 : 1 aqueous 0.5 M NaOH–acetone unless indicated otherwise)

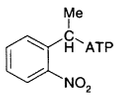
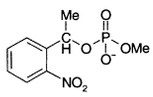
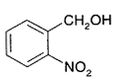
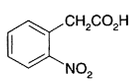
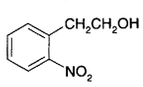
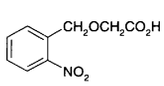
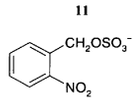
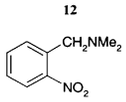
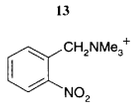
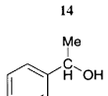
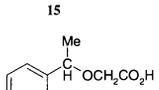
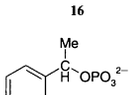
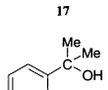
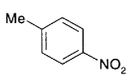
Parent compound	a_N	a_o	a_m	a_p	a_{other}	
 **1**	1.552[b]	0.287[c]	0.108 (1H) 0.116 (1H)	0.294[c]	CH	0.172
 **3**	1.531, (1.578[b])	0.278	0.103 (1H) 0.120 (1H)	0.278	CH Me	0.141 0.017
 **7**	1.594	0.295	0.098 (1H) 0.122 (1H)	0.295	Me	0.246
 **8**	1.430	0.334[c]	0.109 (1H) 0.120 (1H)	0.346[c]	CH_2	0.228
 **9**	1.504	0.313[c]	0.102 (1H) 0.119 (1H)	0.318[c]	CH_2	0.172
 **10**	1.592	0.290[c]	0.098 (1H) 0.120 (1H)	0.299[c]	CH_2	0.133
 **11**	1.470	0.320[c]	0.107 (1H) 0.120 (1H)	0.326[c]	CH_2	0.262
 **12**	1.434, (1.468[b])	0.320[c]	0.114 (1H) 0.118 (1H)	0.324[c]	CH_2	0.268
 **13**	1.601	0.280	0.092 (1H) 0.127 (1H)	0.280	CH_2 N	0.145 0.078
 **14**	1.374, (1.396[b])	0.310	0.116	0.310	CH_2 N	0.156 0.104
 **15**	1.530	0.305[c]	0.106 (1H) 0.121 (1H)	0.311[c]	CH Me	0.148 0.012
 **16**	1.547	0.294	0.115 (1H) 0.117 (1H)	0.294	CH Me	0.12 0.019
 **17**	1.619[b]	0.278	0.102 (1H) 0.120 (1H)	0.278	CH Me	0.141 ~0.01
 **18**	1.639	0.277	0.096 (1H) 0.133 (1H)	0.277	—	—

Table 1 (*Contd*)

Parent compound	a_N	a_o	a_m	a_p	a_{other}	
 **19**	1.405	0.338[c]	0.111	0.361[c]	Me	0.111
 **20**	1.407	0.340	0.115	—	Me	0.380

[a] ± 0.002 mT. [b] a_N measured in aqueous solution instead of in 4 : 1 water–acetone. Where spectra were run in both solvents no significant differences were observed for the other splittings. [c] *ortho* and *para* splittings may be reversed.

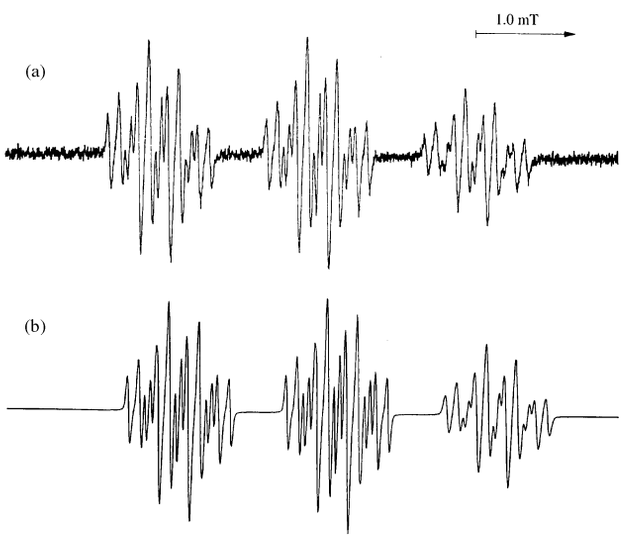
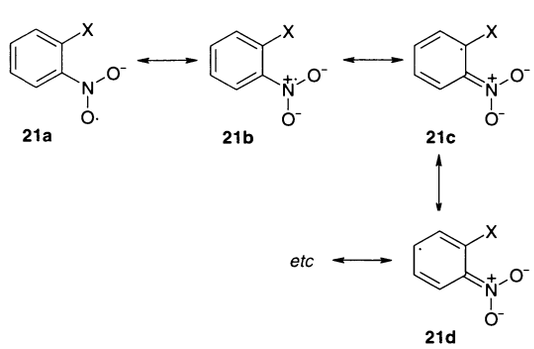


Fig. 3 (a) EPR spectrum of the caged ATP radical-anion **2** formed by reduction of the parent compound **1** (20 mM) by $Na_2S_2O_4$ (40 mM) in basic solution (0.5 M NaOH). (b) EPR spectrum simulation using parameters given in Table 1.

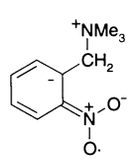
noticeable solvent dependence of the nitrogen splitting which is significantly higher in water than in aqueous acetone [see, for example, those of caged MeP **3** and 2-nitrobenzyl sulfate **12**]. This clearly reflects the increasing importance of the more charged canonical structure **21b** in the more polar solvent. Similar correlation of the nitrogen hyperfine splitting with solvent polarity has been reported for other nitroarene radical-anions.[10]



21a **21b** **21c** **21d**

In analysing the potential steric and electronic effects of *ortho*-substituents, it is helpful to compare the spectra of the *ortho*-nitroaromatic radical-anions described above with those of the radical-anions derived from 3- and 4-methylnitrobenzene, **19** and **20**, respectively (see Table 1). Introduction of the *ortho*-substituent generally leads to an increase in the nitrogen splitting with a concomitant decrease in the splittings from the ring protons, indicating that there is more spin-density on nitrogen and less on the aromatic ring in the 2-substituted analogue. This is attributed to a twist of the nitro group out of the plane of the aromatic ring with a reduction of overlap between the two moieties (*i.e.* steric inhibition of resonance).[11]

On the basis of the observed parameters, the radical-anions fall into three main categories: those which have a nitrogen splitting $a_N \sim 1.4$ mT, relatively close to that of 4-methylnitrobenzene (nitro group in-plane) (**8** and **14**), those with a nitrogen splitting close to that of 2-methylnitrobenzene $a_N \sim 1.6$ mT (nitro group twisted out of plane) (**10** and **13**), and those which fall between these two extremes (**9**, **11** and **12**). For **8**, from the first category, we believe that intramolecular hydrogen-bonding between the alcohol and nitro groups stabilises the nitro group in-plane, and for **14** we propose that the strongly electron-withdrawing ($-I$) trimethylammonium group favours delocalisation of the negative charge and the unpaired electron onto the ring, encouraging planarity (see resonance structure **22**). In the absence of such stabilisation, as in **10** and **13**, the *ortho*-substituent causes the nitro group partly to twist out of plane. In the intermediate cases (**9**, **11** and **12**) which all have electron-withdrawing groups, we propose that the $-I$ effect of the substituent again favours some delocalisation and partially compensates for steric destabilisation. The influence of the inductive effect of the group would be expected to be greatest when the C–X bond is perpendicular to the plane of the aromatic ring thereby maximising the SOMO–σ* interaction **23**. This would reduce overlap of the SOMO with the α-protons, leading to particularly small splittings from these protons (the splitting depends upon the angle θ between the SOMO and the C–H bond with a $\cos^2\theta$ relationship, hence a large angle gives a small splitting).[12] This is particularly noticeable in the radical-anions of **9**, **10**, **13** and **14**.



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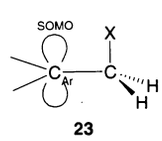


23

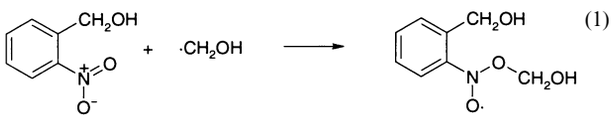
For compounds **1**, **3** and **15**–**17**, where one of the α-benzylic protons has been replaced by a methyl group, the increase in steric bulk also evidently leads to increased twisting and hence to an increased nitrogen splitting. Effects similar to those described above still influence the extent of twisting, as seen in the variations in both the nitrogen and proton splittings. The particularly low α-CH splittings in all these radicals presumably indicate a geometry of the substituent with this proton held close to the plane of the aromatic ring to minimise steric hindrance. Dimethylation of the α-carbon in **18** leads to a further increase in nitrogen splitting due to increased twisting of the nitro group out of plane.[11]

In the context of identifying the species formed on LFP of caged ATP or under continuous irradiation in the absence of DTT (see above),[5] the principal significance of these results is

that the authentic radical-anions have a nitrogen splitting less than that observed (1.66 mT) for the photolytically-generated species. For example, the nitrogen splitting for the radical-anion of caged ATP is 1.552 mT. We considered instead the possibility that the photolytically-generated species with higher a_N is an aryl alkoxy aminoxyl of general structure ArN(O$^{\bullet}$)OR (see below).

(iv) Chemical generation of aryl alkoxy aminoxyls

Aryl alkoxy aminoxyls can be generated by addition of alkyl radicals to nitroaromatics [*e.g.* reaction (1)] and a number have



(1)

8 **24**

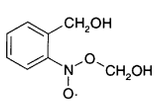
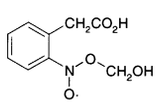
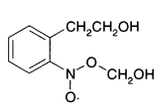
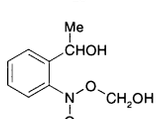
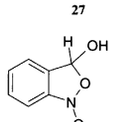
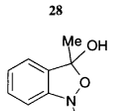
been formed previously by pulse-radiolysis[8] or photolysis[13,14] of nitroaromatics in alcohol or ether solutions. We attempted to generate these radicals by similar routes, firstly by the addition of alkyl radicals to nitroaromatics and secondly, by intramolecular cyclisation of *o*-nitrobenzyl radicals. Our approach involved the use of a three-stream continuous-flow system with the titanium(III)–hydrogen peroxide couple in the presence of a suitable substrate (in excess) and the nitroaromatic. Titanium(III) reacts with hydrogen peroxide to generate the hydroxyl radical [reaction (2)] which then can react with the substrate to generate a suitable alkyl radical [*e.g.* reaction (3)];[15] addition of this radical to the nitro group gives the aryl alkoxy aminoxyl radical.

$$Ti^{III} + H_2O_2 \longrightarrow Ti^{IV} + HO^{\bullet} + HO^{-} \qquad (2)$$

$$HO^{\bullet} + CH_3OH \longrightarrow H_2O + {}^{\bullet}CH_2OH \qquad (3)$$

A limitation of the flow technique is that it requires quite large quantities of nitroaromatic and for practical reasons our study of the reaction with $^{\bullet}CH_2OH$ (from MeOH) was restricted to compounds **8**, **9**, **10** and **15**. (These have adequate water solubility, and **15** is a model for the photolabile portion of caged ATP.) Reaction of $^{\bullet}CH_2OH$ with the nitroaromatics in this way gave spectra assigned to the appropriate aryl alkoxy aminoxyl adducts (**24**–**27**, respectively, see Table 2 and Fig. 4) with significantly higher values of a_N (1.71–1.96 mT) than the nitroaromatic radical-anions; the remaining splittings are again characteristic of delocalisation of the unpaired electron onto the aromatic ring. Small splittings from the OCH_2O protons are also observed in some cases. Similar aryl alkoxy aminoxyls have been reported previously for the addition of α-hydroxyalkyl radicals to *para*-substituted nitrobenzenes.[8] When the experiments were repeated with the α-hydroxybenzyl radical (PhC$^{\bullet}$HOH, generated from reaction of HO$^{\bullet}$ with benzyl alcohol in acidic conditions)[16] nitroaromatic radical-anions were observed rather than adducts. We presume that initial addition to form the aryl alkoxy aminoxyl is followed by a rapid fragmentation to yield the nitro radical-anion and a carbonyl compound [see *e.g.* reaction (4)]. Similar rapid fragmentations have been reported[8] for the adducts of 2-hydroxy-2-propyl radicals to nitroaromatics.

Table 2 EPR splittings (mT)[a] for aryl alkoxy aminoxyls

Aryl alkoxy aminoxyl	a_N	a_o	a_m	a_p	a_{other}	
 **24**	1.725	0.315	0.106	0.315	CH_2 OCH_2	0.203 0.048
 **25**	1.710	0.313	0.104	0.313	CH_2 OCH_2	0.245 0.052
 **26**	1.780	0.315	0.128	0.315	CH_2 OCH_2	0.256 0.046
 **27**	1.965	0.207	0.096	0.207	CH	0.241
 **28**	1.657	0.315	0.109	0.315	CH	0.544
 **29**	1.640	0.338	0.109	0.338	—	—
30	1.66	0.33	0.10	0.33	—	—
31	1.66	0.33	0.10	0.33	—	—

[a] ± 0.002 mT.

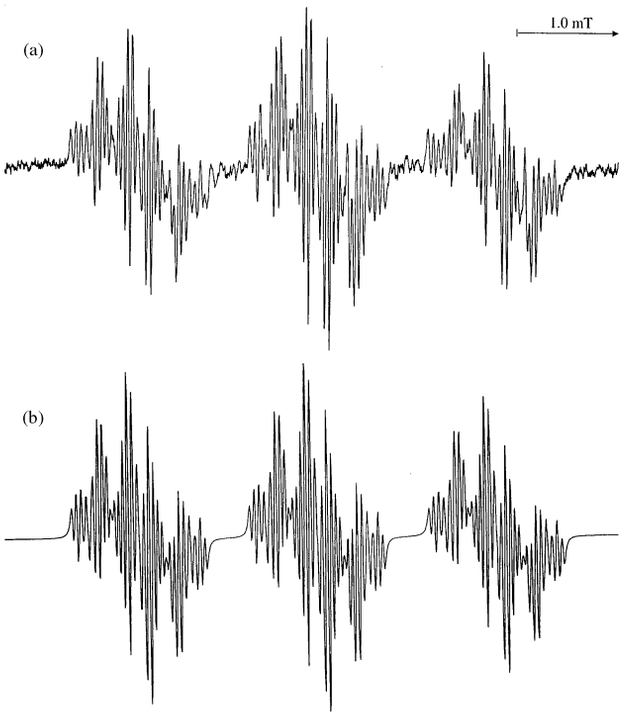
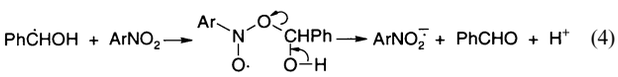
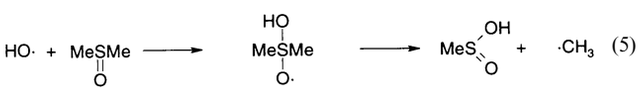


Fig. 4 (a) EPR spectrum of aryl alkoxy aminoxyl **24** obtained on reaction of methanol (1.7% v/v) with titanium(III) (1.67 mM) and hydrogen peroxide (8.3 mM) in the presence of 2-nitrobenzyl alcohol (3.3 mM). (b) EPR spectrum simulation using parameters given in Table 2.



When DMSO was used as the substrate to generate the methyl radical [reaction (5)], the only signals observed were



those of the methyl radical itself. Simple alkyl radicals evidently do not add sufficiently rapidly to the nitroaromatics, in contrast to the behaviour of α-hydroxyalkyl radicals. We have observed a similar selectivity for the related reaction of radical addition to nitroalkanes,[17] a phenomenon understandable in terms of an accelerating effect of +M substituents.

The nitrogen hyperfine splittings of the acyclic alkoxy aminoxyls **24**–**27** (a_N 1.965 mT for **27**) are somewhat higher than for the species seen in the photolysis experiments with caged ATP. We therefore considered the possibility that one of the species observed from caged ATP arises by intramolecular cyclisation onto the nitro group. Hence, the direct reaction of the hydroxyl radical with 2-nitrobenzyl alcohol and 1-(2-nitrophenyl)ethanol was studied. These compounds would be expected initially to produce the substituted benzyl radicals required under the acidic reaction conditions.† 2-Nitrobenzyl alcohol gave a spectrum with a_N 1.657, a_{olp} 0.350, a_{plo} 0.315, $a_{m(2H)}$ 0.109 and a_{1H} 0.544 mT, g 2.0047 that we assign to an alkoxy aminoxyl. The unusually large proton splitting (0.544 mT) is assigned to the α-proton. We propose that the signals are from the cyclic species **28** that arises from intramolecular cyclisation of the initial α-hydroxybenzyl radical onto the oxygen of the nitro group [reaction (6)] (see refs. 18 and 19). Reaction of 1-(2-nitrophenyl)ethanol under similar conditions gave a spectrum (assigned to **29**) with comparable nitrogen and ring-proton splittings (a_N 1.64, a_{olp} 0.338 and a_m 0.109 mT). Identical but more intense signals (see Fig. 5) were obtained by replacing the hydrogen peroxide with sodium persulfate [with a catalytic amount of copper(II)]. The redox couple Cu(I)–$S_2O_8^{2-}$ generates the sulfate radical-anion (rather than HO•) which reacts with the substrate to give an α-hydroxybenzyl radical (*via* an aromatic radical-cation intermediate).[16]

† Addition of the hydroxyl radical to the aromatic ring generates cyclohexadienyl species which undergo rapid acid-catalysed dehydration to form the appropriate benzylic radical.[13]

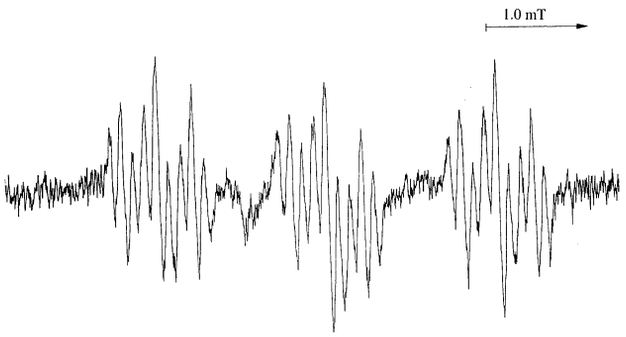
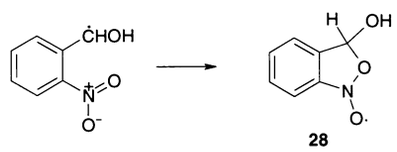
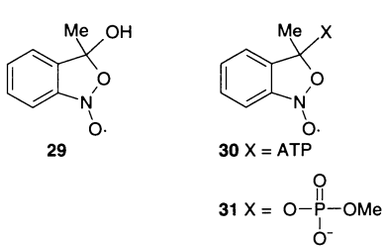


Fig. 5 EPR spectrum obtained on reaction of 1-(2-nitrophenyl)-ethanol **15** (8 mM) with titanium(III) (1.67 mM), sodium persulfate (8.3 mM) and copper(II) sulfate (6.1 μM). The signals are assigned to the cyclic aryl alkoxy aminoxyl.



The cyclic species generated *via* intramolecular addition (**28** and **29**) have nitrogen and proton splittings very close to those observed on photolysis of caged ATP or caged MeP in the laser flash experiments[5] and in the continuous photolysis experiments carried out in buffer without DTT. This strongly supports our assignment of the EPR spectra observed in these photolysis experiments and the LFP experiments[5] to cyclic aryl alkoxy aminoxyls (**30** and **31**).



(v) Related experiments

To obtain a clearer understanding of the processes which lead to radical formation on photolysis of caged compounds (nitroaromatic radical-anions, alkoxy aminoxyls and aryl aminoxyls), we carried out photolysis experiments on 2-nitrobenzyl alcohol **8** and 1-(2-nitrophenyl)ethanol **15**. 4-Nitrobenzyl alcohol was also studied to investigate the importance of the substituent's position.

Photolysis of saturated solutions of **8** and **15** in water gave strong signals from aminoxyls within 1 minute of photolysis, which grew in intensity. In each case, the EPR spectrum showed a large proton splitting (slightly larger in magnitude than the aminoxyl nitrogen splitting) characteristic of monoaryl aminoxyls [ArN(O•)H, **32** and **33**, see Table 3]. Similar species have been detected in the photolysis of 2-nitrobenzaldehyde.[20] Photolysis in EPPS buffer (100 mM, pH 8.5) gave similar signals though of lower intensity (Fig. 6). Photolysis of **8** or **15** in the presence of both EPPS buffer (100 mM, pH 8.5) and DTT (50 mM) gave only an extremely weak EPR spectrum.

Table 3 EPR splittings (mT)[a] for monoaryl aminoxyls generated on photolysis of nitrobenzyl alcohols

Radical	a_N	$a_{\alpha\text{-H}}$	a_{olp}	a_m
32	0.905	1.255	0.320 (2H)	0.115 (2H)
33	1.045	1.325	0.335 (2H)	0.120 (2H)
34[b]	0.924	1.231	0.322 (1H) 0.297 (1H)	0.111 (2H)
	0.971	1.268	0.323 (1H) 0.303 (1H)	0.112 (2H)

[a] ± 0.005 mT. [b] Individual assignment to *cis*- and *trans*-rotamers was not possible.

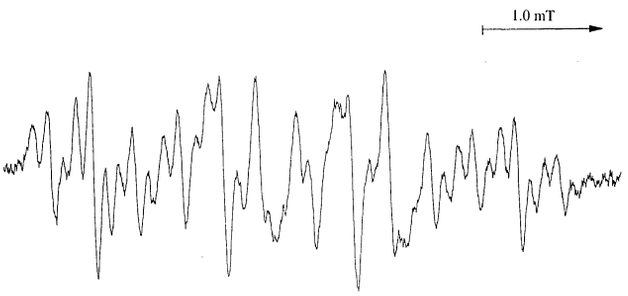
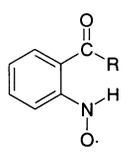


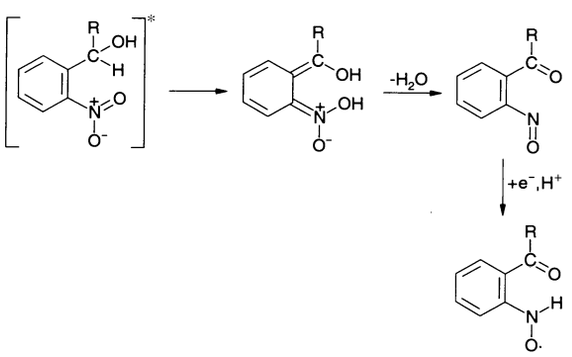
Fig. 6 EPR spectrum obtained on photolysis of 1-(2-nitrophenyl)-ethanol **15** (saturated solution) in EPPS buffer (100 mM, pH 8.5). Signals are assigned to the monoaryl aminoxyl **33**.



32 R = H
33 R = Me

This spectrum appears to differ from that observed in the absence of DTT and may be due to the radical-anion of the starting material but precise assignment is not possible.

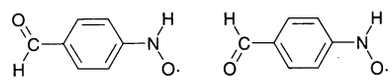
The mechanism of photo-reaction in the absence of DTT evidently involves rapid rearrangement of **8** and **15** to give the corresponding nitroso carbonyl compound; subsequent photo-reduction gives the monoaryl aminoxyls detected (see Scheme 2). The latter step may involve hydrogen-atom transfer



Scheme 2

from the alcohol (presumably *via* radicals of the type RC$^{\bullet}$HOH, *cf*. ref. 14). In the presence of DTT, the nitroso group would be rapidly reduced.[4b]

Photolysis of 4-nitrobenzyl alcohol in water led (within 1 minute) to a complex spectrum of two species with very similar splittings and *g*-values, characteristic of two radicals which vary only in conformation. Both radicals have a relatively small nitrogen splitting and a large proton splitting as well as splittings from two (inequivalent) *ortho* protons and two *meta* protons (see Table 3). They are assigned to the rotamers **34a** and **b**. Generation of the monoaryl aminoxyls is believed to occur *via* a mechanism similar to that of the *ortho*-nitro compounds; photolysis leads to 4-nitrobenzaldehyde[21] which is reduced in a second photo-catalysed step to the monoaryl aminoxyl.



34a **34b**

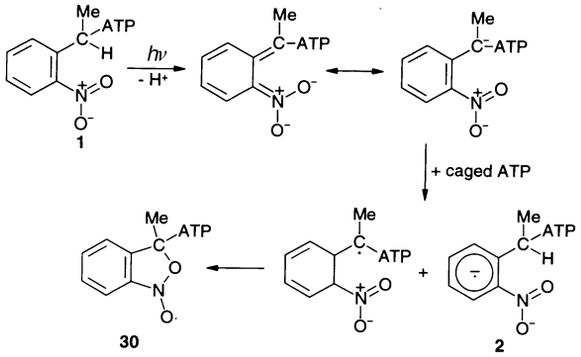
Photolysis of 4-nitrobenzyl alcohol in the presence of EPPS (100 mM, pH 8.5) gave strong signals from the 4-nitrobenzyl alcohol radical-anion; in the presence of DTT signals from the nitro radical-anion were even more intense. Under these conditions, effective electron-transfer occurs from EPPS and/or DTT to the nitroaromatic to generate the radical-anion, which is relatively long-lived. This mechanism is clearly more facile than the intermolecular hydrogen-atom transfer involving another molecule of the substrate. In contrast, for the *ortho*-nitrobenzyl alcohols, intramolecular rearrangement is favoured (Scheme 2); in addition, we note that the *ortho*-substituted radical-anions are shorter lived.

Conclusions

EPR spectroscopy allows different nitrogen-centred radicals to be distinguished and this study aimed to identify radicals observed during photolysis of aromatic 2-nitrobenzyl compounds and specifically of caged compounds: these species are nitroaromatic radical-anions, aryl alkoxy aminoxyls [ArN(O$^{\bullet}$)OR] and aminoxyls [ArN(O$^{\bullet}$)H] that arise by further reaction of nitroso by-products of photolysis. Assignments have been made by comparison with spectra from chemically-generated species. Particularly detailed structural information has been obtained for the radical-anions of *ortho*-substituted nitrobenzenes, including some caged compounds. The results allow assignment of the species generated during photolysis, and on this basis we propose a mechanistic rationale.

At the commencement of continuous photolysis of caged ATP **1**, spectra are dominated by a species assignable to the radical-anion **2** (for photolysis in the presence of a thiol) and to the cyclic aryl alkoxy aminoxyl **30** (formed in the absence of a thiol). The caged methyl phosphate **3** behaved identically. On prolonged irradiation in the absence of thiol, both compounds gave spectra consistent with an aminoxyl such as **33**. In previous work,[5] the species formed by laser flash photolysis of **1** in the presence of thiol had been assigned as the radical-anion **2**, but our results now show that it is the aminoxyl **30**.

These observations can be explained and unified as follows. The generally accepted photolytic process initially generates an anion which, although represented in its *aci*-nitro form, can also be regarded as a benzyl anion since the two are simply different canonical forms (Scheme 1). Simple 2- or 4-nitrobenzyl anions are known to effect single-electron transfer to a second molecule of the parent nitro compound, generating a benzyl radical and a radical-anion.[22] Thus if the benzyl anion generated by photolysis of, for example **1**, undergoes the same reaction, it will produce the radical-anion **2** and a benzyl radical that can cyclise rapidly to the alkoxy aminoxyl **30**. These steps are shown in Scheme 3. We expect that the radical-anion **2** has a shorter lifetime than the cyclic alkoxy aminoxyl **30** under these conditions (pH 8.5); hence in a situation where both are formed at equal rates, the spectrum should be dominated by the alkoxy aminoxyl. Thiols are known to reduce radicals rapidly by hydrogen-atom donation and can also act as electron donors. Inclusion of DTT would be expected to reduce either the cyclic alkoxy aminoxyl or its benzylic radical precursor to non-



Scheme 3

radical products; the nitroaromatic radical-anion would still be present. It is also feasible that the DTT reacts directly with the excited state of caged ATP by electron transfer, again generating the radical-anion **2** (a reaction noted for 4-nitrobenzyl alcohol).

There is nevertheless the apparent contradiction that the cyclic alkoxy nitroxide **30** was observed under LFP conditions in the presence of 10 mM DTT[5] but is only seen in the continuous photolysis experiments when DTT is absent. However, the thiol concentration in the latter experiments was much higher (100 mM DTT) so the lifetime of **30** under these conditions would be much reduced, especially as the continuous photolysis experiments were performed at ambient temperature whereas the LFP data were obtained at 1.5 °C. Further, the continuous photolysis experiment is less well-defined, since it has a constantly changing mix of species as starting material is consumed (and secondary photolysis can also occur), unlike the LFP experiment in which there is only a single exposure to the light flash.

It should be noted that, since **1** and **3** give the same results in continuous irradiation experiments, the purine ring of **1** cannot be implicated in formation of the observed species. Further, there is apparently no specific role for the amine buffer salts which is in accord with previous LFP experiments where the species **30** was formed in similar yield in sodium phosphate buffer as well as in amine buffers.[5] We note that no radical species was observed upon continuous photolysis of caged ATP **1** in aqueous solution that contained neither buffer salts nor DTT. We suggest that, under these conditions, ionisation of the nitronic acid initially formed (Scheme 1, $pK_a \sim 3.7$)[23] is insufficient to provide a high enough concentration of its conjugate base to promote the reactions leading to the radical pathway (Scheme 3). We also note that previous LFP data[5] for photolysis of 10 mM caged ATP indicate that the radical pathway represents a minor proportion (approx. 10%) of the reaction flux. The possibility of damage to biological systems by these radical species should nevertheless be borne in mind during experiments with caged compounds.

Experimental

General

Analyses were performed by MEDAC Ltd, Egham, Surrey. EPR spectra were obtained on a Bruker ESP 300 X-band spectrometer equipped with 100 kHz modulation. NMR spectra were determined on a JEOL FX90Q spectrometer with tetramethylsilane as internal standard for solutions in deuteriochloroform, unless otherwise specified. *J* Values are given in Hz. High resolution FAB mass spectra were obtained on a VG ZAB-SE instrument. Flash chromatography was performed on Merck 9385 silica gel. Light petroleum describes the fraction boiling between 40–60 °C. Organic extracts were dried over Na_2SO_4 and evaporated under reduced pressure. DEAE-cellulose was from Sigma. Triethylammonium bicarbonate buffer (TEAB) was prepared by bubbling CO_2 into a 1 M solution of redistilled triethylamine in water at 4 °C until the pH stabilised at ~7.4. All buffer solutions were prepared from solutions of the specified acids at the indicated molarities and adjusted to the specified pH with concentrated NaOH solution.

Caged ATP **1** (sodium salt),[3] caged MeP **3** (sodium salt),[24] *N*,*N*-dimethyl-2-nitrobenzylamine **13**,[25] 1-(2-nitrophenyl)ethanol **15**,[26] caged phosphate **17**[26] and 2-(2-nitrophenyl)propan-2-ol **18**[27] were prepared as described previously. Compounds **7**–**10** were obtained from Aldrich.

(2-Nitrobenzyloxy)acetic acid 11

A solution of 40% tetra-*n*-butylammonium hydroxide (2.1 ml, 3.23 mmol) in 50% aq. NaOH (12.5 ml, 156 mmol) was added at 10 °C to a solution of 2-nitrobenzyl alcohol (1.96 g, 12.8 mmol) in benzene (25 ml) and the mixture was stirred vigorously for 15 min. *tert*-Butyl bromoacetate (3.75 g, 19.2 mmol) was added dropwise rapidly and the mixture was stirred vigorously for 30 min at 10 °C, then diluted with H_2O (25 ml) and light petroleum (50 ml). The aqueous phase was adjusted to pH 6.5 and stirred for 10 min and the organic phase was separated, washed with H_2O and brine, dried and evaporated to give a brownish oil that was dissolved in TFA (25 ml) and kept at room temperature for 1 h. The TFA was evaporated and the solid residue was dissolved in Et_2O and extracted with 1 M aq. NaOH. The aqueous layer was acidified and extracted with Et_2O, and the extract was dried and evaporated. The residue was recrystallised ($CHCl_3$–light petroleum) to yield the acid **11** (1.44 g, 53%), mp 141.5–142.5 °C (lit.[28] 140–141 °C).

2-Nitrobenzyl sulfate 12

This compound was prepared by the general method of Tserng and Klein.[29] A solution of 2-nitrobenzyl alcohol (153 mg, 1 mmol) in dry DMF (2 ml) was treated with SO_3–Et_3N (200 mg, 1.1 mmol), stirred for 1 h at room temperature and diluted with water to 100 ml. The solution was adjusted to pH 7 and chromatographed on a column of DEAE-cellulose (2 × 30 cm) using a linear gradient of TEAB (10–200 mM, total volume 2000 ml). Fractions were analysed by anion-exchange HPLC (Whatman SAX column, Cat. No. 4621–0505) with a mobile phase of 10 mM sodium phosphate, pH 5.5 plus 3% MeOH (v/v) at 1.5 ml min^{-1}. Retention times for 2-nitrobenzyl alcohol and the sulfate **12** were 1.2 and 3.8 min, respectively. Fractions containing the sulfate were combined and evaporated *in vacuo*, then re-evaporated from MeOH to remove residual triethylamine. For final purification, the recovered material was subjected to preparative reverse-phase chromatography (2 × 30 cm, Waters C_{18} packing, Cat. No. 20594). The column was pre-equilibrated in 20 mM Na phosphate, pH 5.5 and washed with this buffer (~200 ml) after loading. The eluant was then changed to distilled water and the compound eluted when the conductivity of the emerging mobile phase fell to that of water. The recovered material was lyophilised and the residue was dissolved in water (7 ml) to give a 110 mM solution of the sulfate **12** as its sodium salt (based on ε_{265} 5300 M^{-1} cm^{-1} for 2-nitrobenzyl alcohol). The compound had δ_H (D_2O, acetone ref.) 8.16 (1 H, d, *J* 8.3, H-6), 7.51–7.82 (3 H, m, Ar-H), 5.42 (2 H, s, CH_2O). Found (negative ion electrospray MS): M^- 232. $C_7H_6NO_6S$ requires *m*/*z* 232.

(2-Nitrobenzyl)trimethylammonium chloride 14

A solution of *N*,*N*-dimethyl-2-nitrobenzylamine **13** (180 mg, 1 mmol) in MeOH (5 ml) was treated with methyl iodide (0.31 ml, 5 mmol) and refluxed for 2 h. The solvent was evaporated and the residue was dissolved in water (5 ml) and passed through a column of Dowex 1 (Cl form: 1 × 10 cm). The eluate was lyophilised and redissolved in water (5 ml) to give a 191 mM solution of the salt **14** (based on ε_{265} 5300 M^{-1} cm^{-1} for

2-nitrobenzyl alcohol). The compound had δ_H (D_2O, acetone ref.) 8.20–8.32 (1 H, m, H-6), 7.73–7.96 (3 H, m, Ar-H), 5.01 (2 H, s, CH_2N), 3.17 (9 H, s, Me).

[1-(2-Nitrophenyl)ethoxy]acetic acid 16

This compound was prepared from 1-(2-nitrophenyl)ethanol as described for the acid **11**. Yield 52%, mp 119–120.5 °C ($CHCl_3$–light petroleum) (Found: C, 53.3; H, 5.0; N, 6.2. $C_{10}H_{11}NO_5$ requires C, 53.3; H, 4.9; N, 6.2%), δ_H 7.3–8.0 (4 H, m, Ar-H), 5.18 (1 H, q, *J* 6.2, CH), 4.00 (2 H, s, CH_2), 1.60 (3 H, d, Me).

EPR experimental protocols

All solutions were deoxygenated prior to use by purging with oxygen-free nitrogen for at least 10 min.

Photolysis of caged ATP, caged MeP and nitrobenzyl alcohols. Photolysis experiments were carried out using a 300 W Xenon arc lamp equipped with a 250–400 nm filter. In a typical experiment, an aqueous solution of the nitro compound (50 mM), EPPS buffer (200 mM) and, in some cases, DTT (100 mM) was photolysed for 40 s. EPR spectra were recorded both during and after cessation of photolysis.

Generation of nitroaromatic radical-anions. In a typical experiment, the nitroaromatic compound (2×10^{-5} mol) was dissolved in acetone (0.2 ml) and was mixed with 200 mM sodium dithionite in 0.5 M NaOH (0.2 ml). The solution was made up to a final volume of 1 ml by addition of sodium hydroxide solution (0.5 M), final pH *ca.* 13. The resultant solution was placed in a quartz aqueous EPR flat-cell. The recording of the EPR spectrum was commenced 1 min after mixing.

Generation of aryl alkoxy aminoxyls. Generation of aryl alkoxy aminoxyls utilised a continuous flow apparatus[13] which allows simultaneous mixing of three streams approximately 30 ms prior to passage through a quartz aqueous flow flat-cell positioned in the EPR cavity. For $HO^{\bullet}$ generation the three solutions‡ contained Ti^{III} [1.67 mM, added as titanium(III) chloride solution], hydrogen peroxide (8.33 mM) and the substrates [*e.g.* methanol (2% v/v) and the nitroaromatic (3.33 mM)], respectively. For the sulfate radical-anion studies the hydrogen peroxide solution was replaced by a solution of sodium persulfate (8.33 mM) and copper(II) sulfate (6.1 μM) was added to the substrate stream. The flow rate, typically 40 ml s^{-1}, was maintained with a Watson–Marlow 502s peristaltic pump, positioned on the inlet tubes. All experiments were carried out at pH ~ 2.

‡ All concentrations are those after mixing.

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