

APPLICATION OF SPIN LABELING TO DRUG ASSAYS.

I. SYNTHESIS OF 2,2,6,6-TETRAMETHYLPYPERIDIN-4-ONE-1-OXYL-¹⁵N-d₁₆

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SUMMARY

Spin labeled drugs can be utilized for immunoassays and determining the fraction of free drug in serum. To improve the sensitivity we have synthesized triacetoneamine-¹⁵N-d₁₇ by cyclization of phorone-d₁₄ with ¹⁵ND₃. Phorone-d₁₄ was obtained by condensation of acetone-d₆. Triacetoneamine-¹⁵N-d₁₇ gave Tempone-¹⁵N-d₁₆, a nitroxide with just two absorption peaks on the ESR spectrum, whereas Tempone gives 3 peaks. The contribution to line width of methylene and methyl hydrogens of Tempone is evaluated by appropriate substitution of protium with deuterium.

Key Words: Phorone-d₁₄, Tempone-¹⁵N-d₁₆, Drug Spin Labels

INTRODUCTION

We are currently investigating a variety of chemical and instrumental modifications of the spin label technique which would permit optimization of the detection by Electron Spin Resonance (ESR) of drugs spin labeled with nitroxyl radicals and permit the assay of the free fraction of drugs in serum (1). With these modifications, it may be possible to lower the minimal detectable concentration of spin labels whereby the ESR technique would be comparable to other techniques. Since the free fraction may correlate better with therapeutic response, this would greatly enhance the clinical value of such serum drug assays. Two major factors in determining the sensitivity of the technique are the width and number of lines on the ESR spectrum of the radical.

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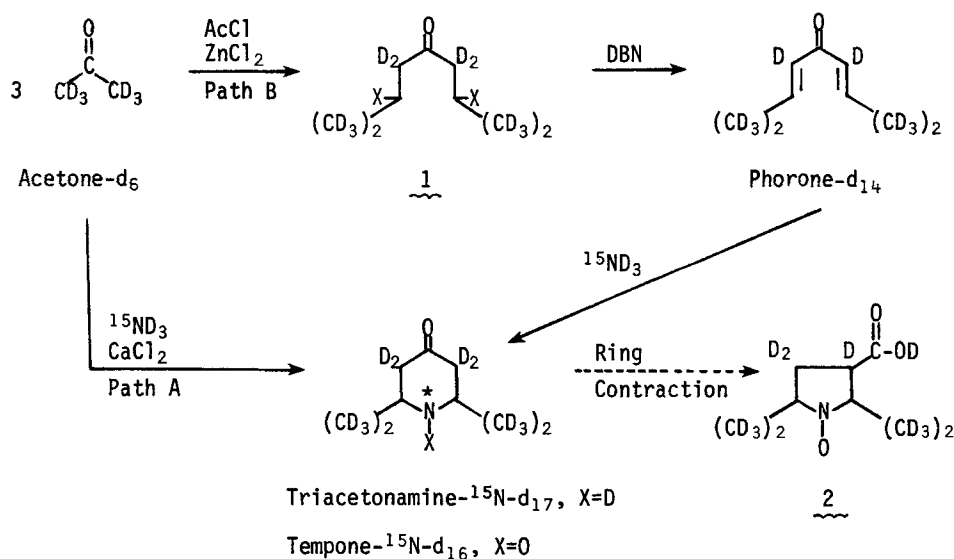
An approach which leads to improved sensitivity is the use of ^{15}N instead of ^{14}N in its natural abundance. The latter isotope has a nuclear spin of 1. The hyperfine interaction of this nucleus with the free electron gives three peaks, whereas ^{15}N ($I = 1/2$) gives only two peaks. With total area of peaks being the same, fewer peaks thus increase the sensitivity by 3/2 when the compounds have the same linewidth as shown by Hedrick *et al.* (2).

For a spin label rotating freely in solution, the width of the individual nitrogen lines is primarily determined by the inhomogeneous broadening due to the superhyperfine interactions of the unpaired electron with nearby protons. Replacement of the protons by deuterons will cause these superhyperfine splittings to be reduced by a factor of six and result in narrower principal peaks (3). Because the area under the peak is constant, this narrowing will increase the signal amplitude of a deuterated relative to that of a protonated probe. Even when the magnetic field modulation is optimized, ultimate sensitivity is still less for protonated compounds (4). Thus, as found by Beth *et al.* (5), deuteration of the spin label leads to an increase in sensitivity by a factor of five. The overall improvement of sensitivity via proposed isotopic substitutions is expected to be about 7-fold.

In this report we describe the synthesis of triacetoneamine- ^{15}N - d_{17} . Triacetoneamine, (2,2,6,6-tetramethylpiperidin-4-one) the precursor of the piperidine and pyrrolidine nitroxides, is ordinarily prepared by the Francis modification of the Heintz-Fischer procedure (6) in which acetone is condensed with an excess of liquid ammonia in the presence of CaCl_2 (Scheme, Path A). It can also be prepared by a Michael-type addition reaction of ammonia to 2,6-dimethyl-2,5-heptadien-4-one (phorone) (7). Despite the cumbersome requirements for aprotic conditions, we elected to prepare triacetoneamine- ^{15}N - d_{17} by first preparing phorone- d_{16} from acetone- d_6 and then cyclizing phorone- d_{16} in a closed system with limiting or equimolar amounts of $^{15}\text{ND}_3$.

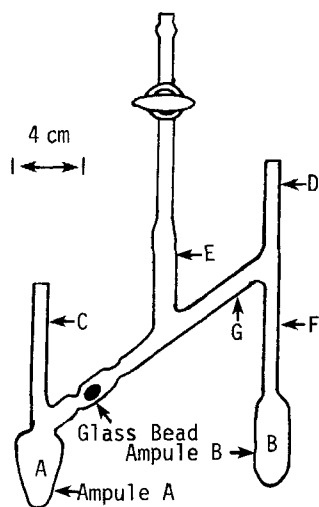
Phorone is commercially prepared by the trimolecular dehydrative condensation of acetone in the presence of an acidic (e.g., HCl) or a basic (e.g., CH_3MgX) catalyst. After numerous attempts with various dehydrating agents

(P₂O₅, molecular sieves, potassium graphite) were unsatisfactory, phorone-d₁₄ was prepared by a modification of Descude's procedure in which acetone-d₆ is condensed in the presence of acetyl chloride and zinc chloride (8) (Scheme, Path B). Acetyl chloride apparently dehydrates the putative intermediate—triacetonedialcohol, 1, X = OH—where the resulting double bond is then apparently hydrohalogenated which in 1 inhibites further (vinylogous) aldol condensations to polyacetones observed previously (9). The hydrochlorinated intermediate 1 was subsequently dehydrohalogenated with 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) to give phorone-d₁₄.



In our current study we have also developed the technique for cyclizing phorone with ammonia to give triacetoneamine in high yield. For this step ¹⁵ND₃ was liberated in one ampule from ¹⁵NH₄Cl with an aprotic base (CaO) and simultaneously enriched with deuterium from D₂O (Figure). Ammonia-¹⁵N-d₃ was then collected over phorone-d₁₄ in the second ampule which was then detached for the cyclization reaction which gave triacetoneamine-¹⁵N-d₁₇. The latter was then oxidized to corresponding nitroxide—Tempone-¹⁵N-d₁₆ (2).

In order to determine the influence of methyl and methylene protons of Tempone on its line width, 2,2,6,6-tetramethylpiperidin-4-one-1,3,3,5,5-d₅ and 2,2,6,6-tetramethyl-d₁₂-piperidin-4-one and the respective nitroxyls were also prepared. The table shows the influence of methyl protons (deuterons) on the



Ammonia Condensation Vessel

line width. Moreover, it was determined by NMR that under the condition of cyclization the vinyl protons of phorone do not exchange with deuterons. However, after cyclization the methylene protons (deuterons) of the resulting piperidine readily exchange either in the presence of a base or an acid.

In conclusion, then, a workable method for preparation of Tempone- ^{15}N - d_{16} on gram scale is described. However, in biomedical experiments it is desirable to use substituted pyrrolidin-1-oxyl or pyrrolin-

Experimental peak-to-peak line widths, ΔH and nitrogen hyperfine splittings a_{N} , of isotopically substituted Tempones*

Compound	ΔH (G)**	a_{N} (G)
Tempone	0.40	0.43*** 16.17
Tempone- d_{16}	0.23	0.33*** 22.68
Tempone- ^{15}N	0.40	
Tempone- ^{15}N - d_{16}	0.23	
Tempone-2,2,6,6-tetramethyl- d_{12}	0.29	
Tempone-3,3,5,5- d_4	0.38	

* Measurements were taken in aqueous 10^{-4} M solution at 25°C . The microwave power was 1 mW and the amplitude of the 100 KHz field modulation was 0.02 G.

** For the natural abundance compounds, the center line, and for ^{15}N compounds, the low field line, respectively, were measured.

*** Ref. 10. Concentration was 10^{-3} M in deoxygenated water.

1-oxyl spin labels, because the 5-membered ring is more rigid. Ring contraction and other steps leading to 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl-d₁₆ 2 will be reported later.

EXPERIMENTAL

UV spectra were recorded with a Beckman DK-2, IR spectra with a Beckman IR-10, and NMR spectra with a Varian A 60D spectrometer. ESR spectra were recorded on a Varian E-4 spectrometer. Gas-liquid chromatography was carried out with a Hewlett-Packard 5750 B Chromatograph. ¹⁵NH₄Cl was obtained from Merck and Company, Incorporated, Rahway, NJ. Other chemicals, including acetone-d₆, were obtained from Aldrich Chemicals, Milwaukee, WI. TLC was performed on 20 x 20 cm plates coated with silica-gel GF₂₅₄, Brinkman Instruments, Westbury, NY. GC phases, the stationary phases, diethylene glycol succinate (DEGS) and methyl silicone phase (oV-1) were purchased from Applied Sciences Laboratories, State College, PA. GC was performed on a Hewlett-Packard 5750 B chromatograph with the following conditions with DEGS column: Injection port 250°C, flame detector 280°C, Argon Flow 30 cc/min, chart speed 0.25/min, temperature program: from 50°C to 200°C at 15°/min; oV-1 column was used at 170°C.

Phorone-d₁₄ (2,6-dimethyl,2,5-heptadien-4-one-d₁₄). Zinc chloride (10 g) in a 300 ml round-bottom flask was melted and then allowed to cool in a nitrogen atmosphere. Trichloroacetyl chloride (57.8 ml, 0.520 mol) was added and the mixture was cooled to 0°C. Acetone-d₆ (50 g, 0.781 mol) was added. The flask was tightly stoppered and the stopper was secured with tape. The mixture was then stirred and allowed to come to room temperature. After six days the resulting red solution was cooled, diluted with ether (150 ml) and then washed with D₂O (2 x 30 ml). More cold D₂O (40 ml) was added; anhydrous sodium carbonate was then added in small portions until the evolution of CO₂ ceased and/or the color of the mixture changed from red to straw-colored. Stirring was continued until the pH of the lower phase was 10. The solution was allowed to rise to room temperature. The organic phase was decanted. The aqueous phase was filtered, the filtrate was saturated with NaCl and organic products extracted with ether (2 x 50 ml). The organic phases were combined, washed with dilute

aqueous NaOD (15 ml), dried with anhydrous Na_2CO_3 and MgSO_4 and the volume was reduced to about 300 ml. The solution was cooled in an ice bath, and 1,5-diazabicyclo[4.3.0]non-5-ene, (DBN) (30 g) was added in small portions. The mixture was then slowly brought to 50°C . The flask with the resulting concentrate was stoppered and kept at room temperature overnight. A white hygroscopic precipitate resulted. It was removed by filtration and washed with ether. The filtrate and the washings were pooled and assayed by GC with DEGS column. After the required amount of DBN had been added, the filtrate had a 55:40 ratio of mesityl oxide (Rt 140 sec) to phorone (Rt 460 sec).

The filtrate was diluted with ether (25 ml). Excess DBN was removed by washing the ether phase with 10% DCl which had been saturated with NaCl until the wash was acidic. The ether solution was then concentrated and the residue distilled at 2 mm pressure over a 10 cm Vigreux column. The temperature of the heating oil bath was not allowed to exceed 80°C . After the residual ether had been collected, six fractions of 1 ml were collected between $25\text{--}70^\circ\text{C}$. The fraction collected at 30°C was mesityl oxide and the fraction collected at 55°C was phorone (2.5 g, 16 mmol, 20% yield); IR ν_{max} neat: 2900 (trace) C-H, 2200, 2180, 2150 and 2040 strong (C-D), 1640 (C=O), 1600 (C=C) cm^{-1} ; NMR (ppm) in CDCl_3 : an envelope at 1.8 and 2.2 ppm (CH_3 of phorone).

Triacetoneamine- $^{15}\text{N-d}_{17}$. Phorone- d_{14} (1.43 g, 9.41 mmol) and a micro spinning bar were added to ampule B and cooled to -78°C . Similarly, $^{15}\text{NH}_4\text{Cl}$ (627 mg, 11.0 mmol), and a micro spinning bar were put into ampule A and then cooled in a Dry Ice-acetone bath. Deuterium oxide (D_2O) (3.0 ml) was added to freeze over the ammonium chloride followed by CaO (450 mg). The vessel was sealed at C and D, the system was evacuated and then flame-sealed at E. Then, with the lower tip of the ampule B at -78°C , the contents of the ampule A were allowed to come to room temperature over a magnetic stirrer. Gaseous ammonia was thus slowly liberated and condensed over the phorone- d_{14} . A slow rate of evolution of $^{15}\text{ND}_3$ was achieved either by warming the ampule A between 0° to 40°C and/or by the level of the cooling acetone-bath at ampule B. Liberation of $^{15}\text{ND}_3$ was considered complete when the condensate over the phorone solidified due to increasing

codistillation of water. Ampule B was then flame-sealed and detached at F or at G while it was being shielded from the flame with aluminum foil. The ampule was then slowly warmed in an oil bath and in 1 hr brought to 85°C while the contents were stirred vigorously. At the end of 8 hr the ampule was cooled to -78°C, the flame-seal was opened and the mixture assayed by GC. If the chromatogram showed less than about 75% yield, the ampule was resealed and stirred for an additional 4 hr at 85°C. Depending on the yield, the product was then isolated by two alternative procedures: 1) GC showed less than 6% of phorone and 2) GC showed more than 6% of the phorone.

(1) The reaction mixture was diluted with ether and then transferred to a 25 ml round-bottom flask. The components of low volatility were removed at reduced pressure to yield the produce as a solid residue. According to GC it contained typically 4 to 5% of phorone.

(2) The reaction mixture was diluted with ether and then treated with N₂ gas for about 5 min. The product was then extracted with 10% DCl until the last extract was acidic. The aqueous phase was made basic by addition of anhydrous Na₂CO₃. This aqueous mixture was at about 50°C brought to near dryness at reduced pressure. The resulting solid residue was washed with anhydrous ethanol-d₁, (2 x 2 ml). This ethanolic solution was then filtered, warmed to 35°C and diluted with anhydrous ether to incipient cloudiness. Upon cooling the product precipitated, 1.03 g (62%) mp 35°C; IR (KBr):2220 (C-D) and 1700 (C=O) and GC on the DEGS column Rt 240 sec. The filtrate was condensed and the oily residue (residue A) by GC was found to contain 85% triacetoneamine ¹⁵N-d₁₆. The solid product was used for ring contraction and residue A for preparation of Tempone-¹⁵N-d₁₆.

2,2,6,6-Tetramethyl-d₁₂-piperidin-4-one. Phorone-d₁₄ (90 mg, 0.59 mmol) and conc. NH₄OH (210 μl, 3 mmol) and a micro spinning bar in a 10 ml ampule were cooled in a Dry-Ice-acetone bath and the ampule was sealed. The mixture was then stirred at 85°C for 4 hr. Ether (2 ml) was then added to the cooled mixture. The aqueous phase was pipetted off and the organic phase was dried (MgSO₄). The ether was removed at reduced pressure and the solid residue was

assayed by NMR (CDCl_3): 2.25 ($-\text{CH}_2-$), 1.45 (N-H) ppm and no visible, i.e., less than 4%, exchange of D with H at $-\text{CD}_3$, (1.25 ppm); GC showed 6% starting material, yet NMR showed no vinyl H (6.10 ppm), hence no exchange occurred.

2,2,6,6-Tetramethyl-1,3,3,5,5-d₅-piperidin-4-one. Phorone (690 mg, 5.0 mmol) conc. ND_4OD (0.75 ml, 11 mmol) were reacted for 20 hr at 85°C. The product was isolated as described above to yield the product as the solid residue (90%); MS: (161, 35%), (160, 70%), (159, 100%) (9); NMR (CDCl_3): 1.25 (CH_3) and no visible peak at 2.25 ppm ($-\text{CH}_2-$). GC with DEGS column showed 5% of phorone, Tempone-¹⁵N-d₁₆. The filtrate residue (residue A, from preparation of triacetoneamine-¹⁵N-d₁₇, 250 mg) was dissolved in MeOD (1 ml), D_2O (3 ml), D_2O_2 (0.10 ml). Phosphotungstic acid (30 mg) was added and the mixture stirred at room temperature (2). GC with DEGS column showed that after 18 hr only trace of starting material (Rt 240 sec) remained. The reaction mixture was concentrated and chromatographed on silica gel GF₂₅₄ (ether:petroleum ether, 3:2). The yellow band, Rf 0.23, gave the product (92%); Rt with DEGS column: 450 sec and with OV-1 column 140 sec.

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