SYNTHESIS OF 4-PROTIO-3-CARBAMOYL-2,2,5,5-TETRAPERDEUTEROMETHYL-3-PYRROLIN-1-YLOXY (mHCTPO): A SELECTIVELY ISOTOPICALLY LABELED COMPOUND FOR USE IN T_2 SPIN LABEL OXYMETRY

Yawares J. Lin and Beverly A. Teicher[1]
Dana-Farber Cancer Institute
44 Binney St., Boston, MA 02115

and

Howard J. Halpern
Pritzker School of Medicine, University of Chicago
Chicago, IL 60637

Summary

The title compound (mHCTPO) has been prepared to enhance sensitivity to molecular oxygen concentration in its Electron Spin Resonance (ESR) spectrum. The synthesis involved a novel variant of the traditional nitroxide synthesis leading to a much improved yield of mHCTPO over the several steps of the synthesis. An enhancement of T_2 spin label oxymetry with independent measurment of spin label concentration (from a spectral feature) has been observed. This molecule represents a new class of potentially valuable compounds for in vivo application and for imaging in ESR oxymetry.

Key Words: 4-Protio-3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy-d_{12}, selectively isotopically spin label, ESR, oxymetry

INTRODUCTION

A wide variety of nitroxide free radicals are readily available as spin labels for producing electron resonance (ESR) signals (1). The methyl protected N-O group contains the unpaired electron necessary to produce an ESR signal, but it is the reactive functional group on the other end of each molecule that provides the chemical handle needed in preparing useful spin labels. It has been shown by others (2-5), with perdeuterated spin labels such as 2,2,6,6-tetramethyl-4-piperidin-4-oxy-1-yloxy d_{16} (TEMPONE) (1) that the inhomogeneous line width could be reduced and spectral amplitude

---

[1]Author to whom correspondence should be addressed.

0362–4803/90/060621–11$05.50

Received July 6, 1989
Revised December 4, 1989

increased by replacing hydrogen with deuterium near the nitroxide functionality.

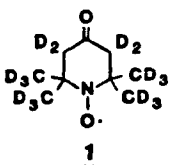
We are involved in a program to exploit the sensitivity of nitroxide ESR spectral width to oxygen in pursuit of an ESR oxymetry. The narrow lines of the deuterated nitroxide compounds are both sensitive to lower oxygen tensions and have increased amplitude relative to hydrogenated compounds. There are disadvantages, however, to the use of fully deuterated compounds for in vivo ESR oxymetry. The height and width of the perdeuterated spin label spectrum is sensitive to both the oxygen tension and the spin label concentration. The concentration dependence of the perdeuterated spectral height (at constant oxygen tension) is not simple. The double integral of the (standard) derivative spectrum is proportional to the number of contributing spins (and hence the concentration of spins) in a sample with uniform spin label distribution. However, the accuracy of the measurement of the double integral will be reduced by details of spectral acquisition - e.g., baseline drift, finite time constant distortion, overmodulation distortion, and sample phase shifts. In a sample consisting of heterogeneous distribution of spin label as would be the case in vivo, label concentration is much more difficult to measure.

To minimize these difficulties, it would be useful to more fully separate the effect of concentration on the spectrum from the effect of line broadening as is the case with the perhydrogenated compound. In order to obtain maximal sensitivity for imaging ambient tissue oxygen tension (in vitro and in vivo), a partially deuterated spin label $\underset{\sim}{2}$ with selective hydrogenation in a single location has been synthesized. By using a partially deuterated nitroxide spin label selectively hydrogenated in a single location we have obtained an oxymetric spin label which possesses the narrow spectrum (and therefore much of the signal to noise advantage) of the perdeuterated compound while at the same time providing sufficient spectral structure to extract information independent of the oxygen concentration about the spin label concentration without double integration.

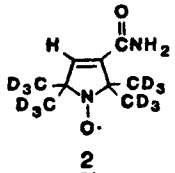
RESULTS

Chemistry

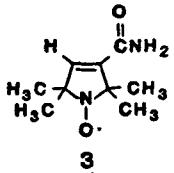
The desired free nitroxyl radical 2 is an analog of the known commercially available CTPO (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy) (3). The key intermediate to this nitroxide 2 is the dibromopiperidone hydrobromide 4. Conversion of 4 to the target molecule 2 can be accomplished by a traditional nitroxide synthesis (6) through the pyrroline-3-carboxyamide 5. This crucial intermediate 4 should come from deuterium/hydrogen exchange of the active α-α'-methine deuterium atoms adjacent to the carbonyl carbon in compound 6 under acidic condition. 3,5-Dibromopiperidone hydrobromide (6) can be obtained from bromination of triacetoneamine-d_{16} (7) with bromine in acetic acid.



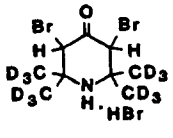
1



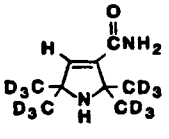
2



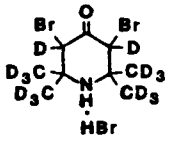
3



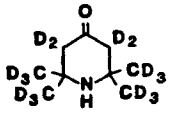
4



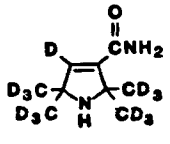
5



6



7



8

Oxymetry with the mHCTPO.

Spectra were taken with 1 x 10^{-4}M of the 4-protio-3-carbamoyl-2,2,5,5-tetraperdeuteromethylpyrrolin-1-yloxy. The spectrometer used for the measurements was a novel low frequency ESR spectrometer operating at 250 MHz, designed for in vivo measurements (11). It therefore was capable of measuring relatively large aqueous sample volumes. Typically the sample volume measured here was 10 ml.

A typical spectrum from the central nitrogen manifold of the 4-hydrogenated (mHCTPO) triplet is shown in Figure 1. This represents the derivative of the radiofrequency energy absorption as is common in ESR spectral presentation. In Figure 2 two spectral parameters are defined. The spectral coupling is defined as the splitting in the absorption spectrum induced by the 4-hydrogen. The coupling a diminishes linearly with spin label concentration in the concentration region applicable for biologic measurements. It is, however, insensitive to oxygen concentration. This allows separation of broadening due to spin label concentration effects from oxygen broadening. The second spectral parameter defined in Figure 2 is the r parameter, the measure of spectral width. It is sensitive to broadening due to both other spin labels and oxygen. It is similar in concept of the k parameter defined by Lai et al. (7).

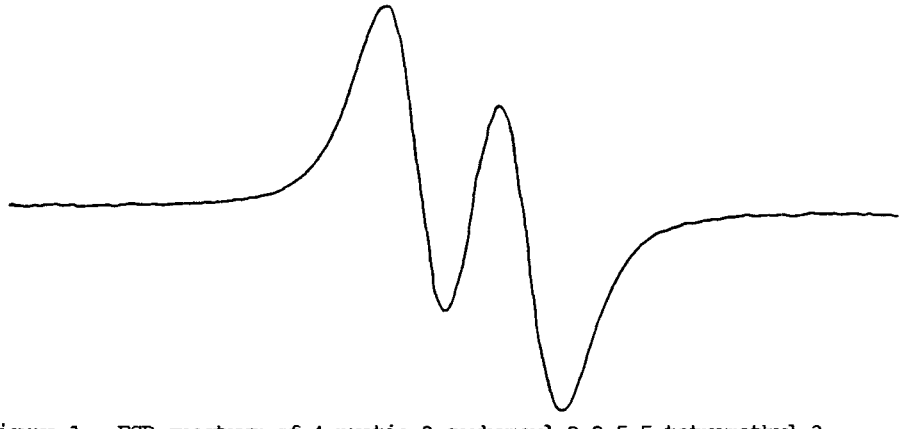


Figure 1. ESR spectrum of 4-protio-3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (mHCTPO). Spectral conditions are as indicated in the text.

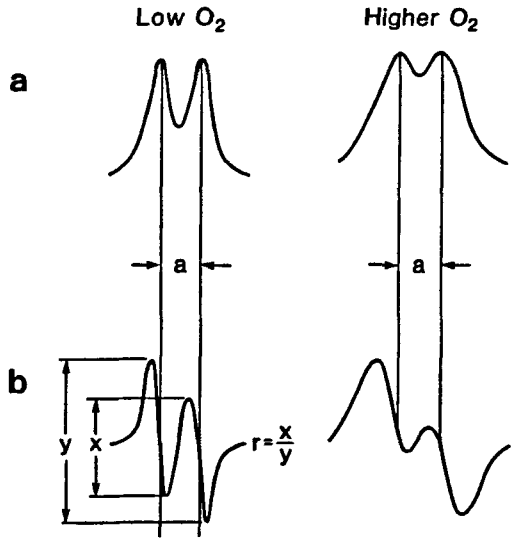


Figure 2. Diagram of the ESR spectra of 4-protio-3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (mHCTPO) indicating the two parameters a and r that characterize the spectrum. The line separation or hyperfine coupling is denoted a and depends on the concentration of mHCTPO but not on the concentration of oxygen. The r value indicated is a measure of overall line width, and decreases with both increasing oxygen and with the concentration of mHCTPO. This allows determination of both oxygen concentration and mHCTPO concentration from intrinsic spectral characteristics.

In Figure 3 data derived from the central nitrogen line of the spectra from a 10 ml, 1 x 10^{-4}M sample of mHCTPO equilibrated with mixtures containing various proportions of oxygen and 99.9% pure nitrogen. The proportions were determined using rotometer flowmeters to adjust and measure component gas flow rates before they were combined and bubbled for two hours through the stopper sealed samples, shaking the samples frequently. Spectra were measured at a temperature of 31°C.

The parameter r is defined as the ratio of the height of the central dip bump feature of the spectrum to the height of the flanking and major bump dip structure as demonstrated in Figure 2. The r values are plotted as a function of oxygen concentration for the spin label mHCTPO concentrations of 1 x 10^{-4}M and 2 x 10^{-4}M. There is a linear relationship of the r value

with oxygen tension over an oxygen concentration range suitable for in vivo measurements.

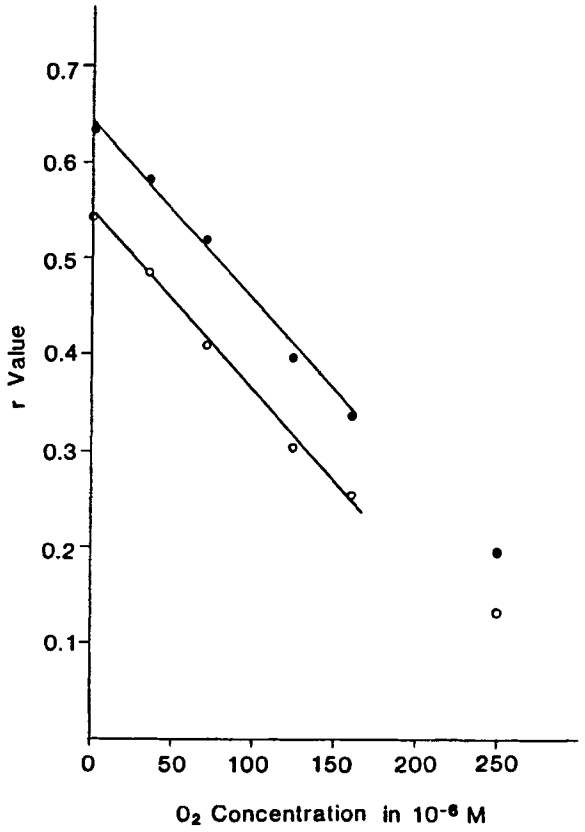


Figure 3. Relationship between the r value derived from ESR spectra of mHCTPO and oxygen concentration for two concentrations of mHCTPO (1 x 10^{-4}M, ●; 2 x 10^{-4}M, ○).

DISCUSSION

Although triacetoneamine-d_{16} (7) has been prepared previously (2,9,10) by the reaction of acetone-d_6 and ammonia-d_3 in the presence of calcium chloride, we hereby report a more convenient method for the preparation of this compound 7 in higher yield (56.7%). A mixture of ammonium-d_4 chloride (which is readily commercially available), acetone-d_6, anhydrous sodium carbonate and magnesium oxide was heated in a tightly capped round-bottomed flask. Deuterated ammonia is generated in situ by

this method. Bromination of triacetoneamine-d_{16} with bromine in acetic acid gave a white precipitate which was not the expected 3,5-dibromo-3,5-dihydrogenated piperidone hydrobromide $\underset{\sim}{4}$ but rather a mixture of 3,5-dibromo-3,5-dideutero-2,2,6,6-tetraperdeuteromethyl-4-oxopiperidine hydrobromide; 3,5-dibromo-3-deutero-2,2,6,6-tetraperdeuteromethyl-4-oxopiperidine hydrobromide and 3,5-dibromo-2,2,6,6-tetraperdeuteromethyl-4-oxopiperidine hydrobromide. This was indicated by the ^{13}C-NMR ($CDCl_3$) at δ 142.04 (s) and 141.88 (t), as a mixture of 2,2,5,5-tetraperdeuteromethyl-pyrroline-3-carboxyamide ($\underset{\sim}{5}$) and 4-deutero-2,2,5,5-tetraperdeutermethyl-pyrroline-3-carboxyamide ($\underset{\sim}{8}$) when the white precipitate was treated with solid potassium hydroxide in aqueous ammonia. Addition of hydrobromic acid after the bromination reaction and allowing the mixture to stir for a longer period (3 days) did not alter the ^{13}C-NMR spectrum of the pyrroline-3-carboxyamide. Eventually, the deuterium/hydrogen atoms were completely exchanged (at least > 99%) when the resulting brominated product was treated with a mixture of 1:1 (v/v) acetic acid and 1N hydrobromic acid at room temperature for 11 days, as indicated by the ^{13}C-NMR spectrum of the pyrroline-3-carboxyamide $\underset{\sim}{5}$ [$CDCl_3$, δ 142.06 (s)]. The structure of $\underset{\sim}{5}$ was also confirmed by the mass spectral data obtained on a VG ZAB-SE double-focusing spectrometer. Oxidation of $\underset{\sim}{5}$ by traditional method for the nitroxide preparation afforded the target 4-protio-3-carbamoyl-2,2,5,5-tetraperdeuteromethyl-3-pyrrolin-1-yloxy ($\underset{\sim}{2}$) in good yield.

Conclusion

We have developed a novel spin label $\underset{\sim}{2}$ taking advantage of the process of selective isotopic labeling to optimize its use as an oxymetry substrate. This selectively isotopically labeled compound has been found to reduce the linewidth of the ESR spectrum relative to that of the fully hydrogenated analog for the hypoxic species by a factor of three and showed a linear dependence on the oxygen tension. Use of mHCTPO will allow relatively simple extraction of quantitative oxymetric information from the very complicated environments of living systems.

Experimental Section

Melting points were determined in Pyrex capillary tube in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA) and are not corrected. IR spectra were obtained on a Perkin-Elmer Model 781 double-beam spectrophotometer. NMR spectra were determined on a Varian XL-400 instrument with tetramethysilane as the reference. Mass spectral data were obtained on a VG ZAB-SE double-focusing spectrometer. The samples were ionized by fast atom bombardment. Ammonium-d_4-chloride was purchased from Sigma Chemical Co., acetone-d_6 (99.5 atom %D) was obtained from Aldrich Chemical Co., magnesium oxide (98%,-325 mesh) was obtained from Alfa Products, Anhydrous sodium carbonate and other chemicals were obtained from Fisher Scientific Co.

2,2,6,6-Tetraperdeuteromethyl-3,3,5,5-tetradeuteropiperidine (Triacetoneamine-d_{16}) (7). A mixture of ammonium-d_4-chloride (3.45 g, 0.06 mole), acetone-d_6 (99.5 atom % D, 12.5 ml, 0.15 mole), anhydrous sodium carbonate (3.18 g, 0.03 mole) and magnesium oxide (3.0 g) was placed in a 250 ml round bottomed flask. The reaction mixture was capped with a rubber septum, wired and heated in an oil-bath at 50°C for 3 days. Acetone (20 ml) was added to the cooled reaction mixture and the mixture was filtered. The recovered solid was crushed into powder, washed with 15 ml of acetone and filtered with suction filtration. The combined filtrates were concentrated to dryness. The resulting red liquid (7.25 g) was distilled under reduced pressure to obtain 4.75 g (56.7%) of a bright yellow liquid (54-55°C/1.9 Hg mm) which solidified when chilled in a dry ice/acetone bath. The product was subsequently used as such. Recrystallization from ether obtained white crystals, mp 57-58°C [lit. (8) 58°C]; IR (KBr): ν3580 (m), 3260 (m), 2220 (m), 1700 (s), 1530 (w), 1265 (s), 1140 (m), 1050 (m), 930 (w) cm^{-1}; ^{13}C-NMR ($CDCl_3$): δ 31.03 (m), 53.50 (m), 54.88 (s), 211.19 (s).

3,5-Dibromo-2,2,6,6-tetraperdeuteromethyl-4-oxopiperidine hydrobromide (4). Triacetoneamine-d_{16} (7) (4.05 g, 23.7 mmol) was disolved in glacial acetic acid (16 ml) and cooled in an ice bath. A solution of bromine (8.14 g, 2.62 ml) in 10 ml of acetic acid was slowly

added to the stirred solution of triacetoneamine-d_{16}. After the addition of bromine was completed, the reaction mixture was stirred at room temperature for 22 h. The precipitate was collected by suction filtration and washed successively with acetic acid, water and ether and was then dried in the air. This gave 7.12 g (74%) of a white powder substance with mp 180-191°C.

The white powder substance (0.32 g, 0.79 mmol) was stirred in a mixture of 1 N hydrobromic acid (5 ml) and acetic acid (5 ml) at room temperature for 11 days. The precipitate was filtered to obtain 0.15 g of product. The filtrate was concentrated, filtered, washed with water (1 ml) and then with anhydrous diethyl ether to give an additional 0.054 g of the product. The total yield was 0.20 g (62.1%).

2,2,5,5-Tetraperdeuteromethylpyrroline-3-carboxyamide (5). To a stirred suspension of 3,5-dibromo-2,2,6,6-tetraperdeuteromethyl-4-oxopiperidine hydrobromide (4) (0.146g, 0.36 mmol) in 28% aqueous ammonia (1.5 ml) was added enough solid potassium hydroxide to saturate the solution. The reaction mixture was stirred overnight. The precipitate of the amide was filtered and dried in air to obtain 0.0506 g (78.3%) of a white solid, mp 183-185°C; IR (KBr): ν3360 (m), 3200(m), 2220 (w), 1665 (s), 1645 (s), 1605 (s), 1415 (m), 1170 (w), 1060 (w) cm^{-1}; ^{13}C-NMR ($CDCl_3$): δ 29.20 (m), 63.02 (s), 66.33 (s), 142.06 (s, 2C), 167.22 (s); mass spectrum: 181 (m+1), 180 (M), 162 (M-CD_3).

4-Protio-3-carbamoyl-2,2,5,5-tetraperdeuteromethyl-3-pyrrolin-1-yloxy (2). To a solution of 2,2,5,5-tetraperdeuteromethylpyrroline-3-carboxyamide (5) (0.031 g, 0.17 mmol) in water (1 ml) was added ethylenediaminetetraacetic acid tetrasodium salt (0.005 g, 0.013 mmol), sodium tungstate dihydrate (0.005 g, 0.015 mmol) and 30% hydrogen peroxide (0.08 ml). The reaction mixture was left in the dark for 4 days. The yellow crystals were filtered and the filtrate was acidified with 1 N hydrochloric acid and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate, filtered, and the filtrate was evaporated to dryness. The total yield of the free nitroxyl radical was 0.025 g (76%), mp 203-204°C (dec.); IR (KBr):

ν3400 (m), 3180 (m), 2230 (w), 1670 (s), 1630 (m), 1420 (m), 1320 (w), 1150 (w), 1100 (w), 1040 (w) cm^{-1}; mass spectrum :197 (M+2), 196 (M+1), 195(M), 181 (M+2-NH_2).

Spectrometer and Spectroscopic Conditions. The spectrometer is discussed in detail elsewhere (11). It consists of a parallel inductor-capacitor resonator wherein the inductor is the sample holder. Power through a directional coupler is supplied to the system capacitively and matched through an electronically controllable capacitive step up network. The reflected signal is extracted using homodyne demodulation. The frequency supplied by the signal generator is locked to the resonator tune through a phase locked loop feeding the frequency modulation of the radiofrequency signal generator. Signal acquisition was done with an IBM AT microcomputer based system for monitoring and control. Lock-in amplifier time constant was typically 0.1 seconds with data point time spacing equal to the time constant. Ten scans sufficed to obtain the spectra for the data shown in Figure 1.

Acknowledgement

This work was supported by NCI grant #RO1-CA36508-05 (BAT) and American Cancer Society Grant PDT-262 (HJH). The authors are grateful to Dr. Steve Santikarn, Department of Nutrition, Harvard School of Public Health (present address: Bangkok, Thailand), for his help in obtaining mass spectra.

References

1. Griffith O.H. and Waggoner A.S. -Accounts Chem. Res. 2:17 (1969).
2. Chiarelli R. and Rassat A. -Tetrahedron, 29:3639 (1973).
3. Dalton L.A., Monge J.L., Dalton L.R. and Kwiram A.L. -Chem. Phys. 6:166 (1974).
4. Hwang J.S., Mason R.P., Hwang L. and Freed J.H. -J. Phys. Chem. 79:489 (1975).
5. Beth A.H., Venkataramu S.D., Balasubramanian K., Dalton L.R., Robinson B.H., Pearson D.E., Park C.R. and Park J.H. -Proc. Natl. Acad. Sci. USA. 78:967 (1981).

6. Rozantsev E.G. -Free Nitroxyl Radicals, Plenum Press, New York-London, 1970, pp. 203-205.

7. Lai C.S., Hopwood L.E., Hyde J.S. and Lukiewicz S. -Proc. Natl. Acad. Sci. USA. 79:1166 (1982).

8. Halpern H.J., Teicher B.A., Bowman M.K., Peric M., Lin Y.J., Nguyen T.D., and Spencer D.P. -J. Magnetic Resonance, submitted, (1989).

9. Graffney B.J. -Spin Labeling: Theory and Applications, Ed. Berliner L.J., Academic Press, New York, 1976, pp. 206-207.

10. Venkataramu S.D., Pearson D.E., Beth A.H., Perkins R.C., Park C.R. and Park J.H. -J. Labelled Compds. Radiopharm. 18:371 (1981).

11. Halpern H.J., Bowman M.K, Spencer D.P., Van Polen J., Dowey E.M., Massoth R.J., Nelson A.C. and Teicher B.A. -Review of Scientific Instruments, 60:1040 (1989).