SYNTHESIS OF PERDEUTERIO-N-(1-OXYL-2,2,6,6-TETRAMETHYL--4-PIPERIDINYL)MALEIMIDE, A HIGHLY SENSITIVE SPIN PROBE

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SUMMARY

Perdeuterio-N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide has been prepared to improve sensitivity and resolution in its ESR spectrum. The synthesis involved modifications of procedures known for the corresponding protonated analog. Spectral data are presented for the product to establish isotopic purity. A five-fold enhancement of the ESR signal for the title compound relative to the proton counterpart has been observed.

Key Words : N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide-d_{17}, deuterium, spin label, ESR

INTRODUCTION

Spin labelled biologically active molecules typically lack definition in their electron spin resonance (ESR) spectra as opposed to unbound labels which give rise to sharp resonances. Previous work by others (1-3) with the spin labels, 2,2,6,6-tetramethyl-4-piperidone-1-oxyl-d_{16} (1) and 2,2,6,6-tetramethyl--4-piperidinol-1-oxyl-d_{18} (2), hinted that ESR spectral features

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0362-4803/81/030371-13$01.00
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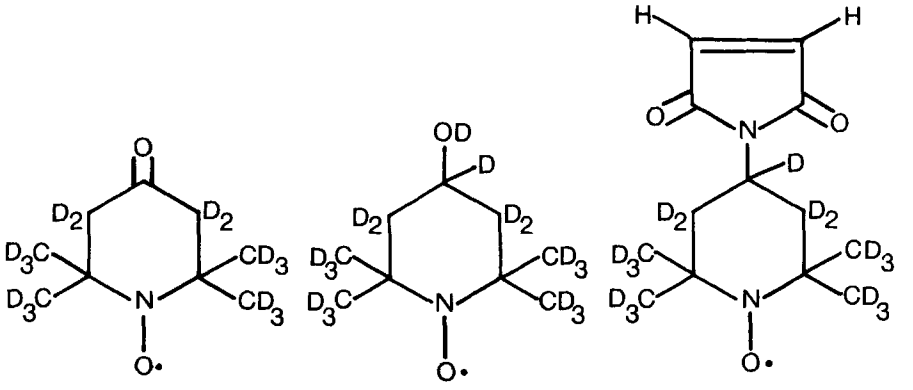
Received September 13, 1979

such as peak heights, line width and homogeneity could all be improved to advantage by having deuterium atoms in place of hydrogens near the nitroxide functionality. In the hope that an analogously deuterium substituted spin label with an additional functional group for covalent attachment of a biologically active molecule might provide some clarification in the ESR spectrum, we have synthesized perdeuterio-N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (3). In preliminary biochemical studies, the



1 2 3

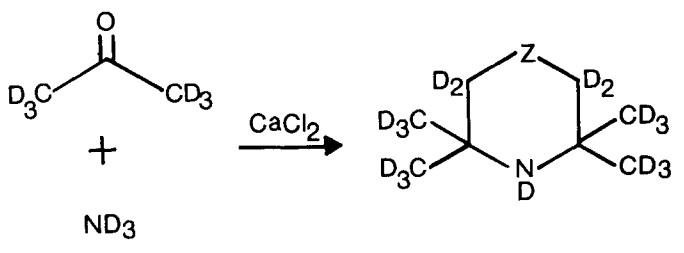
deuterated probe was used to covalently label bovine serum albumin (4) and soluble or membrane bound glyceraldehyde-3-phosphate dehydrogenase. There was a remarkable improvement in ESR spectral resolution and accompanying gains in detectability. It is our contention that the utility of the deuterated maleimide spin label 3 in related areas will rapidly grow. In this report, we wish to describe the synthetic part leading to isotopically pure 3.

RESULTS AND DISCUSSION

Molecular models suggested that both of the olefinic hydrogens present in 3 were at remote positions with respect to the nitroxide

group. For this reason, it appeared unnecessary to label those positions. Consideration of the proton hyperfine couplings reported (2) for undeuterated 2 implied that complete deuteration of the nitroxide bearing heterocyclic ring of 3 was essential to derive maximum benefits in the ESR spectrum. This required optimization of conditions in several of the steps known (5, 6) in the synthesis of the proton analog of 3 both to minimize loss of deuterium and to obtain higher yields.

2,2,6,6-Tetramethyl-4-piperidone-d_{16} (4), the key starting material in the synthesis of 3, has been prepared by Chiarelli and Rassat (1, 5a) by heating acetone-d_6, deuterated ammonia and calcium chloride in an autoclave in 30% yield. For ease of large scale preparation and to obtain a higher yield, the recent modifications of this reaction by Sosnovsky and Konieczny (7-9) seemed attractive. By adopting their procedure (7), followed by workup in a deuterated medium, pure piperidone 4 was realized in 35.8% yield (10). Interaction of ketone 4 in D_2O with slightly over two equivalents of hydroxylamine hydrochloride and one equivalent of sodium acetate furnished the oxime deuterochloride 5 in good yield (11). Reduction of salt 5 with excess of lithium aluminum deuteride in ether proceeded smoothly to provide, after destruction of the excess reducing agent in a deuterated medium, the amine 6 in



4 Z=C=O

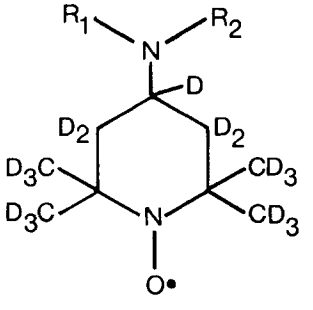
5 Z=C=NOD, DCl

6 Z=C(D)ND_2

7 Z=C(D)NDAc

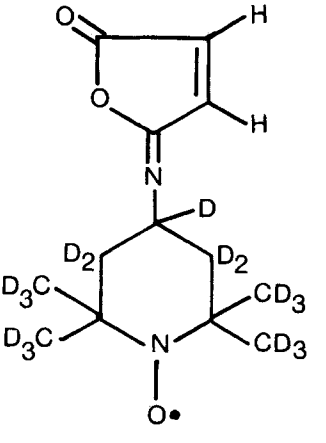
75% yield (12) and was converted to the monoacetate 7 by the standard procedure (6a). A check on the isotopic purity of amine 6 by comparison of the ^1H NMR spectrum with that of an undeuterated amine indicated a high degree of deuterium incorporation in all positions of the piperidine ring. The same trend was also seen in the ^1H NMR spectrum of the monoacetate 7. However, there was some deuterium loss bound to nitrogen atoms in amine 6 which presumably occurred in the conversion 4 → 5. Although this was an anticipated difficulty, fortunately, it had limited effect on the target molecule 3 since all labile deuterium atoms disappeared in subsequent transformations.

Oxidation of acetate 7 by the pertungstate method (6b) followed by saponification (6c) gave the nitroxide radical 8. Reaction of 8 with maleic anhydride by a similar procedure reported (5b) for the proton counterpart gave maleamic acid 9, which cyclized on heating with acetic anhydride and sodium acetate to furnish a mixture of maleimide 3 and isomaleimide 10. Chemically pure 3 required for labelling studies was obtained by column chromatography separation on silica gel and subsequent crystallizations from cyclohexane.



8 $R_1 = R_2 = D$

9 $R_1 = D, R_2 = COCH{=}CHCO_2H$



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The isotopic purity of 3 was ascertained from mass spectral data and ^{1}H NMR spectrum. Authentic undeuterated maleimide 3 was employed for comparison. The mass spectrum of both the labels is reproduced in figure 1. Loss of isobutylene and NO groups, indicated in figure 1, accounted for the base peak. The isotopic composition of 3 was found to be : d_{17} - 70.71% ; d_{16} - 23.86% ; d_{15} - 5.42%. A high degree of

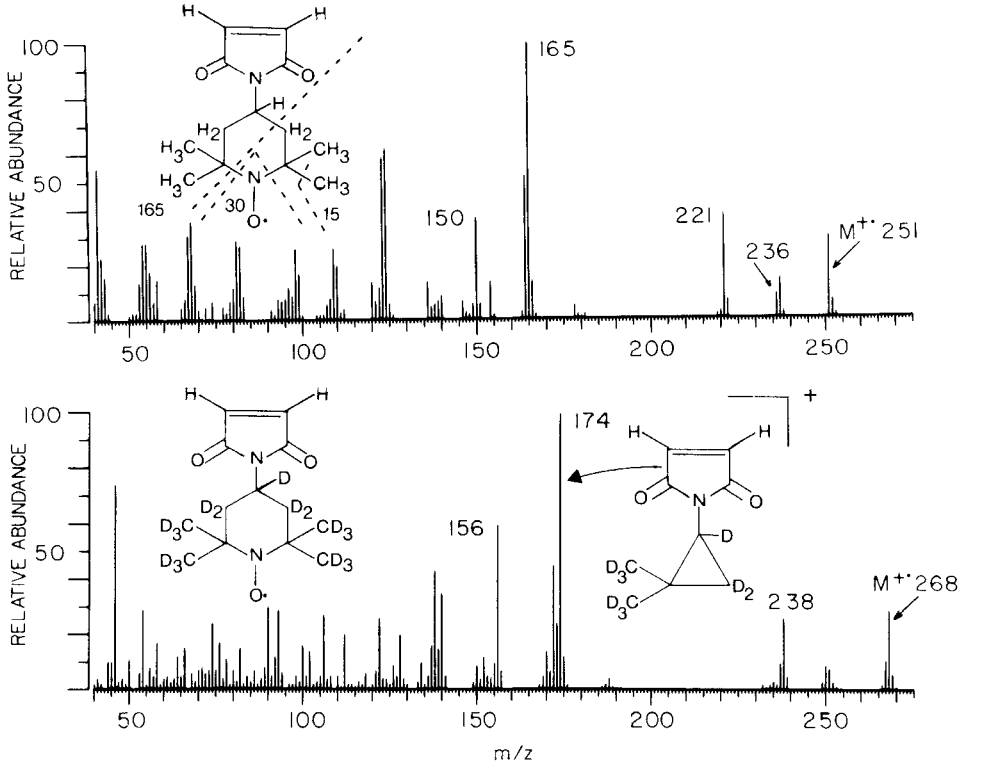

Figure 1. Mass spectrum of undeuterated and deuterated maleimide 3.

deuterium incorporation into the piperidine ring of 3 was inferred from the proton NMR spectrum after in situ reduction of nitroxide radical by the addition of phenylhydrazine (13). Only olefinic protons were discernible in the spectrum.

A comparison of the ESR spectrum of deuterated and undeuterated maleimide 3 is presented in figure 2. Most striking in the spectra is the sharpening of the resonance lines and a five-fold increase in the peak heights resulting from deuteration of the nitroxide containing ring.

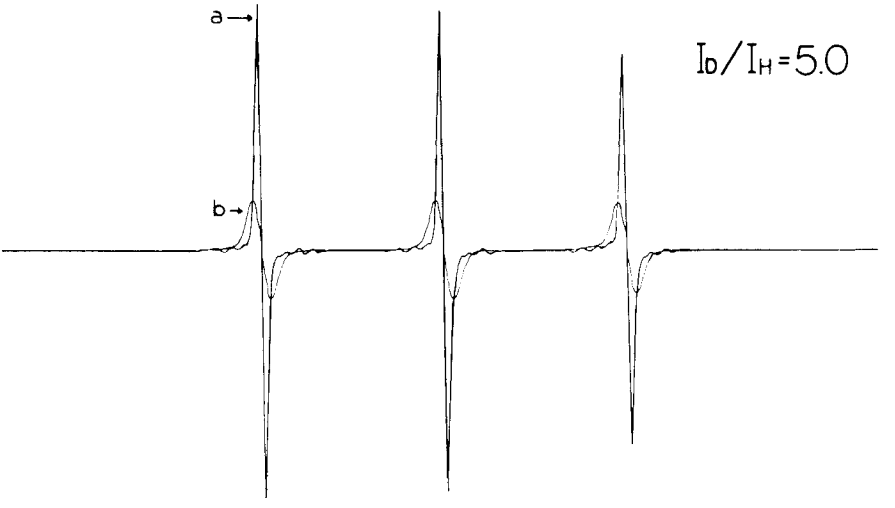

Figure 2. ESR (100 G) spectra of 50 μM (a) deuterated maleimide 3 and (b) its proton counterpart in 5 mM phosphate buffer at pH 8.0. I_D and I_H are the amplitudes of center resonance lines for the deuterium and hydrogen analogues respectively.

EXPERIMENTAL SECTION

Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Ir spectra were recorded on a Perkin-Elmer 727 spectrophotometer. Proton NMR spectra were obtained on a Joel MH-100 nuclear magnetic resonance spectrometer with TMS as an internal standard. Mass spectra (70ev) were recorded on a LKB-9000 mass spectrometer by direct insertion. The deuterium composition was determined on the molecular ion. ESR spectra were recorded on a Varian E-109 spectrometer equipped with a TM_{110} cavity. Thin layer chromatography (TLC) analyses were carried out on Eastman Chromagram 13181 precoated silica gel plates. Spots were revealed by exposure to UV light or iodine vapor. Regents and solvents were purified when necessary. Literature melting or boiling points indicated in parentheses refer to undeuterated compounds.

Acetone-d_6 (99.5% D), deuterium oxide (99.8% D) and lithium aluminum deuteride (98% D) were all purchased from Aldrich Chemical Company. Deuterated ammonia (99% D) was procured from Stohler Isotope Chemicals. Sodium deuterium oxide as a 50% solution in deuterium oxide was purchased from Pfaltz and Bauer Inc.

2,2,6,6,-Tetramethyl-4-piperidone-d_{17} (4). This was prepared following the procedure of Sosnovsky and Konieczny (7). To a mixture of acetone-d_6 (171 g, 2.67 mole) and 4-20 mesh size calcium chloride (62 g) in a 1L three-necked flask fitted with a gas in-let, an efficient mechanical stirrer and a Y-joint carrying a 0-50° thermometer and a dry ice condenser was introduced, over a period of 3 days, deuterated ammonia (25.6 g, 1.28 mole) in approximately

six equal lots. The temperature of the reaction mixture remained for the most part around 30-35° during the introduction of ND_3. After allowing the reaction mixture to remain at room temperature for 4 days, during which time it turned to red and became somewhat thick, unreacted acetone-d_6 (9.5 g, 0.15 mole) was recovered by distillation, bp 35°/100 mm. To the cake, a solution of sodium deuteroxide (30 ml of 50% NaOD diluted with 50 ml D_2O) was added dropwise with vigorous stirring. The liberated oil was thoroughly extracted with ether (5 x 300 ml) and the organic layer was dried ($MgSO_4$). After evaporation of the solvent, the residue was distilled in vacuum. The fraction, bp 85-90°/15 mm, which crystallized in the receiver (54.8 g, 35.8%) was the required piperidone 4 (Lit. (6d) bp 94-90°/14 mm). A small amount of sample recrystallized from CCl_4 at 0° melted at 36-38° (Lit. (6d) mp 35.5-36°). ^1H NMR (CCl_4) δ 1.12 (s, CH_3), 1.50 (b, NH) and 2.12 (d, CH_2) is for the residual protons.

Oxime hydrochloride of 2,2,6,6-tetramethyl-4-piperidone-d_{16} (5). To a solution of the ketone 4 (25.8 g, 0.15 mole) in 25 ml absolute ethanol was added, with ice cooling, a solution of hydroxylamine hydrochloride (25.22 g, 0.36 mole) and anhydrous sodium acetate (14.8 g, 0.18 mole) in 50 ml D_2O. An exothermic reaction ensued and a white solid separated. After allowing the reaction mixture to warm up to room temperature, it was gently heated on a steam bath for 5 hr, diluted with 50 ml absolute ethanol and allowed to stand overnight. The white crystalline hydrochloride that had separated was filtered, washed with cold ethanol and dried in a oven at 100°/30 mm for 6 hr to give the oxime hydrochloride 5, mp 285-290° (dec) (Lit. (11) mp about 300° with dec.). The crystalline white solid amounted to 32 g (95%)

and was used in the next step without further purification. A small amount of sample recrystallized in poor yield from 95% ethanol had mp 290-295° (dec). ^{1}H NMR (D_2O) δ 1.58 (s, CH_3) 2.60 (s, CH_2), and 2.84 (s, CH_2).

2,2,6,6-Tetramethyl-4-aminopiperidine-d_{20} (6). The procedure of Misharin et al. (12) was adapted except that $LiAlD_4$ was used in place of $LiAlH_4$ and excess of the reducing agent was destroyed in a deuterated medium. To a stirred suspension of $LiAlD_4$ (10 g, 0.24 mole) in 500 ml anhydrous ether (distilled over $LiAlH_4$), the oxime hydrochloride 5 (30 g, 0.14 mole) was added cautiously from a solid addition flask at such a rate there was continous boiling of the solvent. After gentle heating for 10 hr, excess $LiAlD_4$ was cautiously destroyed (N_2) by successive addition of 10 ml D_2O, 10 ml NaOD in D_2O and 30 ml D_2O (14). The granular precipitate was filtered, pressed on the filter paper and washed with several portions of anhydrous ether. The organic extract was dried (anhydr. K_2CO_3) and the solvent removed under reduced pressure. Distillation of the residue in vacuum furnished 17.8 g (75%) of amine 6, bp 58-60°/2 mm (Lit. (12) 60-62°/2 mm) as a colorless mobile liquid. ^{1}H NMR (CCl_4) δ 0.62 (b, NH_2), 0.98 (m, CH_3), 1.05 (m, CH_3), 1.60 (m, CH_2) and 2.95 (m, CH). The peak at 0.62δ was very intense relative to the rest of the peaks indicating considerable scrambling of deuterium bound to the hetroatoms.

2,2,6,6-Tetramethyl-4-acetylaminopiperidine-d_{19} (7). This was prepared by the procedure of Rozantsev et al. (6a). Thus, acetic anhydride (21.5 g, 0.23 mole) was added dropwise to a stirred solution of the amine 6 (12 g, 0.068 mole) in 30 ml anhydrous ether held at 10°. The temperature of the reaction mixture was

not permitted to rise above 15°. After stirring for 30 min, the white solid formed was filtered and dried in a desiccator over KOH pellets. The crystalline acetate 7 (14.2 g, 96%) melted at 122-124° (Lit. (6a) mp 120°). The material was pure enough to be used in the next step. ^{1}H NMR ($DCCl_3$) δ 2.05 (s, $COCH_3$). The absence of other peaks in the region 1.2-4.6 δ, seen in the ^{1}H NMR spectrum of the undeuterated compound, indicated a high degree of deuterium substitution in all positions of the piperidine ring.

2,2,6,6-Tetramethyl-4-amino-piperidine-1-oxyl-d_{19} (8). The procedure of Rozantsev et al. (6b,6c) was followed. To a solution of the acetate 7 (12.5 g, 0.058 mole) in 25 ml methanol and 50 ml water were added sodium tungstate (0.9 g), sodium salt of EDTA (0.9 g) and 16 ml of 30% H_2O_2 in 75 ml water. The reaction mixture was allowed to remain in the refrigerator for 6 hr and then for 5 days at room temperature in the dark, at which time it acquired a red color. After saturating the reaction mixture with K_2CO_3, the solution was extracted with ether (5 x 100 ml) and the combined organic layer was dried (K_2CO_3). Removal of the solvent on the rotary evaporator furnished an orange yellow solid (14.5 g). This was suspended in 40 ml of 15% NaOD and the contents stirred and boiled for 12 hr. The cooled solution was suction filtered, saturated with K_2CO_3 and extracted with ether (5 x 100 ml). After drying the organic layer ($MgSO_4$), the solvent was rotoevaporated and the red oil was distilled in vacuum using a short distillation head. The yield of the amino radical 8 was 7.2 g (66%), bp 100-104°/4 mm (Lit. (6c) 97-98°/4 mm). TLC ($HCCl_3$:MeOH, 9:1)

showed a single spot (R_f = 0.66) identical with an undeuterated sample.

N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide-d_{17} (3). Following the reported procedure (5b), amino radical 8 (1.94 g, 10.2 mmole) in 20 ml ether was added to maleic anhydride (1.0 g, 10.2 mmole) dissolved in 60 ml ether and the precipitated orange solid filtered, washed with 10 ml of ether to provide maleamic acid 9 (2.70 g, 92%), mp 160-164° (Lit. (15) mp 170°). The crude acid 9 (2.70 g, 9.40 mmole), anhydrous sodium acetate (0.4 g, 4.87 mmole) and 40 ml of acetic anhydride were heated at 100° for 3 hr. Excess acetic anhydride was removed under vacuum (bp 40°/1 mm) and the residue was dissolved in small amounts of hot benzene. The combined organic extract was passed through a short column of silica gel to remove inorganic material. Removal of solvent in a rotoevaporator afforded a thick red gum (2.5 g). TLC ($HCCl_3$) showed this to be a mixture of maleimide 3 and isomaleimide 10. Separation of the required maleimide from the isomer was achieved by column chromatography on silica gel (75 g, 60-200 mesh size). The column was eluted with hexane-benzene, benzene-chloroform mixtures with increasing amounts of the polar solvent and the separation was monitored by TLC ($HCCl_3$). Maleimide was eluted first and had the R_f value of 0.75. Fractions with this R_f value were pooled together and recrystallized from cyclohexane several times to furnish chemically pure maleimide 3 as an orange yellow solid (1.1 g, 44%). After drying at 80°/30 mm for 12 hr, the maleimide melted at 107-108° (Lit. (15) 108-109°). From the mother liquor, 0.3 g of slightly impure maleimide was obtained, mp 104-106°. Ir (KBr) 1695 cm^{-1}. ^1H NMR ($DCCl_3$) after the

addition of 0.5 equivalent of 0.03 M solution of phenylhydrazine (13) showed peaks at δ 6.72 (s, olefinic) and 7.22 (s, olefinic) and complete absence of peaks in the region 1.1-4.5 δ. The protonated analog exhibited peaks at δ 1.25 (s, 12H), 1.1-1.6 (m, 2H), 2.2 (t, 2H), 4.20 (m, 1H), 6.70 (s, 1H) and 7.22 (s, 1H). The mass and ESR spectra of deuterated 3 and the corresponding proton analog are reported in figures 1 and 2.

ACKNOWLEDGMENT

We thank Dr. Throck J. Watson for providing the mass spectral data made available through partial support from a Biomedical Research Support Grant-RR-05424. This work was supported in part by grants from the U.S. Public Health Services, 5-R01-GM 07884, and the Muscular Dystrophy Association. Dr. Albert H. Beth and Dr. Ray C. Perkins are recipients of postdoctoral fellowships from the Muscular Dystrophy Association.

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