Characterization of Spin Adducts obtained with Hydrophobic Nitrone Spin Traps

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Several 5-alkyl-3,3,5-trimethylpyrroline 1-oxides have been prepared and their ability to trap a number of radicals has been investigated. In each case the addition to the nitrone is stereospecific and affords only one of the two possible geometric isomers of the resulting nitroxyl radical. The addition of a hydrogen atom gives nitroxyls containing two non-equivalent α-protons, the spectra of which are very similar to that observed during the red blood cell haemolysis induced by phenylhydrazine in the presence of a similar spin trap. A re-interpretation of the results obtained in the biological system appears to be warranted.

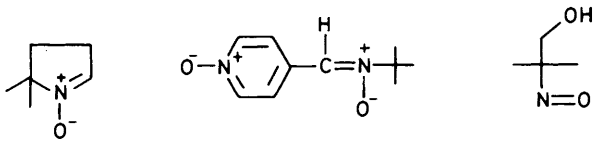
In the last decade, the importance of free radicals in biological processes has been conclusively proven. These species have been implicated in oxidation–reduction processes, many diseases, and the toxicity of numerous environmental contaminants.[1] Owing to their ubiquitous nature, the biological significance of radicals has generated a great deal of interest and new methods of studying them are constantly being sought.

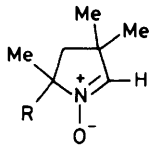
One technique that allows detection and identification of radicals in biological systems is the spin-trapping technique.[2] Most radicals occurring in the biological melieu have a very transient existence and often cannot be directly studied. However, a number of diamagnetic species will rapidly react with the transient radicals to produce long lived radicals, which may be conveniently examined by electron spin resonance spectroscopy. The identity and, under optimum circumstances, the relative concentration of the transient radical can be inferred from the spectral properties of the long lived radical. Typically, either nitroso or nitrone compounds are used as trapping agents, since the addition of a radical results in the formation of a stable nitroxyl radical.

By judicious choice of spin traps, several radicals may be differentiated in a particular biological reaction sequence. For example, Rosen and Rauckman[3] have identified various radicals involved in hepatic microsomal lipid peroxidation. When 5,5-dimethylpyrroline 1-oxide (DMPO) was used as a spin trap, spin adducts from both the hydroxyl radical ($OH^{\bullet}$) and superoxide ($O_2^{-\bullet}$) were observed. Furthermore, it was apparent that the major species produced was $O_2^{-\bullet}$. In contrast to DMPO, *N*-oxylpyridinium-4-ylmethylene t-butyl nitrone (4-OPBN) does not form stable spin adducts with $OH^{\bullet}$ or $O_2^{-\bullet}$. Consequently, when 4-OPBN was used as a spin trap, the peroxyl radical involved in lipid peroxidation ($LO_2^{\bullet}$) could be selectively trapped.

Finally, advantage can be taken of the fact that nitroso spin traps do not normally form stable adducts with any oxygen-centred radicals, *i.e.*, these traps are specific for carbon-centred radicals. Indeed, this difference between 4-OPBN and 2-methyl-2-nitrosopropan-1-ol, (MNPOL), has been utilized to differentiate $LO_2^{\bullet}$ and $L^{\bullet}$ in the linoleic acid–lipoxygenase system.[3,4] In the microsomal system, the sole spin adduct formed with MNPOL was due to the hydrogen atom.

The next desirable advance in spin trapping is the design of traps that will be localized within well defined areas of a cell or biological structure. In order to achieve this goal, nitrones soluble mainly in the aqueous phase (4-OPBN is an example) have been prepared.[5] More recently, spin traps with long alkyl chains have been synthesized. These species will be solubilized with the hydrophobic portion of the system.[6,7] For example, Hill and Thornally have recently reported a study of phenylhydrazine-induced haemolysis in human erythrocytes using several nitrone traps.[7] With DMPO and several other water-soluble traps, both haemolysis and oxygen uptake were inhibited and, concomitantly, the phenyl radical spin adduct could be detected. When a lipid-soluble nitrone (M_3POC_{18}) (**1**) was used, a dramatic decrease in the haemolysis and oxygen uptake was noted. Furthermore, an unusual e.s.r. spectrum was observed and attributed to initial trapping of an allyl-type fatty acid radical followed by rearrangement to an aminium radical.





(1)	M_3POC_{18}	R = $C_{18}H_{37}$
(2)	M_3POC_{16}	R = $C_{16}H_{33}$
(3)	M_3POC_{12}	R = $C_{12}H_{25}$
(4)	M_3POC_7	R = C_7H_{15}
(5)	M_4PO	R = Me

We have been investigating similar lipid-soluble radical traps and wish to report several unusual aspects of the observed spin adducts. Also we draw attention to similarities between some of the observed spectra and that attributed by Hill and Thornally to a rearranged allyl radical, which call into question the assignment of structure to the latter.

Results

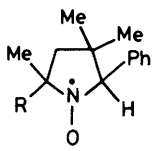
A typical spectrum for the spin adducts is shown in the Figure while values of hyperfine splitting constants (h.f.s.c.) are presented in the Table. It should be noted that, in the unsymmetrical cases, traps are capable, in principle, of affording two isomeric adducts with any one radical. For example, the addition of phenyl radical to M_3POC_7 (**4**) could yield both of the spin adducts (**6**) and (**7**).

The question arises, therefore, of whether such geometric isomers are likely to be distinguishable to e.s.r. spectroscopy. In this connection it is significant that the hydrogen-atom adducts of each of the unsymmetrical traps show the h.f.s.c.s for the two

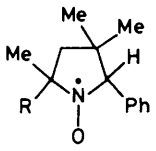
Table. E.s.r. parameters for spin adducts

Radical R•	Source[a]	Solvent[b]	DMPO a_N	DMPO a_H	M_4PO a_N	M_4PO a_H	M_3POC_7 a_N	M_3POC_7 a_H	M_3POC_{16} a_N	M_3POC_{16} a_H	M_3POC_{18} a_N	M_3POC_{18} a_H
Ph•	PAT	Bz	13.9	19.4			14.3	24.8	14.3	24.8	14.3	24.9
Ph•	PAT	Me_2CO	14.2	20.4			14.5	25.3	14.5	25.3	14.5	25.3
Ph•	PAT	MeCN	14.4	21.2			14.8	25.3	14.8	25.2	14.8	25.2
Ph•	ArN_2^+	Me_2CO	14.2	20.4			14.5	25.3				
Ph•	ArN_2^+	MeCN	14.4	21.2			14.8	25.3				
								21.6		21.2		21.6
H•	R_3SnH	MeCy	14.5	18.9	14.4	17.9	14.5		14.5		14.7	
								15.3		15.9		15.9
								21.2		21.3		
H•	NHOH	MeCy			14.3	17.6	14.5		14.6			
								15.3		15.4		
				19.0				20.8		20.2		20.2
D•	R_3SnD	MeCy	14.5				14.2		14.2		14.2	
				2.9(D)				2.3(D)		2.2(D)		2.2(D)
HO•	H_2O_2	H_2O	14.8	14.8	15.5	16.6						
HO•	H_2O_2	MeCN	13.6	13.6	13.0	7.2	13.0	6.0				
$Me_2\dot{C}CN$	AIBN	MeCy	14.6[c]	20.4[c]	12.5	4.7	13.2	5.6	12.8	5.2		

[a] PAT = Ph_3CN_2Ph, Δ; ArN_2^+ = $PhN_2^+BF_4^-$ reduction; NHOH = aerial oxidation of the corresponding hydroxylamine; H_2O_2 = photolysis of hydrogen peroxide; AIBN, Δ. [b] Bz = benzene, Me_2CO = acetone, MeCN = acetonitrile, MeCy = methylcyclohexane. [c] Solvent was *o*-xylene, reference 10.



(6)



(7)

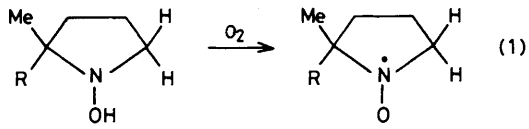
α-protons to be substantially magnetically non-equivalent, presumably because the C_α–H bonds assume different orientations with respect to the semi-occupied orbital. It is reasonable to expect that the C_α–H bonds in isomeric spin adducts such as those illustrated in (**6**) and (**7**) will also have different orientations with respect to the semi-occupied orbital, and will show substantially different values of h.f.s.c. for their α-protons. We conclude that the spectra of isomeric adducts should be readily distinguishable, and therefore that spectra observed represent only one of the two possible isomers.

Discussion

Phenyl Radicals.—The fact that a number of different sources of the phenyl radicals afford essentially the same spectrum for each trap provides compelling evidence that these spectra have been correctly assigned to the phenyl adduct. The small differences noted for each adduct are probably attributable to a solvent effect.[8] Both Janzen and Liu[8] and Hill and Thornally[7] have reported values for the DMPO adduct in agreement with those reported in this work.

The adducts formed from M_3POC_7 (**4**) and other traps containing long-chain alkyl groups have nitrogen h.f.s.c. similar to that for the DMPO adduct but substantially larger hydrogen h.f.s.c. Furthermore, the spectra were consistent with only a single spin adduct; in all cases less than 1% of additional adducts could be detected. Consequently, the addition of the phenyl radical to the nitrone must be stereoselective. The addition must be very sensitive to steric effects and must occur from the less hindered side of the spin trap, *i.e.*, *cis* to the 5-methyl group. Finally, the consistency of the hydrogen h.f.s.c. value as the alkyl chain length increases indicates that the steric bulk felt by the hydrogen does not substantially increase as the size of the alkyl chain increases.

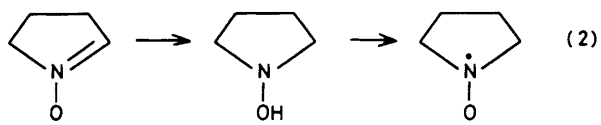
Hydrogen Atom.—The reaction of tributylstannane with the nitrone spin traps results in the ultimate addition of a hydrogen atom. This assignment has been confirmed by three different methods. First, the same radical is produced by the aerial oxidation of the corresponding hydroxylamine as shown in equation (1). Secondly, the reaction of BuSnD with a nitrone



(1)

results in the incorporation of a single deuterium atom. Finally, when M_4PO (**5**) is used, the two α-hydrogens are equivalent so that a triplet of triplets is observed in the spectrum.

From the above discussion, it is apparent that Bu_3SnH is a source of hydrogen atoms. However, the exact mechanism for the hydrogen-atom transfer is not known. Although Janzen and Liu[8] have suggested that the reaction might be photochemically initiated, we have obtained strong spectra from mixtures prepared and run in complete darkness. Besides this photochemically initiated process, several other possibilities exist. First, the nitrone may be reduced to the corresponding hydroxylamine [equation (2)], followed by aerial oxidation to



(2)

the spin adduct. As mentioned previously this hydroxylamine rapidly affords nitroxide when O_2 is bubbled through a solution.

Another alternative involves single-electron transfer and then hydrogen-atom transfer as shown in equations (3) and (4).[9]

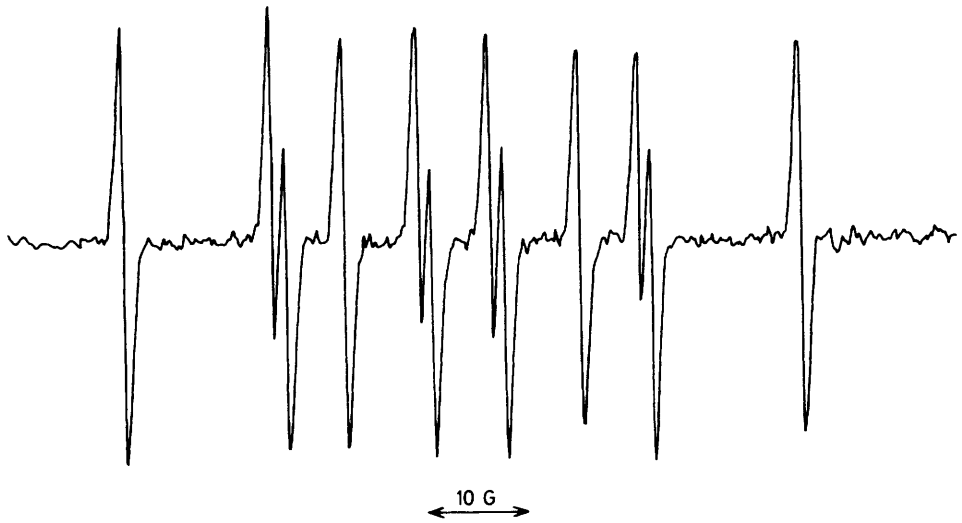


Figure. The e.s.r. spectrum for the spin adduct of M_3POC_{18} (**1**) and the hydrogen atom in MeOH (a_N = 15.8, a_H = 17.5 and 23.3 G)

$$R_3SnH + A \longrightarrow R_3SnH^{+\bullet} + A^{-\bullet} \quad (3)$$

$$R_3SnH^{+\bullet} + Traps \longrightarrow R_3Sn^{+} + H\text{-adduct} \quad (4)$$

However, the identity of the acceptor (A) is not clear; possibly, traces of molecular oxygen could undertake this role.

Whatever the mechanism, it is certain that the resulting e.s.r. spectrum is due to the nitroxyl radical with two non-equivalent α-hydrogen atoms. All of these radicals have spectra remarkably similar to those reported by Hill and Thornally for the trapping of lipid radicals generated in phenylhydrazine-induced haemolysis and attributed to the radical arising from rearrangement of a trapped allyl radical.[7]

However, when we generated allyl radicals by reaction of $Bu^tO^{\bullet}$ with triallyl phosphite or of Me_6Sn_2 with allyl bromide in the presence of the M_3POC_{12} (**3**) or M_3POC_{18} (**1**), the spectra observed were completely different from that reported by Hill and Thornally, but were virtually identical with those given by butyl or hexyl adducts (all of these adducts gave broad spectra having h.f.s.c. for a_N = 13.0 G, and for a_H = 5.8 G).

In the light of these results and the fact that the radical suggested by Hill and Thornally is not a nitroxyl but is an aminium radical cation and is likely therefore to be too unstable to be readily detected under their conditions, we suggest that their assignment of structure is incorrect. Owing to the marked similarity of its spectrum to those reported here we suggest that the radical described by Hill and Thornally is in fact the hydrogen-atom adduct of M_3POC_{18} (**1**). The larger h.f.s.c. reported by these authors (a_N = 16.7, a_H = 18.5 and 24.0 G) compared with those in the Table (a_N = 14.7, a_H = 15.9 and 21.6 G) are apparently due to a solvent effect. In order to substantiate this conclusion, the e.s.r. spectrum for the hydrogen-atom spin adduct with M_3POC_{18} (**1**) was recorded in MeOH. The spectrum, shown in the Figure, revealed a significant increase in h.f.s.c. (a_N = 15.8, a_H = 17.5 and 23.3 G).

We conclude, therefore, that M_3POC_{18} (**1**) traps free hydrogen atoms (or reacts with hydrogen-atom donors) during phenylhydrazine-induced haemolysis. In this connection it is reasonable to expect that intermediates in phenylhydrazine oxidation such as $PhN\dot{N}NH_2$ will be effective hydrogen-atom donors. Finally, it must be emphasized that there may be no connection between the protection to haemolysis afforded by M_3POC_{18} and the mechanism for the formation of the observed radical. Verification of this relationship must await further kinetic studies.

Hydroxyl Radical.—The spin adduct produced by trapping the hydroxyl radical with DMPO is well known. The familiar four-line pattern is often used as a diagnostic aid in identifying $OH^{\bullet}$ in biological systems. For M_4PO (**5**), the nitrogen and hydrogen h.f.s.c. are no longer equivalent so that a six-line pattern results.

Unfortunately, the long alkyl chains in M_3POC_n render these spin traps insoluble in water. Consequently, these were examined in acetonitrile solution. The hydroxyl radical could be produced and trapped by DMPO in this medium with no difficulty. This adduct displayed the normal four-line pattern with a slightly modified coupling constant. When M_3POC_n traps were used, a substantially different behaviour was noted. For the M_4PO (**5**) case in water, the nitrogen and hydrogen h.f.s.c. are similar in magnitude to those observed for the DMPO adduct. However, in acetonitrile solution, the hydrogen h.f.s.c. decreased to 7.2 G, a value that is substantially lower than the 16.6 G observed in water. This small h.f.s.c. is typical for the trapping of carbon-centred radicals by these spin traps (*vide infra*). Consequently, for the M_3POC_n spin traps, we suggest that the spin adducts result from trapping of the $\dot{C}H_2CN$ radical that is produced from the solvent by hydrogen abstraction. The prevalence of this process for these spin traps as compared with DMPO is due to the slower rate of radical addition.[6] However, the addition of this radical again appears to be stereoselective as only a single spin adduct (< 1% of other nitroxyl radicals) could be observed in the e.s.r. spectrum.

2-*Cyano*-2-*propylRadical.*—When2,2′-azobis-(2-methylpropionitrile) (AIBN) is thermally decomposed in the presence of DMPO, a spin adduct is formed whose e.s.r. parameters are shown in the Table. This spin adduct is stable enough to allow purification by column chromatography.[10] In contrast to the large hydrogen h.f.s.c. observed for DMPO, the M_3POC_n spin adducts have abnormally low h.f.s.c. (6—7 G). Similar values of this h.f.s.c. have been noted for the spin adducts obtained with the butyl, hexyl, and allyl radicals. This value is extremely low for carbon-centred radicals. Indeed, the phenyl radical adduct has a hydrogen h.f.s.c. of 25.3 G. The exact cause of this discrepancy is at present unknown but the steric environment of

the hydrogen is a strong possibility. Further studies are currently underway to resolve this dichotomy.

Conclusion

Derivatives of DMPO substituted with long alkyl chains are effective spin trapping reagents. However, care must be exercised in the interpretation of the spectra of the resulting spin adducts. Stereospecific addition apparently is the *modus operandi* although exceptions may exist. Furthermore, the hydrogen-atom adduct has two magnetically non-equivalent α-hydrogens. This adduct has an e.s.r. spectrum very similar to that observed during the phenylhydrazine-induced haemolysis of red blood cells. This fact may necessitate a re-interpretation of the biological results.

Experimental

The spin traps were prepared by modifications of the procedure of Bonnett *et al.*[11] In each case, the physical and spectral properties were consistent with the proposed structure.* Solvents were reagent grade or better and used as received. The e.s.r. spectra were taken on a Varian E-104(A) or Brucker 200-EP spectrometer.

Typically, a solution of the spin trap (usually 0.01M) in the appropriate solvent was employed and the radicals were produced by the methods listed in the Table. In all cases, the background signal before radical production was negligible.

* ^{1}H n.m.r. for M_3POC_7: δ 6.65 (1 H, s), 2.0 (2 H, d × d), 1.45 (3 H, s); 1—2 (broad envelope with peaks at 1.3 due to methyls, 21 H). ^{13}C n.m.r. δ_c 140.7, 77.5, 45.8, 38.7, 37.9, 31.8, 28.7, 29.2, 29.0, 28.4, 26.7, 23.9, 22.6, and 14.1.

Acknowledgements

W. R. C. gratefully acknowledges a summer fellowship while at the Australian National University.

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Received 3rd *September* 1984; *Paper* 4/1518