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Synthesis of Fluorescein Monoglucuronide[1)]

ISAO MATSUNAGA,[a] SHIGETOSHI NAGATAKI,*[,b] and ZENZO TAMURA[c]

New Drug Research Laboratories, Chugai Pharmaceutical Co., Ltd.,[a] 3–41–8 Takada, Toshima-ku, Tokyo 171, Japan, Department of Ophthalmology, School of Medicine, University of Tokyo,[b] 7–3–1 Hongo, Bunkyo-ku, Tokyo 113, Japan and School of Medicine, Keio University,[c] 35 Shinano-machi, Shinjuku-ku, Tokyo 160, Japan

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Fluorescein monoglucuronide (III), the main metabolite of fluorescein (I), was chemically synthesized by means of the Koenigs–Knorr reaction. Nuclear magnetic resonance (NMR) spectra and mass spectra (MS) showed that the synthesized material was monoglucuronidated fluorescein. The changes in absorption spectra with pH indicated that this material was an "ether" glucuronide. The glucuronoside linkage was concluded to be β, since this material was hydrolyzed by β-glucuronidase. Thus, the synthesized material was confirmed to be fluorescein mono-β-D-glucuronide.

Keywords——fluorescein; glucuronidation; β-glucuronidase; ether glucuronide; fluorescein monoglucuronide; Koenigs–Knorr reaction

Fluorescein (I) has been widely used as a tracer to study the blood-ocular barrier properties in human subjects. Problems encountered with the systemic administration of I are that a considerable amount of I is rapidly glucuronidated after administration,[2–4)] and the glucuronidated I still has weak fluorescence which is similar to that of I.[3)] Thus, the fluorescence intensity of glucuronidated I must be differentiated from that of I for studies of the blood-ocular barrier permeability by fluorophotometric techniques, but this is not easy because of the problem of obtaining a pure sample of glucuronidated I.

Chen *et al.*[2,3)] isolated fluorescein monoglucuronide from rabbit and human urine following oral administration of I. However, the extraction of fluorescein monoglucuronide from urinary samples is undoubtedly complex, and the amount that can be extracted is small. Therefore, we have attempted a chemical synthesis of fluorescein monoglucuronide, 6′-hydroxy-3-oxospiro[isobenzofuran-1(3*H*),9′-xanthen]-3′-yl β-D-glucopyranosiduronic acid (III) by using the Koenigs–Knorr reaction. The scheme of the synthesis is illustrated in Chart 1.

Compound I and methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate were stirred in quinoline with freshly prepared silver carbonate to yield fluorescein monoglucuronide tri-*O*-acetate methyl ester, methyl [6′-hydroxy-3-oxospiro[isobenzofuran-1-(3*H*),9′-xanthen]-3′-yl 2″,3″,4″-tri-*O*-acetyl-β-D-glucopyranosid]uronate (II), in about 20—30% yield. Then, the protecting groups in the glucuronic acid moiety were removed by saponification in aqueous potassium carbonate solution at room temperature. The removal of the protecting groups with methanolic potassium hydroxide at room temperature liberated I. Since partial hydrolysis of the synthesized material occurred with hydrochloric acid, neutralization was carried out with Amberlite IR-120 (H^+), and the crude product was purified by elution with chloroform–acetone (1 : 2, v/v) through a Sephadex LH-20 column. Compound III was obtained as a yellow powder. Removal of the solvent by heating (80 °C)

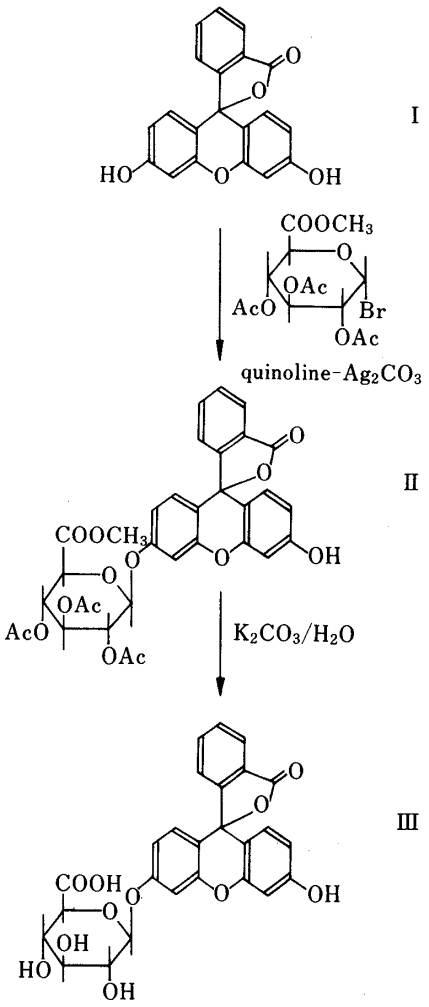

Chart 1

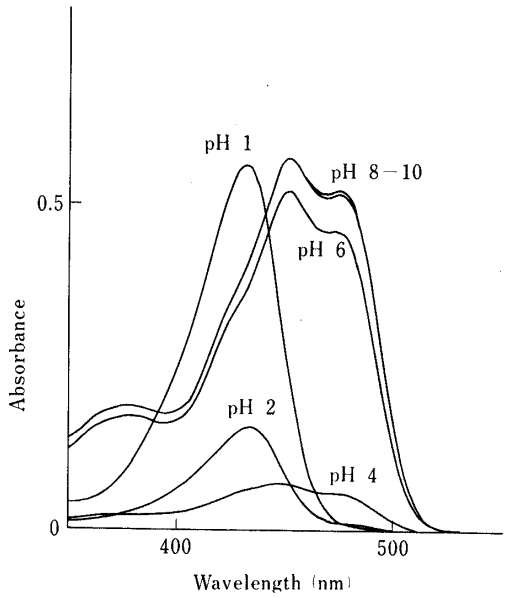

Fig. 1. Absorption Spectra of III
Experimental details are given in the text.

TABLE I. Hydrolysis of III with β-Glucuronidase

		Fluorescence intensity	Hydrolysis (%)
III + β-Gase		77.3	
		79.8	
	Average	78.55	99.6
I + β-Gase		76.9	
		78.5	
	Average	77.7	
β-Gase		8.0	
		8.5	
	Average	8.25	

I: Fluorescite Injection, Alcon Labs. Inc., Lot E-602 β-Gase: β-glucuronidase (260 units, Tokyo Zoki, Type I) Experimental details are given in the text.

for 8 h under reduced pressure decomposed the synthesized material (yielding I and glucuronic acid) to the extent of about 5—10%, so the solvent was removed by freeze-drying.

The nuclear magnetic resonance (NMR) spectra and mass spectra (MS) showed that the synthesized material was monoglucuronidated fluorescein. The absorption spectra of III measured in solutions of various pHs are illustrated in Fig. 1. In strong acid solution, the

absorption spectrum had a maximum at 432 nm, whereas two peaks, at 452 and 475 nm, were observed in solutions of pH 8 and 10. The observed changes in the absorption spectra were consistent with those of monomethylfluorescein described by Chen *et al.*,[5] indicating an "ether" bond between glucuronic acid and I. When the synthesized III was incubated with calf-liver β-glucuronidase, I was detected by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). In addition, the fluorescence intensity of 3.06×10^{-7} M III measured after hydrolysis with β-glucuronidase was almost equal to the intensity of 3.01×10^{-7} M I, as shown in Table I. Thus, the chemical structure of the synthesized material was characterized as fluorescein mono-β-D-glucuronide, identical with the product isolated from urine by Chen *et al.*[2]

Experimental

Melting points were measured with a Yanagimoto micro melting point apparatus. Water content in the synthesized III was determined by Karl–Fischer titration. Optical rotations were measured with a Perkin-Elmer polarimeter, model 241. Solvents were removed in a rotary evaporator under reduced pressure at a temperature below 20 °C. TLC was performed on silica gel plates (Kieselgel 60 F_{245}, Merck) by developing II with $CHCl_3$–AcOEt (1 : 1, v/v), and by developing III with AcOEt–MeOH–H_2O–AcOH (7 : 1 : 1 : 1, v/v). HPLC was carried out on a Shimadzu LC-3A apparatus (μBondapak C_{18} column) with H_2O–MeCN–AcOH (70 : 30 : 5, v/v) at a flow-rate of 2 ml/min; the eluates were monitored at 254 nm. The absorption spectra in the ultraviolet (UV) and visible region were measured with a Shimadzu UV-240-visible recording spectrophotometer. The fluorescence intensity was measured with a Hitachi 650—10(s) spectrophotometer at 492 nm for excitation and at 512 nm for emission; a bandwidth of 4 nm was used. Infrared (IR) spectra were measured on a Hitachi 260-30 spectrophotometer. NMR spectra were recorded on a Hitachi R-24B spectrometer and on a JEOL JNM-FX 100 spectrometer with tetramethylsilane as an internal reference. Mass spectra (MS) were determined on a Shimadzu LKB-9000 spectrometer and a Hitachi M-80 spectrometer.

Methyl [6′-Hydroxy-3-oxospiro[isobenzofuran-1(3*H*),9′-xanthen]-3′-yl 2″,3″,4″-Tri-*O*-acetyl-β-D-glucopyranosid]uronate (II)——I (10 g), methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate (10 g), Ag_2CO_3 (6 g), and Drielite (10 g) were suspended in quinoline (60 ml), and stirred at room temperature for 24 h in a dark room. The reaction mixture was diluted with AcOEt, and after filtration, the organic layer was washed successively with aq. 3 N HCl, aq. $NaHCO_3$, and with H_2O, then dried over anhydrous $MgSO_4$. After evaporation of the solvent, the crude product was purified by elution with $CHCl_3$–AcOEt (4 : 1, v/v) on a silica gel column. Small crystals of II (3.35 g) were obtained from AcOEt–*n*-hexane. mp 188—190 °C. $[\alpha]_D^{23}$ −29.7° (c=0.976, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400, 1745, 1730 (shoulder), 1610, 1240, 1220 (shoulder). UV λ_{max}^{MeOH} nm (log ε): 223 (4.78), 274 (3.87). ^{1}H-NMR (δ in CD_3OD–DMSO-d_6): 2.06 (9H, s, $COCH_3$), 3.73 (3H, s, $COOCH_3$), 4.47 (s, OH), 4.64 (1H, m, CH in sugar moiety), 5.02—5.74 (4H, m, CH in sugar moiety), 6.54—8.14 (10H, m, aromatic H). MS *m/e*: 648 (M^+), 544, 288, 155. *Anal.* Calcd for $C_{33}H_{28}O_{14}$: C, 61.11; H, 4.35. Found: C, 60.66; H, 4.30.

6′-Hydroxy-3-oxospiro[isobenzofuran-1(3*H*),9′-xanthen]-3′-yl β-D-Glucopyranosiduronic Acid (III)——II (3 g) was suspended in aq. K_2CO_3 (3 g in 60 ml H_2O), and stirred overnight at room temperature. After filtration, the saponification product was acidified with dry Amberlite IR-120 (H^+) and extracted with AcOEt. The ion exchange resin was filtered off, and the filtrate was saturated with NaCl, then extracted twice with AcOEt. The extracts were combined and dried over anhydrous $MgSO_4$, then evaporated to dryness. The residue was dissolved in $CHCl_3$–acetone (1 : 2, v/v) and eluted through a Sephadex LH-20 column with the same solvent. The fractions corresponding to the main peak were combined. The solvent was evaporated off, and the residue was dissolved in H_2O and freeze-dried after being passed through a Milipore filter. III (2 g) was obtained as a yellow powder. mp 168—170 °C (dec.). $[\alpha]_D^{23}$ −33.3° (c=0.924, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3700—2300, 1735, 1610. UV λ_{max}^{MeOH} nm (log ε): 223 (4.81), 274 (3.85). ^{1}H-NMR (δ in CD_3OD): 3.4—4.3 (4H, m, CH in sugar moiety), 4.81 (s, OH), 5.09 (1H, dd, J=2, 6 Hz, anomeric H in sugar moiety), 6.52—8.15 (10H, m, aromatic H). ^{13}C-NMR (δ in CD_3OD): 70.9 (d), 72.4 (d), 74.4 (d), 75.1 (d), 83.4 (s), 99.8 (d), 101.6 (d), 103.3 (d), 109.1 (s), 111.7 (d), 112.4 (d), 123.1 (d), 123.8 (d), 125.7 (s), 128.0 (d, ×2), 129.1 (d), 134.6 (d), 151.5 (s), 151.7 (s), 152.2 (s, ×2), 158.2 (s), 158.9 (s), 169.5 (s), 170.0 (s). FD-MS *m/e*: 509 (M^++H), 490 ($M^+ - H_2O$), 333. *Anal.* Calcd for $C_{26}H_{20}O_{11} \cdot nH_2O$ (n=1.72 from 5.73% H_2O determined by Karl–Fischer titration): C, 57.90; H, 4.38. Found: C, 57.68; H, 4.40.

Measurements of Absorption Spectra——The synthesized III was dissolved in MeOH-buffer solutions of various pHs at a concentration of 2.07×10^{-5} M. The MeOH concentration in the solution was 10%, and the buffer solutions were 0.2 M KCl-HCl with pH 1—2, 0.1 M citrate with pH 3—5, 0.1 M phosphate with pH 6—7, and 0.1 M borate with pH 8—10. The absorption spectra are shown in Fig. 1.

Hydrolysis with β-Glucuronidase——The synthesized III was dissolved in 0.4 M acetate buffer (pH 4.5) at a concentration of 1.5×10^{-4} M, and 1300 units of calf liver β-glucuronidase (Tokyo Zoki, Type I) was added. The

solution was incubated at 37 °C for 2 h, then subjected to chromatography. Compound I was detected by TLC and HPLC analysis.

A 0.1 ml (260 units) aliquot of calf-liver β-glucuronidase (×5, Tokyo Zoki, Type I) was added to a mixture of 0.4 ml of 0.2 M acetate buffer (pH 5.0) and 0.2 ml of H_2O containing 3.01×10^{-7} M I or 3.06×10^{-7} M III. The solutions were incubated at 37 °C for 20 min, then diluted with 10.0 ml of 0.2 M carbonate buffer (pH 9.15). The fluorescence intensity values measured at 492 nm for excitation and at 512 nm for emission are listed in Table I.

References and Notes

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