JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2002; **45**: 831–839.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jlcr.587

Research Article

Synthesis of deuterium-labelled coenzyme Q_{10}

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Summary

Deuterium-labelled coenzyme Q_{10} ([2-CD_3-1′-CD_2]coenzyme Q_{10}, coenzyme Q_{10}-d_5) was synthesized by condensation of 2,3-dimethoxy-[5-CD_3]methyl-1, 4-hydroquinone with [1-CD_2]decaprenol. Five positions were selected for deuteration as replacement at these positions allowed examination of every step of the synthesis. This examination was carried out by a combination of ^{1}H- and ^{13}C-nuclear magnetic spectrometry and mass spectrometry. Further, these positions have been proved to be metabolically stable. This reagent makes simultaneous quantification of the source of coenzyme Q_{10} (exogenously supplied or endogenously supplied) possible in biological samples by measurements on gas chromatography–mass spectrometry. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: [2-CD_3-1′-CD_2]coenzyme Q_{10}; [1-CD_2]decaprenol; 2,3-dimethoxy-[5-CD_3]methyl-1,4-hydroquinone; $LiAlD_4$

Introduction

The effects of exogenous coenzyme Q_{10} (Co Q_{10}) on isoprepoid derivatives, including Co Q_{10}, have long been of great interest.[1] The

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Received 19 February 2002
Accepted 7 March 2002

differentiation of exogenous and endogenous Co Q_{10} *in vivo* is a crucial problem in the discussion of the effectiveness of Co Q_{10} administration. The contribution of exogenous Co Q_{10} to total Co Q_{10} levels in tissues warrants investigation. Deuterium-labelled Co Q_{10} is an excellent tool to resolve this issue. Simultaneous quantification of deuterium-labelled and unlabelled compounds by gas chromatography–mass spectrometry was established.[2]

Using deuterium-labelled Co Q_{10} (Co Q_{10}-d_5) **10** the behaviour of exogenous Co Q_{10} has been reported in three papers.[3–5] However, the preparation procedure of the compound has not previously been described. The purpose of this paper is to describe a detailed preparation procedure for the synthesis of [2-CD_3-1′-CD_2]Co Q_{10} **10**. The synthesis of the corresponding radio-labelled compound, [3′-^{14}C] Co Q_{10} was described in the previous article.[6]

Materials and methods

Solanesol (natural) was obtained from Nisshin Flour Milling Co. (Tokyo, Japan). Ethyl decaprenoate (all-*trans* isomer: 95%) was prepared followed the procedure described previously.[6] 3,4,5-Trimethoxybenzoic acid **3** was obtained from Tokyo Kasei Organic Chemicals (Tokyo, Japan). Co Q_{10} was supplied by Eisai Co., Ltd. Silica gel for chromatography (Wakogel C-200) and deuterium oxide were purchased from Wako Chemical Industries, Osaka, Japan. Silica alumina was purchased from Nikki Chemical Co. (Yokohama, Japan). Lithium aluminium deuteride ($LiAID_4$, Art 5664) was purchased from Merck A. G. (Darmstadt, Germany). Thin-layer chromatography (TLC) was performed using Kieselgel GF_{254} (Art 5715, Merck A. G.). Spots were visualized under ultraviolet light in a dark room after applying concentrated sulphuric acid spray followed by heating. All yields given refer to isolated yields obtained after a final purification by column chromatography or recrystallization. Nuclear magnetic resonance (NMR) spectra were measured using $CDCl_3$ solution with a JEOL-GX200 spectrometer (Japan Electron Optics Laboratory, Tokyo, Japan). Tetramethyl silane was used as an internal standard. The conditions were as follows: ^{1}H-NMR: 200 MHz. The data are expressed in δ (ppm). The NMR signals are abbreviated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. The number of protons is shown in parentheses. ^{13}C-NMR: 200 MHz. The data are expressed in hertz (Hz).

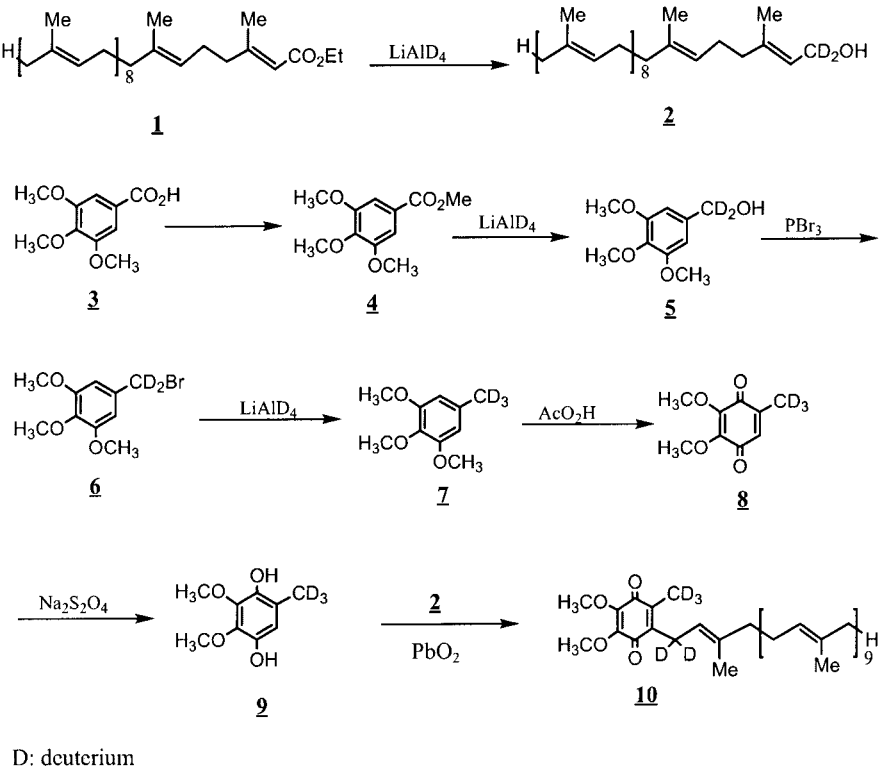
Mass spectra were measured on a JEOL-JMS-D200. Assays for individual determination of Co Q_{10} and [2-CD_3-1′-CD_2]Co Q_{10} **10** were performed on a JEOL JMS-D300 gas chromatograph–mass spectrometer unit equipped with a computer system (JEOL, JMA-2000). The ionizing voltage and ionizing current were 70 eV and 300 μA, respectively. The chamber temperature was maintained at 300°C. The sample temperature was raised linearly from 100°C to 430°C within 3 min. During this time, mass fragments from m/z 824 to m/z 900 were scanned and integrated. The peak ratio was plotted against the internal standard.

2,3,5-Trimethyl-6-decapenyl-p-benzoquinone[7] was used as an internal standard for quantitative analysis of Co Q_{10}.[2] For individual analysis, a multiple ion detector was adjusted to m/z 869 ($[M+2]^+$) for exogenous Co Q_{10} ([2-CD_3-1′-CD_2]Co Q_{10} **10**), m/z 864 ($[M+2]^+$) for endogenous Co Q_{10} and m/z 832 ($[M+2]^+$) for the internal standard.[3]

Results and Discussion

The synthesis of [2-CD_3-1′-CD_2]Co Q_{10} **10** was performed by condensation of deuterated decaprenol **2**, and deuterated Co Qo, hydroquinone (2,3-dimethoxy-[5-CD_3]methyl-1,4-hydroquinone **9**). The total synthetic process, which includes eight steps, is shown in Chart 1.

Basically, the route employed here for the synthesis of deuterium-labelled Co Q_{10} was the same as that we established previously for synthesizing radio-labelled Co Q_{10}.[6] [1-CD_2]Decaprenol **2** was obtained starting from natural solanesol (all-*trans* configuration) via ethyl decaprenoate by the same synthetic route as shown previously.[6] The deuteration of decaprenol was performed by treating ethyl decaprenoate **1** with $LiAlD_4$, and then deuterium oxide. The ^{1}H-NMR of **2** showed no signal at δ 4.24 (allylic methylene protons), even under conditions of 20-fold amplification A nuclear component 2,3-dimethoxy-[5-CD_3]methyl-1,4-hydroquinone **9** was synthesized starting from 3,4,5-trimethoxybenzoic acid **3** in six steps. 3,4,5-Trimethoxybenzoic acid **3** was esterified with methanol to give methyl-3,4,5-trimethoxybenzoate **4**. Reduction of **4** with $LiAlD_4$, gave 3,4,5-trimethoxy-[CD_2]benzylalcohol **5**. The alcohol **5** was brominated with PBr_3 and then reduced with $LiAlD_4$, giving 3,4,5-trimethoxy-[CD_3]toluene **7**. The deuteration ratio of the compound was calculated to be 96.5% based on the residual



D: deuterium

Chart 1. Synthesis of Deuterium - labelled coenzyme Q_{10}

signal intensity at δ 2.3 (ring methyl protons) compared with ring proton intensity at δ 6.30 (2 H) on ^{1}H-NMR spectra. The tri-deutero compound **7** was oxidized by lead dioxide to give 2,3-dimethoxy-[5-CD_3]methyl-1,4-benzoquinone **8**. The deuteration ratio of **8** was calculated to be 96% based on the comparison of the signal intensity at δ 2.08 (ring methyl proton position) with those of ring protons (δ 6.35) on ^{1}H-NMR spectra. The ^{13}C-NMR spectrum of **8** showed no signal at 386 Hz corresponding to the ring methyl signal found in the spectrum of the unlabelled compound. Apart from the differences mentioned above, the labelled and unlabelled compounds were found to give similar signals. Then the benzoquinone **8** was reduced with $Na_2S_2O_4$ to give 2,3-dimethoxy-[5-CD_3]-methyl-1,4-hydroquinone **9**. The ^{13}C-NMR spectrum showed complete disappearance of the signal at 386 Hz, assigned to ring methyl group. Condensation of **9** borate with [1-CD_2]decaprenol **2**, followed by hydrolysis and oxidation with lead dioxide gave a *cis–trans* mixture of [2-CD_3,-1′-CD_2]Co Q_{10} **10**. The crude product was purified by chromatography on silica gel then recrystallized three times

from cooled acetone. The stable isotope chemical yield of the purified [2-CD_3-l′-CD_2]Co Q_{10} **10** from [1-CD_2]decaprenol **2** was 22%. The ^{1}H-NMR spectrum of **10** showed no signal at δ 3.28 (l′-methylene protons), even under conditions of 20-fold amplification. The spectrum was identical to that of the unlabelled Co Q_{10} except for the absence of peaks corresponding to ring methyl and 1′-methylene protons. On ^{13}C-NMR spectrum, no signal was detected at either 292 (ring methyl) or 637 Hz (1′-methylene). Stereospecificity of the final compound **10** was confirmed by performing high-performance liquid chromatography followed by the method described previously.[8] After recrystallizing the silica gel chromatography-purified product, the *cis*-isomer content was found to be 0.5%. The deuteration ratio and stereospecificity were sufficiently high to enable us to use this deuterium-labelled form of Co Q_{10} in metabolic studies.

Experiments

[1-CD_2]Decaprenol **2**

Ethyl decaprenoate **1** 25 g (34 mmol) and $LiAlD_4$, 70 mg (17 mmol) were suspended in 100 ml of absolute ether and 5 ml of deuterium oxide. Reduction was carried out by adding 400 g of silica gel (60–80 mesh). The crude product was purified by chromatography. [1-CD_2]Decaprenol **2** 21.7 g (yield: 92%) was obtained. ^{1}H-NMR: 5.31 (s, 1 H, =CH), 5.01 (m, 9 H, =CH), 1.9–2.1 (m, 38 H, –CH_2-), 1.62 (s, 3 H, =CCH_3), 1.58 (s, 30 H, –CH_3). ^{13}C-NMR: Signal at 1490 Hz, observed for corresponding unlabelled compound, disappeared completely. Mass spectra: m/z 701 ([M^+]), corresponding unlabelled compound: m/z 699, m/z 682 ([M^+]-DHO), corresponding unlabelled compound: m/z 681 ([M^+]-H_2O).

Methyl-3,4,5-trimethoxybenzoate **4**

3,4,5-Trimethoxybenzoic acid **3** was esterified quantitatively in methanol in the presence of concentrated sulphuric acid under refluxing for 5 h. The reaction mixture was poured onto iced water to precipitate needle crystals. After filtration, the crystals were recrystallized from methanol. Methyl-3,4,5trimethoxybenzoate **4** was obtained as crystals.

M.p. 82–84°C. Yield: 92%. R_f value: 0.56 ($EtOAc/C_6H_6$, 1:9); 0.18 (C_6H_6). IR spectra: 1700 cm^{-1} (ester).

3,4,5-Trimethoxy-[CD_2]benzylalcohol **5**

Methyl-3,4,5-trimethoxybenzoate **4** 60 g (265 mmol) was reduced with 8 g (190 mmol) of $LiAlD_4$, in 2 l of absolute ether under stirring and refluxing for 4 h. Excess $LiAlD_4$ was decomposed with 20 ml of deuterium oxide. The product was extracted with ether, and then dried. The solvent was removed *in vacuo*. The residue was distilled under reduced pressure. The purified compound 46.1 g was obtained by vacuum distillation at 164–167°C/1 mmHg. Yield: 87%. R_f value: 0.10 ($EtOAc/C_6H_6$, 1:9); 0.08 (C_6H_6). IR spectra: appearance of OH. ^{1}H-NMR: 6.32 (s, 2 H, Ar-H), 3.78 (s, 9 H, Ar-OMe). The signal at δ 5.46 (benzyl protons) disappeared. The deuteration ratio could not be determined due to overlap with the base line. Mass spectra: m/z 200 ($[M^+]$), m/z 185 ($[M^+]$-CH_3), m/z 183 ($[M^+]$-OH).

3,4,5-Trimethoxy-[CD_2]benzylbromide **6**

3,4,5-Trimethoxy-[CD_2]benzylalcohol **5** 47 g (235 mmol) was dissolved in 1 l of ether and 10 ml of pyridine was added and the temperature was maintained at 0–5°C. To the solution, 20 ml of PBr_3 (350 mmol equivalent) dissolved in ether (total volume 100 ml) was added gradually. The reaction mixture became cloudy. After the completion of addition, stirring continued for further 2 h at the same temperature. After confirmation of the disappearance of the starting material by TLC, the reaction mixture was poured into 1 l of diluted HCI and then extracted twice with 1 l of ether each extraction. The extract was washed with water, and then dried. The solvent was removed *in vacuo*. The crude product 46 g was obtained as a pale yellow oil. Yield: 74%. As the product was unstable, further purification was not attempted and this crude product was directly applied to the next step. R_f value: 0.44 ($EtOAc/C_6H_6$, 1:9); 0.33 (C_6H_6). IR spectra: disappearance of OH.

3,4,5-Trimethoxy-[CD_3]toluene **7**

3,4,5-Trimethoxy-[CD_2]benzylbromide **6** 46 g (175 mmol) was reduced with 3.7 g (88 mmol) of $LiAlD_4$ in 400 ml of absolute ether under stirring and refluxing for 4 h. Excess $LiAlD_4$ was decomposed with

10 ml of deuterium oxide. The product was extracted with ether, and then purified by silica gel chromatography. The purified compound **7** 20.4 g was obtained as a pale yellow oil. Yield: 74%. The product could not be differentiated from corresponding unlabelled compound by TLC or IR spectra comparison. R_f value: 0.50 ($EtOAc/C_6H_6$, 1:9); 0.44 (C_6H_6). ^{1}H-NMR: 6.30 (s, 2 H, Ar-H), 3.80 (s, 9 H, Ar-OCH_3). The deuteration ratio, calculated on the basis of the ratio of the residual signal intensity at δ 7.7 proton to the ring proton intensity at δ 3.68 (2 H), was 96.5%. ^{13}C-NMR: The signal at 546 Hz, observed for the corresponding unlabelled compound, disappeared completely. Mass specta: m/z 185 ($[M^+]$), m/z 170 ($[M^+]$-CH_3).

2,3-Dimethoxy-[5-CD_3]methyl-1,4-benzoquinone ***8***

3,4,5-Trimethoxy-[CD_3]toluene **7** 39.3 g (253 mmol) was dissolved in 200 ml of AcOH. To the solution, 10 ml of 10% sulphuric acid was added. At room temperature, 80 ml of 40% peracetic acid was added gradually to the solution. The colourless reaction mixture changed to red and the temperature of the reaction mixture increased to 50–60°C. After completion of the addition of peracetic acid, stirring was continued for further 16 h at 25–30°C. The disappearance of peracetic acid was checked with potassium iodide starch paper. The reaction mixture was poured onto iced water. The product was extracted with ether, washed with a diluted solution of sodium bicarbonate, and then dried. The solvent was evaporated *in vacuo*. The residual solid was purified by silica gel chromatography. The pure product **8** 24.8 g was obtained as reddish needle crystals. Yield: 54%. M.p.: 75–77°C. The product could not be differentiated from corresponding unlabelled compound by TLC, or IR and UV spectra comparisons. R_f value: 0.36 ($EtOAc/C_6H_6$, 1:9); 0.36 (C_6H_6). ^{1}H-NMR spectra: 6.35 (s, IH, Ar-H), 3.90 (s, 6 H, Ar-OCH_3). Mass spectra: m/z 185 ($[M^+]$), corresponding unlabelled compound: m/z 182, m/z 170 ($[M^+]$-CH_3).

2,3-Dimethoxy-[5-CD_3]methyl-1,4-hydroquinone ***9***

2,3-Dimethoxy-[5-CD_3]methyl-1,4-benzoquinone **8** 24.8 g (134 mmol) was reduced by the usual hydroreduction procedure with 50 g of $Na_2S_2O_4$, 300 ml of ether and 400 ml of water. The resulting mixture was extracted twice, each time with 500 ml of ether and then dried. The solvent was removed *in vacuo*. 2,3-Dimethoxy-[5-CD_3]methyl-1,4-

hydroquinone **9**, 16 g was obtained as colourless sand grain-sized crystals. Yield: 64%. R_f value: 0.21 (EtOAc/C_6H_6, 1:9); 0.18 (C_6H_6); 0.13 (*n*-hexane/$CHCl_3$, 1:1). A red spot was recognized on spraying Emmerie–Engel reagent. ^{1}H-NMR: δ 6.45 (s, 1 H, Ar-H), 5.76 (s, 2 H, Ar-OH), 3.84 (s, 3 H, Ar-OCH_3), 3.01 (s, 3 H, Ar-OCH_3). The ^{13}C-NMR spectrum showed complete disappearance of the signal at 386 Hz, assigned to ring methyl group. Mass spectra: m/z 187 ([M^+]), m/z 172 ([M^+]-CH_3). The product was used immediately in the next reaction.

[2-CD_3-1′-CD_2]Coenzyme Q_{10} **10**

2,3-Dimethoxy-[5-CD_3]methyl-1,4-hydroquinone borate was prepared by allowing a mixture of 2,3-dimethoxy-[5-CD_3]methyl-1,4-hydroquinone **9** 16 g (86 mmol), 5 g of boric acid and 85 ml of toluene to react under stirring and refluxing until 2.3 ml of dehydrated water was obtained. The toluene was then removed *in vacuo* and 65 ml of *n*-hexane and 30 ml of benzene were added to the residue to substitute the solvent. To the solution, [1-CD_2]decaprenol **2** 19.6 g (28 mmol) and 30 g of silica alumina were added. The condensation reaction was carried out at 40–45°C for 4 h under stirring. After completion of addition, the reaction was allowed to continue for 2 hours at the same temperature. The end of the reaction was judged by the appearance of the Co Q_{10} spot as examined by TLC. The reaction mixture was then filtered. The filtrate was oxidized by 7 g of lead dioxide, 5 g of charcoal carbon and 5 ml of acetic acid. After the reaction, the reaction mixture was filtered and the filtrate was washed with water, extracted with toluene and then dried. The solvent was removed *in vacuo*. The red oily residue was purified by chromatography using 800 g of silica gel. The chromatography purified product (14.5 g) was found to give a single spot on TLC. Yield: 60%. After recrystallization three times, each time by addition of a 5-fold excess of cooled acetone compared to the crude product (−2°C), [2-CD_3-1′-CD_2]Co Q_{10} **10** (6.4 g) was obtained. The final product was obtained as a yellow solid. M.p.: 47.8°C. Yield from [1-CD_2]decaprenol **2** was 22%. The *cis*-isomer content of the purified [2-CD_3-1′-CD_2]Co Q_{10} **10** was 0.5%. The Co Q_9 content of **10** was less than 0.1%. R_f value: 0.44 (*n*-hexane-$CHC1_3$, 1:1); 0.46 (C_6H_6). ^{1}H-NMR: 6.78 (s, 3 H, $=CCH_3$), 5.18 (m,10 H, $=CH$), 4.02 (s, 3 H, Ar-OCH_3), 4.00 (s, 3 H, Ar-OCH_3), 1.9–2.1 (m, 38 H, -CH_2-), 1.70 (s, 3 H, $=CCH_3$), 1.60 (s, 27 H, $=CCH_3$). Mass spectra: m/z 869

($[M+2]^+$), m/z 240 (pyrylium ion), m/z 202 (benzylium ion), corresponding unlabelled compound: m/z 864, m/z 235, m/z 197.[9]

Acknowledgements

The authors are grateful to Mr T. Sato, Mr K. Hashimoto and Mr K. Ema for their technical assistance in performing analyses. Critical reading of the manuscript by Dr Ellen Beaumont is highly appreciated.

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