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Synthesis of [1,3,5-$^{13}C_3$]Benzoic Acid and [2,4,6,7-$^{13}C_4$]Benzoic Acid from ^{13}C-Labelled Sodium Pyruvates

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SUMMARY

The synthesis of [1,3,5-$^{13}C_3$]- and [2,4,6,7-$^{13}C_4$]benzoic acid (5a and 5b) from [2-^{13}C]- and [3-^{13}C]sodium pyruvate (1a and 1b), for *in vitro* and *in vivo* tracer studies using ^{13}C nuclear magnetic resonance (NMR) spectroscopy, is reported. After condensation of 1 to [^{13}C]methyldihydrotrimesic acids 2 using sodium hydroxide, decarboxylation to [^{13}C]uvitic acids 3 was carried out using conc. sulfuric acid. Additional decarboxylation of 3 was achieved with cupric oxide and quinoline, and the resulting [^{13}C]toluenes 4 were effectively trapped, using a simplified decarboxylation apparatus. Compounds 4 were subsequently oxidized to 5 using potassium permanganate without further purification. The overall yields of 5 from 1 were 26-28 %, with isotopic purities of 96-98 %. In addition, the assignments for ^{13}C NMR signals of benzoic acid were first substantiated.

Key Words: [1,3,5-$^{13}C_3$]benzoic acid, [2,4,6,7-$^{13}C_4$]benzoic acid, nuclear magnetic resonance spectroscopy, tracer study

INTRODUCTION

The potential of nuclear magnetic resonance (NMR) spectroscopy for *in vitro* and *in vivo* metabolic investigations using ^{13}C-enriched precursors has been widely demonstrated (1). However, there are few examples of the application of ^{13}C labeling/NMR tracer techniques for quantification of metabolites in biological fluids. Recently, we have quantitatively determined [7-^{13}C]hippuric acid (HA) excreted in the urine

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of a volunteer, to whom [7-^{13}C]benzoic acid (100 mg) was orally administered, by NMR spectroscopy (only 10 min accumulation time) using [glycine carbonyl-^{13}C]HA as an analytical standard (2). The time-course of HA excretion was exactly followed by very simple procedures, and this approach was suggested to be useful for diagnosis of liver functions. A similar quantitative approach is basically considered to be feasible for biotransformation of other biologically active compounds including xenobiotics. However, higher sensitivity is desirable for the compounds that are used at low dose. The sensitivity of ^{13}C labeling/NMR tracer techniques is influenced by labeling positions as well as instrumental conditions. The sensitivity is significantly increased by the utilization of compounds labelled in the specific protonated carbons due to the nuclear Overhauser enhancements and short spin-lattice relaxation times.

Numerous compounds of importance in biochemistry and medicine possess a benzene nucleus in the molecule. Therefore, ^{13}C-labelled benzoic acid (BA) is considered to be useful as a key material for ^{13}C labeling of such compounds. Only [1-^{13}C]-, [7-^{13}C]- and [1,2,3,4,5,6-$^{13}C_6$]BA are currently commercially available. These labeling positions are however generally undesirable for tracer studies by NMR spectroscopy because of the low NMR sensitivity of the labelled position (see Fig. 2) or the complexity of spectrum resulting from vicinal ^{13}C-^{13}C couplings. In this paper, we report a convenient method for the preparation of [1,3,5-$^{13}C_3$]- and [2,4,6,7-$^{13}C_4$]BA on a relatively small scale.

EXPERIMENTAL

Proton-decoupled ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian GEMINI-300 instrument, operating at 75 MHz. Spectra were accumulated using a 10.1 μs pulse width, 0.800 s acquisition time and 1.200 s pulse delay. Free induction decays were Fourier transformed with 1.000 Hz line broadening. Mass spectra (electron impact) were recorded on a Hitachi M-80 instrument. M.p.s. were determined on a Yamato MP-1

melting point apparatus and are uncorrected. Flash chromatography was carried out on Wakogel C-300 (silica gel).

[2-^{13}C]Sodium pyruvate 1a (99 atom % ^{13}C) and [3-^{13}C]sodium pyruvate 1b (99.1 atom % ^{13}C) were purchased from Daiichi Kagaku Yakuhin (Tokyo, Japan). Other reagents were purchased from Kanto Kagaku (Tokyo, Japan). All the synthesized products were dried *in vacuo* at room temperature.

[^{13}C]Uvitic acids 3

Compound 1a (2 g, 18 mmol) was dissolved in 31 %-sodium hydroxide solution (5.8 ml) in a 30 ml round-bottom flask at room temperature, by vigorous magnetic stirring. The time required was about 2 h. The resulting pale yellow solution was heated for 3.5 h at 85-90 °C on a steam-bath. A precipitate of sodium oxalate appeared at 30 min after the beginning of heating. The reaction mixture was allowed to cool to room temperature in order to facilitate the filtration. The sodium oxalate was removed by filtration with a glass filter, followed by washing with 12 N sodium hydroxide (3x1 ml). The filtrate was combined with the washings and acidified to pH 1 with conc. hydrochloric acid (*ca.* 8 ml) in an ice bath to prevent the temperature from rising above 20 °C. The precipitated powder was collected on a glass filter, followed by washing with ice-cold water (5x1 ml) to eliminate sodium chloride, and dried to give crude [1,3,5-$^{13}C_3$]methyldihydrotrimesic acid 2a (1.0 g), which was considered to still contain a considerable amount of sodium chloride.

In a 50 ml three-necked flask equipped with a reflux condenser, pressure equalizing dropping funnel, magnetic stirring bar and nitrogen gas inlet with stopcock was placed dry crude 2a (1.0 g). Conc. sulfuric acid (2.8 ml) was run into the nitrogen filled flask from the dropping funnel and the mixture was heated at 120 °C in an oil-bath for about 10 min. After the generation of carbon monoxide (*ca.* 10 min later), a current of nitrogen was started, and the temperature of the bath was raised to 150-155 °C over a period of about 10 min, and held at the temperature for 1.8

h. The reaction mixture was cooled somewhat and poured into water (20-30 ml) to give a gray powdery precipitate. The mixture was allowed to cool to room temperature in order to facilitate the filtration. The powder was collected on a glass filter, and washed with water (5x1 ml), and dried to give crude [1,3,5-$^{13}C_3$]uvitic acid 3a (494 mg, 60 %).

Compound 1b (2 g, 18 mmol) was treated in the same manner as above, to give crude [2,4,6,7-$^{13}C_4$]uvitic acid 3b (454 mg, 56 %).

[^{13}C]Benzoic acids 5.

The apparatus used for the decarboxylation of 3 is shown in Fig. 1. The apparatus consisted of a reaction vessel attached to the joint A, air condenser, bridging tube and trap B with nitrogen outlet, that are all equipped with standard taper 15/25 joints. The reaction vessel was charged with crude 3a (494 mg), powdery cupric oxide (72 mg, 0.33 eq. for the crude 3a) and quinoline (10 ml). The air was swept out of the

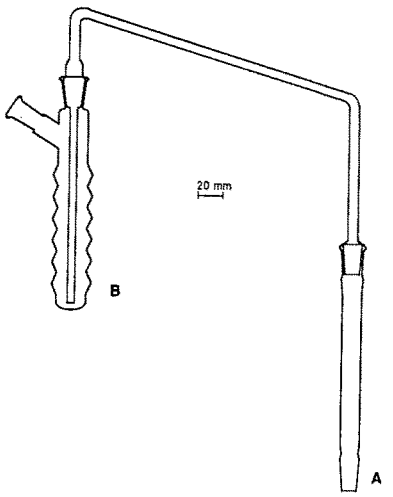


Fig. 1. Reactor and trap assembly used in decarboxylation of 3. Particular attention is given to the correct proportions of various parts of the figure. A 20 ml two-necked flask as reaction vessel equipped with nitrogen gas inlet is connected to joint A. The vessel containing uvitic acid, quinoline and copper catalyst is heated by an oil-bath with magnetic stirring. Entrained vapor is collected in trap B cooled by dry ice-acetone mixture. The apparatus is designed and tested with non-labelled uvitic acid so that contamination of the trapped toluene by quinoline can be minimized.

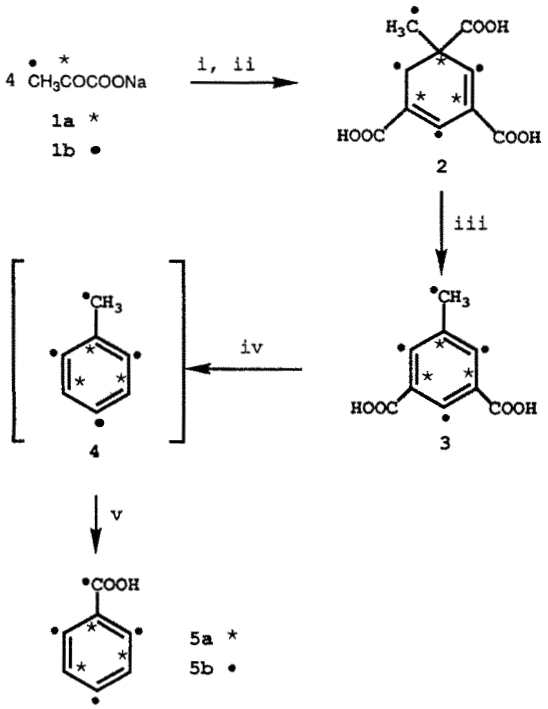
system with nitrogen, and the trap was cooled with a dry ice-acetone mixture. The reaction vessel was heated by an oil-bath, whose temperature was raised from 150 °C to 250-260 °C over a period of about 30 min and held for 3 h, under a nitrogen stream with magnetic stirring. The heating of the oil-bath was assisted with an additional pipe heater. After the trap was removed from the train, the section of the bridging tube† which had been inside the trap was washed with t-BuOH (3x1.5 ml), and the washings were combined with the contents of the trap. To the trap was added potassium permanganate (1.0 g, 6.33 mmol) and water (4.2 ml). The trap was equipped with a reflux condenser and the nitrogen outlet was stoppered. The mixture was refluxed for 16 h with magnetic stirring. The excess potassium permanganate was quenched by heating with methanol (*ca.* 0.5 ml) for a few minutes. The resulting manganese dioxide was filtered off, and washed with 15 ml of hot water. The filtrate and washings were combined and concentrated to about 5 ml under reduced pressure, and acidified to pH 1 with conc. hydrochloric acid in an ice bath to give a white powdery precipitate. The resulting suspension was extracted with chloroform, and the organic layer was dried ($MgSO_4$), and solvent was removed by evaporation to give a crystalline white powder. Flash column chromatography on silica, eluting with chloroform only, and the subsequent recrystallization gave [1,3,5-$^{13}C_3$]BA 5a as white needles (155 mg; 46 % based on uvitic acid; $^{13}C_3$, 97.9 %; 99.3 atom % of ^{13}C), m.p. 120.0-121.4 °C (from hexane); δ_C(75 MHz; $CDCl_3$) 128.4 (C3, C5), 129.2 (C1); *m/z* 125 (M^+, 91 %), 108(100, M-OH) and 80(83, M-COOH).

Crude compound 3b (454 mg) was treated in the same manner as above, to give [2,4,6,7-$^{13}C_4$]BA 5b as white needles (145 mg, 47 % based on uvitic acid; $^{13}C_4$, 96.7 %; 99.2 atom % of ^{13}C), m.p. 120.3-121.5 °C(from hexane); δ_C (75 MHz; $CDCl_3$) 130.1 (C2, C6) 133.7 (C4), 172.1 (C7); *m/z* 126 (M^+, 83 %), 109(100, M-OH) and 80(81, M-^{13}COOH).

† The washings of the remaining section was also oxidized with potassium permanganate, but no BA was obtained.

RESULTS AND DISCUSSION

[1,3,5-$^{13}C_3$]BA 5a and [2,4,6,7-$^{13}C_4$]BA 5b were synthesized from [2-^{13}C]sodium pyruvate 1a and [3-^{13}C]sodium pyruvate 1b, respectively, by the route shown in Scheme 1, modifying the procedure described for [1,3,5-$^{14}C_3$]toluene by Hughes et al. (3). No carriers were used in any synthetic steps. A simplified apparatus (Fig. 1) for the conversion of [^{13}C]uvitic acids 3 to [^{13}C]toluenes 4 was designed so that the trap for 4 could be directly used for the subsequent oxidation to 5. By this device, the tediousness and unavoidable loss in quantity inherent in the small scale purification of volatile compounds were eliminated. Special care was exercised to prevent the trapped 4 from being contaminated by quinoline,



Scheme 1. Synthetic route of 5.
***Reagents*: i, NaOH; ii, HCl; iii, H_2SO_4; iv, CuO, quinoline; v, $KMnO_4$.**
The positions of labeling are marked with * and •.

which is a quencher for potassium permanganate. The overall yields of 5 from 1 were 26-28 %. The isotopic purities of 5a and 5b were found to be 97.9 % and 96.7 %, respectively, by the mass spectrometric analyses. These values were estimated on the basis of the ion intensities in the region of the molecular ions.

The NMR spectra of non-labelled BA and 5 are shown in Fig. 2 and 3, respectively. The assignments for C2(C6), C3(C5) in BA have been previously reported by Ihrig et al. (4) using 3,5-deuteriobenzoic acid, in which the deuterium-bearing carbon atom is identified by the loss of signal intensity. They have concluded that C2(C6) signal is downfield to C3(C5) signal. The data in this paper have first substantiated that the original assignments are correct. As can be seen from Fig. 2, NMR signals

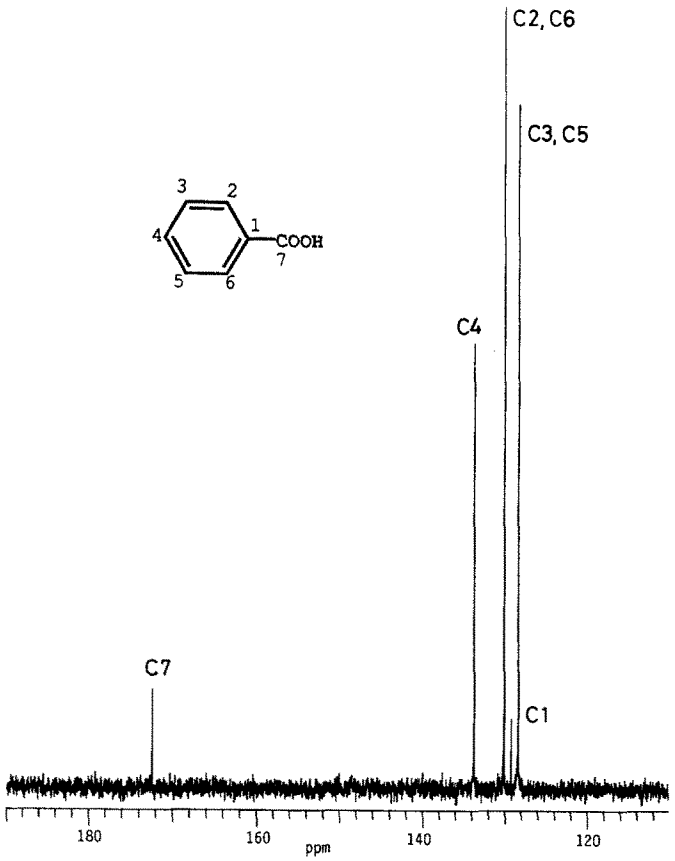


Fig. 2. ^{13}C NMR spectrum of non-labelled BA. Sample concentration, *ca.* 0.3 M; transients, 128.

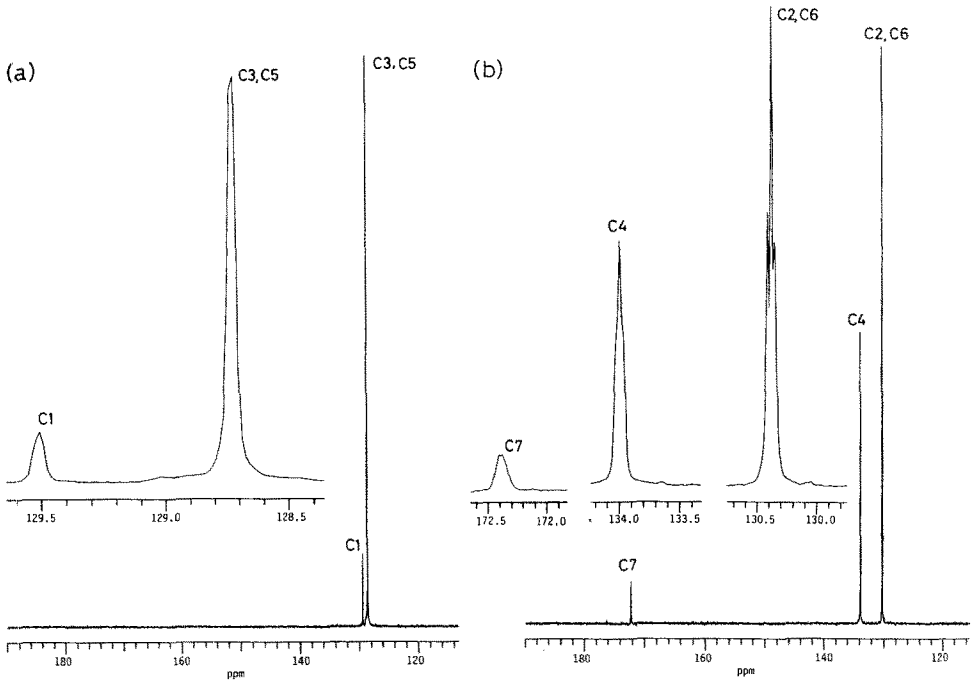


Fig. 3. ^{13}C NMR spectra of 5a(a) and 5b(b). sample concentration, *ca.* 0.1 M; transients, 10. The insets show the expanded signals.

of C2(C6), C3(C5) in BA are more intense than those of C1 and C7 due to the nuclear Overhauser enhancements and short spin-lattice relaxation times. The protonated carbons C2(C6), C3(C5) are therefore desirable as the labeling positions to be monitored in tracer studies. As shown in Fig. 3, some long-range ^{13}C-^{13}C couplings (4,5) were observed under these NMR conditions. The sensitivity of ^{13}C labeling/NMR tracer techniques is considered to be markedly increased by the use of 5 and their derivatives.

Recently, tracer techniques, in which stable isotopically labelled compounds are used as tracers and GC-MS as an analytical tool, have been enjoying a wide application in metabolism studies. In this field, multi-labelled compounds containing at least three stable isotopes with high isotopic purity are required. Compounds 5 will be also useful in tracer studies using GC-MS in this respect.

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