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3-O-β-D-Glucopyranosiduronyl-D-glucaro-1,4-lactone[1]

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3-O-β-D-Glucopyranosiduronyl-D-glucaro-1,4-lactone (VI), a new masked inhibitor of β-glucuronidase, was synthesized by the fusion of dimethyl 2,4-O-benzylidene-5-O-acetyl-D-glucarate (I) and methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate (II) in the presence of mercuric cyanide, followed by removal of the protecting groups and lactonization.

The chemical structure of VI was confirmed to be 3-O-β-D-glucopyranosiduronyl-D-glucaro-1,4-lactone from the chemical and physical data of the products (III, IV, V, and VI) obtained in the course of its synthesis. The inhibitory effect of VI on β-glucuronidase *in vitro* was also examined.

In earlier studies on the inhibition of mammalian β-glucuronidase *in vitro*, Levvy, *et al.*[3] observed that D-glucaro-1,4-lactone was the most powerful inhibitor. Further, Boyland, *et al.*[4] and Kiyomoto, *et al.*[5] found that by oral administration to mammals, D-glucaro-1,4-lactone inhibited β-glucuronidase in the liver, kidney, and urine to prevent the liberation of carcinogenic aminophenols from their glucuronides.

Because of rapid conversion of D-glucaro-1,4-lactone *in vivo*[6] into D-glucaric acid and its 6,3-lactone, which are weaker inhibitors of β-glucuronidase, some masked D-glucaro-1,4-lactones have been investigated. Iida, *et al.*[7] reported that, in oral administration, 2,5-di-O-acetyl-D-glucaro-1,4: 6,3-dilactone possessed more durable inhibitory action on mouse liver β-glucuronidase than D-glucaro-1,4-lactone or D-glucaro-1,4: 6,3-dilactone.[8] We tried to investigate 3-O-β-D-glucopyranosiduronyl-D-glucaro-1,4-lactone (VI) as one of masked inhibitors which would be hydrolyzed by β-glucuronidase to liberate D-glucaro-1,4-lactone.

Synthesis

The synthetic route for VI is illustrated in Chart 1. According to the procedures of previous investigators,[9] we tried to condense dimethyl 2,4-O-benzylidene-5-O-acetyl-D-glucarate[10] (I) with methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate (II) or methyl tetra-O-acetyl-β-D-glucopyranuronate. In most cases, however, the reaction did not progress, and dimethyl 2,4-O-benzylidene-5-O-acetyl-3-O-(methyl-2′,3′,4′-tri-O-acetyl-β-D-glucopyranosiduronate)-D-glucarate (III) was obtained in a poor yield, only when I and II were refluxed in benzene in the presence of mercuric cyanide.

1) Parts of this work were presented at the 90th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, July 1970, and at the 91st Annual Meeting of the Pharmaceutical Society of Japan, Fukuoka, April 1971.
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3) *a*) G.A. Levvy, *Biochem. J.*, **52**, 464 (1952); *b*) G.A. Levvy and C.A. Marsh, *ibid.*, **52**, 690 (1952).
4) E. Boyland, D.M. Wallace, and D.C. Williams, *Brit. J. Cancer*, **11**, 578 (1957).
5) A. Kiyomoto, S. Harigaya, S. Ohshima, and T. Morita, *Biochem. Pharmacol.*, **12**, 105 (1963).
6) I. Matsunaga, T. Imanari, and Z. Tamura, in preparation.
7) R. Iida, S. Nagata, M. Kakimoto, H. Akaike, H. Watanabe, and A. Shioya, *Japan. J. Pharmacol.*, **15**, 88 (1965).
8) S. Harigaya, *J. Biochem.* (Tokyo), **56**, 400 (1964).
9) G.J. Dutton, "Glucuronic Acid," Academic Press, Inc., New York and London, Chapter 1, 1966.
10) I. Matsunaga and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1383 (1969).

A larger amount (16%) of III was obtained by the fusion of I and II (molar ratio, 1: 1) in the presence of mercuric cyanide (1.5—2.0 mole/mole of I) for 10 min (Table I). This is a new procedure for glucuronidation. Fusion with silver salts in place of mercuric cyanide did not give III.

The product separated into needles (IIIa, mp 177.5—178.5°) and prisms (IIIb, mp 144.5—145.5°) by recrystallization from isopropanol.

Benzylidene groups in both IIIa and IIIb were removed by hydrogenation with 5% palladium-charcoal to give dimethyl 5-O-acetyl-3-O-(methyl 2′,3′,4′-tri-O-acetyl-β-D-glucopyranosiduronate)-D-glucarate (IV), which afforded dimethyl 2,4,5-tri-O-acetyl-3-O-(methyl-2′,3′,4′-tri-O-acetyl-β-D-glucopyranosiduronate)-D-glucarate (V) by the usual acetylation. IIIa and IIIb, therefore, were considered to be stereoisomers with respect to the benzylidene group.

Treatment of V with sodium hydroxide in methanol gave 3-O-β-D-glucopyranosiduronyl-Δ^4-D-glucaric acid (IX) by β-elimination (Chart 2).

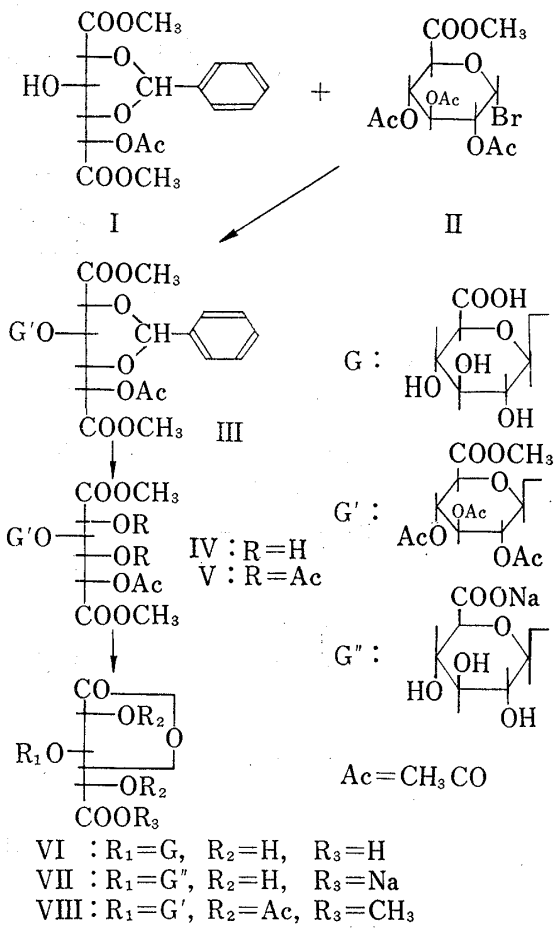

Chart 1. Route of Synthesis of VI

Therefore, using the acetyl, methyl ester, or lactone derivatives of D-glucaric acid as model compounds, we examined the manner of their hydrolysis with 0.2N sodium hydroxide or 0.5M triethylamine in 50% methanol (Chart 2). By measuring the fluorescence intensity of the reaction mixture after treatment with 4′-hydrazino-2-stilbazole,[11] β-elimination was found to be completed within 1 hr at room temperature.

As the by-product gave a peak on a gas chromatogram by reduction followed by trimethylsilylation, both the product (D-glucaric acid) and the by-product obtained by 1-hr hydrolysis were measured by gas chromatography.[12] The results are shown in Table II.

Table I. Condensation of I with II in the Presence of Mercuric Cyanide by Fusion

I (mmole)	II (mmole)	$Hg(CN)_2$ (mmole)	Time (min)	Yield (%) of III from I
10.9	10.9	5.45	10	6.18
10.9	10.9	10.9	10	9.97
10.9	10.9	16.3	10	15.5
10.9	10.9	21.8	10	15.7
10.9	5.45	8.18	10	9.98
10.9	16.3	24.5	10	10.2
10.9	10.9	16.3	5	12.0
10.9	10.9	16.3	20	12.0

11) S. Mizutani, Y. Wakuri, N. Yoshida, T. Nakajima, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **17**, 2340 (1969).
12) I. Matsunaga, T. Imanari, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **18**, 2535 (1970).

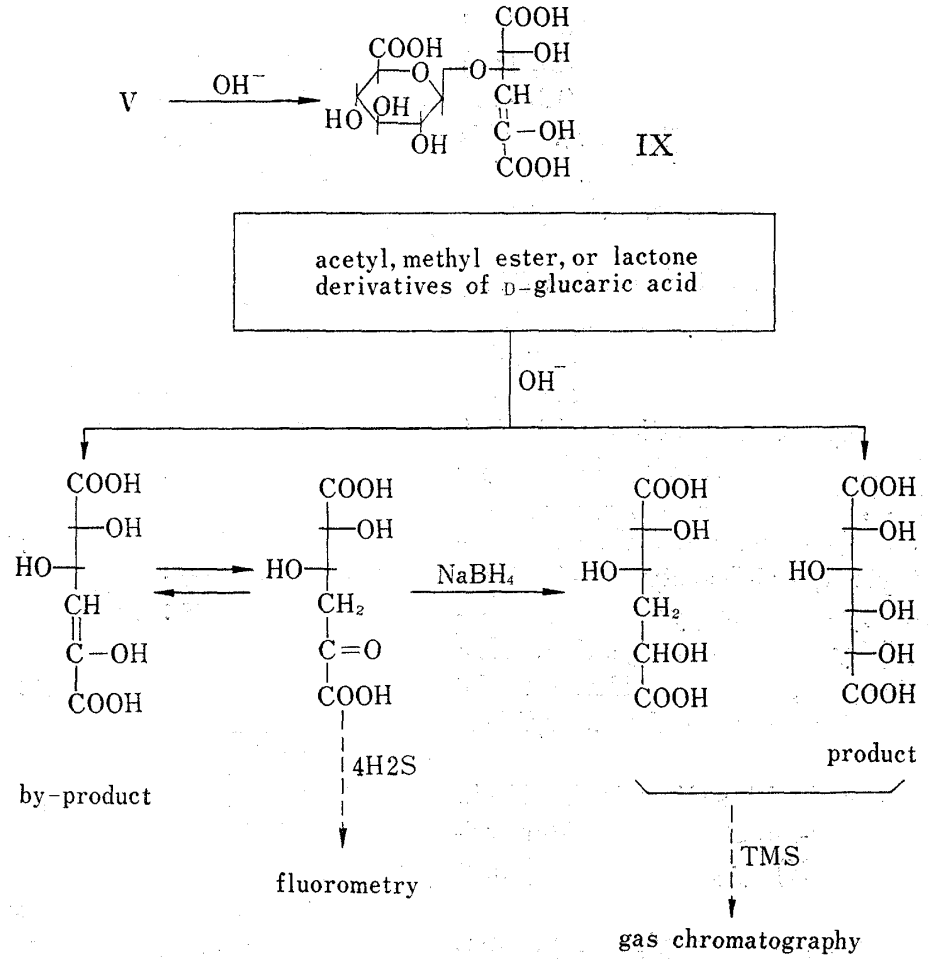

Chart 2

TABLE II. Hydrolysis of D-Glucaric Acid Derivatives with 0.2N Sodium Hydroxide and 0.5M Triethylamine in 50% Methanol (1 hr, 25°)

D-Glucaric acid derivatives	0.2N NaOH in 50% MeOH		0.5M Triethylamine in 50% MeOH	
	Product (%)	By-product (%)	Product (%)	By-product (%)
D-glucaric acid	100	0	100	0
D-glucaro-1,4-lactone	100	0	100	0
D-glucaro-6,3-lactone	100	0	100	0
2,3,5-tri-O-acetyl-D-glucaro-1,4-lactone	97.9	2.1	91.9	2.3
D-glucaro-1,4:6,3-dilactone	9.0	91.0	88.3	11.7
dimethyl D-glucarate	12.3	87.7	92.0	8.0
D-glucaro-1,4-lactone 6-methyl ester	10.0	90.0	92.3	7.7
D-glucaro-6,3-lactone 1-methyl ester	8.9	91.1	91.3	8.7
2,5-di-O-acetyl-D-glucaro-1,4:6,3-lactone	5.4	94.5	72.9	19.5
dimethyl 2,3,4,5-tetra-O-acetyl-D-glucarate	1.6	98.4	70.5	17.0
2,3,5-tri-O-acetyl-D-glucaro-1,4-lactone 6-methyl ester	8.6	91.4	74.9	20.4
2,4,5-tri-O-acetyl-D-glucaro-6,3-lactone 1-methyl ester	6.3	93.7	69.6	26.2

These derivatives of D-glucaric acid were prepared in this laboratory and their purities were confirmed from the results of elemental analyses and from melting points and IR spectra.

Alkaline hydrolysis of the dicarboxylic acid and its monolactone derivatives did not give the by-product, but hydrolysis of the diester, dilactone, or monoester-monolactone derivatives of the dicarboxylic acid with 0.2N sodium hydroxide gave a larger amount of the by-product, whether the derivatives were acetates or not. Use of triethylamine in place of sodium hydroxide suppressed β-elimination during their hydrolysis, especially of non-acetylated derivatives.

Following the above results, hydrolysis of IV, instead of V, was carried out with 0.5M triethylamine in 50% methanol under ice-cooling and then with 0.5N sodium hydroxide in 50% methanol for completion of the reaction. Without β-elimination, VI was produced by removal of the acetyl and methyl ester groups of IV followed by lactonization. Treatment of VI with sodium acetate in methanol gave its disodium salt (VII).

2,5-Di-O-acetyl-3-O-(methyl 2′,3′,4′-tri-O-acetyl-β-D-glucopyranosiduronate)-D-glucaro-1,4-lactone 6-methyl ester (VIII) was also prepared as a syrup by esterification of VI with diazomethane followed by the usual acetylation, and its infrared spectrum ($CHCl_3$) showed a C=O (lactone) band shifted by acetylation to 1809 cm^{-1} in addition to a C=O (ester) band at 1757 cm^{-1}.

Chemical Structure

Because of the drastic condition of the condensation reaction, there might be a possibiltiy of the migration of protecting groups and glucuronic acid moiety. Therefore, the chemical structure of VI was determined from the chemical and physical data of the products (III, IV, V, and VI in Chart 1) obtained in the course of its synthesis.

When VI was hydrolyzed with 2.5N trifluoroacetic acid, D-glucuronic acid and D-glucaric acid were liberated and detected in a ratio of 1: 0.6 by gas chromatography, and there appeared no other peaks. The ratio is acceptable from the fact that saccharic acid is more unstable than uronic acid under acidic conditions.[12]

The nuclear magnetic resonance (NMR) spectra (in $CDCl_3$; 60 MHz) of III, IV, and V showed a distorted doublet (J=10 cps) at τ 5.9—6.0 corresponding to pyranose-C (5) proton and a doublet (J=7 cps) at τ 5.6—5.7 corresponding to pyranose-C (1) proton, similar to that of methyl,[13] ethyl, or n-butyl β-D-glucuronide methyl ester tri-O-acetate, while the NMR spectrum of methyl α-D-glucuronide methyl ester tri-O-acetate[13] was reported to show a sharp doublet (J=10 cps) at τ 5.70 corresponding to pyranose-C (5) proton. The molecular rotations at 589 nm of all III, IV, V, and VI showed negative values (—75.2°, —42.6°, —94.5°, and —73.2°, respectively) and were in good agreement with the theoretical values (—74.9°, —52.3°, —33.5°, and —97.0°, respectively) obtained from the isorotation rule of Hudson[14] assuming a β-configuration. The final evidence for β-linkage was the production of D-glucuronic acid and D-glucaric acid from VI by hydrolysis with bacterial or beef-liver β-glucuronidase.

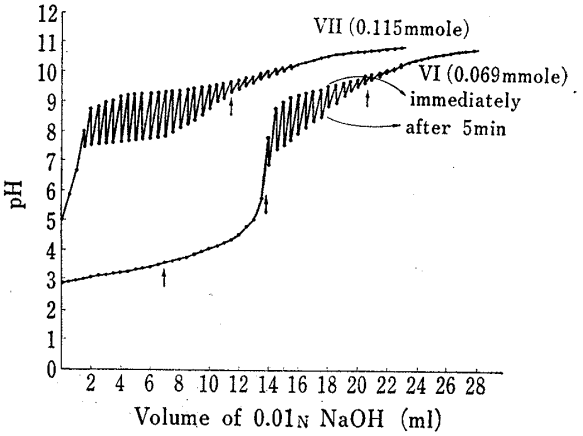

Fig. 1. Titration Curves of VI and VII with 0.01N Sodium Hydroxide

Arrows indicate equivalent points.

Titration curves for VI and its disodium salt (VII) with sodium hydroxide indicated the presence of two moles of carboxyl and one mole of lactone groups in VI (Fig. 1). The infrared

13) M. Matsui and M. Okada, *Chem. Pharm. Bull.* (Tokyo), **18**, 2129 (1970).
14) C.S. Hudson, *J. Am. Chem. Soc.*, 31, 66 (1909).

spectra (KBr) of both VI and VII showed a band at 1780 cm^{-1}, indicating the presence of a γ-lactone. VI exhibited a negative Cotton effect in its optical rotatory dispersion curve (in ethanol) around 220 nm, indicating the presence of a 1,4-lactone.[15]

In order to confirm the position of glucuronide linkage in VI, D-glucarate β-D-glucuronide derived from VI by treatment with triethylamine was oxidized with periodate in a slightly alkaline medium. As shown in Fig. 2, this compound consumed one mole of periodate at 4°, different from oxidation at room temperature. The residual moiety resistant to periodate oxidation afforded D-glucuronic acid L-threonic acid (Fig. 3) by reduction with sodium boro-

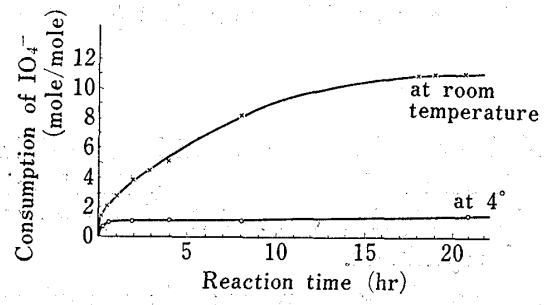

Fig. 2. Periodate Oxidation of 3-O-β-D-Glucopyranosiduronyl-D-glucarate

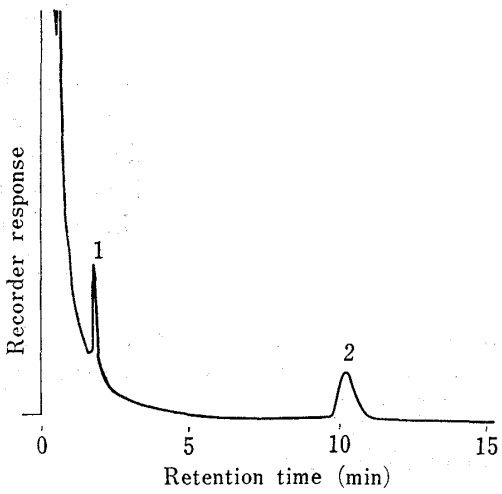

Fig. 3. Gas Chromatogram of Smith Degradation Products of 3-O-β-D-Glucopyranosiduronyl-D-glucarate

peak 1: L-threonic acid
2: L-gulonic acid derived from D-glucuronic acid
conditions: 2% XF-1105 (2.0 m × 4 mm i.d.), 180°, N_2 63 ml/min

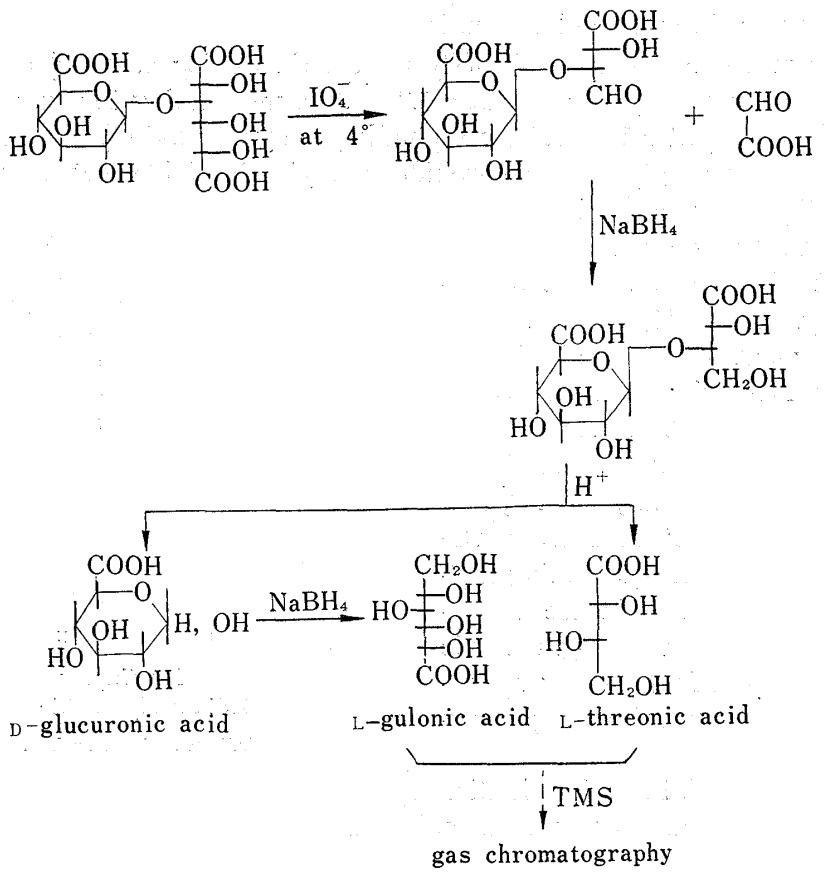

Chart 3

15) T. Okuda, S. Harigaya, and A. Kiyomoto, *Chem. Pharm. Bull.* (Tokyo), **12**, 504 (1964).

hydride followed by acid hydrolysis (Smith degradation). These data demonstrate that D-glucuronic acid is attached to 3-position of D-glucaric acid (Chart 3).

From the above results, VI was confirmed to be 3-O-β-D-glucopyranosiduronyl-D-glucaro-1,4-lactone.

Inhibitory Effect on β-Glucuronidase

Apparent inhibitory activity of VI on hydrolysis of sodium phenolphthalein mono-β-D-glucuronide (PMG) with beef-liver β-glucuronidase was measured by 1-hr incubation and compared with those of D-glucaro-1,4-lactone (X), 3-O-methyl-D-glucaro-1,4-lactone[16] (XI), and D-glucuronic acid. As shown in Fig. 4, VI was located between X and D-glucuronic acid in the degree of inhibition of β-glucuronidase, similar to XI. The effect of pH on this inhibition is illustrated in Fig. 5. Differing from X, VI showed stronger inhibition of the enzyme at around pH 3.5.

From a Dixon plot,[17] VI was found to inhibit beef-liver β-glucuronidase competitively

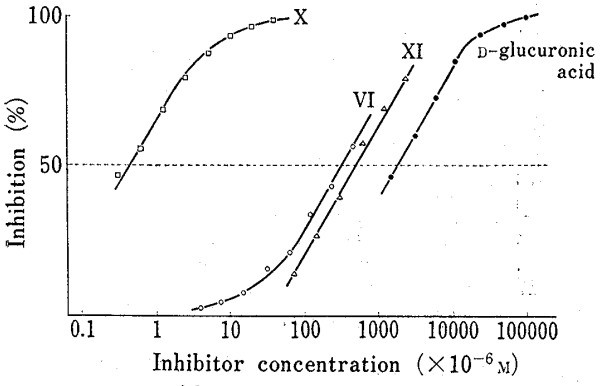

Fig. 4. Inhibitory Effect of 3-O-β-D-Glucopyranosiduronyl-D-glucaro-1,4-lactone (VI), D-Glucaro-1,4-lactone (X), 3-O-Methyl-D-glucaro-1,4-lactone (XI), and D-Glucuronic Acid on Hydrolysis of 50 μM PMG with Beef-liver β-Glucuronidase at pH 4.63 and 38°

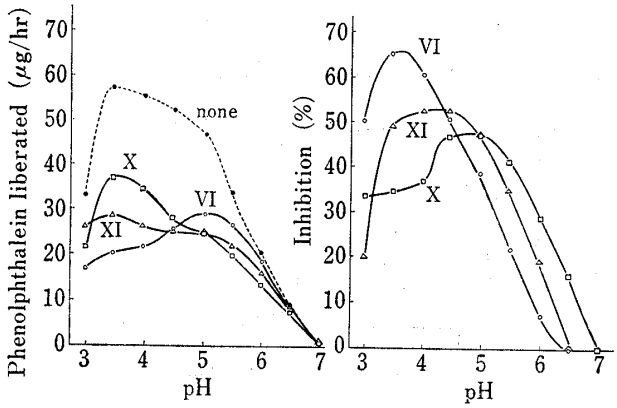

Fig. 5. Effect of pH on the Inhibition of Beef-liver β-Glucuronidase by VI (5×10^{-4}M), X (6.94×10^{-7}M), and XI (5.05×10^{-4}M)

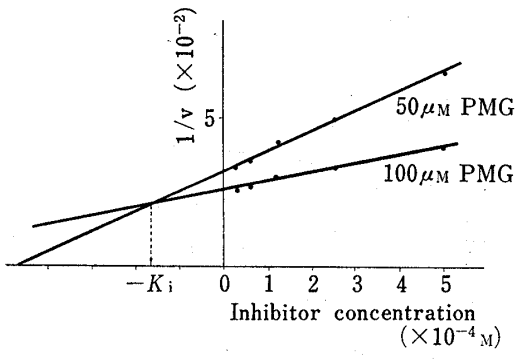

Fig. 6. Plot of Inhibitor Concentration against 1/v (Dixon Plot)

enzyme: beef-liver β-glucuronidase
substrate: sodium phenolphthalein mono-β-D-glucuronide (PMG)
inhibitor: 3-O-β-D-glucopyranosiduronyl-D-glucaro-1,4-lactone (VI)
velocity (v): phenolphthalein liberated (μg/hr) at pH 4.63 and 38°

TABLE III. Apparent Affinities of Various Inhibitors for Beef-liver β-Glucuronidase Compared with That of PMG (pH 4.63)

Compound	K_m, K_i ($\times10^{-6}$M)	Apparent relative affinity
PMG	170	1
VI	162.4	1.06
X	0.58	293.2
XI	185	0.92
D-Glucuronic acid	750	0.23

16) P.A. Levene and G.M. Meyer, *J. Biol. Chem.*, **54**, 805 (1922).
17) M. Dixon, *Biochem. J.*, **55**, 170 (1957).

in appearance, similar to X and XI (Fig. 6). K_i obtained from this graph was compared with K_m of PMG obtained from a Lineweaver plot[18] and the results are shown in Table III. These results show that the direct inhibition of β-glucuronidase by VI is much smaller than that by X, indicating that VI is a masked inhibitor.

When VI was incubated with beef-liver β-glucuronidase for several hours, the inhibitory activity increased, while that of X decreased with time (Fig. 7). The increase in the inhibitory activity of VI suggests the formation of X by hydrolysis with β-glucuronidase. In spite of linear increase of liberated total D-glucarate as measured by gas chromatography (Fig. 8 and 9), inhibitory activity decreased thereafter. This may be due to the transformation of X into D-glucaric acid and its 6,3-lactone.[19] Similar non-enzymic cleavage of the lactone ring was also observed with VI in aqueous solution under the same conditions as enzymic hydrolysis (Fig. 10).

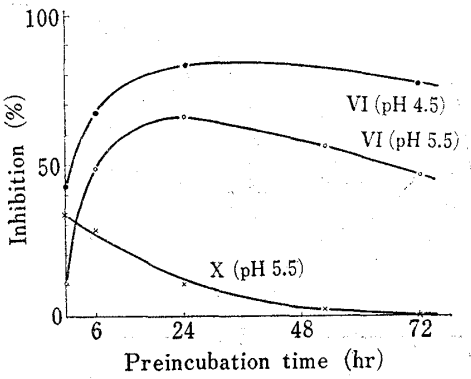

Fig. 7. Time Courses of Inhibition during Incubation of VI (3.5×10^{-4}M) and X (4.5×10^{-7}M) with Beef-liver β-Glucuronidase at 38°

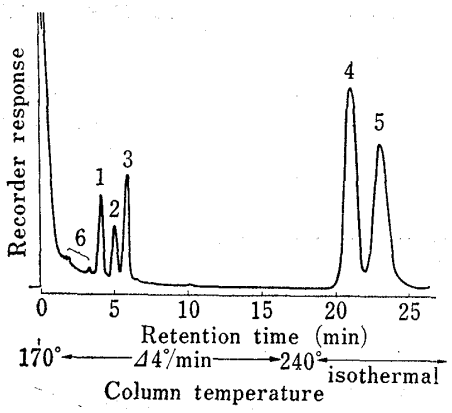

Fig. 8. Gas Chromatogram of the Hydrolysate of VI with Beef-liver β-Glucuronidase

peak 1: L-gulonic acid derived from D-glucuronic acid
2: D-glucaric acid
3: galactaric acid (internal standard)
4: melibiitol derived from melibiose (internal standard)
5: 3-O-β-D-glucopyranosiduronyl-D-glucaric acid derived from VI
6: impurities from enzyme and reagents
column: 2% OV-17

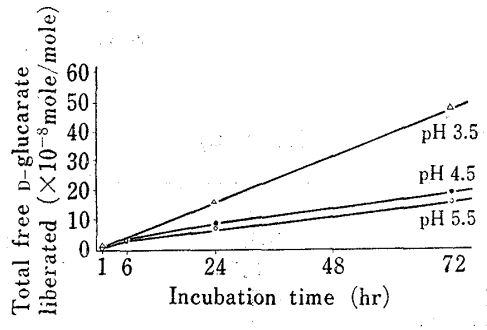

Fig. 9. Time Courses of Total Free D-Glucarate Liberated during Incubation of VI (3.5×10^{-4}M) with Beef-liver β-Glucuronidase

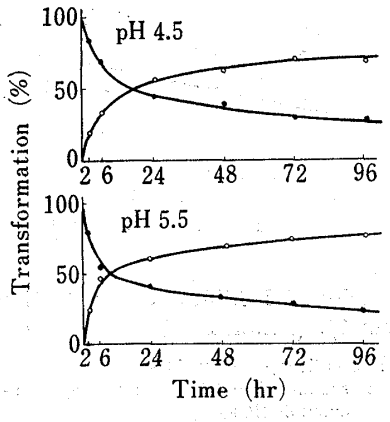

Fig. 10. Transformation of VI (—•—) into 3-O-β-D-Glucopyranosiduronyl-D-glucarate (—○—) at 38°

18) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
19) I. Matsunaga and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **20**, 284 (1972).

From the results of above experiments *in vitro*, VI seems to be a long acting masked β-glucuronidase inhibitor suitable for treatment of increased activity of β-glucuronidase *in vivo*.

Experimental[20]

Dimethyl 2,4-O-Benzylidene-5-O-acetyl-3-O-(methyl 2′,3′,4′-tri-O-acetyl-β-D-glucopyranosiduronate)-D-glucarate (III)——A mixture of I (4 g, 10.9 mmole), II (4.32 g, 10.9 mmole), and finely powdered $Hg(CN)_2$ (4.13 g, 16.3 mmole) was fused on a boiling water bath for 10 min under a reduced pressure (aspirator). When cooled, the residue was extracted with benzene (about 100 ml). The benzene phase was washed with H_2O, dried over anhyd. Na_2SO_4, and decolorized with charcoal. After evaporation of the solvent, the crude product (1.15 g, 15.5%) obtained was recrystallized from iso-PrOH to give IIIa (937 mg) as colorless needles, mp 177.5—178.5°. $[\alpha]_D^{25.5}$ −11.0° (c=0.363, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 1769, 1755, 1742 (C=O). NMR ($CDCl_3$): τ 8.08, 7.97, 7.78 (12H, $COCH_3$), 6.25, 6.17 (9H, $COOCH_3$), 4.60—6.00 (9H, CH in sugar part), 4.05 (1H, CH of benzylidene group), 2.61 (5H, aromatic H). *Anal.* Calcd. for $C_{30}H_{36}O_{18}$: C, 52.63; H, 5.30. Found: C, 52.73; H, 5.30.

The mother liquor was evaporated and gave IIIb (175 mg) as colorless prisms, mp 144.5—145.5°. $[\alpha]_D^{25.5}$ −1.2° (c=0.83, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 1769, 1755, 1745 (C=O). NMR ($CDCl_3$): τ 7.98, 7.88 (12H, $COCH_3$), 6.26, 6.24, 6.21 (9H, $COOCH_3$), 4.60—6.00 (9H, CH in sugar part), 4.09 (1H, CH of benzylidene group), 2.66 (5H, aromatic H). *Anal.* Calcd. for $C_{30}H_{36}O_{18}$: C, 52.63; H, 5.30. Found: C, 52.58; H, 5.31.

IIIa and IIIb gave a spot at *Rf* 0.56 and *Rf* 0.50, respectively, on a thin-layer chromatogram (silica gel) developed with ether and sprayed with NH_2OH–$FeCl_3$ or NH_4VO_3–50% H_2SO_4.

Dimethyl 5-O-Acetyl-3-O-(methyl 2′,3′,4′-tri-O-acetyl-β-D-glucopyranosiduronate)-D-glucarate (IV)——IIIa (3.04 g) was suspended in MeOH (250 ml) and the mixture was shaken with 5% Pd-C (2.9 g) in a stream of H_2 at room temperature. About 200 ml of H_2 was consumed during 6—8 hr. After removal of the catalyst by filtration, the filtrate was evaporated to give a crystalline residue (2.517 g, 95%), which was recrystallized from iso-PrOH to give IV (1.5 g) as colorless needles, mp 133—134°. $[\alpha]_D^{29}$ −7.14° (c=0.98, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1755 (C=O). NMR ($CDCl_3$): τ 7.97, 7.86 (12H, $COCH_3$), 6.93 (OH), 6.27, 6.23 (9H, $COOCH_3$), 4.40—6.10 (9H, CH in sugar part). *Anal.* Calcd. for $C_{23}H_{32}O_{18}$: C, 46.31; H, 5.41. Found: C, 46.47; H, 5.44.

The powder obtained from the mother liquor gave V in an excellent yield by the usual acetylation.

Dimethyl 2,4,5-Tri-O-acetyl-3-O-(methyl 2′,3′,4′-tri-O-acetyl-β-D-glucopyranosiduronate)-D-glucarate (V)——IV (363.4 mg) was dissolved in pyridine (2 ml) and Ac_2O (4 ml) under stirring and, after standing overnight at room temperature, EtOH was added to the reaction mixture and the solution was evaporated to a syrup. Recrystallization of the syrup from EtOH gave V (387.3 mg, 93.5%) as colorless needles, mp 171—172°. $[\alpha]_D^{27.5}$ −13.9° (c=1.08, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 1752—1762 (C=O). NMR ($CDCl_3$): τ 7.99, 7.94, 7.91, 7.87 (18H, $COCH_3$), 6.29 (9H, $COOCH_3$), 4.30—6.10 (9H, CH in sugar part). *Anal.* Calcd. for $C_{27}H_{36}O_{20}$: C, 47.65; H, 5.33. Found: C, 47.71; H, 5.44.

IIIb (81.4 mg) was worked up similarly to IIIa and gave V (52.2 mg, 64.5%), mp 171—172°.

3-O-β-D-Glucopyranosiduronyl-D-glucaro-1,4-lactone (VI)——To a solution of IV (208.3 mg, 0.349 mmole) dissolved in 50% MeOH (10 ml), 0.5M NEt_3 (in 50% MeOH, 6 ml) was added dropwise under ice-cooling. The reaction mixture was allowed to stand in a refrigerator for 2 days. To the reaction mixture, 0.5N NaOH (6 ml) was added dropwise under cooling and, after standing in a refrigerator overnight, the mixture was passed through a column of Amberlite IR-120 (H^+) and the effluent was evaporated to a syrup. The residue was dried over P_2O_5 at 100° for 2—3 hr under a reduced pressure (oil pump). The crude product dissolved in a small volume of EtOH was re-precipitated by the addition of ether to give VI (114.6 mg, 89.4%). $[\alpha]_D^{22}$ −19.9° (c=0.34, EtOH). IR ν_{max}^{KBr} cm^{-1}: 1780 (C=O of lactone), 1740 (COOH). *Anal.* Calcd. for $C_{12}H_{16}O_{13}\cdot H_2O$: C, 37.31; H, 4.70. Found: C, 37.44; H, 4.78.

3-O-(Sodium β-D-glucopyranosiduronate)-D-glucaro-1,4-lactone 6-Sodium Salt (VII)——To a solution of VI (91.2 mg) dissolved in MeOH (4 ml), a methanolic solution (5 ml) of anhyd. AcONa (50 mg) was added dropwise under cooling. The precipitate formed was collected, dissolved in a minimum amount of H_2O, and re-precipitated by the addition of EtOH to give VII (72.8 mg), mp 196° (brown)—215° (bubbling). $[\alpha]_D^{22}$ −20.1° (c=0.945, H_2O). IR ν_{max}^{KBr} cm^{-1}: 1780 (C=O of lactone), 1610 (COO^-). NMR: no signals due to methyl protons of methyl ester and acetyl groups. *Anal.* Calcd. for $C_{12}H_{14}O_{13}Na_2\cdot 3H_2O$: C, 30.91; H, 4.32. Found: C, 31.01; H, 4.75.

2,5-Di-O-acetyl-3-O-(methyl 2′,3′,4′-tri-O-acetyl-β-D-glucopyranosiduronate)-D-glucaro-1,4-lactone 6-Methyl Ester (VIII)——VI (90 mg) dissolved in MeOH was esterified with CH_2N_2 and then acetylated in the usual way to give VIII (33.5 mg) as a syrup. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1809 (C=O of lactone), 1757 (C=O of ester).

20) All melting points are uncorrected. All evaporations were carried out under a reduced pressure, keeping the bath temperature below 40°.

3-O-β-D-Glucopyranosiduronyl-Δ^4-D-glucaric Acid (IX)——V (180.3 mg) was dissolved in 0.2N NaOH (in MeOH, 12 ml) under ice-cooling and then allowed to stand at room temperature for 2—3 hr. The reaction mixture was passed through a column of Amberlite IR-120 (H^+) and the effluent was evaporated to a syrup. The residue dissolved in a minimum amount of EtOH was re-precipitated by the addition of ether to give IX (48.8 mg, 43.2%). IR ν_{max}^{KBr} cm^{-1}: 1740 (COOH), 1660 (C=C). NMR (D_2O): τ 8.82 (3H, triplet, J=7 cps, ethanolic CH_3), 4.30—6.70 (CH in sugar part, ethanolic CH_2, and HOD), 3.32 (1H, doublet, C(4)-H). *Anal.* Calcd. for $C_{12}H_{16}O_{13}\cdot C_2H_5OH$: C, 38.89; H, 5.60. Found: C, 38.51; H, 5.73.

Deacetylation and De-esterification of D-Glucaric Acid Derivatives with Sodium Hydroxide and Triethylamine——i) Fluorometric Measurement of By-product: Dimethyl 2,3,4,5-tetra-O-acetyl-D-glucarate or V (7 mg) was dissolved in 0.1N NaOH (in 50% MeOH) or 0.5M NEt_3 (in 50% MeOH) and the solution was adjusted to 10 ml with the same alkaline solution and allowed to stand at room temperature. At intervals of time, 1 ml of the solution was taken, 0.834M formate buffer (pH 4; 4 ml) and 0.15% 4'-hydrazino-2-stilbazole (4H2S) solution (1 ml) were added to 1 ml of the reaction mixture, and the solution was allowed to stand in the dark at room temperature for 1 hr. To the reaction mixture, 2N HCl was added and the fluorescence intensity was measured at 450 nm excitation and 540 nm of emission.

ii) Gas Chromatographic Measurement of the Product and By-product: The acetyl, methyl ester, or lactone derivative of D-glucaric acid (2—3 mg) was dissolved in 0.2N NaOH (in 50% MeOH, 1 ml) or 0.5M NEt_3 (in 50% MeOH, 1 ml) and the reaction mixture was allowed to stand at room temperature for 1 hr. To the mixed solution, 1% $NaBH_4$ (1 ml) was added and the reaction mixture was allowed to stand at room temperature for 1 hr. After decomposition of the excess reagent with Amberlite CG-120 (H^+), the solution was passed through a small column containing the same resin. The effluent was evaporated to dryness and boric acid was removed as volatile methyl borate by evaporating twice with dry MeOH. The residue was dissolved in distilled water and 3 drops of NEt_3 were added to the solution. After standing at 60° for 5 min, the solution was evaporated to dryness and the residue was dried in a desiccator in vacuum. The sample was trimethylsilylated with pyridine (0.5 ml), hexamethyldisilazane (0.1 ml), and trimethylchlorosilane (0.05 ml) at 60° for 20 min and 1 μl of the reaction mixture was injected directly into a gas chromatograph. The measurement was performed by the peak area ratio method.

Acid Hydrolysis of VI——VI (3 mg) was dissolved in 2.5N trifluoroacetic acid (2 ml) and hydrolyzed in a sealed tube on a boiling water bath for 6 hr. The resulting solution was evaporated to dryness under a reduced pressure. D-Glucuronic acid and D-glucaric acid formed were analyzed by the gas chromatographic method.[12]

Titration of VI or VII with Sodium Hydroxide——VI (29.1 mg, 0.069 mmole) or VII (59.7 mg, 0.115 mmole) was dissolved in distilled water (20 ml) and titrated with 0.01N NaOH (0.5 ml at a time) under bubbling with nitrogen. Immediately and at 5 min after titration, the pH value was read in a pH meter.

Periodate Oxidation of 3-O-β-D-Glucopyranosiduronyl-D-glucarate——i) Conditions: VII (3.4 mg, 6.54 mmole) was dissolved in H_2O (1 ml) containing NEt_3 (1 drop). After standing for 10 min at 60°, the resulting solution was cooled to 4°. A volume of 0.115M $NaIO_4$ (1 ml; previously cooled to 4°) was added and the solution was adjusted to 10 ml with cold distilled water. The mixture was allowed to stand in the dark at 4° or at room temperature.

ii) Estimation of Periodate Consumption[21]: An aliquot of the above oxidation mixture (1 ml) was pipetted into a flask containing a borate buffer (5 ml; 40 mg of $Na_2B_2O_7$ and 30 mg of H_3BO_3 per ml). To the mixture, 20% KI (5 ml) was added and, after standing in the dark for 10 min at room temperature, I_2 produced was determined by titration with 0.01N $NaAsO_2$.

iii) Detection of D-Glucuronic Acid and L-Threonic Acid derived from the Residual Moiety Resistant to Periodate Oxidation[22]: VI (15 mg) was worked up in a similar manner as described in i). After oxidation for 90 min at 4°, an excess of $NaBH_4$ was added to the reaction mixture and the solution was allowed to stand for 2 hr at room temperature. The remaining $NaBH_4$ was destroyed by the addition of Amberlite CG-120 (H^+) and the solution was then passed through a column containing the same resin. The effluent was evaporated to dryness under a reduced pressure and boric acid was removed by evaporating twice with dry MeOH. The reduced material was worked up in a similar manner as described above (acid hydrolysis of VI).

Assay of β-Glucuronidase Activity[23]——To a mixture containing 3.5 ml of 0.1M acetate buffer, 0.5 ml of 1mM sodium phenolphthalein mono-β-D-glucuronide (PMG; Chugai Pharmaceutical Co., Ltd.), and 0.5 ml of an inhibitor solution or distilled water, beef-liver β-glucuronidase (0.5 ml; previously diluted 100-fold with cold distilled water; Tokyo Zoki Chemical Co., Ltd., 13000 Fishman units/ml) was added under ice-cooling. After incubation for 1 hr at 38°, 0.4M glycine buffer (pH 10.5; 5 ml) was added and the intensity of the red color was measured at 550 nm.

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Hydrolysis of VI with β-Glucuronidase——i) With Beef-liver β-Glucuronidase: To a mixture of 0.1M acetate buffer (42 ml; pH 3.5, 4.5, or 5.5) and distilled water (6 ml) or a solution (3.5 mM; 6 ml) of VI, beef-liver β-glucuronidase (×100; 6 ml) was added under ice-cooling, the solution was adjusted to 60 ml with cold distilled water, and incubated at 38°. a) Assay of β-glucuronidase activity: At intervals of time, 2.5 ml of the solution was taken, 1 mM PMG (0.25 ml) was added, and the solution was incubated for 1 hr at 38°. To the resulting solution, 1M glycine buffer (pH 10.5; 2.5 ml) was added and the optical density was read in colorimeter at 550 nm. b) Analysis of D-glucuronic acid and D-glucaric acid liberated, and VI remaining: At intervals of time, 10 ml of the incubated solution was taken and galactaric acid and melibiose as internal standards, and a few drops of NEt_3 were added. The mixed solution was allowed to stand for 20 min at room temperature and 1 ml of 3% $NaBH_4$ was added to the mixture. After standing for 1 hr at room temperature, the excess of $NaBH_4$ was destroyed by the addition of Amberlite CG-120 (H^+) and the solution was then passed through a column containing the same resin. A few drops of NEt_3 were added to the effluent and washings and the solution was evaporated to dryness under a reduced pressure. By evaporating twice with dry MeOH boric acid was removed. The residue was dissolved in distilled water (3 ml) and a few drops of NEt_3 were added to the solution. After standing for 10 min at 60°, the solution was evaporated to dryness and the residue was dried in a desiccator in vacuum. The dried sample was trimethylsilylated in the usual way.[12] Gas chromatographic conditions are shown in Fig. 8. The measurement was performed by the peak area ratio method.

ii) With Bacterial β-Glucuronidase: Bacterial β-glucuronidase (200 mg; Sigma Chemical Co., Type I, 53000 Fishman units/g) was dissolved in cold distilled water and dialyzed overnight at 4° to remove D-glucuronic acid. VI (1 mg), 0.2M acetate buffer (pH 6.5), and a few drops of toluene were added to the enzyme solution, and the solution was incubated overnight at 38°. The solution was worked up in a similar manner as described in i)—b). D-Glucuronic acid and D-glucaric acid liberated were detected similarly by gas chromatography.[12]

Transformation of VI into 3-O-β-D-Glucopyranosiduronyl-D-glucarate during Incubation at 38°——A mixture of 3 mg of VI dissolved in 10 ml of a buffer solution (0.2M pyridine + 0.2M acetic acid) was incubated at pH 4.5 or 5.5 and 38°. At intervals of time, an aliquot of 1 ml of the solution was taken and the concentration of VI and 3-O-β-D-glucopyranosiduronyl-D-glucarate was determined by the gas chromatographic method[19] reported recently for D-glucaric acid and its lactones. Gas chromatographic conditions were as follows: Column, 2% OV-17 (1.5 m×4 mm i.d.); column temperature, 230°; nitrogen flow, 77 ml/min. The measurement was performed by the peak area ratio method.

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