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Synthesis of Δ^8-Tetrahydrocannabinol Glucuronide and Sulfate, and Their Metabolic Disposition in Rats

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Δ^8-Tetrahydrocannabinol (Δ^8-THC) glucuronide and Δ^8-THC sulfate were chemically synthesized as part of a study of the metabolic conjugation of Δ^8-THC. Acidic and enzymatic hydrolyses of these conjugates were examined. Contrary to expectation, Δ^8-THC glucuronide was resistant to both acidic and enzymatic hydrolyses. On the other hand, Δ^8-THC sulfate was readily hydrolyzed by acid, but not at all by arylsulfatase. In addition, this sulfate ester uncompetitively inhibited the hydrolysis of *p*-nitrophenylsulfate by arylsulfatase. The biliary and urinary excretions of these conjugates were also studied. The Δ^8-THC recovered after acid hydrolysis of the 24 hr bile of rats into which Δ^8-THC glucuronide or sulfate had been administered accounted for about 43% and 10% of the dose, respectively. The glucuronide and sulfate of Δ^8-THC both lacked the cataleptogenic effect of the parent compound. Δ^8-THC sulfate exhibited a rather high acute toxicity (LD_{50} 71 mg/kg *i.v.*), but the glucuronide caused no mortality up to a dose of 50 mg/kg *i.v.* in the mouse.

Keywords——Δ^8-tetrahydrocannabinol glucuronide; Δ^8-tetrahydrocannabinol sulfate; acid hydrolysis; enzymatic hydrolysis; acute toxicity; metabolic disposition

Various studies on glucuronide formation in the metabolism of cannabinoids have been presented in recent years.[2-6] Yagen *et al.* have synthesized three glucuronide isomers of Δ^8-tetrahydrocannabinol (Δ^8-THC) and identified the enzymatic product with one of two C-glucuronides.[2] Levy *et al.* have also identified the C-glucuronide of Δ^8-THC as a metabolite in mouse liver *in vivo*.[6] The enzymatic synthesis of cannabinoid glucuronide has been investigated by Lyle *et al.* and the formation of O-glucuronide of Δ^9-THC has been suggested.[4] Harvey *et al.* provided direct evidence by gas chromatography–mass spectrometry (GC–MS) for the *in vivo* formation of O-glucuronides of Δ^8- and Δ^9-THC, cannabidiol (CBD), and cannabinol (CBN).[3,5] Glucuronide formation was regarded as a general metabolic pathway for cannabinoids, but to only a limited extent for THC. On the other hand, there is no conclusive evidence for sulfate formation in the metabolism of cannabinoids, although Widman *et al.* have suggested the possible existence of a very small amount of the sulfate conjugate of Δ^9-THC in rats.[7]

The present study was undertaken to learn to what extent glucuronide and sulfate formation participate in the metabolism of Δ^8-THC in rats. For this purpose, Δ^8-THC glucuronide and sulfate were synthesized, and their responses to acidic and enzymatic hydrolyses, as well as the biliary and urinary excretions of these conjugates in rats, were examined. The pharmacological activity and toxicity were also assessed using mice.

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Materials and Methods

Materials——Animal Experiments: Male rats (Donryu strain) weighing 200—250 g and male mice (ddN strain) weighing 20—25 g were used for the metabolic study, and the pharmacological and toxicological studies, respectively. Sodium Δ^8-THC glucuronide and potassium Δ^8-THC sulfate were dissolved in saline and injected intravenously through the tail vein. Cataleptogenic effect and acute toxicity were assessed according to the methods reported previously.[8] Δ^8-THC was synthesized from Δ^9-THC, isolated from *Cannabis sativa*, by isomerization according to the method of Gaoni and Mechoulam.[9] β-Glucuronidase (Type 1 from the culture fluid of *E. coli*) and arylsulfatase (Type H-1 from *Helix pomatia* and Type III from limpets) were obtained from Sigma Chemical Co. A partially purified β-glucuronidase (bovine liver) was provided by Dr. Kato of the Faculty of Pharmaceutical Sciences, Kyushu University.

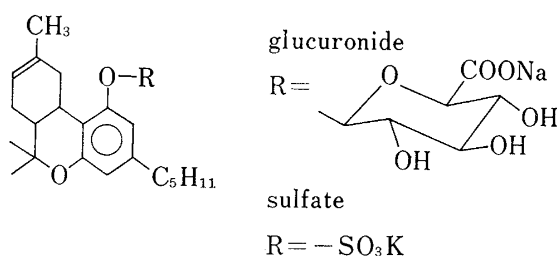

Fig. 1. Structures of Δ^8-THC Glucuronide Sodium Salt and Δ^8-THC Sulfate Potassium Salt

Synthesis of Methyl [Δ^8-THC-1-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid] Uronate——First, 1.48 g of Δ^8-THC (3.2 mmol) and 1.1 g of cadmium carbonate (6.4 mmol) were added to 50 ml of dry toluene, then 25 ml of the solvent was gradually distilled off during 1 hr while a solution of 2.53 g of methyl 2,3,4-tri-O-acetyl-1α-bromo-1-deoxy-D-glucopyranuronate (6.4 mmol) in 50 ml of dry toluene was added dropwise. This acetobromosugar was synthesized from glucuronolactone in three steps. The lactone was esterified to the methyl ester,[10] then acetylated[11] and this tetraacetate was brominated by the method of Bárczai-Martos and Kőrösy.[12] The reaction mixture was refluxed for a further 35 hr. The precipitate was then filtered off, and the solvent was evaporated from the filtrate *in vacuo*. The resulting residue was subjected to a silica gel column chromatography (150 g of Silica gel 60, E. Merck), eluting with *n*-hexane, ethyl acetate (8: 1 and 4: 1). A pale yellow glassy material (188 mg) obtained from the latter solvent was further purified by rechromatography on a column containing 20 g of silica gel. The product was recrystallized from petroleum ether and 118 mg of colorless crystals were obtained. mp 147—148°. *Anal.* Calcd. for $C_{34}H_{46}O_{11}$: C, 64.75; H, 7.35. Found: C, 64.33; H, 7.56. IR cm^{-1}: $\lambda_{C=O}$ 1750 (KBr). $[\alpha]_D^{30}$ $-168°$ (c=0.5, $CHCl_3$). UV λ_{max}^{EtOH} nm (log ε): 275 (3.17), 282 (3.19). NMR ($CDCl_3$) δ: 0.90 (3H, triplet, aliphatic CH_3), 1.08, 1.38 (3H$\times$2, 2 singlets, geminal CH_3), 1.64 (3H, singlet, olefinic CH_3), 1.73 (3H, singlet, $COCH_3$), 2.06 (6H, singlet, 2$\times$$COCH_3$), 3.80 (3H, singlet, $COOCH_3$), 4.10—4.36 (1H, multiplet, C-5′ sugar H), 4.98 (1H, broad doublet, J=8 Hz, C-1′ sugar H), 5.20—5.56 (4H, C_8-H, C-2′, C-3′, C-4′ sugar H), 6.46, 6.74 (2H, 2 singlets, aromatic H). MS *m/e*: 630 (M^+).

Synthesis of the Sodium Salt of Δ^8-THC Glucuronide——Methyl [Δ^8-THC-1-yl-2,3,4-tri-O-acetyl-D-glucopyranosid] uronate (118 mg) dissolved in 15 ml of MeOH was added to 5 N NaOH solution and the mixture was stirred for 2 hr at room temperature. The mixture was concentrated to a small volume, and the resulting precipitate (75 mg) was filtered and washed with small volume of MeOH. mp 180—190° (dec.). *Anal.* Calcd. for $C_{27}H_{39}NaO_8 \cdot 3H_2O$: C, 57.03; H, 7.98. Found: C, 57.39; H, 7.86. $[\alpha]_D^{30}$ $-120°$ (c=0.5, MeOH). IR (KBr): $\nu_{C=O}$ 1610 cm^{-1}.

Synthesis of the Pyridinium Salt of Δ^8-THC Sulfate——Δ^8-THC (370 mg) dissolved in 1 ml of dry pyridine was added to 760 mg of chlorosulfonic acid which had been dissolved in 2 ml of dry pyridine and heated at 70° for 10 min, and the mixture was heated at 70—80° for 1 hr. The resulting precipitate was filtered off and recrystallized from aqueous MeOH (1: 1) to yield 360 mg of Δ^8-THC sulfate pyridinium salt. mp 115—118°. *Anal.* Calcd. for $C_{26}H_{35}NO_5S$: C, 65.93; H, 7.45; N, 2.96. Found: C, 65.82; H, 7.56; N, 2.94. NMR ($CDCl_3$) δ: 0.84 (3H, triplet, aliphatic CH_3), 1.02, 1.34 (3H$\times$2, 2 singlets, geminal CH_3); 1.58 (3H, singlet, olefinic CH_3); 5.34 (1H, triplet, C_8–H), 6.44, 6.90 (2H, 2 singlets, aromatic H); 7.90, 8.41, 8.72 (5H, pyridinium H). MS *m/e*: 394 ($M^+-C_5H_5N$).

Synthesis of the Potassium Salt of Δ^8-THC Sulfate——Δ^8-THC sulfate pyridinium salt (300 mg) was added to alcoholic potassium hydroxide. The mixture was allowed to stand for 1 hr and the solvent was evaporated to dryness until the odor of pyridine had disappeared. The residue was dissolved in a small amount of EtOH and precipitated by the addition of dry ether. A hygroscopic powder (272 mg) was obtained. *Anal.* Calcd. for $C_{21}H_{29}KO_5S \cdot 1/2H_2O$; C, 57.50; H, 6.89. Found: C, 57.91; H, 7.20. IR (KBr): $\nu_{S=O}$ 1250 cm^{-1}. NMR (D_2O) δ: 0.76 (3H, triplet, aliphatic CH_3); 1.02, 1.24 (3H$\times$2, 2 singlets, geminal CH_3); 1.66 (3H, singlet, olefinic CH_3); 5.40 (1H, triplet, C_8–H); 6.48, 6.90 (2H, 2 singlets, aromatic H).

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Acid Hydrolysis of the Conjugates——Δ^8-THC glucuronide (2 μmol) was heated in 5 ml of 5% HCl, 5% H_2SO_4, 5% $HClO_4$ or 10% HCl for 1 hr in an autoclave under a pressure of 1.2 kg/cm^2. Liberated Δ^8-THC was extracted twice with 10 ml portions of *n*-hexane, and the extract was evaporated to dryness, then dissolved in 0.1 ml of MeOH. Δ^8-THC was determined by gas-liquid chromatography (GLC) as described below. Δ^8-THC sulfate (2 μmol) was hydrolyzed with 5% HCl and liberated Δ^8-THC was determined by GLC using the above procedure.

Gas-Liquid Chromatography——The instrument used was a Yanagimoto model G-8 gas chromatograph equipped with a hydrogen flame ionization detector. The column was a 1.5 m × 3 mm i.d. glass column containing 1% XE-60 on Chromosorb W (60—80 mesh) and was maintained at 210°. A methanol solution of the extract was applied; the retention time of Δ^8-THC was 4.3 min. The amount of Δ^8-THC was calculated from the standard curve obtained with an authentic sample.

Enzymatic Hydrolysis of the Conjugates——Δ^8-THC sulfate (2 μmol) in 100 mM acetate buffer (pH 5.0) or the same amount of Δ^8-THC glucuronide in 100 mM phosphate buffer (pH 7.0) was incubated with arylsulfatase (100 units) or β-glucuronidase (20000 units of Type 1 or 15000 units of bovine liver enzyme) in a final volume of 4 ml at 37° for 20 hr. One unit of β-glucuronidase or arylsulfatase activity is defined as the amount of enzyme liberating 1 μg of *p*-nitrophenol or 1 μmol of *p*-nitrocatechol from *p*-nitrophenylglucuronide or *p*-nitrocatechol sulfate per hour at pH 3.8 or pH 5.0, respectively. The hydrolysate was extracted twice with 5 volumes of *n*-hexane. The aqueous layer was further hydrolyzed with 5% HCl and extracted as described above. The extracted Δ^8-THC was determined by GLC.

Inhibition of Arylsulfatase by Δ^8-THC Sulfate——*p*-Nitrophenylsulfate (2.5, 5.0, 7.5, 10, 20 and 40 mM) was incubated with 2 units of arylsulfatase (Sigma Type III) in 100 mM acetate buffer (pH 5.0) (final volume, 1 ml) at 37° for 10 min. The reaction was terminated by addition of 1 ml of 2% phosphotungstic acid in 0.1 N HCl, and the liberated *p*-nitrophenol was determined spectrophotometrically as reported previously.[8]

Biliary and Urinary Excretion of Δ^8-THC Conjugates——Δ^8-THC glucuronide (5 mg/kg) and Δ^8-THC sulfate (3 mg/kg) were injected intravenously into bile duct-cannulated rats or intact rats, and their bile or urine was collected for 24 hr after the injections. The bile and urine were hydrolyzed with 5% HCl as described above and the liberated Δ^8-THC was extracted and determined by GLC. Authentic samples (1 mg) were added to the control bile (5 ml) and subjected to hydrolysis followed by GLC determination in order to determine the percent hydrolysis of the conjugates.

Results

Synthesis of Δ^8-THC Glucuronide and Sulfate

Δ^8-THC glucuronide was synthesized following the method of Conrrow and Bernstein, who have obtained phenolic glucuronides of steroids in good yields.[13] Condensation of Δ^8-THC with methyl tri-O-acetyl-1α-bromo-D-glucopyranuronate in the presence of cadmium carbonate in boiling toluene for 35 hr gave the methyl acetyl derivative of Δ^8-THC glucuronide. The major product obtained by this method was identified as the O-glucuronide derivative, not the C-glucuronide, by Nuclear Magnetic Resonance (NMR) spectroscopy, which showed two singlet peaks attributable to aromatic protons. As a by-product, a small amount of another glucuronide, the mp of which corresponded to that of the methyl acetyl derivative of the C-glucuronide reported by Yagen *et al.*,[2] was also obtained. Since the anomeric proton of the sugar moiety showed a large coupling constant (J=8 Hz) in the NMR spectrum, the glycosidic bond can be assigned the β-configuration. The methyl acetyl derivative was then treated with sodium hydroxide to afford the sodium salt of Δ^8-THC glucuronide as an amorphous powder. Δ^8-THC sulfate was obtained as the potassium salt through the pyridinium salt, which was synthesized by sulfonation of Δ^8-THC with chlorosulfonic acid in pyridine. The structures of both conjugates were well supported by elemental analysis data as well as the infrared (IR), NMR and mass spectral data described in "Materials and Method".

Acid Hydrolysis of Δ^8-THC Glucuronide and Sulfate

The results of acid hydrolysis of Δ^8-THC glucuronide and sulfate are summarized in Table I. To our surprise, the glucuronide was resistant to acid hydrolysis and only 12—17%

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Table I. Acid Hydrolysis of Conjugates

Conjugate	Acid	Hydrolysis (%)[a]	Recovery(%)[b] of Δ^8-THC
Δ^8-THC glucuronide	5% HCl	17.3	90.0
	5% $HClO_4$	15.1	83.1
	5% H_2SO_4	14.7	67.0
	10% HCl	12.1	88.6
Δ^8-THC sulfate	5% HCl	87.6	—

a) See "Materials and Methods" for the conditions used.
b) Recovery of Δ^8-THC after treatment under the conditions of hydrolysis.

Table II. Enzymatic Hydrolysis of Conjugates

Substrate	Enzyme	Hydrolysis (%)[a]	Hydrolysis (%) by 5% HCl[a] after enzyme hydrolysis
Δ^8-THC glucuronide	Bacterial β-glucuronidase	31.6	6.3
	Bovine liver β-glucuronidase	16.4	7.2
Δ^8-THC sulfate	Arylsulfatase (Type H-1)	0	—

a) See "Materials and Methods" for the conditions used.

of the aglycone was liberated. These low recoveries are not due to the degradation of liberated Δ^8-THC during the hydrolytic treatment, because Δ^8-THC itself could be recovered well after similar treatment, as shown in Table I. On the other hand, Δ^8-THC sulfate was hydrolyzed easily by 5% HCl.

Enzymatic Hydrolysis of Δ^8-THC Glucuronide and Sulfate

Table II shows the results of hydrolysis of Δ^8-THC glucuronide and sulfate with β-glucuronidase and arylsulfatase, respectively. The bacterial β-glucuronidase gave the highest percentage hydrolysis among the two enzymes and various acids, although it hydrolyzed only 30% of the glucuronide at most under these experimental conditions. Additional aglycone (6—7%) was recovered on acid treatment of the aqueous layer remaining after enzymatic hydrolysis. On the other hand, Δ^8-THC sulfate was not hydrolyzed at all by arylsulfatase from *Helix pomatia*.

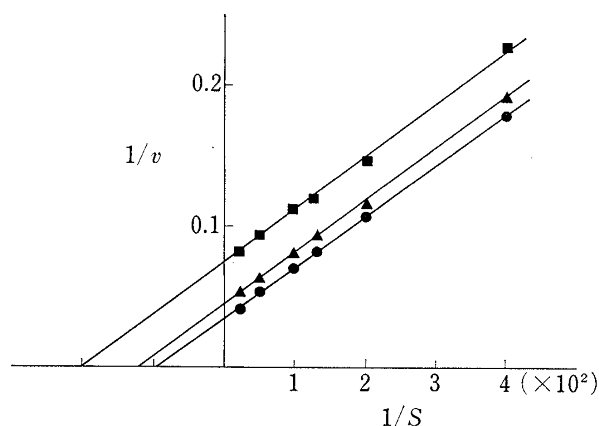

Fig. 2. Double-Reciprocal Plot of Velocity (μg of p-Nitrophenol liberated/10 min) against Concentration of p-Nitrophenylsulfate

●—● : without Δ^8-THC sulfate. ▲—▲ : plus 10^{-4} M Δ^8-THC sulfate. ■—■ : plus 10^{-3} M Δ^8-THC sulfate.

Inhibitory Effect of Δ^8-THC Sulfate on Arylsulfatase

This sulfate ester was not hydrolyzable by arylsulfatase, but exerted an inhibitory effect on the enzyme. Fig. 2 shows a double-reciprocal plot of the effect of Δ^8-THC sulfate on the hydrolysis of p-nitrophenylsulfate by arylsulfatase from limpets. As shown in this figure, addition of the sulfate caused a parallel shift in the rate of hydrolysis of p-nitrophenylsulfate, indicating that this inhibition is uncompetitive. Since arylsulfatase

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from *Helix pomatia* hydrolyzed *p*-nitrophenylsulfate in a manner that was not proportional to the concentration of the substrate in the present system, only the limpet enzyme was used in the inhibition study.

Table III. Recovery of Δ^8-THC after Hydrolysis of Bile and Urine

	Bile		Urine
	Recovery (%) of Δ^8-THC after acid hydrolysis	Corrected biliary[a] excretion (%)	Recovery (%) of Δ^8-THC after acid hydrolysis
Samples (4)[b] (Δ^8-THC glucuronide was injected.)	3.7±0.6[c]	43	ND[d]
Controls (2) [Authentic Δ^8-THC glucuronide (1 mg) was added.]	8.6		10.5
Samples (4) (Δ^8-THC sulfate was injected.)	5.0±0.5[c]	10.2	ND[d]
Controls (2) [Authentic Δ^8-THC sulfate (1 mg) was added.]	49.2		50.4

a) The biliary excretion of Δ^8-THC conjugates was corrected for the hydrolysis of authentic samples.
b) Values in parentheses represent number of determinations.
c) Values represent mean ± S.E. of recovery (%).
d) Not detected by GLC.

Biliary and Urinary Excretion of Δ^8-THC Conjugates after Their Injection

Δ^8-THC glucuronide (5 mg/kg) and sulfate (3 mg/kg) were injected intravenously into intact or bile duct-cannulated rats. The excretion of the unchanged conjugates was calculated by determining the aglycone liberated by hydrolysis with 5% HCl and correcting for the recovery of Δ^8-THC from authentic conjugates subjected to the same procedure. Table III shows the biliary and urinary excretion of Δ^8-THC glucuronide and sulfate during 24 hr after the injection of each conjugate. Free Δ^8-THC could not be detected in the bile of rats injected with Δ^8-THC glucuronide, but 3.7% of Δ^8-THC administered as the glucuronide was extracted after acid hydrolysis. When authentic Δ^8-THC glucuronide (1 mg) was added to the control bile (5 ml), only 8.6% of Δ^8-THC was recovered. Therefore, the glucuronide excreted unchanged into the bile of rats may account for about 43% of the injected dose. Δ^8-THC sulfate was similarly injected and its excretion into the 24 hr bile was examined. No free Δ^8-THC could be detected in the bile samples. The amount excreted unchanged was calculated to be 10.2% of the dose when corrected for the recovery of the authentic sulfate. On the other hand, the urinary excretion of these conjugates was so low that no Δ^8-THC was detected before or after acid treatment.

Acute Toxicity of Δ^8-THC Glucuronide and Sulfate

Table IV shows the acute toxicity of Δ^8-THC conjugates in mice. Δ^8-THC glucuronide showed neither a cataleptogenic effect nor mortality up to a dose of 50 mg/kg *i.v.* However, Δ^8-THC sulfate exhibited an LD_{50} of 71 mg/kg as calculated by the method of Litchfield and Wilcoxon,[14] but showed no cataleptogenic effect up to a dose of 80 mg/kg *i.v.*

Table IV. Acute Toxicity of Δ^8-THC Conjugates

	LD_{50} mg/kg[a]
Δ^8-THC	27.5 (23.1—32.5)[b]
Δ^8-THC sulfate potassium salt	71.0 (65.1—77.4)
Δ^8-THC glucuronide sodium salt	No effect at 50 mg/kg

a) Values in parentheses represent 95% confidence limits according to Litchfield–Wilcoxon.
b) This result is taken from a previous report.[5]

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Discussion

The presence of both O- and C-glucuronides as metabolites of THC has been reported.[2,4-6] The O-glucuronide of THC was synthesized enzymatically by Lyle *et al.*[4] and detected in the liver of mice administered Δ^8-THC by Harvey *et al.*[5] On the other hand, two C-glucuronides were synthesized and one of these was identified as a metabolite of Δ^8-THC *in vivo* and *in vitro* by Yagen *et al.*[2] and Levy *et al.*,[6] respectively. However, no one has yet determined the extent to which these conjugates participate in the metabolism of Δ^8-THC. Therefore, the present study was focused on O-glucuronide, because this type of glucuronide was expected to be easily hydrolyzable by acid, and the amount excreted into the bile and urine could be calculated by determining the aglycone liberated after hydrolysis. Contrary to our expectation, however, the glucuronide of Δ^8-THC was not readily hydrolyzable.

Yagen *et al.* have synthesized the methyl acetyl derivative of Δ^8-THC-O-glucuronide by the condensation of Δ^8-THC with methyl tetra-O-acetyl-β-D-glucopyranuronate in the presence of *p*-toluenesulfonic acid, and also Δ^8-THC C-2 glucuronide by the use of boron trifluoride etherate as the condensing agent.[2] In the present study, the methyl acetyl derivative of Δ^8-THC-O-glucuronide was synthesized by the condensation of Δ^8-THC with methyl tri-O-acetyl-1α-bromo-1-deoxy-D-glucopyranuronate using cadmium carbonate as the condensing agent. Previous workers have not removed the protecting groups,[2] but, in the present study, the sodium salt of Δ^8-THC glucuronide was obtained by treatment with methanolic sodium hydroxide.

Surprisingly, Δ^8-THC glucuronide showed high resistance to acid hydrolysis, but the sulfate ester was readily hydrolyzable. Katzman *et al.* have obtained similar results for estrogen conjugates; the aglycone of estriol glucuronide was not completely recovered by acid hydrolysis, while estrone sulfate was readily hydrolyzable under the same conditions.[15] On the other hand, the glucuronide was hydrolyzed enzymatically to a similar extent, but the sulfate was not hydrolyzed at all by arylsulfatase, and inhibited the hydrolysis of *p*-nitrophenylsulfate by the same enzyme. Although this inhibition might be expected to be competitive, since *p*-nitrophenylsulfate and Δ^8-THC sulfate possess a common phenolic sulfate structure, it was actually found to be uncompetitive, as shown in Fig. 2.

Since Δ^8-THC glucuronide was highly resistant to acid and enzymatic hydrolysis, precise determination of the glucuronide must be performed directly with the glucuronide itself. Direct evidence for glucuronide formation from cannabinoids has recently been obtained by GC-MS.[2-6] Thus, glucuronides of CBD, 11-hydroxy-CBD, CBN and 11-hydroxy-CBN have been identified as major metabolites of CBD and CBN in the mouse liver.[3,5] However, in the case of Δ^8- and Δ^9-THC, only trace amounts of the glucuronides were detected in the mouse liver.[3] These results indicate that glucuronide formation may not contribute greatly to the detoxication of THC, or that the glucuronide formed may be rapidly excreted into the bile, and/or further metabolized. In this connection, Widman *et al.* reported that no sulfate was present in the bile of cannulated rats administered Δ^9-THC.[7] The present results also suggest that the sulfate ester of Δ^8-THC, even if formed *in vivo*, is not significantly excreted into the bile or urine.

Δ^8-THC phosphate exerted a definite cataleptogenic activity, although its acute toxicity was much lower than that of Δ^8-THC,[8] while Δ^8-THC sulfate is devoid of cataleptogenic activity in spite of its rather high acute toxicity. Further studies are required to determine whether the potent inhibitory activity of Δ^8-THC sulfate on arylsulfatase is related to the acute toxicity.

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