[Chem. Pharm. Bull. 32(8)1759—1763(1975)]

UDC 547.751'483.04 : 542.98

Syntheses of Halogen Substituted Indolyl β-D-Glucuronides and Their Hydrolysis by Rabbit Liver β-Glucuronidase

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(Received December 10, 1974)

I and II were synthesized as their barium salts. Methyl (1-bromo-tri-O-acetyl-α-D-glucoside) uronate was condensed with haloindolyl acetate in dry methanol containing sodium methoxide. Acetylation of crude methyl(haloindol-3-yl-β-D-glucoside)uronate thus obtained, followed by deacetylation and demethylation with aq. alkali acetone, afford the desired haloindol-3-yl-β-D-glucuronides.

The hydrolysis of I and II by rabbit liver β-glucuronidase was investigated. The rate of hydrolysis depends on the nature of the buffer, and decreases in the following order: acetate>citrate-phosphate>phosphate. The optimum pH was 4.75 in acetate buffer and 5.0 in both citrate-phosphate and phosphate buffer. K_m for I and II is 8×10^{-4} M and 5×10^{-3} M, respectively. I was applied to the demonstration of β-glucuronidase in disk electrophoresis.

Halogen substituted indolyl glycosides are useful for the detection of glycosidases in tissues.[2-6] The utility of these substrate derives from the rapid deposition of an insoluble, microcrystalline haloindigo (V, VI) at the sites of activity through oxidation of an enzymically released intermediate haloindoxyl (III, IV). In the histochemical study of β-glucuronidase, the methods using 5-bromo-4-chloroindol-3-yl-β-D-glucuronide (I) have been reported,[7-8] however, no method of synthesis of this substrate has been published. Anderson and Leaback[9] reported that the 2-acetamido-2-deoxy-β-D-glucoside, the β-D-glucoside and the β-D-galactoside of 5-bromoindoxyl were synthesized through 1-acetyl-5-bromoindoxyl and the corresponding acetohalogenosugars, but attempts to prepare the β-D-glucuronide under a variety of conditions were unsuccessful. A recent report from our laboratory described application of 5-bromoindol-3-yl-β-D-glucuronide (II) for the demonstration of β-glucuronidase in disk electrophoresis.[10] In the preceding paper, methyl(1-bromo-tri-O-acetyl-α-D-glucoside)uronate was condensed with 1-acetyl-5-bromoindoxyl in alcoholic potassium hydroxide to give a 4.5% yield of ethyl-(5-bromoindol-3-yl-tri-O-acetyl-β-D-glucoside)uronate, which was converted to required substrate (II) by treatment with aq·alkali acetone. Because of the very low yield of the method, other method was investigated. Methyl(1-bromo-tri-O-acetyl-α-D-glucoside)uronate was condensed with 5-bromoindolyl acetate in dry methanol containing sodium methoxide. Attempts to separate reaction product, methyl(5-bromoindol-3-yl-β-D-glucoside)uronate, were unsuccessful. Moreover, attempts to separate desired (II) by demethylation of the crude product were also unsuccessful. Acetylation was therefore executed on the crude product with pyridine-acetic anhydride, methyl(5-bromoindol-3-yl-tri-O-acetyl-β-D-glucoside)uronate, which was converted to II, was isolated in 20% yield. I was also prepared in a similar manner.

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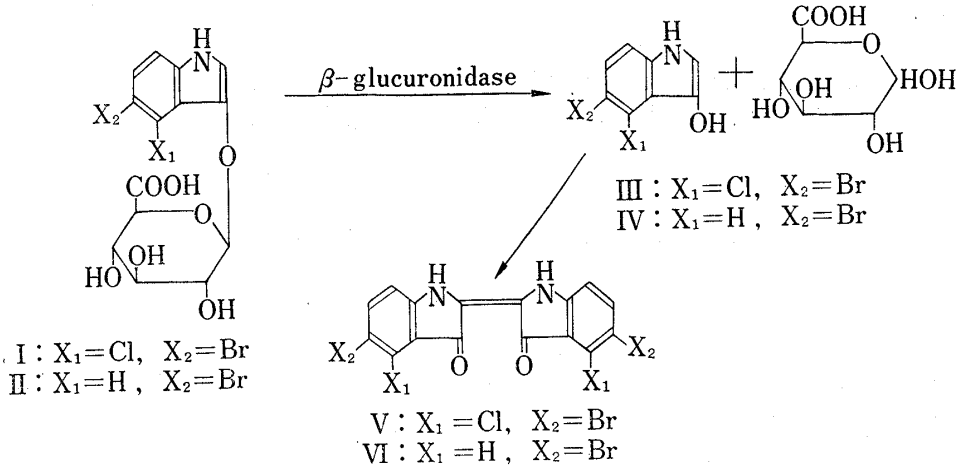

Chart 1

Horwitz and Easwaran[11] reported a kinetics of the hydrolysis of 5-bromo-4-chloroindol-3-yl-β-D-glucoside by almond emulsin β-glucosidase.

The present investigation describes syntheses of I and II and the hydrolysis of both compounds by rabbit liver β-glucuronidase.

Materials and Methods

Methyl(5-bromoindol-3-yl-2,3,4,-tri-O-acetyl-β-D-glucoside)uronate——To 19 ml of a cold (−5°) dry methanol solution containing 0.18 g of sodium was added 2.0 g of 5-bromoindolyl acetate under an atomosphere of nitrogen and the stirred mixture was gassed for 0.5 hr with a stream of nitrogen. The reaction mixture was then cooled to 0° and a solution of 3.1 g of methyl(1-bromo-tri-O-acetyl-α-D-glucoside)uronate in 19 ml of dry methanol was added all at once. The mixture was stirred under an atmosphere of nitrogen for 4 hr, during which time the reaction mixture was allowed to reach room temperature. A stream of air then passed into the mixture for 10 min. The insoluble indigo was removed by filtration, and the filtrate was evaporated. To the gummy residue were added 20 ml of ethyl acetate and 20 ml of water, and the mixture was vigorously shaken. The ethyl acetate layer was then separated, filtered and evaporated. The residue was dried and acetylated with 28 ml of acetic anhydride in 40 ml of pyridine at room temperature. After 2 hr, the reaction mixture was poured into 500 ml of ice water with stirring. The resulting powder was collected, dried, and the product was crystallized from acetone–methanol (charcoal) to give yellow needles, mp 222—224°, yield 0.8 g (20%). The crude product was recrystallized from acetone–methanol to give white needle, mp 226—228°, $[\alpha]_D^{15}$ −67° (c=0.25, chloroform); IR ν_{max}^{Nujol} 3300 (NH) and 1750 cm^{-1} (C=O); NMR ($CDCl_3$) δ: 2.28 (3H, singlet, OAc), 2.01 (6H, singlet, 2OAc), 3.75 (3H, singlet, OCH_3), 6.95—7.38 (3H, multiplet, aromatic protons); Mass Spectrum m/e: 528 (M^+). *Anal.* Calcd. for $C_{21}H_{22}O_{10}NBr$: C, 47.73; H, 4.17; N, 2.65. Found: C, 47.66; H, 4.12; N, 2.21.

Methyl(5-bromo-4-chloroindol-3-yl-2,3,4,-tri-O-acetyl-β-D-glucoside)uronate——Methyl(1-bromo-tri-O-acetyl-α-D-glucoside)uronate was condensed as described above with 5-bromo-4-chloroindolyl acetate to give methyl(5-bromo-4-chloroindol-3-yl-2,3,4,-tri-O-acetyl-β-D-glucoside)uronate in yield of 14%, mp 222°. $[\alpha]_D^{15}$ −118° (c=0.25, chloroform); IR ν_{max}^{Nujol} 3300 (NH) and 1750 cm^{-1} (C=O); NMR ($CDCl_3$) δ: 2.11 (3H, singlet, OAc), 2.03 (6H, singlet, 2OAc), 3.75 (3H, singlet, OCH_3), 6.95—7.40 (2H, multiplet, aromatic protons); Mass Spectrum m/e: 562 (M^+). *Anal.* Calcd for $C_{21}H_{21}O_{10}NBrCl$; C,44.84; H, 3.74; N, 2.49. Found: C, 45.04; H, 3.98; N, 2.67.

Barium 5-Bromoindol-3-yl-β-D-glucuronate——This compound was prepared according to the preceding paper,[10] but methyl (5-bromoindol-3-yl-2,3,4,-tri-O-acetyl-β-D-glucoside) uronate was used instead of ethyl-(5-bromoindol-3-yl-2,3,4,-tri-O-acetyl-β-D-glucoside)uronate.

Barium 5-Bromo-4-chloroindol-3-yl-β-D-glucuronate——To an ice cold acetone (40 ml) containing 0.8 g of methyl (5-bromo-4-chloroindol-3-yl-2,3,4,-tri-O-acetyl-β-D-glucoside) uronate was added 34 ml of ice cold 1N NaOH, and the solution was kept at 0° for 10 min. The solution was adjusted to pH 7.0 by IR-120 (H^+), and then concentrated to about 20 ml under reduced pressure. To this was added a saturated basic lead acetate solution until precipitation was complete. The lead salt was collected by centrifugation, washed with water, and made into a fine suspension in ethanol. The lead was removed by saturation with H_2S and a re-

11) J.H. Horwitz and C.V. Easwaran, *Carbohyd. Res.*, 9, 305 (1969).

sulting PbS was removed, then, the ethanol solution was evaporated to dryness under reduced pressure. To the residue was added 12 ml of water, and the solution was neutralized by saturated $Ba(OH)_2$ solution. The white barium salt of glucuronide was separated immediately, yield 0.5 g, $[\alpha]_D^{15}$ −82° (c=0.1, water). *Anal.* Calcd. for $C_{14}H_{12}O_7NBrCl1/2Ba$: C, 34.36: H, 2.45: N, 2.86. Found: C, 34.29; H, 2.97; N, 2.74.

Spectrophotometric measurements were made with a Hitachi Model EPU-2A spectrophotometer. Rates of hydrolysis of I and II were determined by observing the formation of the indigos, V and VI, at 660 and 600 nm, respectively. Beer-Lambert plots were made according to the procedure of Horwitz and Easwaren.[11]

The procedure of enzymic hydrolysis was as follows: A mixture of 0.8 ml buffer, 0.1 ml of a substrate solution dissolved in 0.5% aqueous polyvinyl alcohol, and 0.1 ml of the appropriately diluted enzyme solution was incubated at 38° for 30 min. At the end of the incubation were added successively 3 ml of 0.4 M glycine buffer (pH 10.45) and 2 ml of 0.5% aqueous polyvinyl alcohol. After standing for 10 min, optical density was estimated.

Disk electrophoresis was performed according to the preceding paper.[10]

Rabbit liver β-glucuronidase was prepared as follows: Rabbit liver was minced and homogenized in 0.25 M sucrose (1: 3 w/v). After removal of nuclei and cell debris by centrifugation at 650 ***g*** for 20 min, the supernatant was centrifuged at 12000 ***g*** for 20 min. The resulting pellet was suspended in 0.025 M sucrose, and the suspension was centrifuged at 105000 ***g*** for 60 min. To one volume of the supernatant was added 1.2 volume of organic solvent mixture (ethanol–acetone–ether, 75: 20: 5, by volume). The mixture was centrifuged at 10.000 ***g*** for 20 min, and the resulting pellet was suspended in 0.02 M phosphate (pH, 7.0). The suspension was centrifuged at 10000 ***g*** for 2 min, the supernatant was used as enzyme solution. Activity was 543 U/mg of protein. One unit of β-glucuronidase was the activity that catalyzed the release of 1 nmole of *p*-nitrophenol/min from *p*-nitrophenyl β-D-glucuronide.

Results and Discussion

Effect of Buffer System

Effect of buffer system on the rate of hydrolysis of I and II is shown in Fig. 1. A comparison of the buffers tested showed that the rate of hydrolysis decreases in the following order: acetate>citrate-phosphate>phosphate.

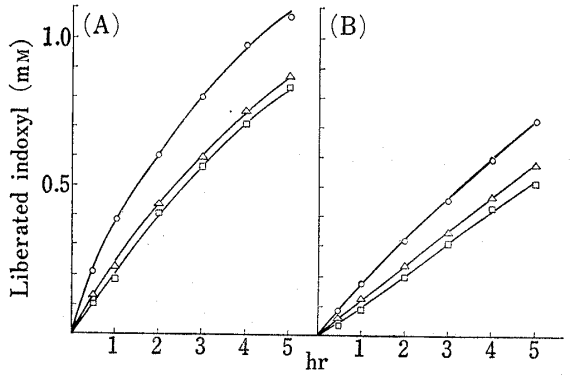

Fig. 1. Rate of Hydrolysis of (A) 5-Bromo-4-chloroindol-3-yl-β-D-glucuronide and (B) 5-Bromoindol-3-yl-β-D-glucuronide in three Buffer Systems

—○— : 0.2M acetate buffer, pH 4.75
—△— : 0.2M citrate-phosphate buffer, pH 5.0
—□— : 0.2M phosphate buffer, pH 5.0

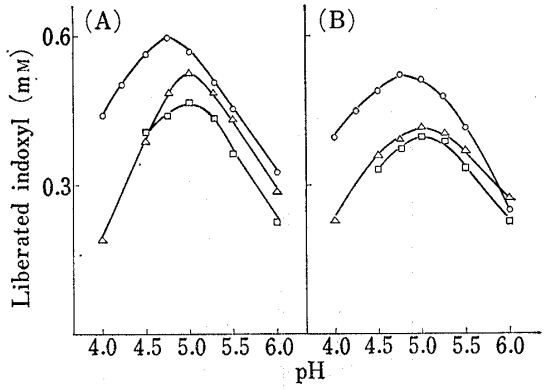

Fig. 2. Effect of pH on the Rate of Hydrolysis of (A) 5-Bromo-4-chloroidol-3-yl-β-D-glucuronide and (B) 5-Bromoindol-3-yl-β-D-glucuronide.

Incubation mixture contained 0.8 ml of buffer, 0.1 ml of 20 mM substrate solution and 0.1 ml of enzyme solution.
—○— : 0.2M acetate buffer
—△— : 0.2M citrate-phosphate buffer
—□— : 0.2M phosphate buffer

Effect of pH

The effects of pH on the rate of hydrolysis of the substrate were determined by use of various buffers ranging from pH 4.0 to 6.0. The results are shown in Fig. 2. For the two substances one-peak activity curves with pH optima between 4.75 and 5.0 were obtained. In acetate buffer the maximal activity was shifted to more acidic region.

Effect of Enzyme Concentration

The hydrolysis of I and II by varying the concentration of rabbit liver β-glucuronidase is shown in Fig. 3. The reaction velocity is proportional to enzyme concentration.

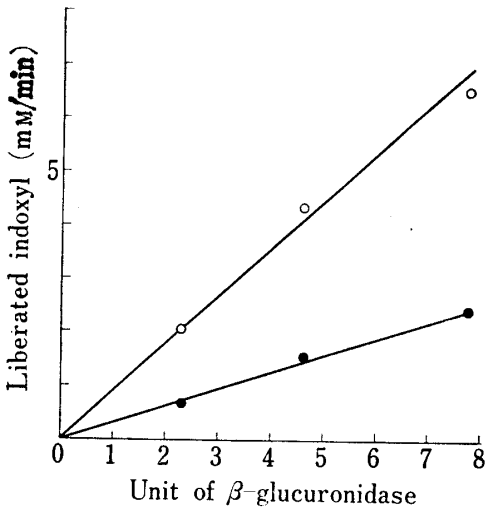

Fig. 3. Effect of Enzyme Concentration on the Hydrolysis of 5-Bromo-4-chloroindol-3-yl-β-D-glucuronide and 5-Bromoindol-3-yl-β-D-glucuronide.

Incubation mixture contained 0.8 ml of 0.2M acetate buffer, pH 4.75, 0.1 ml of 20 mM substrate solution and 0.1 ml of enzyme solution.
—○— : 5-bromo-4-chloroindol-3-yl-β-D-glucuronide
—●— : 5-bromoindol-3-yl-β-D-glucuronide

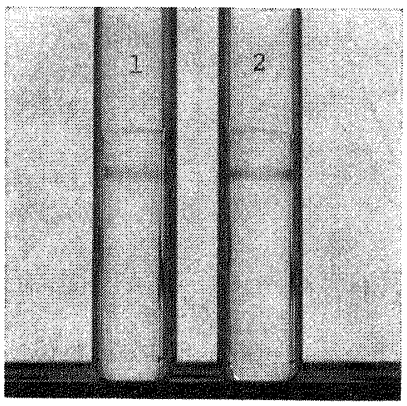

Fig. 5. Photograph of Disk Electrophoresis of Rabbit Liver β-Glucuronidase

1. The developed gel by means of 5-bromo-4-chloroindol-3-yl-β-D-glucuronide.
2. The developed gel by means of 5-bromoindol-3-yl-β-D-glucuronide.

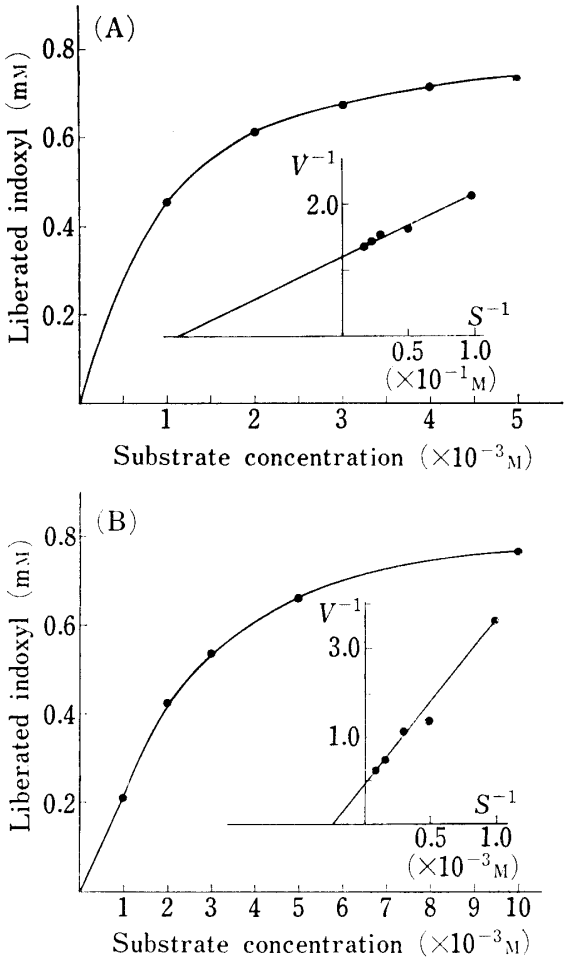

Fig. 4. Effect of Substrate Concentration on the Rate of Hydrolysis of (A) 5-Bromo-4-chloroindol-3-yl-β-D-glucuronide and (B) 5-Bromoindol-3-yl-β-D-glucuronide

Incubation mixture contained 0.8 ml of 0.2M acetate buffer, pH 4.75, 0.1 ml of substrate solution and 0.1 ml of enzyme solution.

Effect of Substrate Concentration

The effects of various substrate concentrations on the reaction rate were studied at the optimal pH of the two substrates in acetate buffer. The results are shown in Fig. 4. The Michaelis constants were obtained from the Lineweaver-Burk plots and are 8×10^{-4}M and 5×10^{-3}M for I and II, respectively.

Application of I to Disk Electrophoresis

According to the preceding paper[10] I was applied to the demonstration of β-glucuronidase in disk electrophoresis. Rabbit liver β-glucuronidase (100 U) was submitted to electrophoresis, and the gels were incubated with I and II, respectively. The results are shown in Fig. 5. In comparison with II, the substrate I showed beautiful blue green bands, corresponding to the enzyme activity, in the gel 2—3 times more rapidly, the visualization reaction was com-

pleted within 10—20 min. Moreover, the blue green band stands out more sharply than in II. Therefore, the results presented here indicated that I is a better reagent than II.

Acknowledgement The authors thank Mr. Tokumitsu Nishiyori for elemental analyses and spectral measurements.