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STUDIES ON THE STEREOSELECTIVE INTERNAL ACYL MIGRATION OF KETOPROFEN GLUCURONIDES USING ^{13}C LABELING AND NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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ABSTRACT:

Internal acyl migration reactions of drug 1β-*O*-acyl glucuronides are of interest because of their possible role in covalent binding to serum proteins and consequent allergic reactions as well as their influence on drug disposition. An approach using ^{13}C labeling and nuclear magnetic resonance (NMR) spectroscopy has been used to investigate *in situ* the kinetics of acyl migration and hydrolysis of 1β-*O*-acyl glucuronides of enantiomeric ketoprofens (KPs) in phosphate buffer solutions at 37°C. Apparent first-order degradation of the 1β-*O*-acyl glucuronide labeled in the ester carbonyl carbon and the sequential appearance of 2-, 3-, and 4-*O*-acyl isomers as both α- and β-anomeric forms were observed for each enantiomer. All of the positional isomers and anomers were characterized using two-dimensional NMR spectroscopy (heteronuclear multiple bond correlation, correlated spectroscopy, totally correlated spectroscopy) of the reaction mixtures. The overall degradation rate constants (hr^{-1}) of (*R*)- and (*S*)-KP glucuronides were 1.07 ± 0.154 and 0.55 ± 0.034, respectively. To evaluate in detail the stereoselective reactivity, a kinetic model describing the rearrangement reactions was constructed, and the kinetics were simulated using a theoretical approach. Only the acyl migration, 1$\beta\rightarrow$2β, was found to have significant stereoselectivity. The rate constants (hr^{-1}) for 1$\beta\rightarrow$2β migration of (*R*)- and (*S*)-KP glucuronides were 1.04 ± 0.158 and 0.52 ± 0.029, respectively. The results may suggest that (*R*)-KP glucuronide could be more susceptible to covalent binding to proteins *via* acyl migration than the corresponding antipode. This stereoselective reactivity may be responsible for the stereoselective pharmacokinetics of KP. The direct approach using ^{13}C labeling and NMR spectroscopy could also provide insight into the reactivities of other labile drug acyl glucuronides and their isomeric glucuronides.

Conjugation with glucuronic acid to yield 1-*O*-acyl-β-D-glucopyranuronates (1β-*O*-acyl glucuronides) is a major metabolic route for many carboxylate drugs including nonsteroidal anti-inflammatory and hypolipidemic drugs (Dutton, 1980). It is well known that 1β-*O*-acyl glucuronides are labile and reactive. These compounds undergo both hydrolysis and internal acyl migration. In the acyl migration, the aglycone is transferred to the C-2, C-3, or C-4 position of the glucuronic acid ring (Spahn-Langguth and Benet, 1992) (fig. 1). In general, acyl migration has been observed to predominate over hydrolysis under physiological conditions (Akira *et al.*, 1997a; Blanckaert *et al.*, 1978; Bradow *et al.*, 1989; Hansen-Moller *et al.*, 1988; Iwakawa *et al.*, 1988; Iwaki *et al.*, 1995; Nicholls *et al.*, 1996; Sidelmann *et al.*, 1996a). Furthermore, the 1β-*O*-acyl glucuronides of many carboxylate drugs have been shown to react with proteins to form covalent adducts (Dickinson and King, 1991; Dubois *et al.*, 1993; Kretz-Rommel and Boelsterli, 1994; Presle *et al.*, 1996; Volland *et al.*, 1991). Covalent binding seems to occur mainly *via* the formation of a Schiff's base linkage between the free aldehyde of the open-chain acyl-migrated glucuronide and a nucleophilic amine group on the protein, in the drug acyl glucuronides that readily undergo acyl migration (Dickinson and King, 1991; Ding *et al.*, 1995; Kretz-Rommel and Boelsterli, 1994; Smith *et al.*, 1990). The isomeric glucuronides differ from one another in reactivity for protein adduct formation (Dickinson and King, 1991). Currently, there is speculation that protein adducts are at least partially responsible for immunological side effects of carboxylate drugs (Spahn-Langguth and Benet, 1992; Worral and Dickinson, 1995; Zia-Amirhosseini *et al.*, 1995).

1β-*O*-Acyl glucuronides are usually excreted in the bile and then hydrolyzed by β-D-glucuronidase in the small intestinal tract, where the unconjugated drugs are reabsorbed into the systemic circulation (enterohepatic circulation). In addition, 1β-*O*-acyl glucuronides are hydrolyzed by tissue and serum esterases. The aglycones formed by these enzymes are then again available for other biotransformations as well as glucuronide conjugation. The isomeric glucuronides formed by acyl migration have often been described as being resistant to β-D-glucuronidase (Blanckaert *et al.*, 1978). Dickinson *et al.* (1986) have suggested that the isomeric glucuronides of valproic acid are resistant to serum esterase. Therefore, the amount of drug regenerated by these enzymes can be decreased if the drug 1β-*O*-acyl glucuronides can undergo rapid acyl migration. Thus, acyl migration is intimately related to drug disposition.

The lability of 1β-*O*-acyl glucuronides themselves has been suc-

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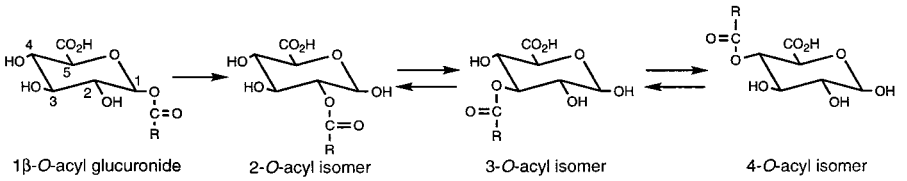

FIG. 1. *Reaction scheme for the acyl migration of 1β-O-acyl glucuronides.*

cessfully investigated by reversed phase high performance liquid chromatography (HPLC)[1] using acidic mobile phase (Spahn-Langguth and Benet, 1992), where the 1β-*O*-acyl glucuronides are relatively stable and the 1β-*O*-acyl glucuronides and the aglycones are easily distinguished from the isomeric glucuronides. However, details of the formation and degradation of each isomer following the degradation of 1β-*O*-acyl glucuronides have been ambiguous because HPLC separation of the various isomers to one another is much more difficult. Moreover, the isomers can ring-open and mutarotate giving α- and β-anomers, which further complicates the HPLC separation. Thus, a more direct and specific method is required to assess the overall reactivities of 1β-*O*-acyl glucuronides, including the formation and degradation of the isomers.

Nuclear magnetic resonance (NMR) spectroscopy is suitable for the determination of such unstable compounds because it allows reaction mixtures to be analyzed without extraction and chromatographic separations. NMR spectroscopy also allows a "real-time" analysis of the reactions that proceed in the NMR tube under defined physicochemical conditions. Bradow *et al.* (1989) have examined the reactivity of 1β-*O*-acyl glucuronide by ^{1}H NMR spectroscopy. However, spectral resolution was not sufficient because of the spectral complexity owing to the formation of many isomers, the small chemical shift range, and the disturbance by residual water. Although ^{19}F NMR spectroscopy has been effectively used to monitor the acyl migration of 1β-*O*-acyl glucuronides of 2-, 3-, and 4-(trifluoromethyl)benzoic acids (Nicholls *et al.*, 1996), it is not a general method as it can not be applied to non-fluorinated compounds. ^{13}C NMR has a chemical shift range that is large and comparable with that of ^{19}F NMR, but in addition the range of drugs that can be studied is virtually unlimited. A major disadvantage is that the sensitivity of ^{13}C NMR is poor because of the low natural abundance (1%) of ^{13}C and the low gyromagnetic ratio. However, ^{13}C NMR can be effectively used together with ^{13}C labeling (approximately 100% enrichment) (London, 1988). We have demonstrated that the NMR approach with ^{13}C labeling is useful in pharmacokinetic research in terms of sensitivity and specificity (Akira and Shinohara, 1996; Akira *et al.*, 1993; Baba *et al.*, 1995). The reactivity of benzoyl glucuronide has been recently elucidated by this ^{13}C NMR approach (Akira *et al.*, 1997a).

Ketoprofen (KP, see fig. 2) is one of the chiral 2-arylpropionic acids (profens), an important group of nonsteroidal anti-inflammatory drugs (NSAIDs), which is clinically used as the racemate and eliminated predominantly as diastereomeric acyl glucuronides in humans (Foster *et al.*, 1988a). Upton *et al.* (1980) were the first to describe the susceptibility of (*RS*)-KP acyl glucuronides to chemical hydrolysis. Subsequently, the hydrolysis half-lives of (*R*)- and (*S*)-KP acyl glucuronides were reported to be greater than 24 hr under physiological conditions (Hayball *et al.*, 1992). However, susceptibility to acyl migration was not investigated in these studies because the glucuronides were not directly analyzed using HPLC. Thus, in the present study, we have investigated in detail the stereoselective reactivity of (*R*)- and (*S*)-KP acyl glucuronides under physiological conditions using the ^{13}C labeling in the ester carbonyl group of the glucuronides and NMR.

[1] Abbreviations used are: HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; KP, ketoprofen; HMBC, heteronuclear multiple bond correlation; COSY, correlated spectroscopy; TOCSY, totally correlated spectroscopy; profens, 2-arylpropionic acids; NSAIDs, nonsteroidal anti-inflammatory drugs; FIDs, free induction decays.

Materials and Methods

Chemicals. (*R*)- and (*S*)-KP acyl glucuronides ^{13}C-labeled in the ester carbonyl carbon [1-*O*-((*R*)-2-(3-benzoylphenyl)-[1-^{13}C]propyl)-β-D-glucuronic acid and 1-*O*-((*S*)-2-(3-benzoylphenyl)-[1-^{13}C]propyl)-β-D-glucuronic acid] were prepared according to the previously reported method (Akira *et al.*, 1997b). N^2H_3 ^2H_2O solution (16 M, 99.0 atom % ^{2}H) was purchased from Aldrich (Milwaukee, WI). Other reagents including ^2H_2O (>99.80 atom % ^{2}H) and 40% NaO^2H ^2H_2O solution (99.0 atom % ^{2}H) were purchased from Kanto Chemical (Tokyo, Japan).

NMR Spectroscopic Monitoring of Degradation. (*R*)-[^{13}C]KP acyl glucuronide (2 mg), (*S*)-[^{13}C]KP acyl glucuronide (2 mg), or a mixture of both labeled diastereomers (each 1 mg) was dissolved in 475 μl of 0.1 M phosphate buffer (pH 7.4). After adding of 25 μl of ^2H_2O to provide a ^{2}H signal for field frequency lock, each solution was transferred to a 5-mm NMR tube with a coaxial capillary tube (1.7-mm o.d., 1.0-mm i.d.) containing 1,4-dioxane as the reference for chemical shifts and quantitation. After the pH of the solution was measured (7.20 ± 0.05), the NMR tube was immediately inserted in the NMR probe set at 310 K, and the reaction was monitored by ^{13}C NMR spectroscopy

^{13}C NMR spectra were obtained using a Bruker AM400 spectrometer at 100 MHz under the ^{1}H-decoupling conditions without sample spinning. An acquisition time of 0.655 sec with 75° pulses and a total pulse recycle time of 2.7 sec were used. Prior to Fourier transformation, an exponential line-broadening of 1.0 Hz was applied to the free induction decays (FIDs), which were also zero-filled to 65536. Chemical shifts were referenced to 1,4-dioxane (δ^{13}C 70). Acquisitions of FIDs were commenced within 20 min after dissolution of the glucuronides. FIDs (216, 10-min accumulation) for (*R*)- and (*S*)-KP acyl glucuronides and FIDs (432, 20-min accumulation) for the mixture of both glucuronides were collected into 32768 computer data points with a spectral width of 25,000 Hz at appropriate intervals over a 12 to 16-hr time period.

Quantitation Method. ^{13}C Resonance heights of [^{13}C]KP acyl glucuronide (ester carbonyl), its isomers (ester carbonyl), and aglycone (carboxyl) in the reaction mixture were measured, and the height ratios of these signals relative to that of dioxane (internal standard) were calculated. These ratios were assumed to reflect the relative proportions of the various compounds contained in the reaction mixture (see text). The ratios were converted to micromoles, assuming that the sum of the ratios at each time point corresponds to the amount of [^{13}C]KP acyl glucuronide initially dissolved (4.6 μmol).

Identification of the Isomeric Glucuronides by Two-Dimensional NMR Spectroscopy. (*R*)- or (*S*)-[^{13}C]KP acyl glucuronide (3 mg) dissolved in 500 μl of ^2H_2O was transferred into a 5-mm NMR tube. To the solution was added 5 μl of 3.2 M N^2H_3 ^2H_2O solution to decompose the [^{13}C]KP acyl glucuronide to its isomers and aglycone. The reaction was followed by ^{13}C NMR spectroscopy. When the relative amounts of the isomers had stabilized, the sample was freeze-dried to remove the alkali and stored at −20°C until analyzed. The residue was reconstituted in 450 μl of ^2H_2O, and then the resultant solution was transferred to a 5-mm NMR tube after filtration, followed by two-dimensional NMR spectroscopy (HMBC, COSY, TOCSY) using a Bruker AM500 spectrometer, operated at 500 MHz. The internal H^2HO signal was used as a lock reference, and shift assignments were made relative to it (δ^{1}H 4.78). The composition of the reaction mixture was almost constant during the time-consuming two-dimensional NMR measurements.

HMBC experiments used 8 scans per increment for 400 increments with a

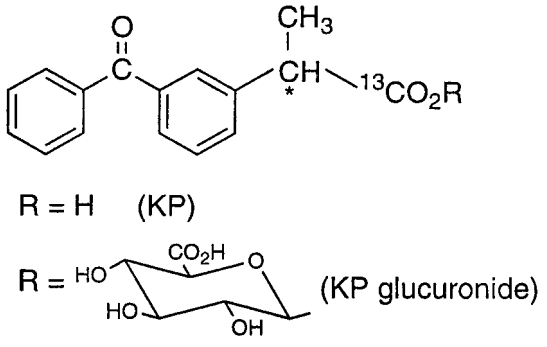

FIG. 2. *Structures of ^{13}C-labeled KP and KP glucuronide.*

The *asterisk* indicates an assymetric center.

spectral width of 3759 Hz in F_2 and 4464 Hz in F_1 and data points of 2048 in F_2 and 1024 in F_1, resulting in a total acquisition time of about 2 hr. COSY experiments used 8 scans per increment for 256 increments with a spectral width of 4000 Hz in F_2 and 2000 Hz in F_1 and data points of 1024 in F_2 and 512 in F_1, resulting in a total acquisition time of about 1 hr. TOCSY experiments used 16 scans per increment for 460 increments with a spectral width of 5000 Hz in F_2 and 2500 Hz in F_1 and data points of 2048 in F_2 and 1024 in F_1, resulting in a total acquisition time of about 4 hr. The mixing time was 80 msec.

Kinetic Analyses. The differential equations fitted to the model (see fig. 8) describing the degradation kinetics of KP glucuronides were constructed, assuming that the acyl migration, hydrolysis, and anomerization follow first-order kinetics (see *Appendix*). The equations were solved by a kinetic simulation program, which uses the Runge-Kutta method as an algorithm and the steepest descent method for the optimization.

Results

The lability of KP glucuronides in phosphate buffer (pH 7.4) at 37°C was directly examined in the NMR tube. With time, the intensity of ^{13}C signals due to the 1β-*O*-acyl glucuronides decreased with concurrent and sequential appearance of several signals at other chemical shifts as shown in figs. 3 and 4. The signal at δ185.8, which was well separated from other resonances, was assigned to the aglycone formed by hydrolysis by comparison of the chemical shift with that of the authentic [^{13}C]KP. Other signals were obviously because of the isomeric glucuronides (2-*O*-acyl, 3-*O*-acyl, and 4-*O*-acyl), as they completely disappeared and the signal due to the aglycone increased by addition of alkaline to the sample (not shown). The signals of the positional isomers were tentatively assigned based on the order in which they were formed in the incubation mixture, assuming that the 2-*O*-acyl isomer is necessarily formed before the 3-*O*-acyl isomer *etc*. This successive acyl migration between the neighboring hydroxyl groups has been demonstrated in numerous drugs (Blanckaert *et al.*, 1978; Bradow *et al.*, 1989; Hansen-Moller *et al.*, 1988; Nicholls *et al.*, 1996; Sidelmann *et al.*, 1996b). Splitting of the signals for the isomeric glucuronides, except for (*R*)-4-*O*-acyl isomer, was observed because of formation of both α- and β-anomers (approximately 1:1 proportion) by mutarotation after acyl migration (Sidelmann *et al.*, 1996a, 1996c). The (*R*)-4-*O*-acyl isomer appeared as a single peak in all the experiments.

The assignments of the acyl ^{13}C signals due to each isomeric glucuronide was confirmed by measurements of the two-dimensional NMR spectra of the reaction mixture. The reaction mixture containing all the isomeric glucuronides was obtained by addition of N^2H_3 to the solution of [^{13}C]KP glucuronide followed by freeze-drying and reconstitution in ^2H_2O. First, the acyl ^{13}C signals were assigned to the signals owing to the protons on the acylated carbons based on the HMBC spectrum (fig. 5). Although the signals owing to the protons

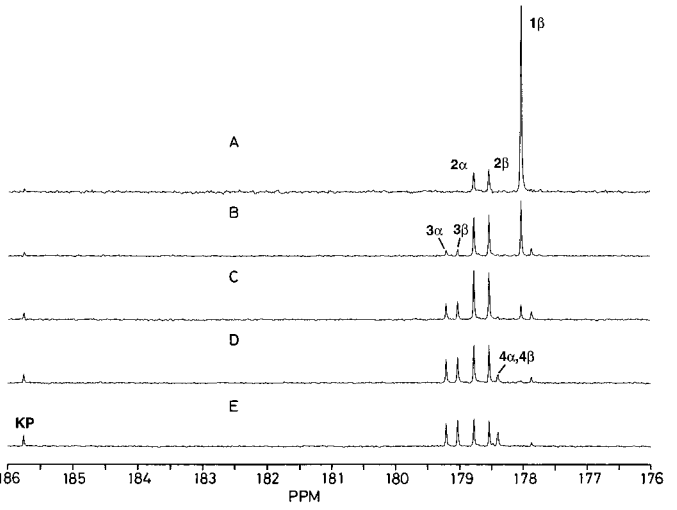

FIG. 3. *^{13}C NMR spectra as a function of time, showing degradation of (R)-[^{13}C]KP glucuronide in phosphate buffer (pH 7.4) at 37°C.*

All spectra were plotted out at the fixed resonance height of the internal standard. The spectra correspond to the following times: *A*, 15–25 min; *B*, 60–70 min; *C*, 135–145 min; *D*, 270–280 min; *E*, 450–460 min.

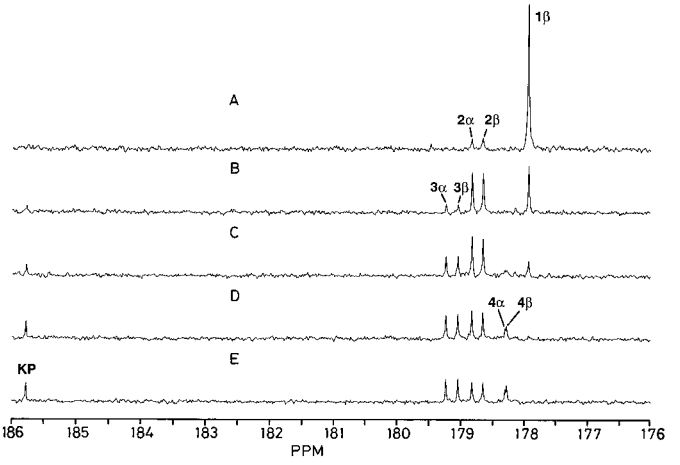

FIG. 4. *^{13}C NMR spectra as a function of time, showing degradation of (S)-[^{13}C]KP glucuronide in phosphate buffer (pH 7.4) at 37°C.*

All spectra were plotted out at the fixed resonance height of the internal standard. The spectra correspond to the following times: *A*, 15–25 min; *B*, 120–130 min; *C*, 240–250 min; *D*, 450–460 min; *E*, 860–870 min.

on the acylated carbons of (*R*)- and (*S*)-2-*O*-acyl isomers were obscured because of the H^2HO resonance, the chemical shifts were determined based on the cross peaks. Subsequently, the ^{1}H-^{1}H COSY and TOCSY spectra of the reaction mixture were measured. The signals owing to protons around the glucuronide ring for each isomeric glucuronide were assigned based on chemical shifts, spin-spin coupling constants, and integrals (Kaspersen and van Boeckel, 1987) and on the connectivity information from the COSY and TOCSY experiments (table 1). The protons on the acylated carbons were thus assigned to the individual isomeric glucuronides. From these experimental results, the acyl ^{13}C signals were assigned to the individual isomeric glucuronides. Consequently, the initial tentative identification based on the order of isomer appearance proved to be correct. Therefore, successive acyl migration from the 1 position to the 4 position for (*R*)- and (*S*)-KP glucuronides can be considered to be established. Also, the splitting of the ^{13}C signals was confirmed as a

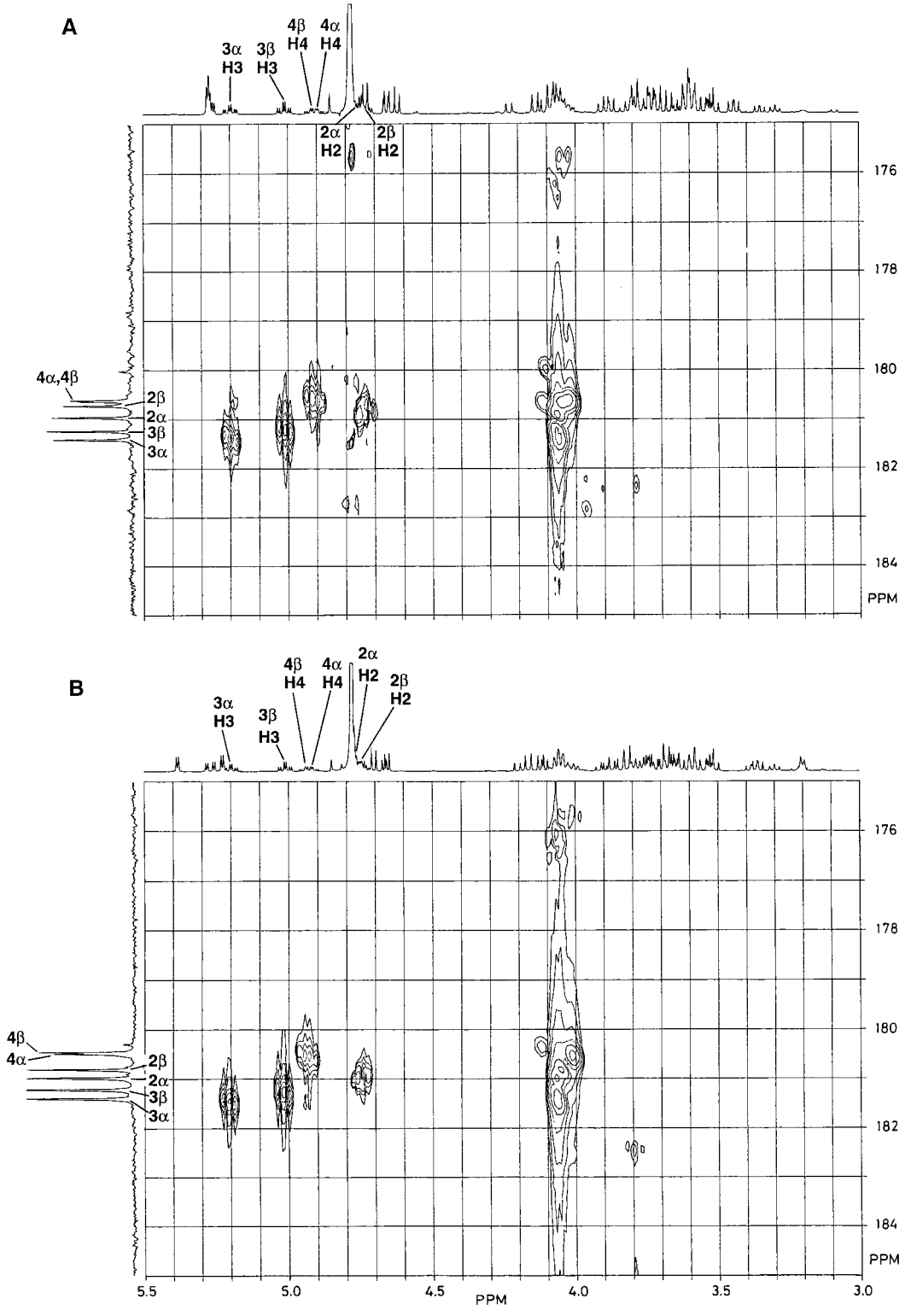

FIG. 5. *HMBC spectra of the equilibrium mixtures of the isomeric glucuronides formed from (R)-[^{13}C]KP glucuronide (A) and (S)-[^{13}C]KP glucuronide (B).*

result of anomerization. The ^{13}C signals for the (*R*)-4α-*O*-acyl and (*R*)-4β-*O*-acyl isomers were found to be spectrally coincident.

The above experimental results demonstrated that almost all the isomeric glucuronides including α- and β-anomers can be discriminated by the combined use of ^{13}C NMR and ^{13}C labeling of the ester carbonyl carbon. Fig. 6 shows ^{13}C NMR spectra of a mixture of (*R*)- and (*S*)-KP glucuronides dissolved in phosphate buffer (pH 7.4). The signals owing to KP glucuronides and their isomers formed by acyl migration were mostly discriminated from one another, although 3β- and 3α-*O*-acyl isomers were not discriminated between their *R*- and *S*-antipodes. These results show that the reactivity of diastereomeric glucuronides can be compared under identical physicochemical conditions using ^{13}C labeling and NMR. It would be impossible to obtain such a high specificity of detection by HPLC.

TABLE 1

^1H NMR chemical shifts of the isomers of (R)- and (S)-[^{13}C]KP glucuronides obtained by two-dimensional NMR analysis of the reaction mixtures in ^2H_2O

Isomer	Glucuronide Ring ^{1}H NMR Signals (δ)									
	(*R*)-[^{13}C]KP Glucuronide					(*S*)-[^{13}C]KP Glucuronide				
	H1	H2	H3	H4	H5	H1	H2	H3	H4	H5
4α	5.28	3.65	3.82	4.90	4.23	5.28	3.68[a]	3.91	4.92	4.20
4β	4.66	3.35	3.62	4.92	3.88	4.67	3.38	3.71	4.94	3.84
3α	5.27	3.73	5.20	3.58	4.14	5.23	3.65[a]	5.20	3.67	4.17
3β	4.73	3.44	5.01	3.60[a]	3.79	4.71	3.36	5.01	3.69	3.82
2α	5.28	4.77	3.90	3.60[a]	4.09	5.39	4.75[a]	3.88	3.58	4.13
2β	4.62	4.74	3.68	3.60[a]	3.71	—[b]	4.74[a]	—[c]	—[c]	—[c]

[a] Although the coupling pattern is obscured because of the complexity of the spectra, the chemical shift is determined based on the correlation signals.
[b] The signal is presumed to be concealed behind the H^2HO signal.
[c] The signal cannot be assigned because of the crowded spectrum.

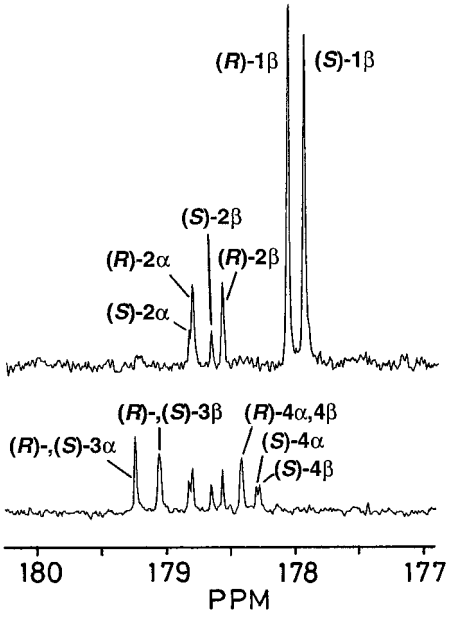

FIG. 6. *^{13}C NMR spectra of an equimolar mixture of (R)- and (S)-[^{13}C]KP glucuronides in phosphate buffer (pH 7.4) at 37°C.*

The spectra correspond to the following times: *upper trace,* 20–40 min; *lower trace,* 420-440 min. The signal due to KP appeared at δ185.8 as shown in figs. 3 and 4.

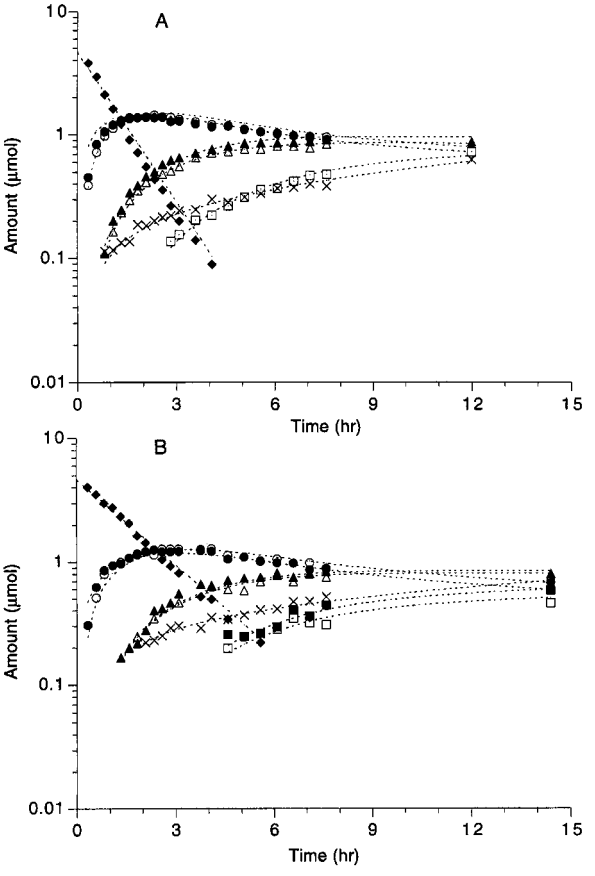

FIG. 7. *An example of profiles for acyl migration, hydrolysis, and mutarotation reactions of (R)-[^{13}C]KP glucuronide (A) and (S)-[^{13}C]KP glucuronide (B) in phosphate buffer (pH 7.4) at 37°C.*

As the individual NMR spectra were accumulated over 10 min, the midpoint was used as the time data point. *A*: ♦, 1β; ●, 2β; ○, 2α; ▲, 3β; △, 3α; ⊡, 4β and 4α; ×, KP. *B*: ♦, 1β; ●, 2β; ○, 2α; ▲, 3β; △, 3α; ■, 4β; □, 4α; ×, KP. The simulated curves obtained from the calculated reaction rate constants are represented by the *dotted lines.*

The amounts of KP glucuronide, its isomers, and aglycone present in the NMR tube were calculated using the resonance-height ratios of these nuclei *vs.* the internal standard (dioxane δ^{13}C 70). Because the monitored nuclei were all quaternary carbons, NMR characteristics (nuclear Overhauser enhancements and spin-lattice relaxation times) and sensitivity were considered to be very similar. In support of this assumption, the sum of the ratios at each time point was almost constant (coefficient of variation 4%). Therefore, the ^{13}C NMR sensitivity of KP glucuronide, its isomers, and aglycone can be regarded as virtually the same and the ratios directly compared to one another. The ratios were converted to micromoles as described in *Methods*. The time-course of the acyl migration and hydrolysis of each KP glucuronide is shown in fig. 7. (*R*)- and (*S*)-KP glucuronides showed pseudo first-order degradation kinetics and apparently disappeared at 4.0 and 5.6 hr after dissolution, respectively. These results indicate that acyl migration from the 1 position to the 2 position of both glucuronides is irreversible, which is consistent with the notion that acyl migration of the 2β-*O*-acyl isomer to the 1β-*O*-acyl isomer is thermodynamically unfavorable. In contrast, the acyl migration reactions between 2-*O*-acyl and 3-*O*-acyl isomers, and 3-*O*-acyl and 4-*O*-acyl isomers are probably reversible. These observations are consistent with those of other workers (Blanckaert *et al.*, 1978; Bradow *et al.*, 1989; Nicholls *et al.*, 1996) except for the study of diflunisal glucuronide isomers assayed with HPLC (Hansen-Moller *et al.*, 1988). Acyl migration of KP glucuronide to the 2β-*O*-acyl isomer was found to be a major pathway of transformation for the degradation, whereas the competing reaction of hydrolysis to KP was only minor under the conditions examined.

The disappearance of (*R*)-KP glucuronide ($t_{1/2}$ = 0.66 ± 0.092 hr) was found to be much faster than that of (*S*)-KP glucuronide

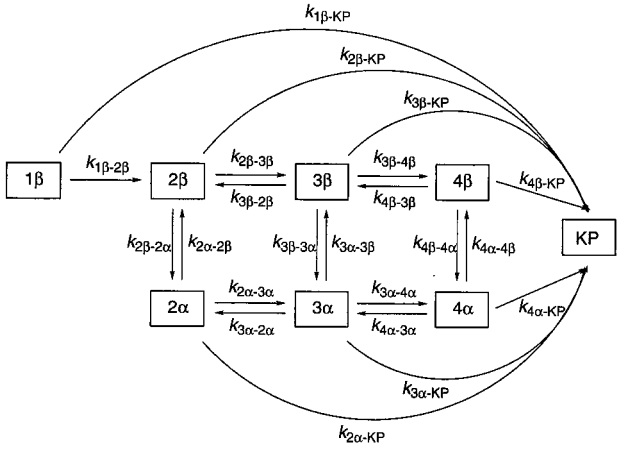

FIG. 8. *Kinetic model for the acyl migration reactions of KP glucuronides.*

In the case of (*R*)-KP glucuronide, the 4α-*O*-acyl and 4β-*O*-acyl isomers are not discriminated because of spectral coincidence, and thus the model is simplified.

($t_{1/2}$ = 1.26 ± 0.074 hr). To evaluate the stereoselective reactivity of (*R*)- and (*S*)-KP glucuronides in detail, the individual rates of acyl migration, hydrolysis, and anomerization were calculated based on the kinetic model shown in fig. 8 using a kinetic simulation program. The model assumes that the acyl migration reactions are reversible except for the initial acyl migration (1β→2β) and occur between the neighboring glucuronic acid hydroxyl groups *via ortho*-acid ester intermediates. In the case of (*R*)-KP glucuronide, the 4α-*O*-acyl and 4β-*O*-acyl isomers were not discriminated because of spectral coincidence. Sidelmann *et al.* (1996b) have constructed a kinetic model describing the degradation kinetics of 1β-*O*-acyl glucuronide of a model drug assuming that no hydrolysis of the isomeric glucuronides occurs. However, in our case, the concentrations of aglycone significantly increased after KP glucuronide had disappeared, implying that the isomeric glucuronides were susceptible to hydrolysis (Volland *et al.*, 1991). Thus, the above kinetic model includes the hydrolysis pathways of isomeric glucuronides. Differential equations fitted to the model were constructed and solved as described in *Methods*. The calculated rate constants for acyl migration, hydrolysis, and anomerization reactions are presented in table 2. The simulated curves obtained from those calculated rate constants are shown as *dotted lines* in fig. 7.

The rate constant for acyl migration of 1β→2β was much larger than that for hydrolysis of 1β→KP in all KP glucuronides. The rate constant of acyl migration 1β→2β of (*R*)-KP glucuronide was two times larger than that of (*S*)-KP glucuronide, whereas the hydrolysis 1β→KP was not stereoselective. Thus, stereoselectivity in the disappearance of KP glucuronide is because of 1β→2β acyl migration. Acyl migration of all the isomeric glucuronides showed relatively similar rate constants, which were smaller than those for the initial 1β→2β acyl migration. The rate constants for hydrolysis were one order of magnitude smaller than those for acyl migration, although 1β→KP hydrolysis was significantly faster than the hydrolysis of other isomeric glucuronides. Anomerization of all the isomeric glucuronides showed similar rate constants for reaction in either direction, α→β or β→α, and were much larger than those for acyl migration and hydrolysis. These results are consistent with the appearance of twin resonances due to α- and β-anomers on the spectra shown in figs. 3 and 4 and indicated that only 1β→2β acyl migration has significant stereoselectivity.

Discussion

The resolution of ^{13}C-labeled compounds by NMR spectroscopy largely depends on the labeled position. In our previous paper (Akira and Shinohara, 1996), the lability of KP glucuronides was investigated using a diastereomeric mixture of [methyl-^{13}C]KP glucuronides and ^{13}C NMR spectroscopy with the aid of methyl-β-cyclodextrin as a shift reagent. The results of this study suggested that the glucuronides were susceptible to acyl migration as well as hydrolysis under physiological conditions, although NMR spectral resolution was poor. Recently, the individual glucuronides of (*R*)- and (*S*)-[carboxyl-^{13}C]KP were prepared in our laboratory (Akira *et al.*, 1997b) to improve spectral resolution between the glucuronide, aglycone, and isomeric glucuronides. In this paper, we have investigated the difference in reactivity between the diastereomers and the reaction kinetics of acyl migration and hydrolysis using these labeled compounds and ^{13}C NMR spectroscopy.

Acyl glucuronides can be considered as reactive metabolites as they can irreversibly bind to endogenous proteins. Stereoselective covalent binding with plasma protein has been established *in vitro* for KP glucuronides (Presle *et al.*, 1996), although covalent binding of KP glucuronides to proteins *in vivo* has not been reported. In contrast, drug-protein adducts have been observed *in vivo* with fenoprofen (Volland *et al.*, 1991). Our results have shown that (*R*)-KP glucuronide is more susceptible to the covalent binding to proteins *via* acyl migration than its corresponding antipode. In healthy humans, KP is extensively metabolized to diastereomeric KP glucuronides and readily excreted in the urine (*ca.* 70% of dose in 24 hr). The diaste-

TABLE 2

Rate constants (hr^{-1}) for acyl migration, hydrolysis, and mutarotation of (R)- and (S)-KP glucuronides and their positional isomers calculated based on the kinetic model shown in fig. 8[a]

Acyl Migration			Hydrolysis			Mutarotation		
	R	*S*		*R*	*S*		*R*	*S*
1β→2β	1.04 ± 0.158	0.52 ± 0.029	1β→KP	0.03 ± 0.005	0.04 ± 0.005	2β→2α	3.53 ± 0.462	4.50 ± 0.866
2β→3β	0.25 ± 0.040	0.29 ± 0.059	2β→KP	0.01 ± 0.002	0.01 ± 0.001	2α→2β	3.17 ± 0.416	3.93 ± 0.723
2α→3α	0.24 ± 0.025	0.14 ± 0.069	2α→KP	0.01 ± 0.005	0.01 ± 0.003	3β→3α	2.83 ± 0.289	3.97 ± 0.666
3β→2β	0.18 ± 0.021	0.17 ± 0.047	3β→KP	0.01 ± 0.001	0.01 ± 0.004	3α→3β	3.07 ± 0.252	4.27 ± 0.839
3α→2α	0.19 ± 0.023	0.11 ± 0.058	3α→KP	0.01 ± 0.001	0.01 ± 0.001	4β→4α	—	3.47 ± 0.681
3β→4β	—	0.14 ± 0.021	4β→KP	—	0.01 ± 0.002	4α→4β	—	3.90 ± 0.608
3α→4α	—	0.21 ± 0.006	4α→KP	—	0.01 ± 0.001			
4β→3β	—	0.21 ± 0.029	4αβ→KP	0.02 ± 0.013	—			
4α→3α	—	0.29 ± 0.035						
3β→4αβ	0.11 ± 0.023	—						
3α→4αβ	0.11 ± 0.012	—						
4αβ→3β	0.11 ± 0.012	—						
4αβ→3α	0.10 ± 0.000	—						

[a] Values are means ± standard deviations of triplicate experiments.

reomeric ratio (*S*/*R*) of urinary KP glucuronides is *ca.* 1.2 (Foster *et al.*, 1988a), which has been suggested to be because of a limited bioinversion of the *R*-enantiomer to the *S*-enantiomer (Jamali *et al.*, 1990). Glucuronidation has been found to be stereoselective for several profens (Hamdoune *et al.*, 1995; Mouelhi *et al.*, 1987; Spahn, 1988). However, the formation ratio of the (*R*)- and (*S*)-KP glucuronides was almost 1 in human liver microsomes (Chakir *et al.*, 1994). Whereas only negligible concentrations of KP glucuronides were present in the plasma of healthy humans after oral administration of racemic KP (Foster *et al.*, 1988b), significant concentrations of KP glucuronides were detected in the plasma of patients with impaired renal function (Foster *et al.*, 1988b; Grubb *et al.*, 1996). In addition, the area under the plasma concentration *vs*. time curve for (*S*)-KP glucuronide was much higher than that for (*R*)-KP glucuronide in the patients. The reason for the stereoselectivity in these patients may be that (*R*)-KP glucuronide is more labile than (*S*)-KP glucuronide, which has been described in this paper. Dubois-Presle *et al.* (1995) have reported the presence of a stereoselective esterase activity toward the (*R*)-KP glucuronide in human serum albumin. Stereoselective hydrolysis could provide an alternative explanation for the lower plasma concentration of (*R*)-KP glucuronide.

The lability of acyl glucuronides of profens including fenoprofen (Volland *et al.*, 1991), naproxen (Iwaki *et al.*, 1995), benoxaprofen (Bradow *et al.*, 1989; Spahn *et al.*, 1989), carprofen (Iwakawa *et al.*, 1988), and flunoxaprofen (Spahn, 1988) has been investigated under physiological conditions (pH 7.4, 37°C) by other workers using HPLC. In all the profen glucuronides, including the KP glucuronides investigated here, the degradation rate constant of the (*R*)-conjugate was 1.5 to 2 times larger than that of its (*S*)-antipode. The glucuronides of carprofen, benoxaprofen, naproxen, and KP underwent predominant acyl migration but only minor hydrolysis similar to most other drug acyl glucuronides (Akira *et al.*, 1997a; Blanckaert *et al.*, 1978; Bradow *et al.*, 1989; Hansen-Moller *et al.*, 1988; Iwakawa *et al.*, 1988; Iwaki *et al.*, 1995; Nicholls *et al.*, 1996; Sidelmann *et al.*, 1996a). (*R*)-Fenoprofen glucuronide undergoes both reactions equally, whereas (*S*)-fenoprofen glucuronide is predominantly subject to hydrolysis. Therefore, (*R*)-profen glucuronide seems to be much more susceptible to acyl migration than the corresponding (*S*)-profen glucuronide.

The present paper has elucidated the stereoselective reactivity of diastereomeric KP glucuronides. Using ^{13}C labeling of the ester carbonyl carbon and NMR, the *R*-conjugate was found to be more susceptible to $1\beta \rightarrow 2\beta$ acyl migration than its *S*-antipode under physiological conditions. This ^{13}C NMR approach is a highly appropriate method to follow the overall reactions of acyl glucuronides because of the high specificity of detection and the lack of a requirement for pretreatment. Although the technique requires the synthesis of ^{13}C-labeled drugs, labeling the carboxyl carbon of carboxylate drugs, such as acidic NSAIDs, is relatively easy (Akira *et al.*, 1997b), and the labeled precursors for such compounds are inexpensive and available. The direct approach of using ^{13}C labeling and NMR as presented in this paper could also provide insight into the reactivities of other labile drug acyl glucuronides and their isomeric glucuronides.

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Appendix

Differential equations for the kinetic model [eqs. 1–7 for (*R*)-[^{13}C]KP glucuronide, eqs. 8–15 for (*S*)-[^{13}C]KP glucuronide] are shown below. $X_{1\beta}$, $X_{2\beta}$, $X_{2\alpha}$, $X_{3\beta}$, $X_{3\alpha}$, and X_{KP} denote the amounts of KP glucuronide, 2β-*O*-acyl, 2α-*O*-acyl, 3β-*O*-acyl, 3α-*O*-acyl isomers and KP, respectively. $X_{4\beta}$, $X_{4\alpha}$, and $X_{4\alpha\beta}$ denote the amounts of (*S*)-4β-*O*-acyl and (*S*)-4α-*O*-acyl isomers and the total amounts of (*R*)-4β-*O*-acyl and (*R*)-4α-*O*-acyl isomers, respectively.

$$\frac{dX_{1\beta}}{dt} = -(k_{1\beta-2\beta} + k_{1\beta-KP}) \cdot X_{1\beta} \quad (1)$$

$$\frac{dX_{2\beta}}{dt} = k_{1\beta-2\beta} \cdot X_{1\beta} - (k_{2\beta-3\beta} + k_{2\beta-2\alpha} + k_{2\beta-KP}) \cdot X_{2\beta} + k_{3\beta-2\beta} \cdot X_{3\beta} + k_{2\alpha-2\beta} \cdot X_{2\alpha} \quad (2)$$

$$\frac{dX_{2\alpha}}{dt} = k_{2\beta-2\alpha} \cdot X_{2\beta} - (k_{2\alpha-3\alpha} + k_{2\alpha-2\beta} + k_{2\alpha-KP}) \cdot X_{2\alpha} + k_{3\alpha-2\alpha} \cdot X_{3\alpha} \quad (3)$$

$$\frac{dX_{3\beta}}{dt} = k_{2\beta-3\beta} \cdot X_{2\beta} - (k_{3\beta-2\beta} + k_{3\beta-4\alpha\beta} + k_{3\beta-3\alpha} + k_{3\beta-KP}) \cdot X_{3\beta} + k_{3\alpha-3\beta} \cdot X_{3\alpha} + k_{4\alpha\beta-3\beta} \cdot X_{4\alpha\beta} \quad (4)$$

$$\frac{dX_{3\alpha}}{dt} = k_{2\alpha-3\alpha} \cdot X_{2\alpha} - (k_{3\alpha-2\alpha} + k_{3\alpha-4\alpha\beta} + k_{3\alpha-3\beta} + k_{3\alpha-KP}) \cdot X_{3\alpha} + k_{3\beta-3\alpha} \cdot X_{3\beta} + k_{4\alpha\beta-3\alpha} \cdot X_{4\alpha\beta} \quad (5)$$

$$\frac{dX_{4\alpha\beta}}{dt} = k_{3\beta-4\alpha\beta} \cdot X_{3\beta} - (k_{4\alpha\beta-3\beta} + k_{4\alpha\beta-3\alpha} + k_{4\alpha\beta-KP}) \cdot X_{4\alpha\beta} + k_{3\alpha-4\alpha\beta} \cdot X_{3\alpha} \quad (6)$$

$$\frac{dX_{KP}}{dt} = k_{1\beta-KP} \cdot X_{1\beta} + k_{2\beta-KP} \cdot X_{2\beta} + k_{2\alpha-KP} \cdot X_{2\alpha} + k_{3\beta-KP} \cdot X_{3\beta} + k_{3\alpha-KP} \cdot X_{3\alpha} + k_{4\alpha\beta-KP} \cdot X_{4\alpha\beta} \quad (7)$$

$$\frac{dX_{1\beta}}{dt} = -(k_{1\beta-2\beta} + k_{1\beta-KP}) \cdot X_{1\beta} \quad (8)$$

$$\frac{dX_{2\beta}}{dt} = k_{1\beta-2\beta} \cdot X_{1\beta} - (k_{2\beta-3\beta} + k_{2\beta-2\alpha} + k_{2\beta-KP}) \cdot X_{2\beta} + k_{3\beta-2\beta} \cdot X_{3\beta} + k_{2\alpha-2\beta} \cdot X_{2\alpha} \quad (9)$$

$$\frac{dX_{2\alpha}}{dt} = k_{2\beta-2\alpha} \cdot X_{2\beta} - (k_{2\alpha-3\alpha} + k_{2\alpha-2\beta} + k_{2\alpha-KP}) \cdot X_{2\alpha} + k_{3\alpha-2\alpha} \cdot X_{3\alpha} \quad (10)$$

$$\frac{dX_{3\beta}}{dt} = k_{2\beta-3\beta} \cdot X_{2\beta} - (k_{3\beta-2\beta} + k_{3\beta-4\beta} + k_{3\beta-3\alpha} + k_{3\beta-KP}) \cdot X_{3\beta} + k_{3\alpha-3\beta} \cdot X_{3\alpha} + k_{4\beta-3\beta} \cdot X_{4\beta} \quad (11)$$

$$\frac{dX_{3\alpha}}{dt} = k_{2\alpha-3\alpha} \cdot X_{2\alpha} - (k_{3\alpha-2\alpha} + k_{3\alpha-4\alpha} + k_{3\alpha-3\beta} + k_{3\alpha-KP}) \cdot X_{3\alpha} + k_{3\beta-3\alpha} \cdot X_{3\beta} + k_{4\alpha-3\alpha} \cdot X_{4\alpha} \quad (12)$$

$$\frac{dX_{4\beta}}{dt} = k_{3\beta-4\beta} \cdot X_{3\beta} - (k_{4\beta-3\beta} + k_{4\beta-4\alpha} + k_{4\beta-KP}) \cdot X_{4\beta} + k_{4\alpha-4\beta} \cdot X_{4\alpha} \quad (13)$$

$$\frac{dX_{4\alpha}}{dt} = k_{3\alpha-4\alpha} \cdot X_{3\alpha} - (k_{4\alpha-3\alpha} + k_{4\alpha-4\beta} + k_{4\alpha-KP}) \cdot X_{4\alpha} + k_{4\beta-4\alpha} \cdot X_{4\beta} \quad (14)$$

$$\frac{dX_{KP}}{dt} = k_{1\beta-KP} \cdot X_{1\beta} + k_{2\beta-KP} \cdot X_{2\beta} + k_{2\alpha-KP} \cdot X_{2\alpha} + k_{3\beta-KP} \cdot X_{3\beta} + k_{3\alpha-KP} \cdot X_{3\alpha} + k_{4\beta-KP} \cdot X_{4\beta} + k_{4\alpha-KP} \cdot X_{4\alpha} \quad (15)$$