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AN IMPROVED SYNTHESIS OF [^{15}N] 16-DOXYL STEARIC ACID

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SUMMARY

An improved method for the synthesis of [^{15}N] 16-Doxyl stearate, a fatty-acid spin label, is reported. The method requires only one mole of [^{15}N] 2-amino-2-methyl propanol per mole of methyl keto stearate with an overall yield of about 50%. In contrast, the previous method produced only 1-3% yield. The great improvement in yield was achieved mainly through the reduction of reaction volume which increases the reaction rate by the law of mass action.

Key Words: 16-Doxyl stearic acid, ^{15}N, spin label, ESR

INTRODUCTION

The [^{14}N] Doxyl fatty-acid spin labels (FASL) with the nitroxide group attached to various positions along the acyl chain are widely used for structural studies of biological membranes by means of electron spin resonance (ESR) spectroscopy (1). The use of [^{15}N] FASLs instead of [^{14}N] FASLs provides an enhancement of spectral sensitivity and resolution because of the reduction of nuclear manifolds and of line overlap. The [^{15}N] FASL should therefore be a better probe than its [^{14}N] analogue for fluidity studies of biological systems. In addition, the simultaneous use of [^{15}N] FASL and [^{14}N] FASL has been shown to improve greatly the sensitivity of ESR methods in the measurement of lipid-lipid collision frequencies in membranes (2-5). The wider application of these experiments, however, is hampered by the lack of the availability of [^{15}N] FASLs.

The synthesis of [^{15}N] FASL was first reported by Venkataramu et al. (6) who adapted the synthetic route for [^{14}N] FASL developed by Keana et al. (7) to

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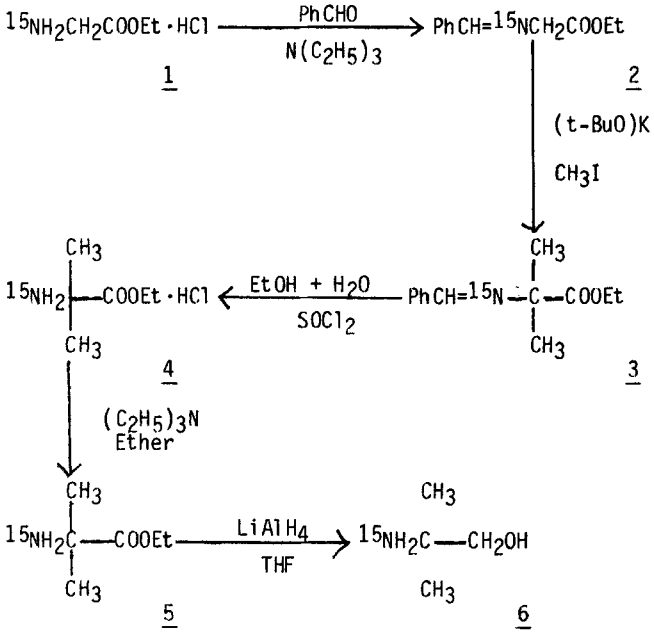
prepare the [^{15}N] analoque. The method has an overall yield of only 1-3% (8) which has become a major restraint in the cost-effective production of [^{15}N] FASLs. In this communication, we report an improved method for the synthesis of [^{15}N] 16-Doxyl FASL which produces an overall yield of 50% with respect to 2-amino-2-methyl propanol.

RESULTS AND DISCUSSION

The synthesis of [^{15}N] 2-amino-2-methyl propanol

The synthetic routes for Doxyl FASLs developed by Keana et al. (6) require a substantial amount of [^{15}N] 2-amino-2-methyl propanol. The synthesis of the latter was reported by Venkataramu et al. (7). However, our attempt in preparing [^{15}N] 2-amino-2-methyl propanol following their approach encountered some difficulties. In their synthetic procedure, the Schiff's base 3 (see Scheme I) was hydrolyzed by using $SOCl_2$ in absolute ethanol. The use of absolute ethanol



Scheme I

as a solvent during the hydrolysis might have been a mistake because the hydrolysis requires water molecules. We found that inclusion of a small quantity of water facilitates the complete hydrolysis. The rest of the synthetic

routes for 4 through 6 (see Scheme I) were then developed in our laboratory. After the hydrolysis reaction, the liberated benzaldehyde was extracted with pentane. By evaporating ethanol, 4 was obtained in a solid form. Neutralization of 4 with triethylamine in ether generated 5 which was reduced with $LiAlH_4$, and then refluxed in tetrahydrofuran (THF) to give rise to 6. There are two major differences between our procedure and Venkataramu et al.'s approach for the syntheses of 4 through 6. The amino acid ester 5 is known to exhibit high water solubility (9). We prepared 5 in dry ether to minimize its losses whereas water was present in their method. In addition, our procedure does not require the distillation of 5 which is known to be heat-labile at atmospheric pressure (9). The synthetic routes described here are highly reproducible and produce an overall yield of about 60% of 2-amino-2-methyl propanol with respect to [^{15}N] glycine.

The synthesis of Doxyl fatty acid spin label

The synthetic procedure for Doxyl FASLs developed by Keana et al. (7,8) involves the refluxing of the keto ester of the fatty acid in toluene with about ten-fold excess of 2-amino-2-methyl propanol and a catalytic amount of p-toluene sulfonic acid for 8-10 days. The resulting oxazolidine is then oxidized to the corresponding Doxyl FASL which is further purified by chromatography. This method produced about 1-3% of FASL based on the amount of 2-amino-2-methyl propanol used (8). Thus, an enormous proportion of 2-amino-2-methyl propanol was wasted during the synthesis. Since [^{14}N] 2-amino-2-methyl propanol is inexpensive, Keana's method has been used for commercial-scale production of [^{14}N] FASLs. However, the cost of [^{15}N] 2-amino-2-methyl propanol has become a major limitation in the [^{15}N] FASL synthesis using Keana's approach. Attempts were made in our laboratory to improve the overall yield of this synthetic route. It was found that by reducing the reaction volume by a factor of 8, a 20-fold increase in the yield of FASL could be accomplished using a one-to-one molar ratio of the keto ester of the fatty acid and 2-amino-2-methyl propanol. Thus, reduction in reaction volume increases the efficiency of the reaction probably as a result of the law of mass action. In a typical experiment, the

reaction of 2 mmoles of methyl 16-keto stearate and 2 mmoles of [^{15}N] 2-amino-2-methyl propanol in 3 ml toluene for 6 days in the presence of 10 mg of p-toluene sulfonic acid produces about 1 mmole of pure 16-Doxyl FASL. The amount corresponds to a 50% yield based on the amount of [^{15}N] 2-amino-2-methyl propanol used.

In conclusion, we have described an improved synthetic route for [^{15}N] 16-Doxyl FASL which increases the yield by a factor of 20 when compared to the yield reported previously. Our method should be useful for the preparation of other [^{15}N] FASLs as well as other short acyl chains containing the Doxyl moiety. The wider availability of these [^{15}N] probes should stimulate research in the field of spin-label studies of biological systems.

EXPERIMENTAL

[^{15}N] glycine was purchased from MSD Isotopes (St. Louis) and other chemicals used were obtained from Aldrich Chemical (Milwaukee). For thin layer chromatography (TLC), the samples were run on Kodak silica gel sheets, developed by a mixture of hexane:ether:acetic acid (70:30:2), and detected by using a sulfuric acid spray (50% sulfuric acid). Doxyl derivatives were detected by UV light. 16-Keto stearic acid methyl ester was synthesized by the procedure of Marsh and Watts (10).

Hydrolysis of Schiff's base and isolation of ethyl [^{15}N] 2-amino-2-methyl propionate

The synthetic routes for 1 through 3 (see Scheme 1) were as described by Venkataramu et al. (6); 19.5 g (88 mmol) of ethyl 2-(benzylidine-[^{15}N] amino) propionate, 3, was obtained starting with 7.5 g of [^{15}N] glycine. The Schiff's base 3 (88 mmol) was then dissolved in 200 ml of absolute ethanol containing 3 ml of water. To the solution was added 16.5 g (138 mmol) of $SOCl_2$ at a rate such that the temperature remained at -5 to 0°C. After warming up to ambient temperature for 2-4 hours with gentle stirring, the solution was rotary evaporated at 30°C to reduce the volume to about 40 ml which was then extracted with 3 x 50 ml of pentane. The pentane extract containing benzaldehyde was discarded. The

alcoholic solution was further rotary evaporated and kept under vacuum overnight to produce a light brown solid of the amino acid ester hydrochloride 4 (12.5 g, 74 mmol).

The solid ethyl [^{15}N] 2-amino-2-methyl propionate hydrochloride, 4, was dissolved in 25 ml of dry methanol containing 8.0 g (80 mmol) of triethylamine. The solution was basic as tested with a moist litmus. To the solution was added 300 ml of ether, which was stirred at 0°C for 30 min. The precipitated amine hydrochloride was filtered off. The filtrate was then rotary evaporated and dried under vacuum to yield a light brown liquid of [^{15}N] 2-amino-2-methyl propionate, 5 (9.7 g, 73 mmol).

The reduction of the amino ester 5 was carried out with the use of $LiAlH_4$ (5.3 g, 140 mmol) in refluxing tetrahydrofuran. The reaction yielded 6.0 g of the crude product which was distilled at 50 Torr at 75°C to produce 5.5 g of viscous [^{15}N] 2-amino-2-methyl propanol, 6. The overall yield was 62% based on the amount of [^{15}N] glycine used.

Synthesis of [^{15}N] 16-Doxyl stearic acid

To 3 ml of dry toluene was added methyl 16-keto stearate ester (0.624 g, 2 mmol), [^{15}N] 2-amino-2-methyl propanol (0.18 g, 2 mmol), and p-toluene sulfonic acid (10 mg). The reaction mixture was refluxed for 6 days in a 25-ml flask using a Dean-Stark water separator containing a few grams of anhydrous K_2CO_3 for 6 days. The TLC result showed no trace of the free keto stearate ester, suggesting that the reaction reached completion. The resulting yellow liquid was diluted with 50 ml of ether; the solution was then washed twice with 10 ml of saturated $NaHCO_3$ solution and the residual moisture was removed with $MgSO_4$. M-chloroperoxybenzoic acid (0.5 g) in 25 ml of ether was added dropwise to the above solution kept cold in an ice bath. After stirring at ambient temperature for 24 hours, the deep yellow liquid formed was washed with 3 x 25 ml of saturated $NaHCO_3$ solution, 2 x 25 ml saturated NaCl, and then dried with $MgSO_4$. The solution was rotary evaporated and dried under vacuum to yield a yellow liquid of the [^{15}N] 16-Doxyl stearate ester. It was further chromatographed on silica gel (70-230 mesh, 40 g) and eluted with hexane:ether (70:30). To the

ester dissolved in 2 ml of dioxane was added 5 ml of a 4% NaOH solution, which was then kept at ambient temperature for 24 hours. After the solution pH was adjusted to pH 2-3 with 2 N HCl, the final product was extracted with chloroform, dried with $MgSO_4$ and rotary evaporated to yield a yellow solid of [^{15}N] 16-Doxyl stearic acid (0.390 g, 1.0 mmol). The product was pure as determined by both TLC and ESR measurements. It migrated as a single spot on the TLC plate to the same position as that of the [^{14}N] analogue (Aldrich Chemicals). In addition, the ESR spectra of the probe in synthetic phospholipid membranes (not shown) are identical to those reported previously (2,3).

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