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Preparation of 1-*O*-acylglucuronides of ^{13}C-labelled (*R*)- and (*S*)-ketoprofens

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SUMMARY

The preparation of enantiomeric [1-^{13}C]ketoprofens (KPs) and their acylglucuronides has been reported for the nuclear magnetic resonance (NMR) spectroscopic studies on the stereoselective pharmacokinetics and reactivities of KP acylglucuronides. The racemic [1-^{13}C]KP was prepared by a three-step synthetic scheme from [^{13}C]potassium cyanide in overall yield of 23 %. The racemate was optically resolved by the formation of diastereomeric amides with (*R*)-(+)-α-phenylethylamine, separation of the amides by column chromatography on silica gel, and nonhydrolytic cleavage of the amide bond using nitrogen tetroxide. The yields of KP enantiomers were 30 % based on the racemate. The acylglucuronides of (*R*)- and (*S*)-[1-^{13}C]KP were isolated from human urine after dosing of each labelled KP (100 mg) using preparative high-performance liquid chromatography following Sep-Pak C_{18} pretreatments. The yields of the conjugates from 0-4 h post-dose urine were roughly 50 mg.

Key words: [^{13}C]ketoprofen, acylglucuronide, NMR, reactivity

INTRODUCTION

The 2-arylpropionic acids (2-APAs), an important group of nonsteroidal anti-inflammatory drugs (NSAIDs), possess a chiral center and exist as racemates. The 2-APAs exhibit stereoselective pharmacokinetics and also undergo a unidirectional bioinversion of the less active *R*-enantiomer to the active *S*-enantiomer (1). The 2-APAs are conjugated with glucuronic acid to form diastereomeric 1-*O*-acylglucuronides. This is the major metabolic pathway in several cases. Acylglucuronides of xenobiotic carboxylic acids are unstable and reactive. At physiological pH, they undergo hydrolysis, internal acyl migration and covalent binding to proteins (2). Therefore, the pharmacokinetics and reactivities of diastereomeric 2-APA acylglucuronides have become of interest because they are

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intimately related to the stereoselective disposition and pharmacological activities of 2-APAs (2).

To assess the pharmacokinetics and reactivities of 2-APA acylglucuronides, simple sample handling techniques are necessary which minimize the artificial chemical hydrolysis and acyl migration of acylglucuronides during analysis. The use of ^{13}C labeling and nuclear magnetic resonance (NMR) spectroscopy has found a broad application in metabolic investigations (3,4). We have recently demonstrated the usefulness of the approach in pharmacokinetic research (5-7). The application of the technique provides the ability to analyze biological fluids and reaction mixtures without extraction and chromatographic separations owing to the high specificity of detection. Therefore the technique is simple and convenient, and suitable for the analysis of labile compounds such as acylglucuronides. Ketoprofen (2-(3-benzoylphenyl)propionic acid, KP, see Chart 1) is an NSAID which is eliminated predominantly as diastereomeric acylglucuronides in humans. In this paper, we have synthesized enantiomeric [1-^{13}C]KPs and their diastereomeric acylglucuronides as probes to investigate the stereoselective pharmacokinetics and reactivities of the acylglucuronides.

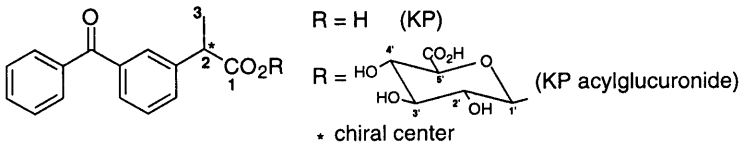


Chart 1.

EXPERIMENTAL

[^{13}C]Potassium cyanide (99.6 atom % ^{13}C) was purchased from Nippon Sanso (Tokyo Japan). KP, benzoyl peroxide and Wakogel C-300 (silica gel) were purchased from Wako Pure Chemical Industries (Osaka, Japan). (*R*)-(+)-α-phenylethylamine (optical purity, 96.4 %) and tetrabutylammonium bromide (TBA) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Water-soluble carbodiimide hydrochloride (WSCD-HCl), 1-hydroxybenzotriazole (HOBT) and trifluoroacetic acid (TFA) were purchased from Peptide Institute (Osaka Japan). Nitrogen tetroxide was purchased

from Takachiho Shoji (Tokyo, Japan). Dehydrated dimethylformamide, 3-methylbenzophenone and other reagents were purchased from Kanto Chemical (Tokyo, Japan). 3-Bromomethylbenzophenone **1** was synthesized from 3-methylbenzophenone according to the literature (8), except for the use of chloroform as the reaction solvent. Each synthetic step was investigated using unlabelled compounds and the structure of each product was confirmed by the ^{1}H NMR spectroscopy prior to the syntheses of labelled compounds.

^{1}H NMR and ^{1}H-decoupled ^{13}C ($^{13}C\{^{1}H\}$) NMR spectra of KP acylglucuronides were recorded in $^{2}H_2O$ on a Bruker (Tsukuba, Japan) AM 400 spectrometer, operating at 400 and 100 MHz, respectively, and chemical shifts were referenced to that of sodium 3-(trimethylsilyl)-[2,2,3,3-$^{2}H_4$]-propionate. ^{1}H NMR and $^{13}C\{^{1}H\}$ NMR spectra of other compounds were recorded in $CDCl_3$ on a Varian (Tokyo, Japan) GEMINI-300 spectrometer, operating at 300 and 75 MHz, respectively, and chemical shifts were referenced to those of the solvent ($\delta^{1}H$ 7.26, $\delta^{13}C$ 77.0). Mass spectra (MS) were recorded on a ThermoQuest (San Jose, CA, USA) TSQ 7000 spectrometer. Melting points were determined on a Yamato (Tokyo, Japan) MP-1 melting point apparatus and were uncorrected. Flash column chromatography was carried out on Wakogel C-300. Optical purities of labelled KPs were determined using the high-performance liquid chromatography (HPLC) system equipped with Waters (Milfold, MA, USA) M600E multisolvent delivery system, Waters U6K injector, Waters Lambda-Max Model 481 LC Spectrophotometer set at 254 nm, and CHIRALCEL OJ column (250 x 4.6 mm I.D., 10 μm, Daicel Chemical, Tokyo, Japan). The mobile phase was hexane-2-propanol-acetic acid (9/1/0.5, v/v/v) at a flow rate of 1 ml/min. Purification of KP acylglucuronides was performed using the HPLC system equipped with M600 multisolvent delivery system, Rheodyne (Cotati, CA, USA) 7125 injector, Shimadzu (Kyoto, Japan) SPD-6A UV Spectrophotomeric Detector set at 254 nm, and Inertsil PREP-ODS (250 x 30 mm I.D., 10 μm, GL Sciences, Tokyo, Japan). The mobile phase was acetonitrile-10 mM TFA aqueous solution (pH 2.0) (3/7, v/v) at a flow rate of 25 ml/min. Purities of KP acylglucuronides were determined using the HPLC system equipped with M600E multisolvent delivery system, U6K injector, Lambda-Max Model 481 UV detector set at 254 nm, and Inertsil ODS (250 x 4.6 mm I.D., 5 μm, GL Sciences). The mobile

phase was acetonitrile-10 mM TBA in 0.1 M citrate buffer adjusted to pH 4.3 (3/7, v/v) at a flow rate of 1 ml/min

(*RS*)-2-(3-Benzoylphenyl)-[1-^{13}C]-propionic acid ((*RS*)-[1-^{13}C]KP) (*RS*)-**4**.

3-Bromomethylbenzophenone **1** (9.38 g) dissolved in 120 ml of hot ethanol was added dropwise to a stirred solution of [^{13}C]potassium cyanide (2.00 g) in 75 ml of water and the mixture was heated under reflux for 1.5 h. The solvent was evaporated in vacuo. To the oily residue was added 200 ml of water followed by extraction with chloroform. The organic layer was washed with water, dried over sodium sulphate, and evaporated in vacuo. The oily residue was purified by flash column chromatography over 300 g of silica gel with hexane-ethyl acetate (5/1) as the eluent. The eluate was evaporated in vacuo to give 5.57 g (82 % based on [^{13}C]potassium cyanide) of 3-benzoylphenyl-[1-^{13}C]-ethanenitrile **2** as a colorless oil.

Potassium tert-butoxide (3.10 g) was added to a solution of **2** (5.57 g) in 200 ml of anhydrous benzene, and the mixture was stirred at room temperature for 30 min. To the suspension was added methyl iodide (7.16 g), followed by stirring at room temperature for an additional 2 h. To the reaction mixture was added 200 ml of water followed by extraction with benzene. The organic layer was washed with water, dried over sodium sulphate, and evaporated in vacuo. The oily residue was purified by flash column chromatography over 250 g of silica gel with hexane-ethyl acetate (5/1) as the eluent. The eluate was evaporated in vacuo to give 2.60 g (44 %) of (*RS*)-2-(3-benzoylphenyl)-[1-^{13}C]-propanenitrile **3** as a colorless oil.

Compound **3** (2.60 g) dissolved in 170 ml of 1.5 % potassium hydroxide aqueous solution was heated under reflux for 48 h. The reaction mixture was diluted with 300 ml of water, washed with diethyl ether, acidified to pH 3 with 1 N hydrochloric acid, and extracted with chloroform. The organic layer was washed with water, dried over sodium sulphate, and evaporated in vacuo. The oily residue was purified by flash column chromatography over 100 g of silica gel with chloroform-methanol (20/1) as the eluent. The eluate was evaporated in vacuo to give 1.74 g (62 %) of (*RS*)-[1-^{13}C]KP (*RS*)-**4** as a yellow oil. The oily product solidified on standing in a desiccator for several days. m.p. 95°C; ^{13}C{^{1}H} NMR: intense signal at δ^{13}C 179.80 (C1); ^{1}H NMR δ^{1}H 1.56 (3H, dd, $J_{H3,C1}$=5.1 Hz, $J_{H3,H2}$=7.2 Hz, H3), 3.83 (1H,

dq, $J_{H2,H3}$=7.2 Hz, $J_{H2,C1}$=7.9 Hz, H2), 7.4-7.8 (9H, aromatic protons); MS (electron impact) m/z 255(M^+, 82 %), 209(66, M-^{13}COOH), 178(83, M-phenyl), 105(100, benzoyl), 77(72, phenyl).

Optical resolution of (*RS*)-[1-^{13}C]KP (*RS*)-**4**.

Compound (*RS*)-**4** (1.63 g) was dissolved in 15 ml of dehydrated dimethylformamide, and then HOBT (1.30 g), WSCD-HCl (1.84 g), 4-dimethylaminopyridine (1.17 g) and (*R*)-(+)-α-phenylethylamine (1.2 ml) were added to the solution with magnetic stirring. After stirring at room temperature for 2 h, the solvent was removed in vacuo. The oily residue was dissolved in ethyl acetate, washed with 10 % citric acid aqueous aolution, water, 5 % sodium hydrogen carbonate aqueous solution, and water, dried over sodium sulphate, and evaporated in vacuo. The oily residue was subjected to flash column chromatography over 180 g of silica gel with hexane-ethyl acetate (4/1) as the eluent. The first eluate was evaporated in vacuo to give (*R*)-(+)-α-phenylethylamide of (*S*)-[1-^{13}C]KP **5** as a white solid (0.96 g, 42 %); ^{13}C{^{1}H} NMR: intense signal at δ^{13}_C 172.51 (C1); ^{1}H NMR δ^1_H 1.38 (3H, d, $J_{H2',H1'}$=6.9 Hz, H2'), 1.51 (3H, dd, $J_{H3,C1}$=4.6 Hz, $J_{H3,H2}$=7.1 Hz, H3), 3.59 (1H, dq, $J_{H2,C1}$=6.0 Hz, $J_{H2,H3}$=7.1 Hz, H2), 5.09 (1H, ddq, $J_{H1',C1}$=2.1 Hz, $J_{H1',H2'}$=6.9 Hz, $J_{H1',NH}$=7.9 Hz, H1'), 5.76 (1H, broad s, NH), 7.2-7.8 (14H, aromatic protons). The second eluate was evaporated in vacuo to give (*R*)-(+)-α-phenylethylamide of (*R*)-[1-^{13}C]KP **6** as a colorless oil (1.03 g, 45 %); ^{13}C{^{1}H} NMR: intense signal at δ^{13}_C 172.44 (C1); ^{1}H NMR δ^1_H 1.43 (3H, d, $J_{H2',H1'}$=6.9 Hz, H2'), 1.52 (3H, dd, $J_{H3,C1}$=4.7 Hz, $J_{H3,H2}$= 7.1 Hz, H3), 3.63 (1H, dq, $J_{H2,C1}$=6.6 Hz, $J_{H2,H3}$=7.1 Hz, H2), 5.08 (1H, ddq, $J_{H1',C1}$=2.1 Hz, $J_{H1',H2'}$=6.9 Hz, $J_{H1',NH}$=8.1 Hz, H1'), 5.76 (1H, broad s, NH), 7.1-7.8 (14H, aromatic protons).

Nitrogen tetroxide (ca. 1.2 g) in a bomb was liquefied by cooling in an ice bath and directly poured into 34 ml of carbon tetrachloride contained in a precooled beaker. The resulting green solution was transferred into a 200-ml two-necked round-bottomed flask, and anhydrous sodium acetate (5.83 g) was added to the solution. The flask was equipped with a pressure equalizing dropping funnnel and a thermometer, and cooled to -15°C in a mixture of salt and ice. A solution of **5** (0.94 g) in 20 ml of carbon tetrachloride was added dropwise to the suspension through the dropping

funnnel with magnetic stirring, followed by stirring for additional 2 h at -15°C. The reaction mixture was poured into a mixture (150 g) of water and crushed ice followed by addition of 5 % sodium hydrogen carbonate (130 ml), and then extracted with carbon tetrachloride. The organic layer was washed with water, dried over sodium sulphate, refluxed for 2 h, and evaporated in vacuo. The oily residue was purified by flash column chromatography over 130 g of silica gel with chloroform-methanol (50/1) as the eluent. The eluate was evaporated in vacuo to give (*S*)-[1-^{13}C]KP (*S*)-**4** as a colorless oil, which was solidified on standing in a desiccator for several days (0.48 g; 72 %; 99.5 atom % of ^{13}C); m.p. 69.5-71.0°C; $[\alpha]_D$ +52.1° (c=0.76, in dichloromethane); optical purity 92 %. Anal Calcd. for $C_{15}{}^{13}C_1H_{14}O_3$: C, 75.28; H, 5.53. Found: C, 75.07; H, 5.56.

Compound **6** (1.01 g) was treated in the same manner as above to give (*R*)-[1-^{13}C]KP (*R*)-**4** as a colorless oil, which was solidified on standing in a desiccator for several days (0.48 g; 67 %; 99.2 atom % of ^{13}C); m.p. 69.0-70.5°C; $[\alpha]_D$ -52.3° (c=0.88, in dichloromethane); optical purity 95 %. Anal Calcd. for $C_{15}{}^{13}C_1H_{14}O_3$: C, 75.28; H, 5.53. Found: C, 75.02; H, 5.62.

The ^{1}H and ^{13}C{^{1}H} NMR spectra and mass spectra of (*R*)-**4** and (*S*)-**4** were identical to those of (*RS*)-**4**.

Preparation of (*S*)- and (*R*)-[1-^{13}C]KP acylglucuronides **7**, **8**

A healthy male volunteer (72 kg weight) received a single oral dose of (*R*)- or (*S*)-[1-^{13}C]KP (each 100 mg) with 100 ml of water after overnight fast. Informed consent was obtained from the volunteer. Urine samples were collected at 0-2, 2-4, and 4-6 h postdose. Immediately after collection, the urine was acidified (pH 3-4) with 85 % phosphoric acid in order to stabilize the acylglucuronides, and then stored at -20°C until isolation of the acylglucuronides. The volume of urine collected for each period was in the range of 30-80 ml.

The urine (~10 ml) was applied to a Sep-Pak C_{18} cartridge which was washed with 3 ml each of methanol, acetonitrile and 10 mM TFA aqueous solution immediately before use. The cartridge was washed with 3 ml of 10 mM TFA aqueous solution and then eluted with 3 ml of acetonitrile-10 mM TFA aqueous solution (4/1, v/v). The eluate was evaporated by a rotary evaporator below 35°C. The oily residue

was dissolved in 220 μl of acetonitrile, filtered with DISMIC-3cp (0.45 μm, Advantec Toyo, Tokyo, Japan). The filtrate (30-80 μl per injection) was injected into the HPLC column, and the eluates (ca. 50 ml per injection) corresponding to the acylglucuronide were obtained. The retention times of (*S*)- and (*R*)-KP acylglucuronides were 19.4 and 20.6 min, respectively. The eluates were evaporated by a rotary evaporator below 35°C in order to eliminate most of the acetonitrile, and then the concentrated aqueous solution (pH 2-3) was freeze-dried to give (*S*)- **7** or (*R*)-[1-^{13}C]KP acylglucuronide **8** as a white foam. **7** : $^{13}C\{^1H\}$ NMR: intense signal at $\delta^{13}C$ 178.02 (C1); ^1H NMR δ^1H 1.54 (3H, dd, $J_{H3,C1}$=5.5 Hz, $J_{H3,H2}$=7.2 Hz, H3), 3.5-3.6 (3H, m, H2',3',4'), 3.86 (1H, d, $J_{H5',H4'}$=9.7 Hz, H5'), 4.07 (1H, dq, $J_{H2,H3}$=7.2 Hz, $J_{H2,C1}$=8.2 Hz, H2), 5.58 (1H, dd, $J_{H1',C1}$=3.1 Hz, $J_{H1',H2'}$=8.1 Hz, H1'), 7.6-7.8 (9H, aromatic protons); MS (electron spray ionization) m/z 432($M+H^+$), 454($M+Na^+$), 470($M+K^+$). **8**: $^{13}C\{^1H\}$ NMR: intense signal at $\delta^{13}C$ 178.15 (C1); ^1H NMR δ^1H 1.55 (3H, dd, $J_{H3,C1}$=5.4 Hz, $J_{H3,H2}$=7.2 Hz, H3), 3.4-3.6 (3H, m, H2',3',4'), 3.95 (1H, m, H5'), 4.07 (1H, dq, $J_{H2,H3}$=7.2 Hz, $J_{H2,C1}$=7.9 Hz, H2), 5.57 (1H, dd, $J_{H1',C1}$=3.1 Hz, $J_{H1',H2'}$=8.2 Hz, H1'), 7.6-7.8 (9H, aromatic protons); MS (electron spray ionization) m/z 432($M+H^+$), 454($M+Na^+$), 470($M+K^+$).

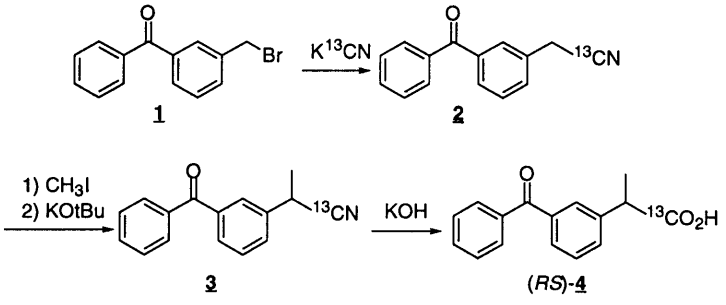
RESULTS AND DISCUSSION

In the preparation of ^{13}C-labelled 1-*O*-acylglucuronide of ketoprofen, special attention was paid to get sufficient spectral resolution in ^{13}C NMR spectroscopy. The ^{13}C labeling position largely affects the resolution (6). The 1-*O*-acylglucuronides of 2-APAs undergo internal acyl migration and the acyl (aglycone) moiety is transferred to the C2', C3' or C4' position of the glucuronic acid ring (2). The carboxyl carbon (C1) of KP is the reaction site and is most favorable for the separation of the positional isomers. Therefore, the C1 was chosen for the labeling position.

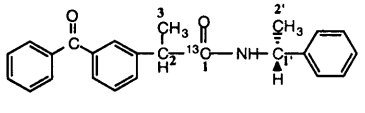
Isotopically labelled racemic KP (*RS*)-**4** was synthesized in an overall yield of 23 % using [^{13}C]potassium cyanide as the labeling source according to the route diagrammed in Scheme 1. The optical resolution of (*RS*)-**4** was performed according to the method described by Blazevic et al (9), modifying the procedures. The method consisted of preparation of the diastereomeric amides (**5**, **6**) with (*R*)-(+)-α-phenylethylamine, separation of the amides by column chromatography on silica gel,

and the subsequent nonhydrolytic cleavage of the amide bond using nitrogen tetroxide (10). Blazevic et al. have reported that only one spot of KP was observed on a thin-layer chromatogram after the cleavage reaction of the amide bond. In contrast, in our experiments another less polar spot other than the desired product was observed in both of the cleavage reactions for **5** and **6**. The less polar spot was speculated to consist of the diastereomeric α-phenylethyl esters of (*R*)- or (*S*)-KP as shown in Chart 2, based on the White's report (11) as well as the NMR and mass spectroscopic analyses (data are not shown). The yields of (*R*)- and (*S*)-KP (ca. 70 %) based on the amides were similar to those reported by Blazevic et al., and the yield of the byproducts was 30 % in both of the cleavage reactions for **5** and **6**.

Keglevic et al. (12) have developed a useful synthetic method for acylglucuronides, in which benzyl 2,3,4-tri-*O*-benzylglucopyranuronate is condensed with carboxylic acid to form glycosidic linkage followed by catalytic hydrogenation for deprotection. However, this method is unsuitable for the preparation of KP acylglucuronides because the benzoyl carbonyl moiety is reduced under the deprotection conditions (13). Thus the labelled KP acylglucuronides were prepared *in vivo* and isolated from urine using preparative HPLC. Although the diastereomeric acylglucuronides of 2-APAs can be separated by HPLC (2), the resolution becomes poor when large amounts of samples are injected into preparative columns. If



Scheme 1. Synthetic route of racemic [1-^{13}C]ketoprofen.



5, **6**

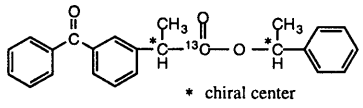


Chart 2.

comparable amounts of both diastereomers are contained in the sample after dosing of racemic KP (14), the HPLC peaks overlap to a much greater extent, which markedly decreases the isolation yields. Thus, each KP acylglucuronide diastereomer was isolated from the urine obtained after dosing of the corresponding KP enantiomer. The KP acylglucuronides were concentrated in the 0-2 h and 2-4 h post-dose urine in the subject, from which roughly 50 mg of KP acylglucuronides were obtained. The C1 resonances of the diastereomers were found to be resolved with each other ($\Delta\delta$=0.13 ppm). The lability of the KP acylglucuronides is now under investigation using the labelled compounds and ^{13}C NMR spectroscopy.

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