NOTIZEN

Synthesis and Electron Spin Resonance Study of Spin-Labelled Compounds Related to Tumour-Growth Inhibitory Nitroarylaziridines

N. J. F. Dodd

Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester

and

R. G. Harcus and P. N. Preston

Department of Chemistry, Heriot-Watt University, Riccarton, Currie, Edinburgh

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Spin Labels, Antitumour Agents

Three stable free radicals have been prepared which are akin to 5-aziridino-2,4-dinitrobenzamide (CB 1954); these compounds all contain a nitroxide function. The metabolism and excretion of two such compounds in mice has been monitored by electron spin resonance (ESR) spectroscopy and compared with that of the simpler nitroxide, 4-keto-2,2,6,6-tetramethylpiperidino-1-oxyl (tempone).

The ESR technique has been used extensively [1] in efforts to elucidate the role of free radicals in carcinogenesis although results obtained are notoriously difficult to interpret. Recently Sosnovski *et al.* [2] described the synthesis of spin-labelled analogues **1** of the antineoplastic drugs [3] TEPA (**2a**) and thio TEPA (**2b**). They believe [2] that compounds of this type could be useful for two reasons: first, they may exhibit scavenging properties on free radicals and/or other paramagnetic species in tumour tissues; and secondly the mode of such interactions might be amenable to investigation by ESR spectroscopy. It is also of interest that certain nitroxides have been shown to act as radiation sensitizers *in vitro* [4] and *in vivo* [5]. In this paper we describe our results on the synthesis of spin-labelled nitroarylaziridine analogues (**3a – c**; see scheme) and our attempts to monitor their activity by ESR spectroscopy both *in vitro* and *in vivo*. We also report related ESR studies using the readily available [6] 4-keto-2,2,6,6-tetramethylpiperidino-1-oxyl (tempone).

Requests for reprints should be sent either to Dr. N. J. F. Dodd, Paterson Laboratories, Christie Hospital and Holt Radium Institute, *Manchester M20* 9BX or to Dr. P. N. Preston, Department of Chemistry, Heriot-Watt University, Ricarton, Currie, *Edinburgh EH14* 4AS.

Synthesis of the spin labels

The new compounds **3a – c** are analogues of 5-aziridino-2,4-dinitrobenzamide [7] (CB 1954) (**3d**) which exhibits [7] potent tumour-growth inhibitory activity against Walker carcinoma 256. The free radicals labelled at the amido group (**3a, b**) were prepared by nucleophilic displacement of chloride ion from **3e** by aziridine and piperidine respectively; the chloride **3e** was synthesised by reaction of the known [8] aroyl halide **3f** with 4-amino-2,2,6,6-tetramethylpiperidino-1-oxyl in tetrahydrofuran at $-46\,^{\circ}C$. A nucleophilic displacement was also employed to synthesise the nitroxide **3c** from the halide **3g**.

ESR results

ESR spectra were recorded using a Varian E-9 spectrometer. The spectra of aqueous solutions of the spin labels **3a** and **3c** were indistinguishable, showing three lines with $a_N = 1.71 \pm 0.02$ mT and $g_0 = 2.0063 \pm 0.0003$. Their solubility in water is of the order of 5×10^{-6} M. Therefore 2.5 mg of label was dissolved in DMSO and diluted with 9 volumes of arachis oil [7]. This suspension (0.25 ml) was injected intraperitoneally into each of a number of 4 month old female RFM/Un mice. The spin labels could not be detected, by ESR, in the blood, liver or kidney, even within 10 min of injection. In one case, sacrifice of a mouse 10 min after an intravenous injection of label **3c** in DMSO also failed to reveal any signal in the blood. However, urine samples taken between 30 min and 4.5 h after injection showed the presence of a signal indistinguishable from that of the injected spin label and representing a concentration of $10^{-5} - 10^{-6}$ M.

Further experiments were carried out using the water soluble spin label tempone. 0.2 ml of a 5×10^{-3} M solution of tempone in physiological saline was injected into each mouse. In this case a three line spectrum was detected in the blood, 10 min after injection, representing a concentration of about 10^{-7} M. The concentration fell by approximately 50% within 1 h and was no longer detectable after 4 h. An identical, but more intense, signal was detected in the urine. This decreased in concentration from about 10^{-4} M, 1 h after injection, to 10^{-6} M, 4 h after injection. The signal had a g-value of 2.0064 ± 0.0003 and $a_N = 1.71 \pm 0.02$ mT. This differs from the signal in water, which was found to have $a_N = 1.61 \pm 0.02$ mT and $g_0 = 2.0063 \pm$

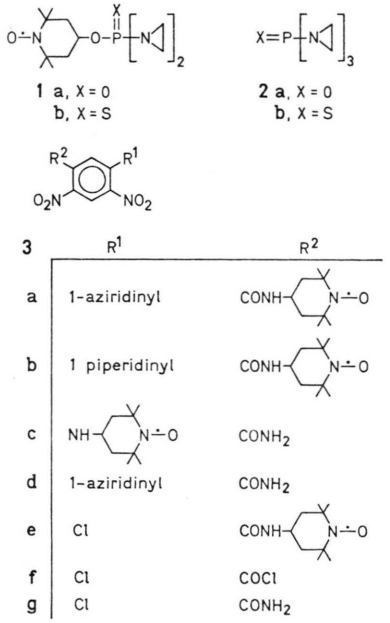
0.0003. Addition of tempone to whole blood, plasma or urine *in vitro* gave a signal with $a_N = 1.61$ mT. Consequently the species present in the blood and urine of tempone injected mice is not tempone. Further studies showed that the signal from tempone added to whole blood or plasma was barely detectable after 3 h storage on ice. In contrast, storage at 0 °C or room temperature, of blood from injected animals produced an increase in the ESR signal.

It can be concluded that tempone is rapidly metabolized in mice, following intraperitoneal injection. One possible reaction is conversion of the keto group to hydroxyl and subsequent formation of a glucuronide[9], but this suggestion is tentative until we complete our studies using simple spin labels containing a variety of functional groups. The increase in signal with time *in vitro* suggests that tempone or its metabolite complexes reversibly *in vivo* with some component of blood, thereby broadening the ESR signal beyond detection. The reduction of several drugs is known[9] to involve the oxidation of haemoglobin and we attempted to monitor such an interaction. However, low temperature ESR examination of blood from mice injected with tempone or the label **3c** failed to show the presence of a complex of haemoglobin and the nitroxide and showed that there was no increase in methaemoglobin.

The absence of a detectable signal in blood of animals injected with the spin labels **3a** and **3c** is probably due to the low solubility of the labels in aqueous media and to the formation of a complex in blood, which lowers, still further, the concentration of free label or its metabolite.

The preliminary experiments reported here indicate the potential value of the ESR technique in determining the fate of spin-labelled drugs *in vivo*. This is of considerable importance since the metabolism and binding of spin labels will affect their possible anti-tumour activity or radiosensitizing action.

Scheme



Antitumour evaluation

The nitroxides **3a** and **c** were evaluated for effect on Walker tumour cells *in vitro*, a system in which CB 1954 **3d** kills 99% of tumour cells at a concentration of 1 $\mu g \cdot ml^{-1}$. Compound **3a** was effective (99% tumour cell kill) only at 100 $\mu g \cdot ml^{-1}$ while **3c** had no effect even at 100 $\mu g \cdot ml^{-1}$.

Experimental

4-Keto-2,2,6,6-tetramethylpiperidino-1-oxyl (tempone) was prepared by a reported[6] procedure. 4-Amino-2,2,6,6-tetramethylpiperidino-1-oxyl was purchased from Aldrich Chemical Co. and used without further purification.

5-Chloro-N-[4-(2,2,6,6-tetramethylpiperidino-1-oxyl)]-2,4-dinitrobenzamide (**3e**)

4-Amino-2,2,6,6-tetramethylpiperidino-1-oxyl (1.0 g, 5.9 mmol) and triethylamine (0.5 g) in dry tetrahydrofuran (15 ml) was added dropwise with stirring to a solution of 5-chloro-2,4-dinitrobenzoyl chloride (1.5 g, 5.8 mmol) and triethylamine (0.35 g) in tetrahydrofuran (10 ml) at −46°. After 4.5 h the mixture was warmed to room temperature and the product was filtered. The filtrate was evaporated to leave a red oil which was purified chromatographically (silica gel, 3 : 1, benzene : chloroform eluant). This material was recrystallised from chloroform/hexane to give **3e** as yellow needles (0.9 g, 34%), m.p. 230 – 231 °C, $\lambda_{max.}^{EtOH}$ 229 nm (sh), 252 (ε, 12,600), M^+ found 399.1061; req. 399.1072. Found: C, 48.01; H, 5.11; N, 13.86%. $C_{16}H_{20}N_4O_6Cl$ requires: C, 48.07; H, 5.04; N, 14.01%.

5-Aziridinyl-N-[4-(2,2,6,6-tetramethylpiperidino-1-oxyl)]-2,4-dinotrobenzamide (**3a**)

5-Chloro-N-[4-(2,2,6,6-tetramethylpiperidino-1-oxyl)]-2,4-dinitrobenzamide (0.2 g, 0.5 mmol) in tetrahydrofuran (6 ml) was added dropwise over 40 min to a mixture of aziridine (0.02 g, 0.48 mmol), triethylamine (0.05 g) and tetrahydrofuran

(3 ml). After stirring for 3 h the product was filtered and chromatographed (silica gel, THF eluant). Fractions containing the required material were treated with petroleum ether (60 – 80 °C) to precipitate the product **3a** (0.18 g, 81%) as yellow needles m.p. 218 – 220 °C (dec.), $\lambda_{max.}^{EtOH}$ 235 nm (ε, 13,100), 271 (11,200), 329 (11,000); ESR a_N = 1.55 mT (benzene solvent); M^+ found 406.1738; $C_{18}H_{24}N_5O_6$ requires 406.1727. Found: C, 53.42; H, 6.10; N, 16.98; $C_{18}H_{24}N_5O_6$ requires C, 53.20; H, 5.95; N, 17.23%.

5-(1-Piperidinyl)-N-[4-(2,2,6,6-tetramethyl-piperidino-1-oxyl)]-2,4-dinitrobenzamide (**3b**)

This was synthesised in a manner analogous to the method above. The initial product was chromatographed (silica gel, 1 : 1 THF/petroleum ether eluant) and the eluted oil was recrystallised from THF/petroleum ether to give **3b** as yellow needles, 0.19 g (68%), m.p. 204 – 205 °C. $\lambda_{max.}^{EtOH}$ 236 nm (ε, 12,000), 255 (sh), 380 (11,500); ESR a_N = 1.55 mT (benzene solvent); M^+ found, 448.2215; $C_{21}H_{30}N_5O_6$ requires 448.2196. Found: C, 56.36; H, 6.73; N, 15.43; $C_{21}H_{30}N_5O_6$ requires: C, 56.24; H, 6.74; N, 15.62%.

5-[N-4-(2,2,6,6-Tetramethyl-piperidino-1-oxyl)]-amino-2,4-dinitrobenzamide (**3c**)

5-Chloro-2,4-dinibrobenzamide (0.3 g, 1.2 mmol), 4-amino-2,2,6,6-tetramethylpiperidino-1-oxyl (0.2 g, 1.2 mmol) and tributylamine (0.25 g) were heated under reflux in butan-1-ol (25 ml) for 6 h. The product was evaporated to approx. 15 ml and the product was purified chromatographically to give **3c** as yellow needles, 0.29 g (63%), m.p. 233 – 234 °C. $\lambda_{max.}^{EtO}$ 227 nm (ε, 10,800), 271 (9,800), 349 (12,400), 400 (sh); ESR a_N = 1.575 mT (benzene solvent); M^+ found 380.1574; $C_{16}H_{27}N_5O_6$ requires 380.1570. Found: C, 50.39; H, 5.89; H, 18.20. $C_{16}H_{22}N_5O_6$ requires: C, 50.52; H, 5.83; N, 18.41%.

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[1] *cf.* H. M. Swartz, Biological Applications of Electron Spin resonance, (H. M. Swartz, J. R. Bolton, and D. C. Borg, eds.), Wiley-Interscience, 1972, Ch. 4.
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