SYNTHESIS OF RADIOACTIVELY METHYL-LABELLED (ℓ)-CARNITINE

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SUMMARY

Commercial (ℓ)-carnitine chloride was N-demethylated by the action of sodium benzenemercaptide in warm N,N-dimethylformamide. The product 4-(N,N-dimethylammonio)-3-hydroxybutanoic acid chloride salt was isolated in good yield by ion exchange chromatography. Methylation of the product by ^{14}C -iodomethane in dry methanol produced biologically active 4-N- Me-^{14}C -(ℓ)-carnitine chloride of high specific activity in excellent yield.

Key words: carnitine, (ℓ)-carnitine, norcarnitine, N-demethylation, N-alkylation

INTRODUCTION

Carnitine (3-hydroxy-4-N-trimethylammoniobutanoate) (I) is an obligate in the transport of long-chain fatty acids across the mitochondrial membrane as acylcarnitine esters (1,2). Only the (ℓ)-stereoisomer is biologically active (3). Despite considerable interest in the biosynthesis and metabolism of this compound, only one simple method of synthesis of 4-N-[Me-labelled]-(ℓ)-carnitine chloride has been published (4). *d,ℓ*-Carnitine has been resolved chemically (5,6) but the low recoveries limit the usefulness of these methods in radiochemical synthesis. Radioactive *d,ℓ*-carnitine has been resolved enzymatically (7). We have described a two stage synthesis of radioactive (ℓ)-carnitine (8) which although developed primarily for carboxy- and chain-labelled (ℓ)-carnitine is also useful for [Me-labelled](ℓ)-carnitine synthesis. Our own requirement for 4-N-[Me-labelled]-(ℓ)-carnitine

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0362-4803/82/040535-07$01.00

Received July 6, 1981
Revised September 18, 1981

led us to attempt the published specific chemical synthesis (4). Using the reported N-demethylation procedure we were unable to produce any 4-(N,N-dimethylammonio)-3-hydroxybutanoic acid chloride salt (II, norcarnitine), the immediate precursor of 4-N-[Me-labelled] carnitine. Review of the literature (9,10,11) suggested that the known S_N2 rate enhancement effects of polar, aprotic solvents could be applied with advantage to this problem. After some experimentation, the following reliable method of synthesis was developed.

EXPERIMENTAL

Instrumentation

All proton magnetic resonance spectra were recorded by a Varian A-60 instrument. Solutions of samples (2% w/v) were prepared in ^2H_2O and the sample pH adjusted to 10 with NaO^2H. Chemical shift values are reported relative to external tetramethylsilane (TMS). Liquid scintillation counting was performed as previously described (8).

Reagents

Benzenethiol was purchased from Eastman Organic Chemicals (Rochester, N.Y.), distilled at reduced pressure, and stored under nitrogen before use. N,N-Dimethylformamide (DMF) (Fisher Scientific Co., Cleveland, OH) was distilled from calcium hydride (2g/L) at reduced pressure and stored under nitrogen. Scintillation grade toluene was purchased from Research Products International Corp. (Elk Grove Village, IL) and used without purification. [Me-^{14}C] Iodomethane was purchased from New England Nuclear Corp. (Boston, MA). The (ℓ)-carnitine chloride was a generous gift of the Otsuka Pharmaceutical Factory, Naruto, Tokushima, Japan. d,ℓ-[Me-^{14}C]Carnitine (specific activity = 47.5 mCi/mmol) was purchased from Amersham (Arlington Hts., IL).

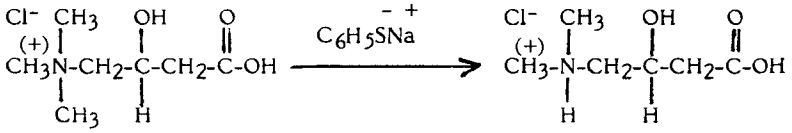
Sodium benzenemercaptide

Sodium benzenemercaptide was prepared according to a published procedure (12). It should be noted that water must be removed completely from the reaction vessel by azeotropic co-distillation with toluene. Dry toluene was added as required to the distillation pot and the distillation continued for one hour after the distillation head temperature reached 115°. The sodium benzenemercaptide product was isolated as a finely

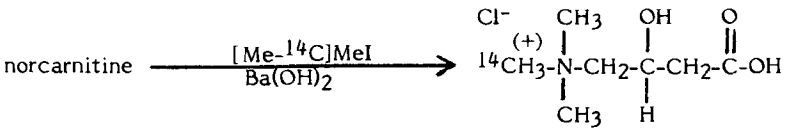
divided white powder which may be handled rapidly in air. The compound should be stored under vacuum in darkness. Sodium benzenemercaptide entirely suitable for N-demethylation of carnitine may be prepared without the additional purification steps recommended (12). The yield of product was nearly quantitative with respect to sodium hydroxide in three preparations.

Synthesis Scheme

The general scheme for the two steps in the synthesis of methyl-labelled (ℓ)-carnitine is shown below:



(ℓ)-carnitine (I) norcarnitine (II)



(ℓ)-[Me-^{14}C]carnitine (III)

4-(N,N-dimethylammonio)-3-hydroxybutanoic acid chloride salt

The demethylation of (ℓ)-carnitine chloride must be performed under nitrogen and in darkness to prevent the formation of colored impurities. The reaction vessel was a 250 ml two-necked boiling flask fitted with a gas inlet tube, water cooled reflux condenser, and drying tube. In this apparatus 2.0g (1 x 10^{-2}mol) (ℓ)-carnitine chloride was dissolved in 100 ml DMF at 80° with continual magnetic stirring. The flask was cooled to room temperature and 6.6g (5 x 10^{-2} mol) sodium benzenemercaptide was added in a single portion with efficient stirring. After 20 minutes the flask was again heated to 100° for 8 hours. At the conclusion of the heating period, the flask was cooled to room temperature and the entire contents poured into a 500 ml separatory funnel containing 150g ice and 4.5 ml conc. HCl. The solution was extracted four times with 100 ml portions of diethyl ether to remove benzenethiol, and the DMF removed by co-distillation with water in a vacuum rotary evaporator over a 40° water bath. Water was added to the evaporation flask as required to effect removal of the DMF at bath temperatures not greater than 40°.

When the volume of the solution had been reduced to 5 ml, its pH was adjusted to 10 with sodium hydroxide and the entire solution applied to a 2 cm x 30 cm column of Dowex 2X8, 200-400 mesh (OH^- form) anion exchange resin equilibrated with 0.05M NaOH. The column was washed with 200 ml 0.05M NaOH at a flow rate of 1.5 ml/min, and then with water until the pH of the effluent was neutral. The resin was transferred to a beaker containing ice water and concentrated HCl added slowly with stirring until the supernatant liquid reached pH 1. The resin was again poured into the column and all effluent collected during the packing of the resin bed. A final wash of the column with 200 ml of 0.1M HCl removed the remaining product. The combined acidic eluates were concentrated to near dryness in vacuo.

The isolated product was applied to a 2 cm x 50 cm column of Dowex 50X8, 200-400 mesh (H^+ form) cation exchange resin. The column was eluted by a linear acid concentration gradient formed by mixing 300 ml each of 0.5M HCl and 2.5M HCl; 10 ml fractions were collected. Fractions reacting positively to iodoplatinate spray reagent (13) were pooled and evaporated to yield a colorless, hygroscopic glass amounting to 1.7g (9.3×10^{-3} mol, 93%) after lyophilization. Although the compound once fortuitously crystallized from a mixture of water, ethanol and acetone, no reliable crystallization solvent mixture was found.

The product's identity as 4-(N,N-dimethylammonio)-3-hydroxybutanoic acid (chloride salt) was confirmed by ^1H n.m.r. spectroscopy. Observed chemical shifts (relative to external tetramethylsilane) were: 2.9 δ (doublet (J=6Hz), rel.int. 2 ($-C\underline{H}_2CO_2^-$)), 3.45 δ (singlet, rel. int. 6 (($C\underline{H}_3)_2NCH_2^-$)), 3.75 δ (doublet (J=6Hz), rel. int. 2 (($CH_3)_2NC\underline{H}_2^-$)), and 4.75 δ (multiplet, rel. int. 1 ($-CH_2C\underline{H}(OH)CH_2^-$)). (Cf: carnitine: 3.15 δ (doublet (J=6Hz), rel. int. 2 ($-C\underline{H}_2CO_2H$)), 3.75δ (singlet, rel. int. 9, (($C\underline{H}_3)_3\overset{+}{N}C\underline{H}_2^-$)), 3.95$\delta$ (doublet (J=6Hz), rel. int. 2 (($CH_3)_3\overset{+}{N}C\underline{H}_2^-$)), and 4.9 δ (multiplet, rel. int. 1 ($-CH_2C\underline{H}(OH)CH_2^-$)).)

The 4-(N,N-dimethylammonio)-3-hydroxybutanoic acid chloride salt was found to be uncontaminated by carnitine on Eastman #13181 silica thin layer chromatographic plates with two development systems: MeOH/acetone/conc. HCl (90:10:4 V:V:V), R_f product 0.45, R_f carnitine 0.22, α = 2.05; and MeOH/acetone/conc. HCl (10:90:4 V:V:V), R_f product 0.25, R_f carnitine 0.14, α = 1.78.

(ℓ)- Me-^{14}C carnitine chloride salt

The preparation of III was undertaken according to Lindstedt and Lindstedt (14) with modification (8,15). In a 2.5 ml round bottom boiling flask were combined 1 ml water, 50 mg (2.7 x 10^{-4} mol) (II), and 150 mg (4.8 x 10^{-4} mol) $Ba(OH)_2 \cdot 8H_2O$. To this solution was added 2.0 mCi [^{14}C]-iodomethane in 1 ml methanol. Four additional 1 ml methanol washes were required to effect complete transfer of the iodomethane. The flask was stoppered tightly and stirred 15 h. The flask contents were then evaporated, redissolved in 2 ml water, and barium precipitated by addition of 0.5 ml 1M H_2SO_4. After centrifugation, the supernatant liquid was decanted, the barium sulfate precipitate washed with 0.1M H_2SO_4, and the wash liquor centrifuged again. The combined supernatants were adjusted to pH 7 with KOH and applied to a 1.2 cm x 19 cm column of Dowex 1X8, 200-400 mesh (OH^- form) ion exchange resin. The column was eluted with water and 5 ml fractions were collected. Fractions containing radioactivity (fractions 3-6) were pooled, adjusted to pH 4 with HCl and evaporated. The residue was then applied to a 1.2 cm x 19 cm column of Dowex 50X8, 200-400 mesh (H^+ form) ion exchange resin. This column was washed first with 60 ml 1M HCl and then with 200 ml of 2M HCl; 5 ml fractions were collected. Fractions containing radioactivity (fractions 4-9) were pooled and evaporated.

Overall recovery of radioactivity was 81% based upon the manufacturer's stated contents of the [^{14}C]-iodomethane sample, and 95% based upon measured radioactivity within the reaction vessel at the outset of the synthesis. The product (ℓ)-[Me-^{14}C] carnitine chloride (III) co-chromatographed with authentic (ℓ)-carnitine chloride as a single radioactive spot on the thin layer chromatographic systems described above. Each preparation (20,000 dpm with [^{14}C] and 50,000 dpm with ^{3}H) was quantitatively recovered; radioactivity was determined as described previously (8). The synthetic product co-chromatographed with authentic (ℓ)-carnitine as a single sharply defined band on a reversed-phase high performance liquid chromatography column with quantitative recovery of injected radioactivity (16). The thin layer chromatographic conditions employed allow detection of 100 dpm of [^{14}C] or 250 dpm of ^{3}H. Carnitine was determined spectrophotometrically by a published method (17). The specific radioactivities of the products of several preparations are shown in the table below.

Table 1. Specific radioactivity of (ℓ)-carnitine preparations

Preparation	Iodomethane	(ℓ)-Carnitine
	mCi/mmol purchased	mCi/mmol determined
	[Me-^{14}C]MeI	
A	52.6	58
B	52.6	58.3
C	31	43.9
D	52.3	54.0
	[Me-^{3}H]MeI	
E	63.8	61.5

The (ℓ)-[Me ^{14}C]- and (ℓ)-[Me-^{3}H]carnitine preparations were compared to commercial d,ℓ-[Me-^{14}C]carnitine as a substrate for carnitine palmitoyltransferase. The synthesis of palmitoyl-(ℓ)-carnitine was measured as previously described (2,18). Only (ℓ)-carnitine is bioactive (3); the synthesized radioactively-labelled (ℓ)-carnitine and commerical d,ℓ-[Me-^{14}C]carnitine were incorporated as expected and previously observed (4,8).

Table 2. (ℓ)-[Me-^{14}C]- and (ℓ)-[Me-^{3}H]carnitine incorpration into palmitoyl-(ℓ)-carnitine by mitochondria

Label added	μCi per incubation	dpm in butanol	nmol palmitoyl-ℓ-carnitine formed/min·mg[a]
d,ℓ-[Me-^{14}C]carnitine	.0475	7982	75.2[b] ± 3.1
(ℓ)-[Me-^{14}C]carnitine	.054	4356	72.7 ± 2.6
(ℓ)-[Me-^{3}H]carnitine	.0615	5461	80.0 ± 5.7

[a] mean ± S.D. for 4 determinations

[b] assuming only 50% of the d,ℓ-[Me-^{14}C]carnitine is the bioactive isomer.

The method described for the synthesis of radioactively methyl-labelled (ℓ)-carnitine is simple, reliable, and efficent. N-demethylation of (ℓ)-carnitine has been performed by this procedure on several occasions with consistently good results. The subsequent methylation with radioactive iodomethane has been achieved in excellent yield on a scale ranging from 50 μCi (^{14}C) to 25 mCi (^{3}H) employed. The radioactively methyl-labelled (ℓ)-carnitine produced contains one labelled methyl group and can be synthesized with the same specific radioactivity as the iodomethane used in the preparation.

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