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Syntheses of [^{13}C,^{15}N]-Labeled Polyamines

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Summary

[1,4-$^{13}C_2$,1,4-$^{15}N_2$]butanediamine (**1**), a key compound in the syntheses of [5,8-$^{13}C_2$,1,4,8-$^{15}N_3$]spermidine (**2**) and [5,8-$^{13}C_2$,1,4,8,12-$^{15}N_4$]spermine (**3**), has been prepared as part of a 6-step process from 1,2-dibromoethane using potassium [^{13}C]cyanide and potassium [^{15}N]phthalimide. In the course of the syntheses, it was found that 1,4-dibromobutane was generated from tetrahydrofuran when bromination using triphenylphosphine and tetrabromomethane took place. A high-yield preparation of monobenzyloxycarbonyl (Z) derivative of **1**, a precursor for **2**, was obtained using a water-soluble Z reagent, Z-DSP, in a two-phase system of alkaline solution and chloroform. All the steps for **1**, **2**, and **3**, were aimed at minimizing the loss of stable isotopes.

Keywords: spermidine, spermine, 1,4-dibromobutane, monobenzyloxycarbonyl putrescine, internal standard, IS-MS

Introduction

Two major polyamines in mammalian cells, spermidine and spermine, as well as the diamine putrescine are biosynthesized and metabolized in cells in response to growth signals, differentiation, etc.[1] They pass through cell membranes with the aid of an ATP-dependent polyamine transport system. The presence of such a transport system suggests that extracellular polyamines may play a role in maintaining a cellular polyamine level.[2] To obtain more information on the role of extracellular polyamines, especially in the whole animal, we planned the use of ^{15}N-labeled polyamines as tracer compounds, the syntheses of which had been reported using ^{15}N-labeled ammonium sulfate.[3] Recently, we developed a unique method for the

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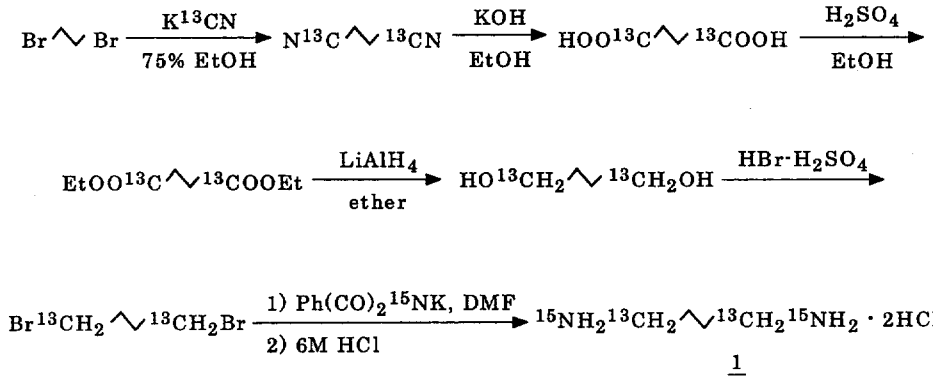
separation and determination of heptafluorobutyryl (HFB) derivatives of polyamines by ion spray ionization–mass spectrometry (IS-MS), in which ^{15}N-labeled polyamines were used as internal standards for the determination of natural polyamines.[4] This methodology will be applied in the tracer experiments using internal standards for polyamines having a higher mass than ^{15}N-labeled polyamines. This paper deals with the syntheses of such internal standards.

Results and Discussion

Potassium [^{13}C]cyanide and potassium [^{15}N]phthalimide were used as the sources of ^{13}C and ^{15}N.

The synthetic route for **1** is shown in Scheme 1. The first step for the synthesis of



Scheme 1. Synthesis of [1,4-$^{13}C_2$,1,4-$^{15}N_2$]butanediamine

[1,4-$^{13}C_2$] succinodinitrile proceeded readily using a 1:1 ratio of potassium [^{13}C]cyanide and 1,2-dibromoethane.[5] The conversion of [1,4-$^{13}C_2$] succinodinitrile to succinic acid followed by esterification to obtain [1,4-$^{13}C_2$]ethylsuccinate proceeded satisfactorily. Reduction of [1,4-$^{13}C_2$]ethylsuccinate to [1,4-$^{13}C_2$] butanediol was conducted with $LiAlH_4$ in dry ether. After evaporation, the butanediol was extracted from the residue with EtOH in a yield of 90%.

For the preparation of [1,4-$^{13}C_2$]-1,4-dibromobutane from [1,4-$^{13}C_2$]-1,4-butanediol, we first tried bromination using tetrabromomethane and triphenylphosphine in THF,[6] since preliminary trials using unlabeled 1,4-butanediol gave a high yield of more than 95%. IS-MS analysis of the resulting labeled 1,4-dibromobutane revealed significant contamination of unlabeled 1,4-dibromobutane. It was shown by GLC that the amount of unlabeled 1,4-dibromobutane increased during the bromination reaction in the absence of

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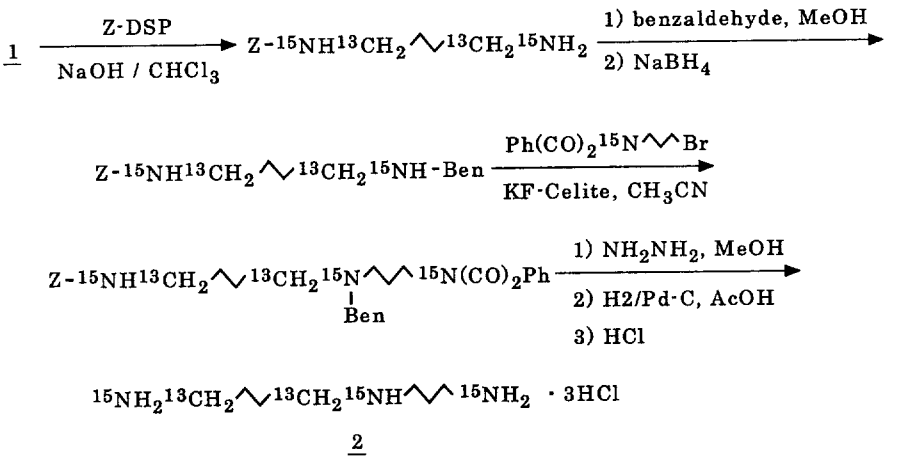
1,4-butanediol, hence, the contamination must have been from the THF. Therefore, an alternative bromination route for labeled 1,4-butanediol using concentrated hydrobromic acid and sulfuric acid[7] was examined and found to give pure [1,4-$^{13}C_2$]-1,4-dibromobutane with a yield of 80-90%. The next steps for **1**·2HCl, involving reaction with potassium [^{15}N]phthalimide, hydrolysis in 6M hydrochloric acid, and purification by cation exchange chromatography, have already been described.[3]



Scheme 2. Synthesis of [5,8-$^{13}C_2$,1,4,8-$^{15}N_3$]spermidine

Scheme 2 shows the synthetic route for **2**. The first two steps were improved over those reported previously.[3] To overcome the low yield of mono-Z derivative of **1** caused by preferential formation of the N,N'-bis-Z derivative when ZCl is used, we planned the immediate extraction of the mono-Z derivative prepared during the reaction of **1** with a water-soluble Z-reagent, Z-DSP,[8] in a two-phase system using sodium hydroxide solution and chloroform under vigorous stirring. In a mixture of putrescine (3 mmol) and Z-DSP (3.6 mmol) in 3M sodium hydroxide (5 ml) and chloroform (20 ml), it was found by HPLC that 75% of the putrescine was converted to its mono-Z derivative and 5% to its di-Z derivatives. Unreacted **1** and the bis-Z derivative of **1**, which eventually formed after removal of Z by a hydrochloric acid treatment, were recovered as **1**·2HCl through a cation exchanger. Because the Z group of the mono-Z derivative was not removed under the conditions used for $NaBH_4$-reduction to introduce the benzyl group to the primary amine, the next step was adopted to produce a reasonable yield of the protected precursor of **2**, prepared by alkylation with [^{15}N]-(3-bromopropyl)phthalimide. The protected precursor, after

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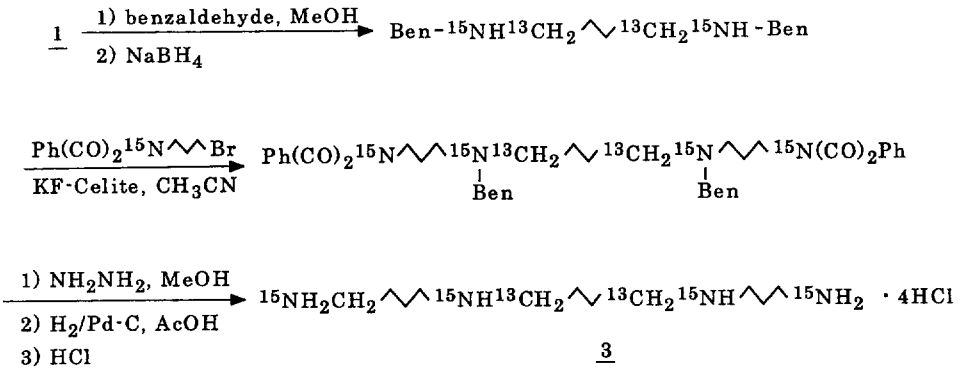
purification by silica gel column chromatography, was deprotected by the usual procedures to obtain **2**·3HCl, which could, if necessary, then be further purified through a small cation exchange column.

The synthetic route for **3** was essentially the same as previously reported for ^{15}N-labeled spermine (Scheme 3).



Scheme 3. Synthesis of [5,8-$^{13}C_2$,1,4,8,12-$^{15}N_4$]spermine

In the final process, a mixture of synthesized **1**, **2** and **3** was subjected to HFB derivatization and analyzed by IS-MS.[4] As is shown in Fig.1, the three HFB derivatives carrying ammonium ions appeared at the calculated m/z values.

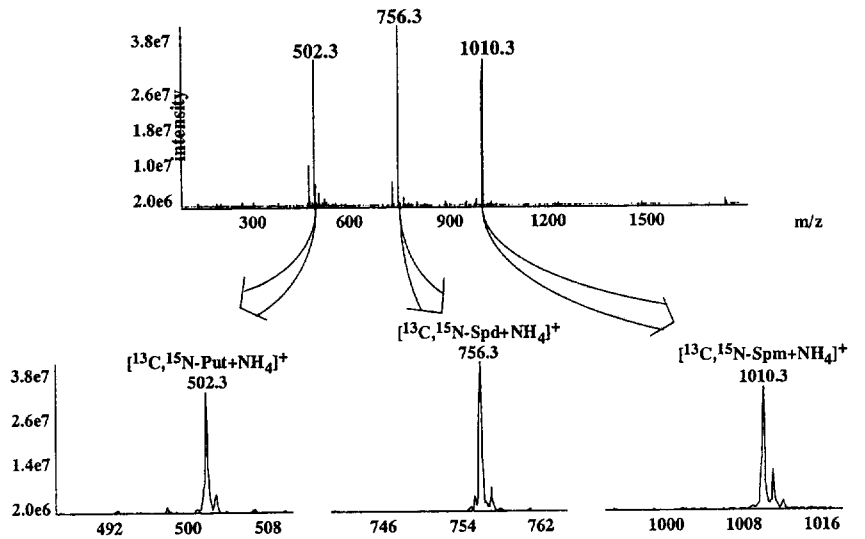


Fig. 1 Simultaneous mass analysis of HFB derivatives of 1, 2, and 3.

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Experimental

General. Potassium [^{13}C]cyanide (99atom%) and potassium [^{15}N]ammonium sulfate (99atom%) were purchased from ISOTEC INC. Potassium [^{15}N]phthalimide and [^{15}N]-(3-bromopropyl)phthalimide were prepared in our laboratory.[3] A water soluble Z-reagent, [*p*-(benzyloxycarbonyloxy)phenyl]dimethylsulfonium methylsulfate (Z-DSP) was purchased from WAKO Pure Chemicals Co., Ltd. GLC analyses were performed on a Shimadzu GC-9A Gas Chromatograph with a Pyrex glass column (1.6 m×3 mm I.D.) packed with 10% PEG (80/100 mesh Uniport HP). Heptafluorobutyryl (HFB) derivatization of the polyamines was conducted according to the method reported previously.[4] MS analyses were performed on a PE-SCIEX API 300 mass spectrometer with an ion spray ionization interface. A sample of HFB-polyamines dissolved in 50% acetonitrile-water containing 0.5% ammonium acetate was introduced to the spectrometer with a syringe drive (HARVARD APPARATUS 22).[4]

[1,4-$^{13}C_2$,1,4-$^{15}N_2$]Butanediamine (1). Potassium [^{13}C]cyanide (1.65 g, 25.0 mmol) and 1,2-dibromoethane (4.69 g, 25.0 mmol) were refluxed for 5 h in 18 ml of 70% EtOH. Acetone was then added to the reaction mixture to precipitate potassium bromide, and the filtrate was evaporated to dryness in vacuo at 30℃ (11.6 mmol). The yield of [1,4-$^{13}C_2$]cyanide was 93%, as measured by GLC. A mixture of [1,4-$^{13}C_2$]succinodinitrile (11.6 mmol) dissolved in 90 ml of EtOH and potassium hydroxide (3.9 g) dissolved in 45 ml of EtOH was then refluxed for 12 h, and allowed to cool. To precipitate KCl, 9 ml of conc. HCl was added to the solution, and the filtrate was evaporated to dryness. The resulting [1,4-$^{13}C_2$]succinic acid was then dissolved in 50 ml of EtOH containing 150 mg of conc. H_2SO_4. The solution was refluxed for 3 h in the presence of molecular sieves to absorb water, then neutralized with 260 mg of sodium bicarbonate.[9] The filtrate was evaporated to dryness to obtain [1,4-$^{13}C_2$]ethylsuccinate (10.3 mmol by GLC).

The ethylsuccinate (10.3 mmol) was dissolved in 25 ml of dry ether, then was added over the course of 20 mins to a stirred suspension of 1.0 g of $LiAlH_4$ in 25 ml of dry ether. Stirring was continued for an additional 2 h. Water and dil. H_2SO_4 under ice were then added to the reaction mixture. The resulting solution was evaporated to dryness, and repeated extractions of the labeled 1,4-butanediol from the residue with EtOH were conducted until butanediol was no longer observed in the final extract by GLC, resulting in a satisfactory yield of [1,4-$^{13}C_2$] butanediol (9.1 mmol by GLC).

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The next bromination step was first examined using an unlabeled sample of butanediol. After careful addition of conc. H_2SO_4 (9.5 mmol) followed by conc. HBr (27 mmol) to an unlabeled butanediol (9.1 mmol) sample over ice, the mixture was refluxed for 1 h. The reaction mixture to which 10 ml of water was added was extracted twice with 10ml of dichloromethane. After evaporation, 1,4-dibromobutane (8.1 mmol by GLC) was obtained. When the labeled 1,4-butanediol (9.1 mmol) was similarly treated, the reaction was incomplete, and 4-bromobutanol was observed. The initially extracted mixture was therefore again subjected to the H_2SO_4-HBr treatment to obtain [1,4-$^{13}C_2$] dibromobutane (5.1 mmol by GLC).

Potassium [^{15}N]phthalimide (12 mmol) was added to the labeled dibromobutane (5.1 mmol) dissolved in 10 ml of DMF, and the mixture was kept at 80℃ for 3 h. Ice-cold water was then poured into the reaction mixture to precipitate the doubly labeled phthaloyl putrescine. The collected precipitate was suspended in conc. HCl-AcOH (1:1) and was hydrolyzed in a sealed tube at 120℃ for 5 days. After evaporation, the residue was dissolved in distilled water and was applied to a column packed with DOWEX 50W-X8 cation exchanger (6.5×1.6 cm I.D.), then eluted with 1.5 M HCl after successive washings with 0.5 M and 1.0 M HCl to collect the putrescine fraction. After evaporation, the residue was recrystallized from 95% EtOH to obtain a pure **1**·2HCl (4.4 mmol). The overall yield from [1,4-$^{13}C_2$]succinodinitrile to **1**·2HCl was approximately 38%.

[5,8-$^{13}C_2$,1,4,8-$^{15}N_3$]Spermidine (2). **1**·2HCl (0.6 mmol, 100 mg) dissolved in 1 ml of 3 M NaOH was vigorously stirred with 4 ml of chloroform at rom temperature. To the stirred suspension, Z-DSP (0.7 mmol, 280 mg) dissolved in 0.12 ml of water was added in three parts, one per minute. After 30 min of stirring, the chloroform phase was taken out, washed twice with 1 ml of water, and extracted three times with 1.5 ml of 1 M HCl to a neutralized solution, then made alkaline with 1.5 ml of conc. ammonia and re-extracted three times with 2 ml of chloroform to obtain the mono-Z derivative of **1** (0.3 mmol by weight).

The mono-Z derivative (0.3 mmol) was then allowed to react with benzaldehyde (0.36 mmol) in 3 ml of MeOH in the presence of triethylamine (0.3 ml) and $MgSO_4$ (50 mg), followed by reduction with $NaBH_4$ (35 mg). After the reaction, the MeOH was evaporated, and the residue was extracted with water and ether. Mono-Z, the mono-benzyl derivative of **1** (0.27 mmol by weight) was obtained after evaporation of the ether extract.

The derivative (0.27 mmol) was then dissolved in 1 ml of acetonitrile and was refluxed for 20 h in the presence of [^{15}N]-(3-bromopropyl)phthalimide (0.27 mmol) and KF-celite (180 mg). The protected precursor of labeled spermidine **2** was

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purified by silica gel column chromatography with a solvent system of benzene and acetone to give 0.2 mmol by weight. Deprotection procedures by hydrazine treatment followed by catalytic reduction were conducted in the usual manner.[3] **2**·3HCl was recrystallized from EtOH-water (0.1 mmol).

[5,8-$^{13}C_2$,1,4,8,12-$^{15}N_4$]Spermine (3). The synthetic route for **3** was principally the same as described previously for ^{15}N-labeled spermine, except for the use of **1**·2HCl (0.2 mmol) as the initial putrescine. In this small-scale preparation, a recrystallized **3**·4HCl (0.07 mmol, 25 mg) was obtained.

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