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STEREOSELECTIVE SYNTHESIS OF L-[4-^{13}C]CARNITINE

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SUMMARY

The stereoselective synthesis of L-[4-^{13}C]carnitine was achieved in 5 steps. The label was introduced from K^{13}CN into an easily separated diastereomeric pair of 3-deoxy-D-[1-^{13}C]aldohexoses. Reductive amination of the labeled aldohexose yielded the corresponding D-1-(dimethylamino)[1-^{13}C]alditol which was oxidized in two steps and alkylated with iodomethane to yield L-[4-^{13}C]carnitine. The stereochemical integrity at C-2 of the 3-deoxy-D-[1-^{13}C]glucose precursor was maintained throughout the synthesis of L-[4-^{13}C]carnitine.

Keywords: L-[4-^{13}C]carnitine, 3-deoxy-D-*ribo*-[1-^{13}C]hexose, 3-deoxy-D-[1-^{13}C]glucose, 3-deoxy-D-*arabino*-[1-^{13}C]hexose, 3-deoxy-D-[1-^{13}C]mannose.

INTRODUCTION

L-Carnitine [(3*R*)-3-hydroxy-4-(trimethylamino)butanoic acid] is widely distributed in organisms and tissues that utilize fatty acids as a source of energy[1]. L-Carnitine serves to transport fatty acids from the cytosol across the mitochondrial membrane[2,3]. Interest in the biological role of carnitine has been stimulated by studies supporting the pharmacological use of carnitine in the treatment of a wide variety of diseases[4]. In addition, carnitine metabolism is of interest to us for evaluation of an important metabolic regulatory parameter: the acetyl-Coenzyme A to Coenzyme A ratio. This application is based on the equilibrium of the carnitine acetyl transferase system which transfers acetyl groups between carnitine and Coenzyme A[5-8]. In this context, the substantially greater cellular concentrations of carnitine, when compared to Coenzyme A concentrations, make

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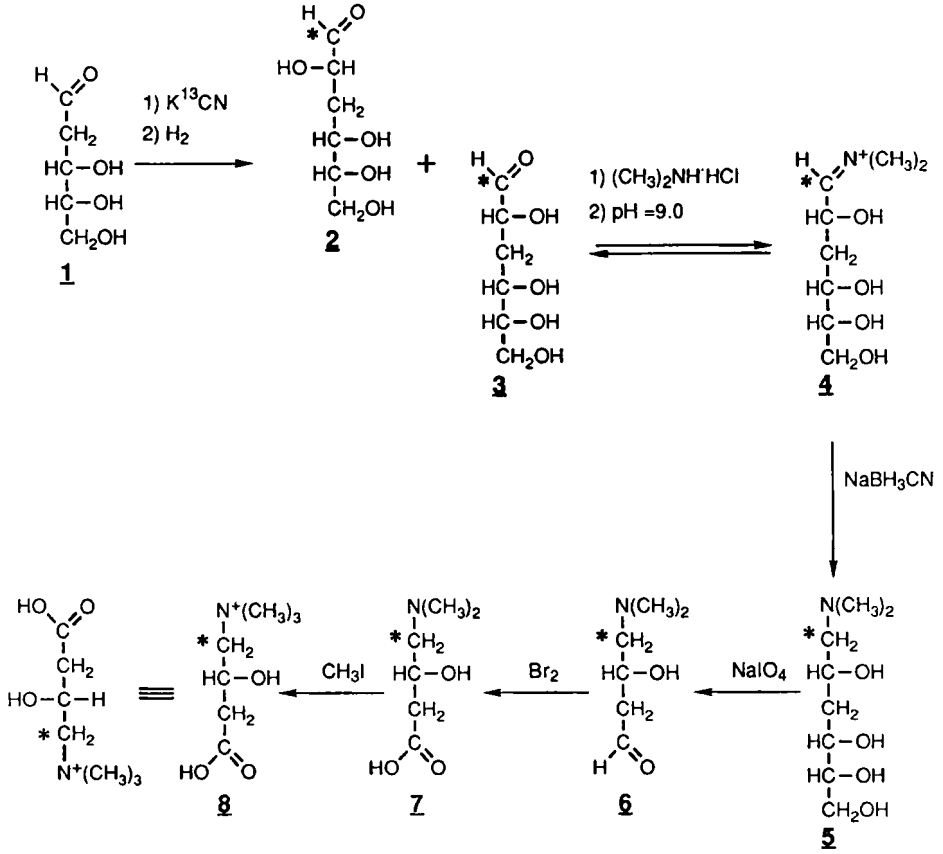
carnitine attractive as an indirect probe of the acetyl-Coenzyme A to Coenzyme A ratio using *in vivo* NMR spectroscopy. We report a stereoselective synthesis of L-carnitine suitably labeled with ^{13}C for *in vivo* NMR studies.

RESULTS AND DISCUSSION

There were two major considerations in our selection of a synthetic route to labeled carnitine. First, it has been established that the biologically important form of carnitine has the L configuration[9] and that D-carnitine is a competitive inhibitor of L-carnitine acyl transferases[8,10]; consequently, we sought a stereospecific synthesis of L-carnitine. Second, because the principal metabolic transformations which we desire to probe are the acylations and acetylations of carnitine, it was necessary to label carnitine at C-2, C-3, or C-4; acetylation of carnitine causes a significant ^{13}C chemical shift at these carbons.

Hoppel and coworkers reported two routes to the stereoselective synthesis of radiolabeled L-carnitine[11,12]. The first route involves the N-demethylation of carnitine



and alkylation with [^{14}C]iodomethane[11] and is not useful for the synthesis of carnitine labeled in the butyryl group. The second route involves the hydroxylation of 4-N-trimethylaminobutanoate by liver butyrobetaine hydroxylase[12]. The enzymatic step was carried out on 50-60 μmoles of precursor and appeared difficult to implement for the larger quantities required here. Sih and coworkers[13] developed an asymmetric synthesis of carnitine that involves the reduction of 4-chloroacetoacetic acid, n-octyl ester using Bakers yeast. This route produces the desired L-isomer in an enantiomeric excess of greater than 98%, and appears useful for the synthesis of carnitine labeled in the butyryl group. As we required enantomerically pure carnitine, we chose to investigate the route described below.

We have developed a stereospecific synthetic route to L-carnitine. In our synthesis, the labeled carbon is introduced from K^{13}CN by addition to 2-deoxy-D-ribose (1) generating the diastereomeric pair of 3-deoxyaldohexoses, 3-deoxy-D-[1-^{13}C]glucose (3-deoxy-D-*ribo*-[1-^{13}C]hexose, 3) and 3-deoxy-D-[1-^{13}C]mannose (3-deoxy-D-*arabino*-[1-^{13}C]hexose, 2). This mixture of the C-2 epimers of 3-deoxyaldohexoses was separated chromatographically. The chirality at C-2 of the sugar precursor is transferred to carnitine using the synthetic scheme shown in Scheme 1. The 3-deoxy-D-[1-^{13}C]glucose (3) was reductively aminated by treatment with dimethylamine and $NaBH_3CN$[14] to the corresponding 1,3-dideoxy-1-(dimethylamino)alditol (5). Periodate cleavage of the aminoalditol between C-4 and C-5 (and C-5 and C-6) followed by treatment with bromine yielded L-3-hydroxy-4-(dimethylamino)-[4-^{13}C]butanoic acid (7). Quaternization of the dimethylamino group was achieved by treatment with excess iodomethane. Using this route, we produced L-carnitine (8) in 30 % overall yield from 3-deoxy-D-[1-^{13}C]glucose (3). Carnitine (8) was shown to be in the L-configuration by its optical rotation[10] and by the fact that it serves as a substrate for carnitine acetyltransferase[7]. Starting with 3-deoxy-D-mannose, D-carnitine could be prepared using the synthetic route described here.

EXPERIMENTAL

Chemicals-- Chemicals were obtained from the following commercial sources: [*acetyl*-^{3}H]acetyl-Coenzyme A from New England Nuclear (Boston, MA); 2-deoxy-D-ribose, $Pd/BaSO_4$ (5%), carnitine acetyltransferase (Acetyl-Co-A: carnitine-O-

acetyltransferase; EC 2.3.1.7), L-carnitine, and acetyl-Coenzyme A from Sigma Chemical Co. (St. Louis, MO). $K^{13}CN$ (99.2% ^{13}C) was obtained from the Los Alamos National Stable Isotopes Resource. Ion exchange resins were from Bio-Rad Laboratories (Richmond, CA).

Carnitine Acetyltransferase Activity-- L-[4-^{13}C]Carnitine preparations were tested as substrates for carnitine acetyltransferase using commercial enzyme preparations and radiolabeled acetyl-Coenzyme A. Assays were carried out as described by Idell-Wenger and coworkers[7].

NMR Methods-- Proton decoupled FT ^{13}C NMR spectra were obtained at 25.2 MHz using a Varian XL-100 spectrometer interfaced to a Nova 1210 computer. Acquisition parameters were as follows: 5 KHz sweep width, 8 K data points, 0.819 s acquisition time, 1 s relaxation delay, and 25 °C. Samples were dissolved in ^2H_2O, and the spectrometer was locked on the deuterium signal. Chemical shifts are reported in ppm downfield from external TMS=0 ppm.

*Synthesis of 3-deoxy-D-[1-^{13}C]glucose(**3**)*-- Compound **3** was synthesized by the addition of $K^{13}CN$ to 2-deoxy-D-ribose (**1**) using a modification of the method developed for the synthesis of [1-^{13}C]aldoses[15,16]. Specifically, 130 mmol $K^{13}CN$ was dissolved in water (25 ml) and the pH of the solution adjusted to 7.5 by the addition of acetic acid. An aqueous solution (25 ml) of 2-deoxy-D-ribose (130 mmol, **1**) was added, and the pH was maintained at 7.5 by the addition of 3 M acetic acid or 3 M NaOH. The disappearance of $K^{13}CN$ and the appearance of the C-2 epimeric pair of 3-deoxy-D-[1-^{13}C]aldononitriles was monitored by ^{13}C NMR spectroscopy. When the $K^{13}CN$ was consumed (30 m), the reaction mixture was acidified to pH 4.0 by adding acetic acid. The crude reaction mixture was catalytically reduced at room temperature in a 250-ml Parr pressure apparatus by treatment with H_2 at 2.8 Bars (40 psi) and $Pd/BaSO_4$ (5%, 66 mg/mmol of nitrile) that had been pre-reduced with H_2. The reaction was carried out with vigorous agitation and continued until H_2 consumption ceased. The catalyst was then removed from the reduction mixture by filtration through Celite. Cations were then removed by treatment with Dowex 50-X8 (H^+). The resulting aqueous solution contained the 3-deoxy-D-[1-^{13}C]aldohexoses, unreacted 2-deoxy-D-ribose and acetic acid. The sugars were concentrated to a syrup and stored in a desiccator over NaOH pellets for several days.

The 2-deoxy-D-ribose and the pair of C-2 epimeric 3-deoxy-D-[1-^{13}C]aldohexoses were separated by column chromatography on Dowex AG 50-X8 (200-400 mesh, 9 x 130 cm) in the Ba^{++} form using water as an eluent[17]. Column fractions were analyzed for aldoses by gas chromatography[15]. Compound **3** eluted first, then 3-deoxy-D-[1-^{13}C]mannose (**2**), which was followed by 2-deoxy-D-ribose (**1**). The 3-deoxyaldoses were pooled separately and concentrated to syrups. The recovery of carbon-13 was 38% as the gluco-epimer (**3**) (50 mmol) and 52% as the manno-epimer (**2**) (68 mmol). Compound **3** was used without further purification as the precursor for the synthesis of L-carnitine.

*Synthesis of 1,3-Dideoxy-1-(dimethylamino)-D-[1-^{13}C]glucitol (**5**)*-- Compound **3** was reductively aminated to the corresponding 1-deoxy-(dimethylamino)-D-alditol by treatment with dimethylamine and sodium cyanoborohydride[14]. Specifically, **3** (50 mmol) and dimethylammonium chloride (500 mmol) were dissolved in water at room temperature, and the pH of the resulting solution was adjusted to 9.0 with the addition of 3 M NaOH. After 3.0 h at 25°C, $NaBH_3CN$ (100 mmol) was added to the reaction mixture. The pH of the reaction mixture was maintained at 9.0 by adding 3 M NaOH. The progress of the reaction was followed by $^{13}C\{^1H\}$ NMR. The disappearance of resonances from C-1 of **3** (103.0, 99.2, 97.7 and 92.2 ppm) was accompanied by an increase in resonances from the imino forms (104.4 ppm), the product aminoalditol (64.3 ppm) and a side product 3-deoxy-D-glucitol (65.8 ppm). After nine days, the only ^{13}C-labeled compounds detected by NMR were **5** (80%) and 3-deoxy-[1-^{13}C]glucitol (20%). Anions were exchanged with bicarbonate ion by treatment with a 1.2 molar excess of Dowex AG2-X4 (50-100 mesh) in the bicarbonate form. Water and dimethylammonium bicarbonate were removed at reduced pressure on a rotary evaporator. The resulting syrup (9.8 g) was dissolved in water (50 ml) and chromatographed on a column containing Dowex AG 50-X8 in the H^+ form (200-400 mesh, 1.2 X 50 cm). After loading the sample, the column was washed with water (200 ml) to remove the 3-deoxy-[1-^{13}C]glucitol. Compound **5** bound to the column and was eluted with 0.4 M NH_4OH (2 l). The pH of the column fractions was monitored, and **5** was contained in the fractions that followed an increase in the pH from 3.5 to 9.0. These fractions were pooled and concentrated by rotary evaporation. The resulting syrup was dried in a desiccator overnight and was used for the next step without further purification (yield, 7.5 g, 78 %).

Synthesis of L-3-Hydroxy-4-(dimethylamino)-[4-^{13}C]butanoic acid (7)-- Compound **5** was converted to **7** in two steps. First, $NaIO_4$ (43 mmol) was added to a solution of **5** (39 mmol) in H_2O (500ml). Progress of the reaction was monitored by the disappearance of periodate[18], which was complete in 15 m. Iodite was first precipitated as $Ba(IO_3)_2$ by adding a molar equivalent of barium acetate and then removed by filtration. Bromine (200 *mmol*) was added to the filtrate and the reaction mixture stirred for several hours. Compound **7** was recovered from the reaction mixture by chromatography on Dowex 50-X8 in the H^+ form; **7** bound to the column and was eluted with 0.4 M NH_4OH. The pH of the column fractions was monitored; **7** was contained in the fractions that followed an increase in the pH from 3.5 to 7.0. Solvent was removed by rotary evaporation to yield a solid (yield, 2.5 g, 43 %).

Synthesis of L-[4-^{13}C]Carnitine (8) -- Compound **7** was converted to L-[4-^{13}C]carnitine (**8**) by treatment with iodomethane. A ten-fold molar excess of methyl iodide (10.5 ml / 25 ml methanol) was added to a vigorously stirred aqueous solution that contained **7** (2 g/100 ml) and Ag_2CO_3 (5.0 g). Stirring was continued, and the progress of the reaction was monitored by ^{13}C NMR (C-4 of **7**, 63.9 ppm; C-4 of **8**, 71.4 ppm). After two hours, AgI and excess Ag_2CO_3 were removed by filtration. Compound **8** was purified by chromatography on Dowex AG 50-X8 in the H^+ form. Compound **8** bound to the column and was eluted with 2 M HCl. The fractions that contained carnitine were identified by spotting a small sample on paper and staining with Dragendorff's reagent[19]. The fractions positive to Dragendorff's reagent were pooled; water and HCl were removed by rotary evaporation. Compound **8** was crystallized as its hydrochloride from ethanol-acetone (yield, 3.0 g, 90%). $^{13}C\{^1H\}$ NMR: C1, 179.2; C2, 44.1; C3, 65.1; C4, 71.3; Me, 55.26 ppm; $^1J_{C4\text{-}C3}$, 60.2 ; $^3J_{C4\text{-}C1}$, 3.2;$^1J_{N\text{-}C4}$, 2.7; $^1J_{N\text{-}Me}$, 3.6 Hz. Optical Rotation: $[\alpha]_D$= -23.8 (c=0.40, H_2O) lit $[\alpha]_D$= -23.9[10]. The isotopic enrichment at C-4 of **8** was estimated from fractional intensity of the ^{13}C satellites on the natural abundance ^{13}C NMR signal at C-3. As expected the isotope was incorporated from K^{13}CN into **8** without dilution (^{13}C enrichment at C-4 was >99%.)

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