Syntheses of [5,8-$^{13}C_2$]- and [1,12-$^{13}C_2$]Spermine Using Potassium [^{13}C]Cyanide as the ^{13}C Source

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[5,8-$^{13}C_2$]Spermine and [1,12-$^{13}C_2$]spermine were prepared using [^{13}C]KCN as the source of the label. By reaction of the latter with 1,2-dibromoethane and ethylene chlorohydrin, the corresponding [1,4-$^{13}C_2$]succinodinitrile and [CN-^{13}C]ethylene cyanohydrin were respectively obtained. The reaction conditions were carefully adjusted so as to optimize the yields of the ^{13}C-enriched intermediates. The nitrile residues were then reduced using sodium trifluoroacetoxyborohydride in tetrahydrofuran. [1,4-$^{13}C_2$]Putrescine and [3-^{13}C]3-aminopropanol were thus obtained. The latter was transformed into its [3-^{13}C]3-carbobenzyloxyamidopropyl bromide derivative. The syntheses of [5,8-$^{13}C_2$]spermine from the [1,4-$^{13}C_2$]putrescine precursor and *N*-(3-bromopropyl)phthalimide, and of [1,12-$^{13}C_2$]spermine from *N,N'*-bisbenzylputrescine and the [3-^{13}C]3-carbobenzyloxyamidopropyl bromide precursor were then carried out using our previously reported methods, which were modified so as to maximize the yields of the ^{13}C-enriched products.

Key words polyamine; spermine; ^{13}C-NMR; nucleic acid; ^{13}C-succinodinitrile synthesis; ^{13}C-ethylene cyanohydrin synthesis

Naturally occurring polyamines bind to biological macromolecules and regulate their physiological functions. The polyamines spermidine and spermine are widely distributed in mammalian tissues, and spermine is known to bind more tightly than spermidine to nucleic acids. To study polyamine–nucleic acid interactions at the molecular level, NMR techniques have been widely used.[1] These studies required polyamines specifically enriched with stable isotopes. Several N-15 enriched polyamines were prepared in our laboratory,[2] and were used during NMR studies on polyamine–tRNA interactions.[1d] We report below the syntheses of two symmetrically C-13-enriched spermines, [5,8-$^{13}C_2$]spermine and [1,12-$^{13}C_2$]spermine for use in further NMR studies. Preliminary runs using 1,2-dibromoethane or ethylene chlorohydrin and [^{13}C]KCN gave poor results. Reaction conditions were therefore chosen to allow for efficient use of the [^{13}C]KCN reagent.

Results and Discussion

Synthesis of [5,8-$^{13}C_2$]Spermine Putrescine (1,4-diaminobutane) was the first labeled precursor used for [5,8-$^{13}C_2$]spermine synthesis. Synthetic methods for [1,4-$^{13}C_2$] and [1,4-$^{14}C_2$]putrescine have been reported, and are based on the reaction of labeled KCN or NaCN with 1,2-dibromoethane in aqueous ethanol.[3] The resulting succinodinitrile[3a–c] was then reduced with diborane in THF to putrescine.[3d] To avoid the formation of 1-bromo-2-cyanoethane due to a monosubstitution reaction, several of the reported procedures used the stoichiometric 2:1 ratio of KCN:1,2-dibromoethane to enhance the yield of succinodinitrile. Khan and Robins[3d] used 3 mol of [^{13}C]KCN to 1 mol of 1,2-dibromoethane, but the yield of [1,4-$^{13}C_2$]succinodinitrile was only 35% based on the amount of [^{13}C]KCN. When we followed the procedure of Clarke *et al.*,[3b] where a 2:1 ratio of [^{14}C]KCN:1,2-dibromoethane was used, low yields of the expected succinodinitrile were obtained. The reaction mixture became deep brown and smelled of isonitrile at increasing reaction times. Frydman *et al.*[3c] used a 1-to-1 molar ratio of 1,2-dibromoethane and KCN, and obtained a good yield of crude succinodinitrile. We therefore reexamined the reaction using gas-liquid chromatography (GLC) in order to find the best reaction conditions and to check whether or not 1-bromo-2-cyanoethane was formed when an excess of 1,2-dibromoethane was used. As can be seen in Fig. 1A, no 1-bromo-2-cyanoethane was formed even when a 1-to-1 molar ratio of 1,2-dibromoethane and potassium cyanide was used. On the other hand, when the theoretical molar ratio of the reactants was used, a significant coloring of the reaction mixture was observed with increased formation of by-products (Fig. 1B). These results clearly showed that the first substitution reaction is the limiting step for the formation of succinodinitrile. The reason for the lower yields when the stoichiometric ratio is used is unknown, but the strong basicity of potassium cyanide in aqueous ethanol may lead to the formation of hydrolysis products. Based on these results, we chose to use an equimolar ratio of 1,2-dibromoethane to potassium cyanide. Under these conditions an incorporation of more than 90 % of potassium cyanide into succinodinitrile was achieved (see Experimental). Extraction of the succinodinitrile into organic solvents from its aqueous solution[3a–c] was laborious and time-consuming. Furthermore, a purification step using silica gel column chromatography had also been attempted.[3d] In order to omit these steps, we explored direct reduction of the succinodinitrile using sodium acyloxyborohydride.[4] The crude [1,4-$^{13}C_2$]succinodinitrile, after removal of KBr and evaporation of the solvent, was reduced using sodium trifluoroacetoxyborohydride in tetrahydrofuran (THF) under the conditions

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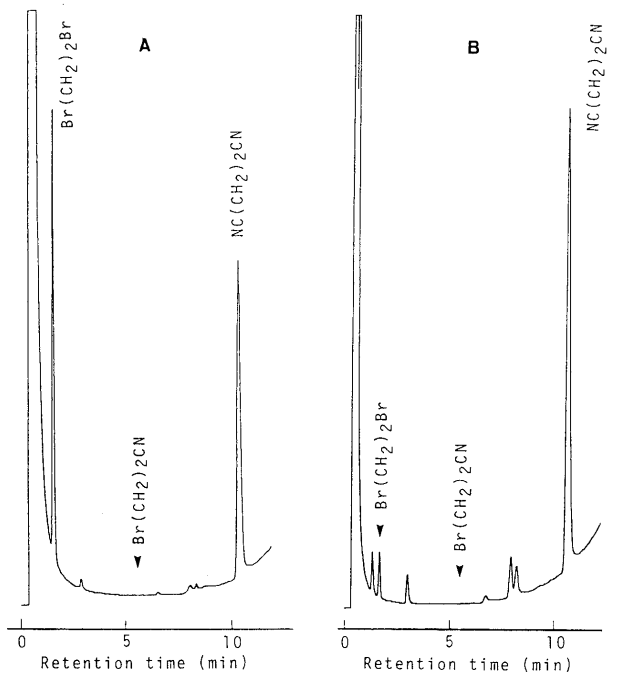

Fig. 1. Gas Chromatograms of the Reaction Products of 1,2-Dibromoethane and Potassium Cyanide

A mixture of 1,2-dibromoethane and potassium cyanide, 7 mmol and 7 mmol (A), or 3.5 mmol and 7 mmol (B), respectively, was refluxed in 5 ml of 75% ethanol for 4 h. An aliquot of each reaction mixture was injected into the gas chromatograph.

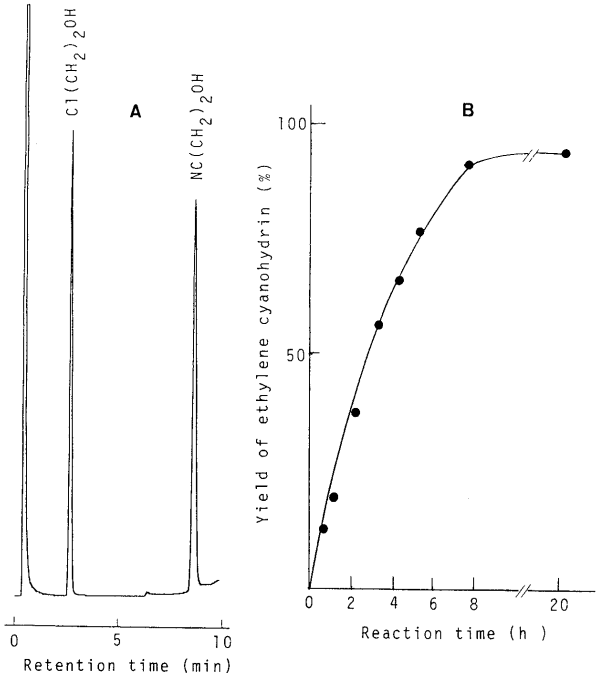

Fig. 2. Gas Chromatographic Determination of Ethylene Cyanohydrin Derived from Ethylene Chlorohydrin and Potassium Cyanide

A solution of 7 mmol of potassium cyanide and 10 mmol of ethylene chlorohydrin in 0.7 ml of water were mixed and stirred at 50 °C. An aliquot of the reaction mixture was injected into a gas chromatograph. A typical gas chromatogram of the reaction mixture heated for 6 h (A); time-dependent formation of ethylene cyanohydrin (B).

described in Experimental. The reaction mixture, after having been treated with water and concentrated hydrochloric acid, was evaporated to dryness. The residue was dissolved in water and was applied to a cation exchange column in order to separate putrescine dihydrochloride. A 65% yield of putrescine dihydrochloride was obtained after the two steps of reduction and cation exchange.

The synthesis of [5,8-$^{13}C_2$]spermine tetrahydrochloride from [1,4-$^{13}C_2$]putrescine dihydrochloride was carried out according to our published procedures,[2] which included alkylation of *N*,*N*′-bisbenzyl-[1,4-$^{13}C_2$]putrescine with *N*-(3-bromopropyl)phthalimide, followed by elimination of the protecting groups, and afforded [5,8-$^{13}C_2$]spermine tetrahydrochloride in good yield.

Synthesis of [1,12-$^{13}C_2$]Spermine An *N*-protected 3-bromopropylamine was considered to be the best choice as the label-carrying precursor for [1,12-$^{13}C_2$]spermine, since established methods[2] for the synthesis of the latter could be used. The synthetic pathway leading to a [1-^{13}C]*N*-protected 3-bromopropylamine included ethylene cyanohydrin formation from ethylene chlorohydrin and potassium cyanide, followed by a reduction of the nitrile group, protection of the resulting primary amino group, and finally bromination of the alcohol group. Ethylene cyanohydrin preparation from ethylene chlorohydrin and sodium cyanide on a large scale has been described.[5] Scaling down the procedure one-thousand-fold resulted in failure due to a temperature-dependent increase of color and release of an unpleasant-smelling gas. This problem was overcome when the reaction mixture, containing much precipitated NaCN or KCN, was transformed to a homogeneous solution by addition of water. The best yields of ethylene cyanohydrin were then established by using GLC (Fig. 2). The conditions given in Experimental led to a nearly quantitative use of potassium cyanide. The ethylene cyanohydrin was extracted with dichloromethane, and reduced by the afore-mentioned method using sodium trifluoroacetoxy borohydride. Isolation of the resulting aminopropanol was difficult owing to its polarity and its low boiling point; direct derivatization with carbobenzoxy chloride was therefore chosen. The reaction was carried out directly on the previous reaction mixture. The resulting *N*-carbobenzoxyamidopropanol was easily extracted with chloroform, but was contaminated with benzyl alcohol. They were easily separated by a silica gel column chromatography using benzene: acetone. The overall yield of pure *N*-carbobenzoxyamidopropanol was about 50% based on ethylene cyanohydrin, suggesting an average 80% yield for each step. Bromination of the hydroxy group of *N*-carbobenzoxyamidopropanol proceeded smoothly using tetrabromomethane and triphenylphosphine.[6] The pure *N*-carbobenzoxyamidopropyl bromide was obtained in good yield from the alcohol precursor after silica gel column chromatography.

The known synthetic procedures for spermine[2] were then applied, but using [^{13}C]*N*-carbobenzoxyamidopropyl bromide instead of *N*-(3-bromopropyl)phthalimide. The established reaction conditions for the alkylation of *N*,*N*′-bisbenzylputrescine with the phthalimide derivative, followed by reflux for 24 h in acetonitrile in the presence of KF-Celite, resulted in poor yields of the spermine precursor. The formation of by-products was significantly reduced when the reaction temperature was lowered; 72 h at 20 °C was found to afford a good yield of the spermine

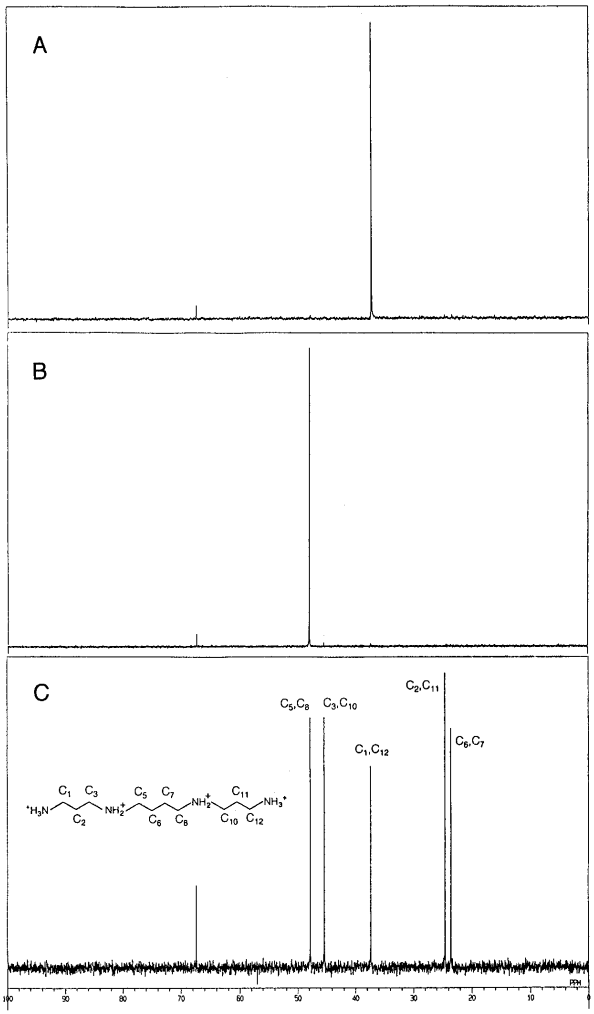

Fig. 3. ^{13}C-NMR of Spermine

Each sample was dissolved in 0.5 ml of D_2O, and the ^{13}C-NMR spectrum was recorded as described in Experimental. A: [1,12-$^{13}C_2$]spermine·4HCl, 2 mg, 100 scans; B: [5,8-$^{13}C_2$]spermine·4HCl, 2 mg, 100 scans; C: natural spermine·4HCl, 20 mg, 500 scans. δ values: 37.39 ppm for C_1 and C_{12}, 24.56 ppm for C_2 and C_{11}, 45.37 ppm for C_3 and C_{10}, 47.83 ppm for C_5 and C_8, and 23.59 ppm for C_5 and C_7.

precursor. The elimination of the protecting groups to give spermine was now easier than in the published procedures,[2] since the carbobenzoxy group was also cleaved during the hydrogenolysis of the benzyl groups. [1,12-$^{13}C_2$]Spermine tetrahydrochloride was thus obtained in 47% yield based on [^{13}C]*N*-carbobenzoxyamidopropyl bromide.

^{13}C-NMR of Spermine ^{13}C-NMR spectra of [5,8-$^{13}C_2$]spermine and [1,12-$^{13}C_2$]spermine as well as natural spermine are shown in Fig. 3. Chemical shifts were assigned using the reported data.[7]

Experimental

Materials Potassium cyanide-^{13}C (>99 atom% ^{13}C) was obtained from Isotec Inc., U.S.A. KF-Celite and *N,N'*-bisbenzylputrescine·2HCl were prepared in this laboratory.[2] Free *N,N'*-bisbenzylputrescine was prepared by extraction with 4 M ammonium hydroxide and chloroform. All organic solvents and reagents were of analytical reagent grade. *N*-(3-Bromopropyl)phthalimide, 98%, was obtained from Aldrich Chem. Co., Inc.; 10% palladium on charcoal from Kojima Chem. Co., Ltd., and carbobenzoxy chloride (Z-Cl) from Kokusan Chem. Works, Ltd. 1,2-Dibromoethane and trifluoroacetic acid were obtained from Tokyo Kasei Co., Inc., and sodium borohydride and triphenylphosphine from Kanto Chem. Co., Inc. Hydrazine monohydrate, ethylene chlorohydrin, triethylamine, and tetrabromoethane were obtained from Wako Pure Chem. Ind., Ltd.

For column chromatography, a cation exchange resin (Dowex 50W-X8, 200—400 mesh, Dow Chem. Co.) and a silica gel (Wako gel C-300, Wako Pure Chem. Ind., Ltd.) were used. For TLC, pre-coated silica gel plates (Silica gel 60 F-254, E. Merck) were used.

GLC A GC-9A gas chromatograph (Shimazu, Kyoto) equipped with a flame ionization detector was employed. A Pyrex glass column (1.6 m × 3 mm i.d.) packed with 10% PEG-20M on 80—100 mesh Uniport HP (GL Sciences, Tokyo) was used. Helium was used as the carrier gas at a flow-rate of 40 ml/min. The temperatures of the injector and the detector were 250 °C and the column oven temperature was programmed from 100 °C to 200 °C at a rate of 10 °C/min.

^{13}C-NMR ^{13}C-NMR spectra were recorded with a JEOL EX-270 spectrometer operating at 67.8 MHz. Samples were dissolved in D_2O and dioxane was used as an internal standard (67.40 ppm).

[1,4-$^{13}C_2$]Succinodinitrile A mixture of potassium [^{13}C]cyanide (460 mg, 7 mmol) and 1,2-dibromoethane (1.32 g, 7 mmol) in 5 ml of a 75% EtOH solution was heated under reflux for 4 h. The reaction mixture was allowed to cool, and acetone was added until no further precipitation of potassium bromide was observed. The filtrate was evaporated to dryness *in vacuo* at 30 °C. The yield of [1,4-$^{13}C_2$]succinodinitrile was 93% based on potassium [^{13}C]cyanide, as measured by GLC.

[1,4-$^{13}C_2$]Putrescine Dihydrochloride [1,4-$^{13}C_2$]Succinodinitrile (400 mg, 5 mmol) dissolved in 1 ml of THF was added dropwise to a stirred suspension of $NaBH_4$ (0.75 g, 20 mmol) and trifluoroacetic acid (2.3 g, 20 mmol) in 15 ml of THF at 0 °C. Stirring was continued for 12 h at room temperature, then 3 ml of concentrated HCl was carefully added at 0 °C, and the mixture was evaporated to dryness. The residue was dissolved in water and separated on a Dowex 50 (H^+) column (10 ml) by stepwise elution using increasing concentrations of hydrochloric acid (0.5, 1.0, 1.5, and 2.0 M). The fractions containing putrescine were eluted with 1.5—2.0 M hydrochloric acid. They were collected and evaporated to dryness. The crystalline residue was recrystallized from ethanol–ethyl ether to give 530 mg (3.3 mmol) of [1,4-$^{13}C_2$]putrescine·2HCl; 65% yield based on [1,4-$^{13}C_2$]succinodinitrile.

[5,8-$^{13}C_2$]Spermine Tetrahydrochloride Benzaldehyde (220 μl, 2 mmol) was added to a stirred suspension of [1,4-$^{13}C_2$]putrescine·2HCl (160 mg, 1 mmol), triethylamine (0.4 ml) and $MgSO_4$ (0.3 g) in 4 ml of MeOH at room temperature. After 2 h, the reaction mixture was cooled on ice, and $NaBH_4$ (0.4 g) was carefully added. Stirring was continued for 1 h, after which time, MeOH was removed by distillation *in vacuo*. The residue dissolved in water was extracted with ethyl ether. The latter was then distilled off and the resulting crude oil (180 mg), mostly composed of *N,N'*-bisbenzyl[1,4-$^{13}C_2$]putrescine, was directly subjected to the next alkylation step.

It was added, together with *N*-(3-bromopropyl)phthalimide (540 mg, 2 mmol) and KF-Celite (0.7 g), to 7 ml of acetonitrile, and the mixture was heated under reflux for 20 h, then cooled to 20 °C. The KF-Celite was filtered off, the solvent was evaporated, and the residue was dissolved in *ca.* 15 ml of benzene–acetone (40 : 1). This solution was applied to a silica gel column (15 g) equilibrated with the same solvent. The column was successively eluted with 100 ml of benzene–acetone (40 : 1), 100 ml of benzene–acetone (20 : 1), 50 ml of benzene–acetone (10 : 1), and 80 ml of benzene–acetone (5 : 1). The protected spermine, *N,N'*-bisbenzyl-*N,N'*-bis-(3-phthalimidopropyl)[1,4-$^{13}C_2$]putrescine, (400 mg, 0.6 mmol) was eluted with benzene–acetone (5 : 1). The protecting groups were sequentially eliminated.[2] Heating under reflux for 3 h in 6 ml of MeOH containing 0.4 ml of hydrazine monohydrate cleaved the phthalimide group, and MeOH was removed *in vacuo*. The residue was shaken well with $CHCl_3$ and 4 M ammonium hydroxide. The $CHCl_3$ extract was evaporated and the resulting residue of 4,9-bisbenzylspermine was subjected to hydrogenolysis in 10 ml of acetic acid at 60 °C with Pd/C to cleave the benzyl groups. By addition of a calculated amount of HCl to the catalyst-free filtrates, [5,8-$^{13}C_2$]spermine tetrahydrochloride (200 mg, 0.59 mmol) was obtained (60% yield from [1,4-$^{13}C_2$]putrescine). ^{13}C-NMR (D_2O) δ 47.83 ppm (Fig. 3).

[CN-^{13}C]Ethylene Cyanohydrin A homogeneous solution of potassium [^{13}C]cyanide (400 mg, 7 mmol), ethylene chlorohydrin (800 mg, 10 mmol), and 0.7 ml of water was stirred for 10 h at 50 °C. Precipitated KBr was then dissolved by addition of 1.5 ml of water, and the solution was extracted with dichloromethane (5 × 3 ml). The combined dichloromethane extracts were paper-filtered and evaporated to dryness *in vacuo*. The yield of [CN-^{13}C]ethylene cyanohydrin was 93% based on potassium [^{13}C]cyanide as estimated by GLC (Fig. 2B).

[3-^{13}C]3-Carbobenzyloxyamidopropanol [CN-^{13}C]Ethylene cyanohydrin (400 mg, 5.6 mmol) dissolved in 0.5 ml of THF was added dropwise to a stirred suspension of $NaBH_4$ (506 mg, 13.4 mmol) and trifluoroacetic acid (1.53 g, 13.4 mmol) in 11 ml of THF on ice. As $NaBH_3(OCOCF_3)$ reacts with the alcoholic hydrogen of ethylene cyanohydrin, an excess of $NaBH_3(OCOCF_3)$ was used. Stirring was continued for 12 h at 0 °C, and 2 ml of water was carefully added to the reaction mixture, followed by triethylamine (0.9 ml, 6.5 mmol) and carbobenzyloxy chloride (1.0 ml, 6.5 mmol). After 2 h at 20 °C, the THF was removed by distillation *in vacuo*, and the residue was partitioned between water and $CHCl_3$. After removal of the $CHCl_3$, the [^{13}C]alcohol was purified by column chromatography (15 g of silica gel), using sequential elution with benzene–acetone at 20 : 1, 10 : 1, and 5 : 1 ratios. The fraction containing the reaction product was detected by TLC [benzene–acetone (4 : 1), *Rf* 0.35]. Pure [3-^{13}C]3-carbobenzoxyamidopropanol (0.57 g, 2.7 mmol) was obtained in 50% yield based on [CN-^{13}C]ethylene cyanohydrin.

[3-^{13}C]3-Carbobenzyloxyamidopropyl Bromide A solution of tetrabromoethane (1.0 g, 3 mmol) in 4 ml of THF was slowly added to a mixture of [3-^{13}C]3-carbobenzyloxyamidopropanol (0.53 g, 2.5 mmol) and triphenylphosphine (0.79 g, 3 mmol) in 12 ml of THF. The reaction mixture was stirred for 12 h at 20 °C. After removal of the precipitate, the filtrate was evaporated, and the oily residue was dissolved in benzene–acetone (30 : 1) and applied to a silica gel column (5 g), using the same solvent as the eluant. The eluates were evaporated, and the resulting oily residue redissolved in cyclohexane–ethyl acetate (9 : 1) was applied to a silica gel column (10 g), and eluted with the same solvent system. The fraction containing the compound was detected by TLC [cyclohexane–ethyl acetate (9 : 1), *Rf* 0.2]. Pure [3-^{13}C]3-carbobenzyloxyamidopropyl bromide (500 mg, 1.84 mmol) was obtained in 73% yield from the protected 3-aminopropanol.

[1,12-^{13}C]Spermine Tetrahydrochloride A suspension of [3-^{13}C]3-carbobenzyloxyamidopropyl bromide (500 mg, 1.84 mmol), *N*,*N*′-bisbenzylputrescine (240 mg, 0.9 mmol), and KF-Celite (600 mg) in 6 ml of acetonitrile was stirred in a flask at 20 °C for 72 h. The reaction mixture was then filtered, the filtrate was evaporated to dryness, and the oily residue was dissolved in benzene–acetone (20 : 1) and applied to a silica gel column (15 g) equilibrated with the same solvent. The column was sequentially eluted with 130 ml of benzene–acetone (40 : 1), 20 ml of benzene–acetone (10 : 1), and 100 ml of benzene–acetone (5 : 1). The protected spermine precursor, 1,12-biscarbobenzyloxy-4,9-bisbenzyl[1,12-$^{13}C_2$]spermine (275 mg, 0.42 mmol), was eluted with benzene–acetone (5 : 1) [TLC: benzene–acetone (4 : 1), *Rf* 0.15 with tailing]. A 47% yield based on *N*,*N*′-bisbenzylputrescine was obtained. The protecting groups were eliminated by hydrogenolysis under the same conditions as described above for 4,9-bisbenzylspermine, with frequent exchanges of hydrogen in the reaction vessel, in order to remove carbon dioxide. By addition of a calculated amount of HCl to the catalyst-free filtrate, [1,12-$^{13}C_2$]spermine tetrahydrochloride (140 mg, 0.4 mmol) was obtained (40% yield based on *N*,*N*′-bisbenzylputrescine). ^{13}C-NMR (D_2O) δ 37.39 ppm (Fig. 3).

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