A New Method for Obtaining ^{1}H NMR Structural Information on Nitroxide Spin Labels Through Reduction to Hydroxylamine by Radiolysis of their Methanolic Solutions

M. E. Brik,*[a] H. Remita[b] and M. O. Delcourt[b]

[a] *Laboratoire de Chimie Structurale Organique, ICMO URA 1384, Université de Paris-Sud, Bât. 410, 91405 Orsay, Cedex, France*

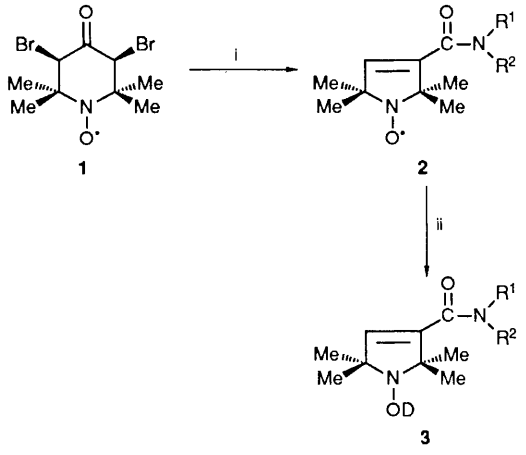
[b] *Laboratoire de Physico-Chimie des Rayonnements, URA 75, Université de Paris-Sud, Bât. 350, 91405 Orsay, Cedex, France*

A preliminary description of a convenient method for the reduction of nitroxide spin labels by a radiolysis technique is described; the main advantage of this procedure is the ease with which nitroxides are converted to the corresponding non-paramagnetic hydroxylamines, the ^{1}H NMR spectra of these hydroxylamines gives structural information on the parent spin labels.

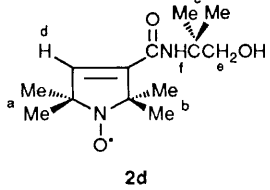
Nitroxides are widely employed as spectroscopic probes for observing binding sites and molecular motion in marcomolecules[1–3] using ESR spectroscopy. Because of the presence of an unpaired electron, nitroxides (R_2N–$O^\bullet$) have been recently used to enhance contrast in magnetic resonance imaging[4–6] and thereby increase the diagnostic usefulness of this new technique. However, owing to the paramagnetic nature of nitroxide spin labels, one cannot conveniently get detailed structural information on these molecules by NMR spectroscopy.

Stable nitroxide radicals used as spin labels have two t-alkyl substituents on the nitroxide nitrogen, often incorporated into a cyclic structure. The paramagnetism of these radicals results in substantial broadening of ^{1}H NMR absorptions, especially for those nuclei close to the radical centre. This line broadening would be removed by selective reduction of the nitroxides to the corresponding hydroxylamines (*e.g.* **2**→**3**), for which NMR characterisation should be straightforward.

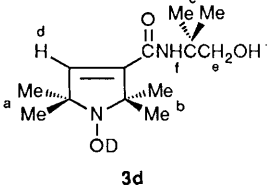
The reduction of nitroxides to disubstituted hydroxylamines is an easy reaction and can be accomplished by a variety of mild reducing agents. The most usual ones are phenylhydrazine and ascorbic acid.[7,8] But such a process using reductive chemical agents is hardly selective, these compounds not only reduce nitroxides but also react with other functional groups, such as ketones, aldehydes or acids. Looking for a selective



Scheme 1 *Reagents and conditions:* i, HNR^1R^2, $CDCl_3$; ii, ^{60}Co, γ-rays, CD_3OD



2d



3d

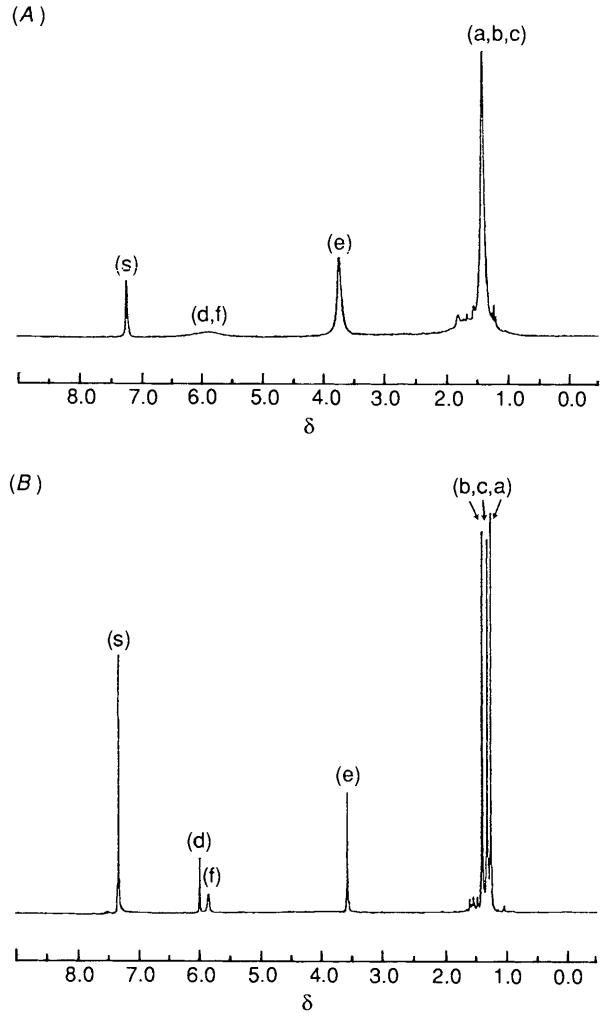


Fig. 1 ^{1}H NMR spectra ($CDCl_3$) of (*A*) nitroxide **2d** before radiolysis (*B*) hydroxylamine **3d** after radiolysis

reduction, we have investigated the radiolysis of methanolic solutions (CD_3OD) of a series of nitroxides **2**.

The nitroxides used in this study were 2,5-dihydropyrrol-1-oxyls **2**, obtained from *cis*-3,5-dibromo-4-oxo-2,2,6,6-tetramethylpiperidin-1-oxyl[9] **1** by Favorskii rearrangement promoted by amines[10] (Scheme 1).

Radiolysis of polar liquids[11-12] generates transient species (solvated electrons e_s^- and radicals): in the case of alcohols,[12] (for instance CD_3OD) the primary radiolytic species (e_s^-, D$^\bullet$, OD$^\bullet$, $^\bullet CD_2OD$) are very rapidly converted to the alcoholic radical $^\bullet CD_2OD$, which is highly reductive. This reductive power of an irradiated alcohol can be used without additional reducing agent. When $R_2NO^\bullet$ molecules are present in the solution, one expects the selective reduction of the nitroxide function according to eqn. (1).

$$\begin{matrix} R \\ R \end{matrix}\!\!>\!N\text{–}O^\bullet + \begin{cases} e^-_s \\ D^\bullet \\ {}^\bullet CD_2OD \end{cases} \longrightarrow \begin{matrix} R \\ R \end{matrix}\!\!>\!N\text{–}OD \qquad (1)$$

The reduction can be adjusted by varying the radiation dose and the dose rate.† As an example, we present ^{1}H NMR spectra for irradiated **3d** and non-irradiated **2d** 2,5-dihydro-3-(2-hydroxy-1,1-dimethylethylcarbamoyl)-2,2,5,5-tetramethyl-1*H*-pyrrol-1-oxyl (Fig. 1). For nitroxide **2d**, the ^{1}H NMR spectrum shows, in the δ 1.2–1.5 region, a broad line which corresponds to six methyl groups, it is not possible to distinguish the a, b and c positions. Reduction of nitroxide to its corresponding hydroxylamine removes the paramagnetism of this species and consequently a high resolution spectrum of **3d** is obtained (Fig. 1). We now clearly observe three different methyl groups a, b and c that we can assign to the different methyl groups as shown in Fig. 1 and at the same time the narrowing contraction of the line related to the $-CH_2-$ group e at δ 3.58. Also at δ 6 the ethylenic proton d and at 5.8 the amide proton f are well resolved after reduction. Comparable results are presented for a series of hydroxylamine compounds.‡

These results show that reduction of nitroxides by a radiolysis technique presents several advantages over conventional reduction procedures using chemical agents. First, the radiolytic method itself is very convenient, the reduction can be achieved after a few hours irradiation in a suitable solvent, so that no further extraction or separation is required before NMR measurements. Secondly, the hydroxylamine is the only product of the reduction reaction, indeed no other product arising from the nitroxide has been detected by NMR.

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† The radiation source was a ^{60}Co-cell exhibiting a maximum rate of 0.8 Mrad h^{-1} (8 kGy h^{-1}). The dose rate can be changed by increasing the distance of the sample from the centre of the source. Calibrated solutions of nitroxides were prepared in pyrex tubes (φ = 15 mm; degassed by N_2) and then exposed to γ rays. The irradiation of the tube does not disturb the NMR signals. After irradiation, the solvent was evaporated and the ^{1}H NMR spectra were recorded on a Bruker 250 MHz (in $CDCl_3$). Chemical shifts are given relative to $SiMe_4$.

‡ *^{1}H NMR data* ($CDCl_3$) for hydroxylamines after reduction of nitroxides by radiolysis of their methanolic solutions.

3a $R^1 = -CH_2CH_2OH$, R^2 = H: δ 1.1 (s, 6H), 1.3 (s, 6H), 3.3 (m, 2H), 3.5 (m, 2H), 5.9 (s, 1H) and 6.5 (m, 1H); **3b** $R^1 = -CH_2(CH_2)_3OH$, R^2 = H: δ 1.3 (s, 6H), 1.5 (s, 6H), 1.65 (m, 4H), 3.35 (m, 2H), 3.7 (m, 2H), 6.05 (s, 1H) and 6.3 (m, 1H); **3c** $R^1 = -CH_2(CH_2)_5OH$, R^2 = H: δ 1.25 (s, 6H), 1.3 (m, 4H), 1.4 (s, 6H), 1.45 (m, 2H), 3.2 (m, 2H), 3.45 (m, 4H), 5.4 (m, 1H) and 6.5 (m, 1H); **3d** $R^1 = -C(Me)_2CH_2OH$, R^2 = H: δ 1.27 (s, 6H), 1.30 (s, 6H), 1.4 (m, 6H), 3.58 (m, 2H), 5.8 (s, 1H) and 6 (s, 1H); **3e** $R^1 = -C(CH_2OH)_2Me$, R^2 = H: δ 1.20 (s, 6H), 1.25 (s, 3H), 1.4 (s, 6H), 3.65 (m, 4H), 6.08 (s, 1H) and 6.45 (m, 2H); **3f** $R^1 = -CH_2OH$, $R^2 = -CH_2OH$: δ 1.3 (s, 6H), 1.4 (s, 6H), 3.4 (m, 4H), 3.65 (m, 4H), 6.05 (s, 1H) and 6.6 (m, 2H); **3g** $R^1 = -CH(Me)CH(OH)Ph$, R^2 = Me: δ 1.05 (s, 3H), 1.10 (s, 6H), 1.20 (s, 3H), 1.22 (s, 6H), 2.62 (m, 1H), 2.9 (m, 1H), 4.65 (s, 1H), 5.25 (s, 1H) and 7.1 (m, 5H); **3h** R^1, $R^2 = -CH_2CH_2OCH_2CH_2-$: δ 1.30 (s, 6H), 1.4 (s, 6H), 3.6 (m, 8H) and 5.8 (s, 1H).