Synthesis of Selectively ^{13}C-Labelled Benzoic Acid for Nuclear Magnetic Resonance Spectroscopic Measurement of Glycine Conjugation Activity

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SUMMARY

The synthesis of [4-^{13}C]benzoic acid (BA) labelled in a single protonated carbon, for use as a probe to measure glycine conjugation activity by nuclear magnetic resonance (NMR) spectroscopy, has been reported. The labelled compound was prepared by a seven-step synthetic scheme on a relatively small scale using [2-^{13}C]acetone as the source of label in overall yield of 16 %. The usefulness of [4-^{13}C]BA was demonstrated by the NMR spectroscopic monitoring of urinary excretion of [4-^{13}C]hippuric acid in the rat administered with the labelled BA.

Key words: [4-^{13}C]benzoic acid, [4-^{13}C]hippuric acid, nuclear magnetic resonance spectroscopy, glycine conjugation, liver function.

INTRODUCTION

It is well recognized that liver failure influences the disposition of many drugs that are metabolized by the liver because of the impaired drug-metabolizing-enzyme activities (1). There have been many studies on the influence of liver failure on mixed-function oxygenase reactions. In contrast, such studies on conjugation reactions have been limited so far, although some studies on glucuronidation and *N*-acetylation have been performed (1). The authors have undertaken a study on the relationship between liver failure and the activity of glycine conjugation that is one of the major biotransformations of carboxylate-containing xenobiotics. Benzoic acid (BA), a food preservative that is also used in the treatment of hyperammonemia, appears to be an ideal substrate for the study because BA is quantitatively conjugated with

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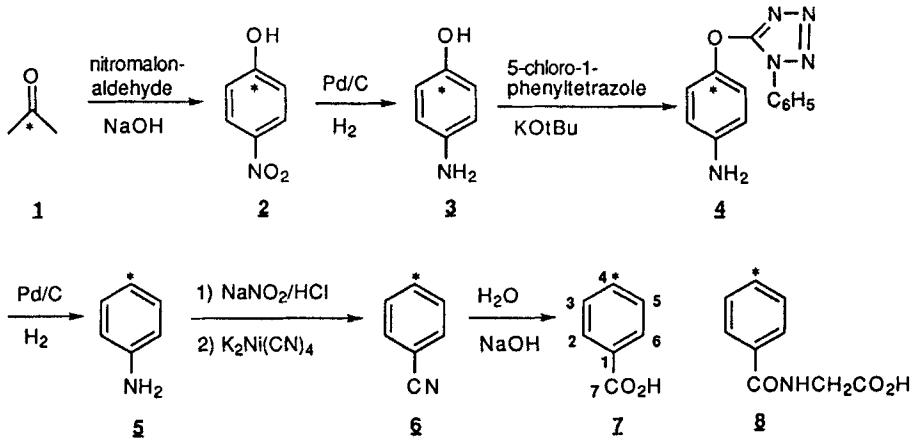
glycine to form hippuric acid (HA) mainly in the liver and excreted in urine in normal humans, rhesus monkeys, rabbits, and rats (2).

Tracer techniques are essential for the detection of BA conversion to HA because HA is endogenously formed from BA that originates from food. The authors reported a simple and convenient approach to follow the biotransformation in a human subject, where [7-^{13}C]BA (2 mg/kg) was orally administered as a substrate followed by urinalysis using 100-MHz ^{13}C NMR spectroscopy (10 min accumulation time) without any separation procedure (3). Subsequently, the same biotransformation in the rat was followed under the similar NMR conditions with an approximately ten-fold higher sensitivity using newly synthesized [2,4,6,7-$^{13}C_4$]BA as a substrate (4,5). [1,3,5-$^{13}C_3$]BA was also synthesized and demonstrated to afford a sensitivity similar to [2,4,6,7-$^{13}C_4$]BA in our laboratory (unpublished work). The improvement of sensitivity was due to the large nuclear Overhauser enhancements and short spin-lattice relaxation times (T_1) of the protonated carbon nuclei (C2,6, C4, and C3,5). However, the synthetic yields of those compounds were relatively low, and ^{13}C labels were introduced into plural positions with different chemical environments as well, which caused long-range ^{13}C-^{13}C couplings (4). The long-range couplings broadened the resonances to a significant extent. Thus, the authors have investigated alternative synthetic routes to introduce ^{13}C labels into only the protonated carbons, i.e., C2,6, C4 or C3,5 that are useful for the NMR spectroscopic monitoring of BA conversion to HA. In this paper, [4-^{13}C]BA has been synthesized from [2-^{13}C]acetone in a higher yield for use as a probe to investigate glycine conjugation activity. In addition, the usefulness of [4-^{13}C]BA has been demonstrated by the NMR spectroscopic monitoring of urinary [4-^{13}C]HA excretion in the rat administered with the labelled BA.

RESULTS AND DISCUSSION

Isotopically labelled BA **7** was prepared using the synthetic route diagrammed below. Each intermediate except for 4-nitro[1-^{13}C]phenol **2** was used for the subsequent step without purification in order to increase the isotopic yield. Compound **2** was prepared by the condensation of [2-^{13}C]acetone **1** with nitromalonaldehyde according to the previously reported method (6). The reduction of **2** to 4-amino[1-^{13}C]phenol **3** proceeded smoothly using 10 % Pd/C at room temperature and at atmospheric pressure. The aromatic hydroxy group of **3** was eliminated to give [4-^{13}C]aniline **5** by the formation of 1-phenyl-5-tetrazolylether **4** and the subsequent hydrogenolytic cleavage (7,8). The hydrogenolysis reaction was completed in a



Scheme 1. Synthetic route of [4-^{13}C]benzoic acid.
The position of labeling is marked with *.

much shorter time than described in the literature (7), by using a relatively large amount of 10 % Pd/C. The reactions from 2 to 5 proceeded almost quantitatively, which was examined by the TLC analyses of reaction mixtures. Compound 5 was easily converted to benzonitrile 6 according to the method described by Takino *et al.* (9), and then hydrolyzed to give 7 in overall yield of 16 % (77 % per step) based on [2-^{13}C]acetone.

The isotopic purity of 7 was estimated to be 95.8 % on the basis of the ion intensities in the region of the molecular ion in mass spectrometric analyses. Compound 7 showed a single intense resonance of C4 at $\delta^{13}C$ 133.8 in the ^1H-decoupled ^{13}C NMR spectrum ($CDCl_3$), whereas the C2,6 ($\delta^{13}C$ 130.2) and C3,5 ($\delta^{13}C$ 128.4) resonances appeared in natural abundances. This means that the label was exclusively introduced into the C4 position without rearrangement of the label (10). In addition, the C4 resonance of 7 was much sharper than that of above-mentioned [2,4,6,7-$^{13}C_4$]BA as shown in Fig.1 because the former has no long-range coupling. Thus, the NMR spectrum of 7 was extremely simplified, compared with those of [2,4,6,7-$^{13}C_4$]- and [1,3,5-$^{13}C_3$]BA. The overall isolated yield of 7 was two times higher than those (~150 mg) of [2,4,6,7-$^{13}C_4$]- and [1,3,5-$^{13}C_3$]BA, using almost the same moles of the starting materials. The isolated yield of 7 would be probably increased if sublimation is used as a means of separation. Also, the starting material 1 of 7 is much less expensive than those of the multi-labelled BAs, i.e., [^{13}C]pyruvates. Therefore, the present method is of greater advantage than the previous method for producing BA labelled in the specific protonated carbons.

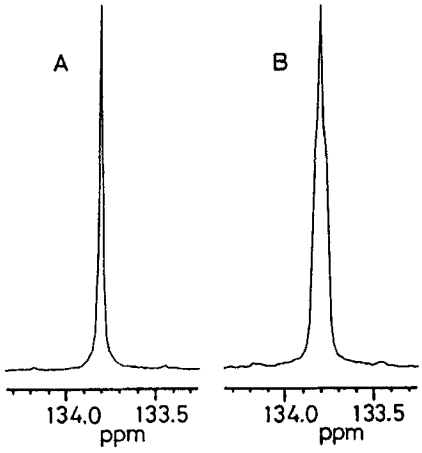


Fig. 1. C4 resonances of [4-^{13}C]BA (A) and [2,4,6,7-$^{13}C_4$]BA (B) detected by ^{1}H-decoupled ^{13}C NMR spectroscopy.
Parameters were pulse width, A 45°, B 55°; acquisition time, 0.80 s; pulse delay, A 0.20 s, B 1.20 s; transients, A 2, B 10; and line broadening, 1 Hz. sample concentration, A 0.2 M, B 0.1 M.

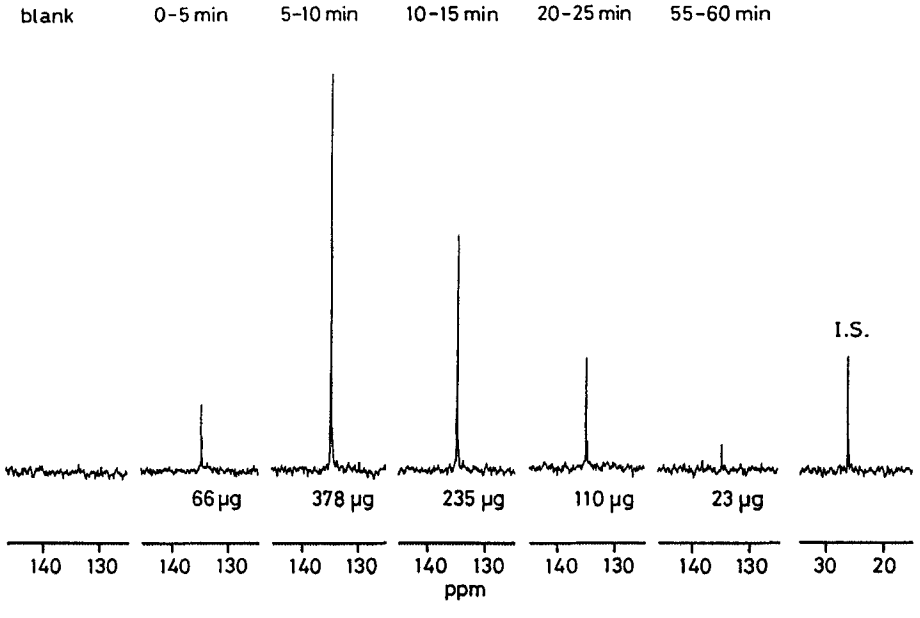


Fig. 2. Partial $^{13}C\{^1H\}$ NMR spectra of urine from a rat after iv administration of [4-^{13}C]BA (2 mg/kg). The resonances at 134.9 ppm are due to C4 of [4-^{13}C]HA. Collection periods are indicated at the top. The amounts of [4-^{13}C]HA determined are given below the resonances. All spectra were plotted out at the fixed resonance height of the spiked I.S. (202 µg).

[4-^{13}C]BA 7 was administered to rats, and the excreted urine was analyzed with NMR spectroscopy under the conditions described in the experimental section in order to demonstrate the usefulness of the labelled compound. Consequently, only resonance at $\delta^{13}C$ 134.9 was observed in the aromatic region of the spectra, and the resonance was due to C4 of

[4-^{13}C]HA **8** formed from the administered BA. The C4 resonances of BA (δ^{13}C 134.0) and HA were confirmed to be well separated on the spectra by the analysis of the urine sample spiked with [4-^{13}C]BA. These results mean that little, if any, BA administered is excreted as the unconjugated form, which is in agreement with the previous data (2,5). The time course of urinary [4-^{13}C]HA excretion was followed with sufficient signal-to-noise (S/N) ratios as shown in Fig. 2. [4-^{13}C]HA was quantitated using [2-^{13}C]acetate as an internal standard (I.S.) (see Fig. 2). The validity of the use of [2-^{13}C]acetate was demonstrated in the previous paper (5). The calibration of NMR sensitivity for [4-^{13}C]HA (C4) and [2-^{13}C]sodium acetate (C2) was performed using those non-labelled analogues. The advantage of this calibration is that no preparation of the authentic labelled metabolite is required. The C4 resonance of 55-60 min postdose urine was 23 μg. Thus, the lower limit of measurable amounts seemed to be ~50 nmol, which is comparable to that of [2,4,6,7-$^{13}C_4$]HA (5). Almost all the BA administered was excreted as HA within 2 h, which was similar to the previous data using [2,4,6,7-$^{13}C_4$]BA as a substrate (5). Thus, the present NMR approach using [4-^{13}C]BA is considered to be applicable to the investigation of glycine conjugation activity in the model rat with liver failure. In addition, [4-^{13}C]BA might be useful for the detection of glycine conjugation reaction using *in vivo* ^{13}C NMR spectroscopy because of the relatively high sensitivity and the simplicity of spectra.

EXPERIMENTAL

^{1}H NMR and ^{1}H-decoupled ^{13}C NMR spectra of synthesized compounds were recorded in $CDCl_3$ on a Varian GEMINI-300 instrument, operating at 300 and 75 MHz, respectively. Mass spectra (electron impact) were recorded on a VG Autospec E instrument. Melting points were determined on a Yamato MP-1 melting point apparatus and were uncorrected. Column chromatography was carried out on Wakogel C-300 (silica gel). Thin-layer chromatography (TLC) was done on silica gel 60 F_{254} plates (Merck, Darmstadt) using benzene-dioxane-acetic acid-1-butanol (90:25:4:4) as the eluent, unless otherwise stated. [2-^{13}C]Acetone **1** (99 atom % ^{13}C) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). [2-^{13}C]Sodium acetate (99.1 atom % ^{13}C) was purchased from ISOTEC Inc. (Miamisburg, OH, USA). Dehydrated dimethylformamide was purchased from Kanto Kagaku (Tokyo, Japan) and further dried with Molecular Sieves 4A 1/16 (Wako, Tokyo, Japan) prior to use. Sodium nitromalonaldehyde monohydrate was prepared as described by

Fanta (11) and could be stored in a desiccator for several months at room temperature. Each synthetic step was investigated using unlabelled compounds and the structure of each product was confirmed by the comparison of the ^1H NMR spectra with those of the authentic compounds prior to the syntheses of labelled compounds.

4-Nitro[1-^{13}C]phenol **2**.

A solution of sodium nitromalonaldehyde monohydrate (3.2 g) and [2-^{13}C]acetone **1** (1 g, 17.2 mmol) in 200 ml of water was made in a 300-ml round-bottomed flask equipped with a magnetic stirrer and cooled in an ice-water bath. To the solution was added dropwise with stirring an ice-cold solution of sodium hydroxide (4.4 g) in 20 ml of water. The resulting orange solution was tightly stoppered and stored in a refrigerator at 4-5 °C for 6 days, after which time the color of the solution turned black. Hydrochloric acid (6 N, 30 ml) was added dropwise to the cold solution. The resulting brown/black precipitate was removed by gravity filtration. The precipitate was suspended in 30 ml of 6 N hydrochloric acid. It was then boiled for 10 min and subjected to gravity filtration while hot. The combined acidic filtrates were then extracted with diethyl ether (100 ml x 6). The ether layers were then dried with magnesium sulphate and evaporated under vacuum to give 1.37 g of dark yellow solid. The solid was purified by column chromatography over 50 g of silica gel with chloroform-methanol (50:1) as the eluent. The eluate was evaporated to dryness in vacuo after filtration to give 1.18 g (49 %) of **2** as a yellow solid. TLC showed a single spot at an R_f identical with that of authentic unlabelled 4-nitrophenol.

4-Amino[1-^{13}C]phenol **3**.

Compound **2** (1.18 g) was dissolved in 15 ml of methanol and then 10 % Pd/C (150 mg) was added to the solution in a 50-ml round bottomed flask equipped with a reflux condenser that was connected to a balloon containing hydrogen gas through a three-way tap. The appratus was evacuated with a water aspirator and then filled with hydrogen gas through the three-way tap. The mixture was vigorously stirred with a magnetic stirrer for 6 h. The Pd/C was filtered off followed by washing of the catalyst with 20 ml of methanol. The filtrate was combined with the washing and evaporated to dryness in vacuo to give 897 mg (97 %) of crude 4-amino[1-^{13}C]phenol **3** as a dark brown crystalline solid. TLC showed a single spot at an R_f identical with that of authentic unlabelled 4-aminophenol.

4-Amino[1-^{13}C]phenol 1-phenyl-5-tetrazolylether **4**.

To a suspension of 897 mg of crude **3** in 20 ml of dehydrated dimethylformamide was added potassium *tert*-butoxide (1.1 g) under a dry-nitrogen stream followed by magnetic stirring at room temperature for 1.5 h. To the suspension was added a solution of 1.5 g of 5-chloro-1-phenyltetrazole in 6 ml of dehydrated dimethylformamide followed by magnetic stirring at room temperature for 1.5 h. The reaction mixture was poured into 200 ml of ice water. The precipitated brown powder was collected by filtration followed by washing with water (20 ml x 3), and dried in vacuo to give 1.95 g (94 %) of crude product **4**. TLC showed a single spot at an R_f identical with that of authentic unlabelled 4-aminophenol 1-phenyl-5-tetrazolylether.

[4-^{13}C]Benzoic acid **7**.

Crude **4** (1.95 g) and 10 % Pd/C (1.95 g) were suspended in 150 ml of benzene in a 300-ml round-bottomed flask connected to a balloon containing hydrogen gas similarly to the apparatus used for the reduction of compound **2**. The mixture was vigorously stirred under a hydrogen atmosphere at 35 °C with a magnetic stirrer for 3.5 h, after which time TLC of the reaction mixture showed a single spot a little less polar than the starting material, which was considered to be due to 1-phenyltetrazolone. The Pd/C was filtered off. The filtrate was poured into 150 ml of 0.5N sodium hydroxide aqueous solution followed by extraction with benzene (200 ml x 2) in order to remove 1-phenyltetrazolone. The combined organic layers were dried over magnesium sulphate, and evaporated on a rotary evaporator in vacuo to give 596 mg of crude [4-^{13}C]aniline **5** as a brown oil.

To a mixture of crude **5** (596 mg) and 220 ml of 0.4 % hydrochloric acid was added dropwise with magnetic stirring a solution of sodium nitrite (540 mg) in 35 ml of water followed by stirring for an additional 2 h keeping the temperature below 5 °C by an ice-water bath. The mixture was neutralized with a saturated aqueous solution of sodium carbonate in an ice-water bath to prevent the temperature from rising above 5 °C. The reaction mixture was added to a solution of potassium tetracyanonickelate(II) in 75 ml of 1M NH_3-NH_4Cl buffer (pH 10) in a 500-ml of round-bottomed flask equipped with a reflux condenser, followed by magnetic stirring at 50-60 °C for 15 min. The resulting precipitate was filtered off followed by washing of the precipitate with 50 ml of water. The filtrate was combined with the washing and extracted with diethyl ether (200 ml x 5). The organic layer was dried over magnesium

sulphate and evaporated on a rotary evaporator to give 545 mg of crude [4-^{13}C]benzonitrile **6** as a reddish brown oil.

A suspension of crude **6** (545 mg) in 25 ml of 1N sodium hydroxide aqueous solution was refluxed with vigorous magnetic stirring for 4.5 h. The reaction mixture was diluted with 25 ml of water and washed with 50 ml of chloroform. The aqueous layer was acidified with 4N hydrochloric acid to pH 2, followed by extraction with chloroform (50 ml x 3). The organic layer was dried over magnesium sulphate and evaporated to dryness to give 469 mg of crude **7** as pale yellow powder. The subsequent recrystallization gave [4-^{13}C]BA as crystalline powder. (333 mg; 16 % based on acetone; 95.8 atom % of ^{13}C), m.p. 120.7-121.3 °C (from water); ^{1}H NMR δ^{1}_{H} 7.48 (t, 2H, H3 and H5, J=7.8 Hz), 7.62 (dt, 1H, H4, J=7.8 Hz, $^{1}J_{C4,H4}$ 161.1 Hz), 8.13 (t, 2H, H2 and H6, J=7.8 Hz); ^{1}H-decoupled ^{13}C NMR for 20 mg of the title compound with 8 scans: intense signal at δ^{13}_{C} 133.8 (C4); m/z 123(M^{+}, 92 %), 106(100, M-OH) and 78(70, M-COOH). Anal Calcd. for $C_6{}^{13}C_1H_6O_2$: C, 68.29; H, 4.91. Found: C, 68.36; H, 4.89. TLC using benzene-dioxane-acetic acid-1-butanol (90:25:4:4) and chloroform-methanol (100:1) gave single spots at R_f values identical with those of authentic unlabelled BA.

Animal experiments and NMR measurements.

The tracer studies using rats were performed essentially as described previously (5). Briefly, [4-^{13}C]BA was administered into the jugular vein through a catheter at a dose of 2 mg/kg, followed by urine collection from both ureters through catheters in 5-min periods for 1 h and then in the following 1 h, while infusing 5 % mannitol at a rate of 3 ml/h in order to stimulate urine excretion. Whole urine sample (100-200 μl) collected in each 5-min period and a portion (400 μl) of the 1-2 h urine were subjected to the NMR analysis. To each urine sample were added sodium 3-trimethylsilyl-[2,2,3,3-$^{2}H_4$]-propionate (5 mg), $^{2}H_2O$ (50 μl), [2-^{13}C]sodium acetate (202 μg) as an I.S. for quantitation, and a drop of 10 N sodium hydroxide aqueous solution. The volume of sample was made up to approximately 0.5 ml by the addition of water, and the sample was transferred to a 5-mm NMR tube. ^{1}H-decoupled ^{13}C NMR measurements were carried out at 300K on a Bruker AM400 (9.4 T) spectrometer. With this spectrometer 216 free induction decays (10 min accumulation time) were collected, after 75° (5 μs) pulses, using a relaxation delay of 2 s and data acquisition time of 0.655 s. Free induction decays were Fourier transformed with 10.0-Hz line broadening.

Quantitation method.

A standard mixture of non-labelled sodium hippurate (460 mM) and sodium acetate (506 mM) dissolved in water was quadruplicately analyzed by ^{13}C NMR spectroscopy under the same conditions as those for urine samples. The relative sensitivity for equal numbers of C4 ($\delta^{13}c$ 134.9) of sodium hippurate and C2 ($\delta^{13}c$ 26.1) of sodium acetate was calculated to be 3.85 (C4/C2) from those resonance heights. The amount of urinary [4-^{13}C]HA was calculated based on the relative sensitivity, the ratio of the resonance heights [C4 of [4-^{13}C]HA/C2 of [2-^{13}C]acetate], and the amount of [2-^{13}C]acetate added.

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