SYNTHESIS OF ^{15}N-5-DOXYLSTEARIC ACID FOR IMPROVED EPR CHARACTERIZATION OF LIPID MOTION IN BIOMEMBRANES

Sindhaghatta D. Venkataramu,[a,b] Donald E. Pearson[a*], Albert H. Beth,[b] K. Balasubramanian,[b] Charles R. Park[b] and Jane H. Park[b*]

([a]Chemistry and [b]Physiology)
Nashville, Tennessee 37232

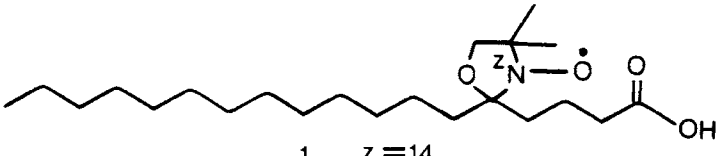
SUMMARY

^{15}N-5-Doxylstearic acid has been synthesized to improve EPR spectral characterization of the motional properties of the lipid in biological membranes. The EPR spectrum of the title compound displayed a 1.73 fold gain in sensitivity compared with the corresponding label substituted with the common isotope of nitrogen.

Key Words : 5-Doxylstearic acid, ^{15}N, spin label, EPR

INTRODUCTION

Lipid spin labels possessing a "Doxyl" group (1), of which 5-doxylstearic acid (1) is representative, have been widely used



1, z = 14
2, z = 15

*To whom correspondence may be addressed.

0362-4803/83/040433-13$01.30

Received October 5, 1982

in the electron paramagnetic resonance (EPR) and saturation transfer electron paramagnetic resonance (ST-EPR) investigations of the motional characteristics and ordering of lipids in biological membranes (2). In these and related studies such as the interaction of lipids with proteins and mechanisms of fatty acid permeation across cell walls, the low signal to noise ratio is a limiting factor in the experimental design. In addition, the overlap of resonances results in inhomogeneous line broadening which hinders motional characterization from conventional EPR spectra by quantitative simulation. Even determination of the rotational correlation time, τ_c, by measurement of intensity ratios, H"/H and L"/L, at turning points from ST-EPR spectra may be difficult.

We have recently shown that spectral sensitivity and resolution can be significantly enhanced for biological investigations by deuteration and ^{15}N-substitution in the $\backslash N \rightarrow O^{\cdot}$ group in N(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (3-5). The spectral gains resulted from the replacement of strong electron proton hyperfine interactions in the unmodified label by weak magnetic interactions by deuterium substitution and from reduction of the nuclear manifolds from three to two by ^{15}N-substitution.

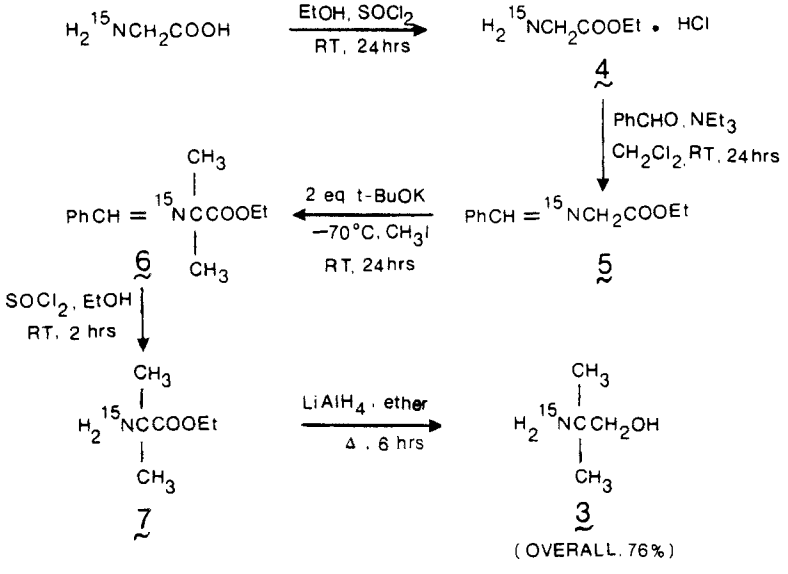
In order to improve EPR spectral analysis for motional studies of lipids in biological membranes, we have synthesized ^{15}N-5-doxylstearic acid (2). The new analog has shown increases in resolution and sensitivity when bound to bovine serum albumin at a variety of correlation times (6,7). Improvements were also observed when the label was used as a probe in plasma membranes of

erythrocytes (6,7). In this communication, we describe the synthesis of ^{15}N-5-doxylstearic acid (2) and comparative EPR data for ^{14}N and ^{15}N labels as freely tumbling entities.

RESULTS AND DISCUSSION

Keana et al. (8) have developed two synthetic routes for lipid spin labels containing a doxyl group. Consideration of both approaches implied that ^{15}N-2-amino-2-methyl-1-propanol (3, Scheme I) was required in a substantial quantity to prepare 2 and related members. The classical procedure (9) for the corresponding amino alkanol with the common isotope of nitrogen is not satisfactory

SCHEME I



for ^{15}N-modification because of unavailability of the required ^{15}N-labeled precursor and yield considerations. For the same reasons, the alternative procedure (10) for the fully deuterated ^{14}N-analog of 3 is not suitable. Starting from ^{15}N-glycine, we have prepared ^{15}N-2-amino-2-methyl-1-propanol (3) in 76% overall yield. The key step in the conversion, shown in Scheme I, is the sequential alkylation of benzylidene glycine ester 4 which is based on the findings of Stork et al. (11).

The Schiff base 5 was prepared in high yield from ^{15}N-glycine via the ester hydrochloride 4. Deprotonation of 5 with two equivalents of potassium t-butoxide at -70°C produced the anion which reacted smoothly with excess methyl iodide to afford alkylated derivative 6 in high yield. Removal of the protective group by treatment with thionyl chloride in ethanol followed by basification gave the corresponding α-substituted amino acid ester 7. Lithium aluminum hydride reduction of the ester 7 furnished pure 3. The procedure described here for 3 is simple, proceeds under mild conditions with high yield in each step, and can be readily adopted for deuterium modification also.

The synthesis of ^{15}N-5-doxylstearic acid (2), formulated in Scheme II, was accomplished by a general procedure (8,12). Thus acid catalyzed condensation of methyl-5-keto stearate (8) with a ten-fold excess of ^{15}N-2-amino-2-methyl-1-propanol (3) with azeotropic removal of water formed afforded the oxazolidine 9. Oxidation of 9 followed by saponification gave ^{15}N-5-doxylstearic acid (2).

SCHEME II

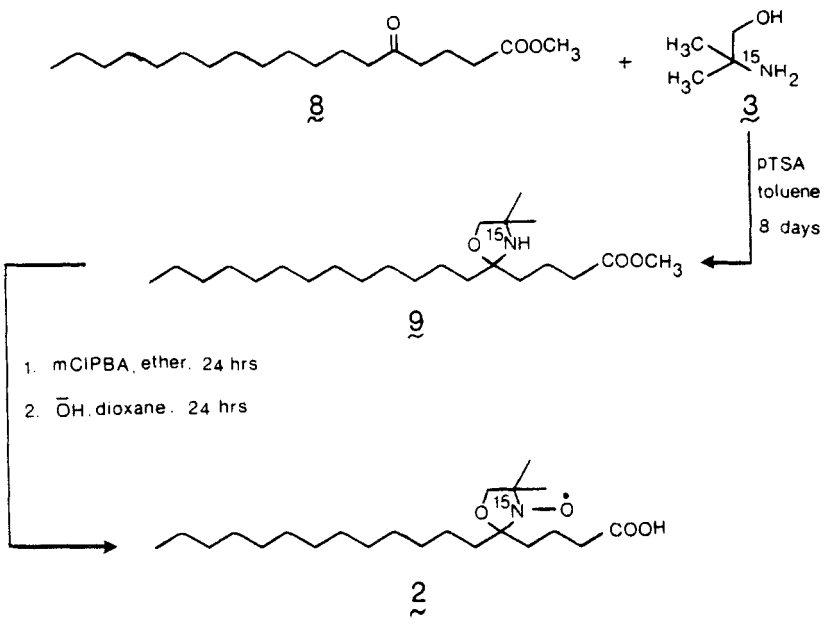


Fig.1. (next page) compares the EPR spectrum for the conventional label, 5-doxylstearic acid (1) with that of the newly synthesized ^{15}N-analog 2 as freely tumbling entities at equal concentrations. The ratio of the low field signal amplitude in the ^{15}N-spectrum to that of the center line in the ^{14}N-spectrum is 1.73. A value of 1.58 for sensitivity enhancement has been reported (13) for ^{15}N-2,2,6,6-tetramethyl-4-oxo-piperidine-1-oxyl compared with the corresponding ^{14}N label. The increase in sensitivity is primarily due to reduction in the number of lines from three to two (4,13-15).

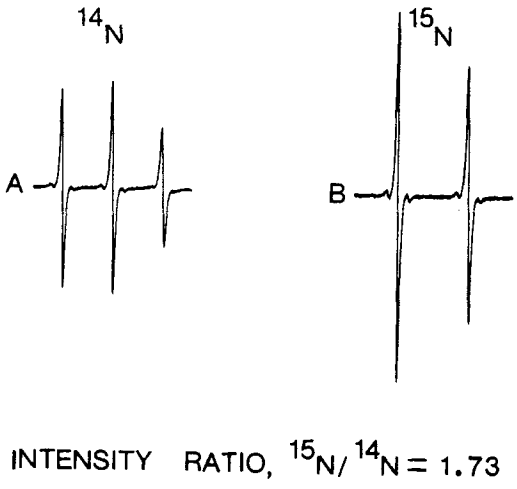


Fig. 1. X-band EPR spectra of freely tumbling (A) 5-doxylstearic acid and (B) 15-N-5-doxylstearic acid. The 50 G spectrum of each spin label at a concentration of 10 μMolar in 5mM phosphate buffer, pH 7.5, was recorded at 23°C with identical instrument settings of 10 mWatt power, 0.5 G field modulation and 100 kHz frequency.

In conclusion, a method is presented for the alteration of the basic unit responsible for the paramagnetic property in lipid spin labels which results in improved EPR spectral resolution and sensitivity. Further enhancements in sensitivity and resolution should be possible by replacement of hydrogens with deuterium in the vicinity of the paramagnetic group. We are currently exploring this possibility.

EXPERIMENTAL SECTION

The physical data were obtained as follows : melting points in a Thomas-Hoover melting point apparatus (uncorrected); IR spectra on a Perkin-Elmer 727 ; PMR on a Joel MH-100 with TMS as internal standard; EPR spectra on a Varian E-109 spectrometer equipped with TM_{110} cavity. Thin layer chromatography (TLC) analyses were carried out on Eastman Chromagram 13181 precoated silica gel plates with fluorescent indicator. Spots were revealed by exposure to UV light or iodine vapor. Reagents and solvents were purified when necessary. Literature melting or boiling points indicated in parentheses refer to unlabeled compounds.

Potassium t-butoxide was purchased from Aldrich. ^{15}N-Glycine was obtained from Merck Co. and was converted to ^{15}N-glycine ethyl ester hydrochloride 4, mp 142-144°C, by treatment with thionyl chloride and ethanol (16). A near quantitative yield was realized by precipitating the hydrochloride with anhydrous ether.

5-Doxylstearic acid (1) was purchased from Syva. Methyl-5-keto stearate (8) was prepared by the general procedure of Hubbell and McConnell (17).

Benzylidene Derivative of ^{15}N-Glycine Ethyl Ester (4). The procedure of Stork et al. (11) was followed. Thus, to a magnetically stirred suspension of ^{15}N-glycine ethyl ester hydrochloride 4 (27.6 g, 0.196 mole), freshly distilled benzaldehyde (21.5 g, 0.202 mole), anhydrous magnesium sulfate (20 g) and 400 ml CH_2Cl_2, cooled in ice, was added triethylamine (60 ml, 0.42 mole) and the stirring continued at room temperature for 24 hr. The slurry was

suction filtered and the solid was washed with several portions of CH_2Cl_2. After solvent removal in vacuum at room temperature, the residue was cooled in ice, diluted with 100 ml ice cold water. The oil was extracted with ether (3 x 100 ml) and the combined organic extract was washed with brine and dried (anhyd. $MgSO_4$). Removal of the solvent by rotoevaporation and finally at the vacuum pump afforded the Schiff base 5 as a pale yellow oil (37.1 g, 98.5%). PMR (CCl_4) δ 1.26 (3H, t, $COOCH_2CH_3$), 4.02 - 4.24 (2H, q, $COOCH_2CH_3$), 4.24 (2H, s, $^{15}NCH_2$), 7.29 - 7.42 (3H, m, Ar), 7.62 - 7.78 (2H, m, Ar), 8.16 - 8.22 (1H, d,-CH=^{15}N-). The Schiff base 5 was used in the next step without further purification.

Ethyl 2-(benzylidene-^{15}N-amino)propionate (6). To a magnetically stirred suspension of potassium t-butoxide (50.3 g, 0.45 mole) in 300 ml dry THF, cooled to -70°C, was added Schiff base 5 (37.1 g, 0.193 mole) in 100 ml dry THF during 15 min (N_2). The orange yellow solution was stirred for half hour and then freshly distilled methyl iodide (75.6 g, 0.53 mole) in equal volume of THF was added dropwise. The mixture was allowed to attain room temperature and stirring continued for 16 hr. Most of THF was rotoevaporated below 35°C and the resulting slurry was cooled in ice, and 100 ml ice cold water was added with vigorous stirring. The oily layer was separated and the aqueous layer extracted with ether (3 x 100 ml). The combined organic phase was washed with water, brine and the organic extract dried (anhyd. $MgSO_4$). Removal of ether in a rotoevaporator and finally at the pump (0.1 mm) afforded 6 as a pale yellow oil (40.3g, 94.8%). PMR (CCl_4) δ 1.10 - 1.25 (3H, t, $COOCH_2CH_3$), 1.40 - 1.44 (6H, d, $2CH_3$), 4.02 -

4.22 (2H, q, $COOCH_2CH_3$), 7.28 - 7.40 (3H, m, Ar), 7.62 - 7.78 (2H, m, Ar), 8.18 - 8.24 (1H, d,-CH=^{15}N-). The product was pure as judged by PMR and was used in the next step without purification.

Ethyl 2-^{15}N-amino-2-methyl propionate (7). To a solution of the benzylidene ester 6 (40.3 g, 0.183 mole) in 200 ml absolute ethanol, cooled to -5°C, was added thionyl chloride (32.72 g, 0.275 mole) during half hour. After stirring at 0°C for 1 hr and then at room temperature for 2 hrs, most of ethanol and thionyl chloride were removed under reduced pressure. The golden yellow oil was extracted with pentane (3 x 100 ml) and the pentane layer was washed with ice cold 1 N hydrochloric acid. The combined acid extract was cooled in ice-salt bath and cautiously saturated with solid K_2CO_3. The liberated amine was extracted with ether (4 x 75 ml) and the organic phase dried (K_2CO_3). Removal of the solvent afforded a pale yellow oil (22 g) with a strong amine odor. Distillation at atmospheric pressure gave pure amine 7, bp 145 - 148°C, as a colorless mobile liquid (18.5 g, 77%). The pot residue was dissolved in ether and passed through a short column of silica gel (2 x 6 cm) and eluted with ether to give 0.8 g more of amine raising the yield to 80%. PMR (CCl_4) δ 1.18 - 1.32 (9H, m, $COOCH_2CH_3$ and $2CH_3$), 1.40 (2H, b, $^{15}NH_2$), 4.00 - 4.22 (2H, q, $COOCH_2CH_3$).

^{15}N-2-Amino-2-methyl-1-propanol (8). To a well-stirred suspension of $LiAlH_4$ (9 g, 0.24 mole) in 300 ml dry ether was added a solution of the amino ester 7 (19.3 g, 0.146 mole) in 100 ml ether at such a rate as to maintain a uniform boiling of ether. After stirring at room temperature for 6 hr, the mixture was refluxed

for 6 hr, cooled in ice-salt bath, and excess $LiAlH_4$ was destroyed by successive addition of 9 ml water, 9 ml of 15% sodium hydroxide solution, and 27 ml of water. The ether layer was decanted and washed several times with ether. The combined organic extract was dried (anhyd. K_2CO_3) and the solvent removed in vacuum. The viscous oil was distilled at atmospheric pressure using a short column. The fraction boiling between 162 - 166°C was the required ^{15}N-2-amino-2-methyl-1-propanol (3). PMR (CCl_4) δ 1.02 - 1.04 (6H, d, $2CH_3$), 2.80 (3H, b, $^{15}NH_2$ and OH), 3.16 - 3.20 (2H, d, OCH_2).

^{15}N-5-Doxylstearic acid (2). The reported procedure (12) for the corresponding ^{14}N-analog was followed. A solution of methyl-5-ketostearate (8, 624 mg, 2 mmole) and amino alkanol 3 (1.8 g, 20 mmoles) in 25 ml xylene containing 15 mg p-toluenesulfonic acid was stirred and refluxed in a Dean-Stark apparatus containing anhyd. K_2CO_3 for 8 days. The cooled solution was diluted with an equal volume of ether and washed successively with saturated sodium bicarbonate solution (3 x 25 ml), water (3 x 25 ml) and brine (2 x 25 ml). The organic extract was dried (anhyd. $MgSO_4$) and the solvent was removed in a rotoevaporator and finally at the pump (0.1 mm) to afford the oxazolidine 9 as a pale yellow oil (0.8 g).

The crude amine 9 (0.8 g) was dissolved in 100 ml ether, cooled in ice, and a solution of m-chloroperbenzoic acid (0.5 g) dissolved in 20 ml ether was added dropwise. After stirring at room temperature for 36 hrs, the organic phase was washed with saturated sodium bicarbonate solution (4 x 25 ml), water (3 x 25 ml) and dried (anhyd. $MgSO_4$). Removal of the solvent in vacuum

gave the radical as a pale oil (0.6 g). Chromatography on silica gel (40 g, 60 - 200 mesh size) and elution with hexane-ether mixtures with increasing amounts of the polar solvent furnished the radical ester (0.5 g). This was rechromatographed on silica gel (10 g) and eluted with ether. Approximately 2-ml fractions were collected. Purity of each fraction was assessed by TLC (hexane-benzene, 70 : 30) employing the corresponding ^{14}N-analog for comparison. Homogeneous fractions were pooled, taken to dryness, to give pure radical ester (0.37 g) as an orange yellow oil.

The radical (0.37 g) was dissolved in 2 ml dioxane and 5 ml 4% sodium hydroxide solution and let stand at room temperature for 3 hrs. The solution was acidified with conc. HCl to pH 2 and diluted with 25 ml ice cold water. The acid was extracted with ethyl acetate (2 x 25 ml) and the organic phase was washed with water (3 x 25 ml) and dried (anhyd. Na_2SO_4). Removal of the solvent in vacuum and finally at the pump (0.1 mm) afforded ^{15}N-5-doxylstearic acid (2, 220 mg) as a pale yellow solid, mp 48 - 49°C. The corresponding ^{14}N-analog melts at 47 - 48 °C.

ACKNOWLEDGMENT

We gratefully acknowledge financial support of this work by the U. S. Public Health Service, 5-R01-GM 07884, and the Muscular Dystrophy Association. Dr. K. Balasubramanian is a recipient of postdoctoral fellowship from the Muscular Dystrophy Association.

REFERENCES AND NOTES

1. Doxyl refers to the 4',4'-dimethyl-oxazolidine-N-oxyl group
2. Berliner L. J. - Spin Labeling I : Theory and Applications, Academic Press, New york, 1976
3. Venkataramu S. D. Pearson D. E. Beth A. H. Perkins R. C. Park C. R. and Park J. H. -J. Labeled Compds. and Radiopharm. 18: 371 (1981)
4. 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)
5. Beth A. H. Balasubramanian K. Wilder R. T. Venkataramu S. D. Robinson B. H. Dalton L. R. Pearson D. E. and Park J. H. - Proc. Natl. Acad. Sci. USA. 78: 4955 (1981)
6. Venkataramu S. D. Pearson D. E. Park C. R. and Park J. H. - Fed. Proc. 41: 1389 (1982)
7. Beth A. H. Venkataramu S. D. Balasubramanian K. Dammers A. J. Pearson D. E. Park C. R. and Park J. H. - Int. Symposium on EPR, Denver, CO, 24, 85 (1982)
8. Keana J. F. W. - Spin Labeling II ; Theory and Applications, Ed., Berliner L. J. Academic Press, New York, 1979, p 115
9. Johnson K. and Degering E.F. - J. Org. Chem. 8: 7 (1943)

10. Chiarelli R. and Rassat A. - Tetrahedron. 29: 3639 (1973)
11. Stork G. Leong A.Y.W. and Touzin A.M. - J. Org. Chem. 41: 3493 (1976)
12. Jost P. Libertini L.J. Hebert V.C. and Griffith O.H. - J. Mol. Biol. 59: 77 (1971)
13. Hedrick W.R. Mathew A. Zimbrick J.D. and Whaley T.W. - J. Magn. Reson. 36: 207 (1979)
14. Yost Y. Polnaszek C.F. Mason R.P. and Jordan L. - J. Labeled. Compds. and Radiopharm. 18: 1089 (1981)
15. Gaffney B.J. Elbrecht C.H. and Scibilia P.A. - J. Mag. Reson. 44: 436 (1981)
16. Brenner M. and Huber W. - Helv. Chim. Acta. 36: 1109 (1953)
17. Hubbell W.L. and McConnell H.M. - J. Am. Chem. Soc. 93, 314 (1971)