STABLE ISOTOPE SUBSTITUTED SPIN LABELS. 2. AN IMPROVED SYNTHESIS OF PERDEUTERIO-^{15}N-(1-OXYL-2,2,6,6-TETRAMETHYL-4-PIPERIDINYL)MALEIMIDE

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SUMMARY

Stable isotope substituted spin labels increase the resolution and sensitivity of EPR measurements. An improved synthesis of ^{15}N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide has been devised and utilized. This improved synthesis is accomplished in five steps instead of the normally required seven or more.

Key Words: ^{15}N, ^{2}H spin labels, synthesis, EPR

INTRODUCTION

Stable isotope substitution in the immediate vicinity of the free electron in a spin label should result in significant improvement in ESR signal resolution and enhancement (1, 2). Deuterium substitution on the carbons of the piperidine ring have been shown to result in sharpening of the signal and a five-fold enhancement of the signal for N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide-d_{17} has been reported by Venkataramu, et al. (3). Substituting the ^{15}N isotope for the naturally occurring ^{14}N in the spin label has a more dramatic effect on the ESR signal. The number of nuclear spin states decreases from three (m, = -1, 0, +1) to two (m, = $-\frac{1}{2}$, $+\frac{1}{2}$) (4).

Preliminary studies in our laboratory utilizing this perdeuterio, ^{15}N-substituted spin label at 9 GHz EPR operation have shown an increase of about one order of magnitude in the signal-to-noise over non-isotopically substituted spin label at 35 GHz operation. However, the use of stable isotopes and 35 GHz operation is additive and the combination provided the most powerful approach to resolving complex EPR spectra (5). We report herein an improved method for the synthesis of a Mal-6 spin label requiring only five steps. This

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method was utilized for the synthesis of ^{15}N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide-d_{17}, 5. This approach is summarized in Scheme 1.

RESULTS AND DISCUSSION

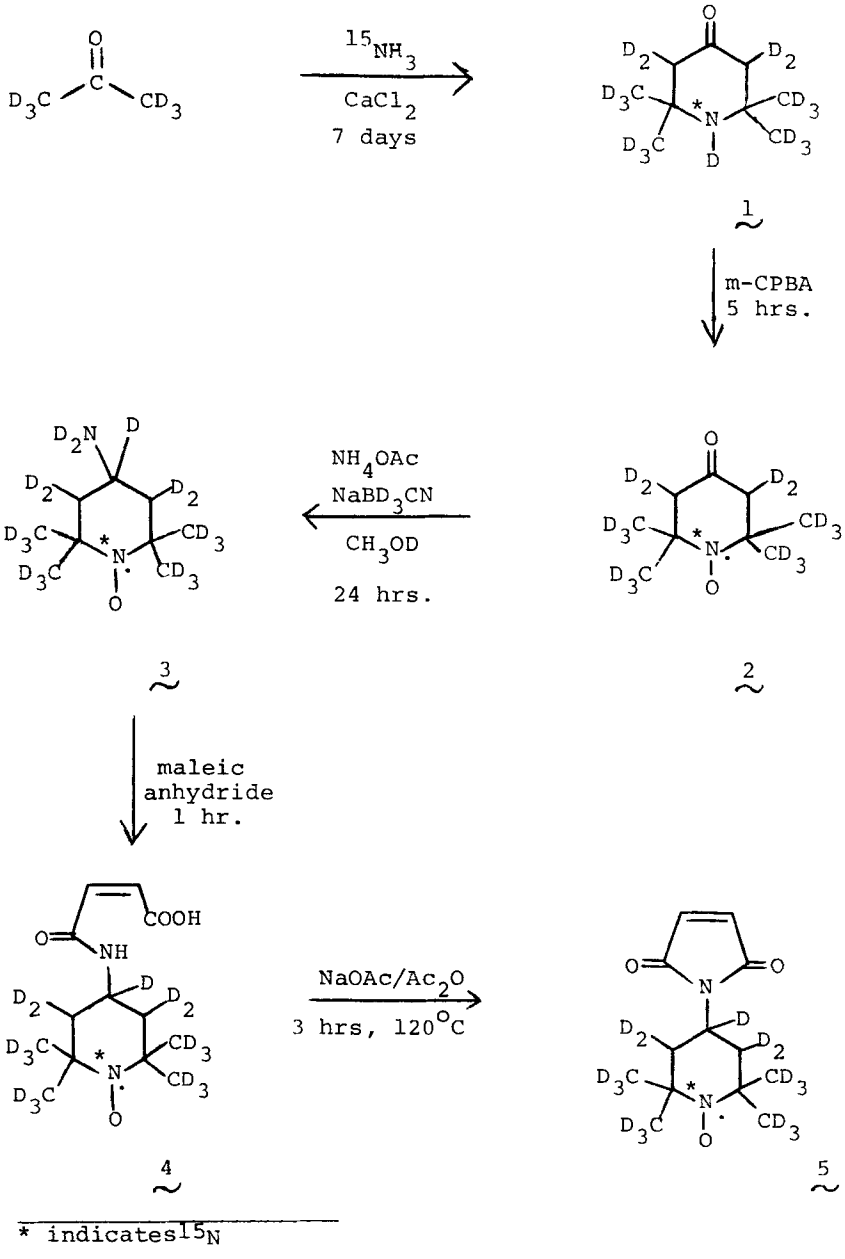
The incorporation of ^{15}N into the Mal-6 type of spin label requires the introduction of ^{15}N-ammonia in the initial step. This step is the preparation of ^{15}N-2,2,6,6-tetramethyl-4-piperidine-d_{16} according to the method of Chiarelli and Rossat (6). Due to the necessity of working on a small scale with the ^{15}N isotope and to optimize the yield of the isotopically substituted triacetoneamine, the modified procedure of Sosnovsky and Konieczny (7) at room temperature was utilized.

Ammonia substituted by both ^{15}N and ^{2}H is available only by custom synthesis in sealed glass flasks at atmospheric pressure. This makes multiple additions of ammonia to the reaction mixture very cumbersome. Preliminary studies showed no significant difference in the deuterium substitution in the final product if $^{15}NH_3$ was used instead of $^{15}ND_3$. $NaOD/D_2O$ was utilized in the workup. This allowed for the introduction of several additions of $^{15}NH_3$ to the reaction mixture since $^{15}NH_3$ was available in a pressurized lecture bottle.

The isotopically substituted triacetoneamine, 1, was directly oxidized to the corresponding nitroxyl, 2, using m-chloroperbenzoic acid essentially by the method of Rauckman, et al. (8). Reductive amination, using ND_4OAc and $NaBD_3CN$, by a modification of the method of Rosen (9) produced 3. Conversion to the maleimide derivative by the usual method (10) completed the synthesis of perdeuterio, ^{15}N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide, 5. This synthetic approach allowed for a 5.3% overall yield of the pure crystalline ^{15}N, ^{2}H-isotopically labelled product.

The approach described herein is comprised of five steps. The previous approaches reported for the deuterio or non-isotopically labelled derivatives have utilized seven or more steps. The shortened reaction sequence is beneficial since the ^{15}N-isotope is necessarily introduced in the first step and must be carried throughout the sequence. This approach was also carried out on a reasonable scale which produced 1.25 g of final product. This is a sufficient quantity for many biochemical protein labelling experiments.

Scheme 1. Synthesis of perdeuterio, ^{15}N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide



* indicates ^{15}N

EXPERIMENTAL METHODS

Melting points were determined on a Thomas-Hoover Mel-Temp apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 710 spectrophotometer. Proton NMR spectra were obtained on a Varian FT-60 NMR spectrometer with a Nicolet TT-7 fourier transform accessory. Spectra were determined in $CDCl_3$ with tetramethylsilane as internal standard. Mass spectra (70 eV) were obtained on a Varian MAT-112S mass spectrometer by direct insertion. The deuterium composition was determined on the molecular ion relative to the non-deuterated standard. EPR spectra were recorded on a Varian E-4 spectrometer. Thin layer chromatography was carried out on precoated silica gel plates (250 μ) from E. Merck. All reagents were analytical reagent grade and were used without further purification. All non-deuterated solvents except anhydrous ether were redistilled prior to use.

Acetone-d_6 (99.5% D), deuterium oxide (99.8% D) and $NaBD_3CN$ were purchased from Aldrich. $^{15}NH_3$ was purchased from Merck and Co. Sodium deuterium oxide and DCl were obtained from Kor Isotopes.

^{15}N-2,2,6,6-Tetramethyl-4-piperidone, d_{17} (1). To a well-stirred mixture of 24.37 g (.38 m) of d_6-acetone (99.5% D) and 9.05 $CaCl_2$ (4-20 mesh) was introduced approximately 5 ml (.17 m) of $^{15}NH_3$ over 3 days in 5 approximately equal portions. The reaction was carried out in a 100 ml 3-neck roundbottom flask fitted with a gas-inlet tube and a dry-ice condensor. After addition of the $^{15}NH_3$ over 3 days the reaction mixture was allowed to sit undisturbed for 4 days at room temperature. During this time the reaction mixture turned red and thickened. After four days, unreacted acetone was removed by distillation under reduced pressure (35°-38°C; 125-140 mm) and 25 g of 40% NaOD in D_2O was diluted to 50 ml with D_2O and added with vigorous stirring. This mixture was then extracted several times with a total of 1000 ml of diethyl ether (anhydrous). After drying over $MgSO_4$ and evaporation of solvent in vacuo, the reddish-yellow oil was dissolved in a small amount of carbon tetrachloride and cooled in the freezer overnight. Colorless needles were collected and three other crops were obtained in the same way. A total of 7.60 g (34.6% based on starting acetone) slightly yellow crystals were finally collected. TLC showed about 90% purity, and this material was used without further purification. Proton NMR indicated less than 5% protons in the methyl and methylene positions.

^{15}N-1-Oxyl-2,2,6,6-tetramethyl-4-piperidone-d_{16} (2). To 5.86 g (.0339 m) of 90% triacetoneamine dissolved in 75 ml of anhydrous methylene chloride was added, over 1 hr with stirring, 13.02 g (.064 m) of 85% meta-chloroperbenzoic acid. The reaction mixture was allowed to stand for 5 hours. The reaction mixture turned dark yellow-red as the oxidant was added. The reaction mixture was then extracted 3x with equal volumes of cold 5% sodium carbonate solution. The organic layer was then dried over $MgSO_4$ and solvent was removed to yield 6.41 g of crude oil. Thin layer indicated about sixty percent purity (67.4% yield), and was utilized directly in the next step.

4-Amino-1-oxyl-2,2,6,6-tetramethyl-^{15}N-piperidine-d_{19} (3). To a solution of 17.99 g (.21 m) of 90% ND_4OAc dissolved in about 60 ml MeOD was added 6.41 g of 60% 4-oxo-2,2,6,6-tetramethyl piperidinooxyl (.0206 m) and .97 g (.015 m) $NaBD_3CN$. ND_4OAc was prepared by dissolving NH_4OAc in MeOD and evaporating the MeOH/MeOD three times *in vacuo*. About 5-10 g of 3A molecular sieves were also added to the solution to absorb water. The reaction mixture was stirred magnetically at room temperature for 24 hrs. After filtering, solvent was removed under vacuum and the red oil taken up into a minimum of D_2O. The pH of the solution was lowered to 5-6 with dilute DCl in D_2O and extracted 3x with $CDCl_3$. The solution was then made basic with NaOD, saturated with NaCl and extracted 3x with $CDCl_3$. After drying over $MgSO_4$, solvent was removed under vacuum to leave a red oil (3.20 g). Thin layer chromatography indicated about 90% purity (73.2% yield).

^{15}N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide-d_{17} (5). Maleic anhydride (1.67 g, .017 m) was dissolved in 20 ml anhydrous ether with stirring. 4-Amino-2,2,6,6-tetramethyl piperidinooxy (3.20 g, .015 m) was dissolved in 10 ml of anhydrous ether and added dropwise to the maleic anhydride solution *via* a dropping funnel. After addition of the piperidinooxy solution the reaction mixture was stirred magnetically for about 1 hr. The product was then collected by filtration and washed with 20 ml anhydrous ether to yield 3.27 g (80% yield).

Cyclization was accomplished by heating a mixture of the maleamic acid derivative (3.27 g, .0114 m), anhydrous NaOAc (0.47 g, .0057 m) and 25 ml of acetic anhydride for 2 hrs at 100°C and 1 hr at 120°C in an oil bath. Acetic anhydride was removed by distillation *in vacuo* and the residue was triturated in benzene. Insoluble solids were removed by

filtration. The crude product was purified on a silica gel column with methylene chloride and increasing amounts of ethyl acetate as eluent. Pure maleimide eluted first. The combined maleimide fractions were dried over $MgSO_4$ and solvent was removed. Recrystallization of the residue from hexane gave 1.25 g (.0046 m) of pure maleimide (40.3% yield) m.p. 104-106°C (lit. (10) mp 99°C). The IR showed no trace of isomaleimide. Mass spectra obtained gave a molecular ion at m/e 269 and the deuterium composition was determined to be: d_{17}, 52.0%; d_{16}, 32.2%; d_{15}, 11.3%; d_{14}, 4.5%. EPR spectra studies showed that the ^{15}N composition was 99.8%.

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