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Preparation of *p*-Nitrophenyl 2-*O*-Acetyl-β-D-glucopyranoside and *N*-Acetyl-β-D-glucosaminidase Activity toward It. (Essential Requirement of 2-Acetamide Group of Substrate for *N*-Acetyl-β-D-glucosaminidase Hydrolysis)

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Paranitrophenyl 2-*O*-acetyl, and 2,3-di-*O*-acetyl-β-D-glucopyranoside (VI, VII) were synthesized in order to investigate the effect of the substitution of the amide-nitrogen of the substrate by oxygen on the *N*-acetyl-β-D-glucosaminidase hydrolysis. Paramethoxybenzylidene was used as an *O*-blocking group for the preparation, and it was found that this blocking group could be easilly removed without the destruction of *p*-nitrophenyl glycoside. The evidence that VI could not be hydrolysed with this enzyme indicated the essential requirement of the amide-nitrogen of the substrate for the enzyme action.

Several *p*-nitrophenyl 2-acylamino-2-deoxy-β-D-glucopyranosides containing several *N*-acyl substituents were synthesized recently in order to investigate the *N*-acyl specificity of *N*-acetyl-β-D-glucosaminidase [EC. 3. 1. 2. 30]. The *N*-acyl specificity of this enzyme was fairly well clarified in a previous study.[1] An attempt to investigate the effect of replacing the amide-nitrogen of the substrate with oxygen on the enzyme action led to the synthesis of 2-*O*-acetyl-β-D-glucopyranoside.

The present report will describe the preparation of *p*-nitrophenyl 2-*O*-acetyl-β-D-glucopyranoside (VI) and *p*-nitrophenyl 2,3-di-*O*-acetyl-β-D-glucopyranoside (VII) as the reference derivative, and the susceptibilty of VI toward the enzymic hydrolysis. *para*-Nitrophenyl glycoside was chosen as a consequence of the previous investigation. The greater reactivity of the 2-hydroxyl group as compared to the other secondary hydroxyl groups has been noted.[3] Therefore, it is most probable that the 2-*O*-acetyl derivative of II results in the reaction of II with a limited amount of acetic anhydride. The selective 2-*O*-acetylation of *p*-nitrophenyl 4,6-*O*-(*p*-methoxybenzylidene)-β-D-glucopyranoside (II) under limited conditions and the chromatographic purification of debenzylidenated products gave a pure *p*-nitrophenyl 2-*O*-acetyl-β-D-glucopyranoside (VI). The location of the acetyl group was confirmed by periodate-oxidation analysis. *para*-Methoxybenzylidene as the *O*-blocking group was used for the preparation because *p*-nitrophenyl glycoside was usually very acid-labile. This new blocking group, which has been reported to be smoothly cleaved under mild acidic conditions,[4] was removed more easily without the destruction of glycoside. The product III substituted with di-*O*-(*p*-methoxybenzylidene) was found in the case of the prolonged treatment.

The synthetic scheme and the compounds synthesized are shown in Fig. 1.

With regard to the enzymic hydrolysis, this sub-

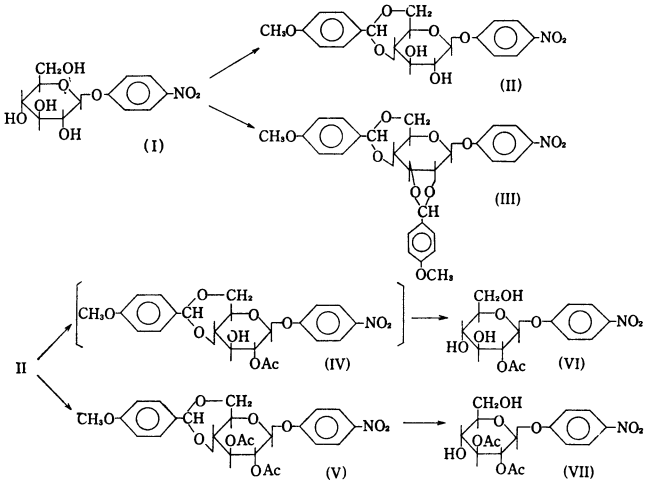

Fig. 1.

1) K. Yamamoto, *J. Biochem.*, **73**, (1973).
2) E. Glaser and W. Wulwek, *Biochem. Z.*, **145**, 514 (1924).
3) J. M. Sugihara, *Ad. Carbohyd. Chem.*, **8**, 1 (1953).
4) S. Chladek and J. Smrt, *Coll. Czech. Chem. Comm.*, **28**, 1301 (1963).

strate VI was not affected by *N*-acetyl-*β*-D-glucosaminidase. This evidence suggests that the –NH– group of the amide bond of 2-acetamide-2-deoxy-D-glucopyranoside is essential for the action of this enzyme.

Experimental

The melting points were determined by a micro-melting-point apparatus (Yanagimoto MFG.), and the optical rotations were measured by means of a recording spectropolarimeter (Yanagimoto MFG, ORD-185).

para-*Nitrophenyl 4,6-O-(p-methoxybenzylidene)-β-D-glucopyranoside (II).* To 50 ml of dimethylformamide solution of (I)[2] (1.0 g) we added 3 ml of anisaldehyde, 6 ml of ethyl-orthoformate, and 2 ml of trifluoroacetic acid.[4] After the reaction had continued for 8 hr at room temperature, the reaction mixture was evaporated *in vacuo* to dryness. The residue, suspended in a cold sodium bicarbonate solution, was collected by filtration. The crystallization of the dried raw product from toluene afforded a crystalline (II) (1.1 g, 79%); mp 190—192°C $[\alpha]_{589}^{20}=-21.7°$ (C, 0.773 in dimethylformamide) Found: C, 57.17; H, 5.08; N, 3.30%. Calcd for $C_{20}H_{21}O_9N$: C, 57.28; H, 5.05; N, 3.34%.

The same reaction mixture as above was kept for 20 hr. at room temperature and subsequently treated in the same manner. The first crystallization of the dried raw products from toluene and methanol gave 0.51 g (27%) of a crystalline product (III), and the mother liquor of III afforded 0.42 g of II upon further crystallization. Mp 288—289°C, $[\alpha]_{589}^{20}=+13.7°$ (*C*, 1.47 in dimethylformamide) Found: C, 62.68; H, 5.14; N, 2.61%. Calcd for $C_{28}H_{27}O_{10}N$: C, 62.56; H, 5.06; N, 2.61%.

para-*Nitrophenyl 2,3-di-O-Acetyl-4,6-O-(p-methoxybenzylidene)-β-D-glucopyranoside (V).* Five hundred and fifty mg of II were acetylated with 10 ml of acetic anhydride in 60 ml of pyridine. The evaporated residue of the reaction mixture was then crystallized from ethanol, thus giving 530 mg (88%) of V. Mp 252—253°C $[\alpha]_{589}^{20}=-5.82°$ (*C*, 0.923 in dimethylformamide). Found: C, 57.49; H, 5.05; N, 2.81%. Calcd for $C_{24}H_{25}O_{11}N$: C, 57.25; H, 5.01; N, 2.78%.

para-*Nitrophenyl 2,3-di-O-Acetyl-β-D-glucopyranoside (VII).* A 400 mg portion of V, dissolved in 45 ml of 66% acetic acid, was heated for 5 min in boiling water, and then the acetic acid solution was evaporated *in vacuo* to a heavy syrup. The crystallization of the heavy syrup from ethanol gave 190 mg (62%) of VII. Mp 183—185°C $[\alpha]_{589}^{20}=-24.9°$ (*C*, 0.553 in dimethylformamide). Found: C, 49.75; H, 5.04; N, 3.62%. Calcd for $C_{16}H_{19}O_{10}N$: C, 49.87; H, 4.97; N, 3.63%. The concentration of the mother liquors gave a further 90 mg (29%) of (VII). Mp 182—183°C.

Selective O-Acetylation of II and Preparation of (VI). A chloroform–pyridine solution (1:1, 60 ml) of II (460 mg) was cooled to −10°C, and to this solution we added 230 mg of acetic anhydride in 10 ml of chloroform, drop by drop, over a 10 hr period. The residue, after the evaporation of the reaction mixture, showed three spots on a thin-layer chromatogram. As the fastest of them corresponded to V and the slowest to II, the middle spot was assumed to be the mono-*O*-acetylated product(IV). The residue was debenzylidenated in 66% acetic acid for 5 min at 100°C; the subsequent evaporation of the hydrolysate gave a heavy syrup. The chromatographic purification of VI from the contaminants was performed on a Bio-gel-P-2 column (2.0×200 cm). The heavy syrup, which had been washed three times with ether, was dissolved in 45 ml of water and then applied to a Bio-gel column (2.0×200 cm) and eluted with 0.01M acetic acid. The effluent was monitored by measuring the absorbance at 300 mu. The main peak, which was assumed to be VI from the thin-layer chromatographic identification, was collected and dried by lyophillization. It afforded 120 mg (33%) of the amorphous solid, which showed $[\alpha]_{589}$ and the following elemental analytical result; $[\alpha]_{589}^{20}=-36.9°$ (*C*, 0.430 in dimethylformamide). Found: C, 48.17; H, 4.99; N, 4.00%. Calcd for $C_{14}H_{17}O_9N$: C, 48.98; H, 4.99; N, 4.08%.

In the periodate oxidation, which was performed in 10 mM of sodium periodate (pH 5.5) at room temperature by measuring the amount of periodate consumed acording to Avigad's method.[5] VI Consumed 1 mole equivalent periodate; its analytical data are shown in Table 1. This evidence indicates that the specimen had the structure of VI.

Table 1. Periodate-oxidation analysis for VI

Compound	Amount for analysis (mol)	$NaIO_4$-Consumption: Moles of $NaIO_4$ consumed	Molar equivalent: observed	Molar equivalent: calculated
VI	2.15×10^{-6}	2.11×10^{-6}	0.98	1.00
	1.07×10^{-6}	1.18×10^{-6}	1.10	1.00
VII	2.08×10^{-6}	0.12×10^{-6}	0.058	0.00
	1.04×10^{-6}	0.00	0.00	0.00

N-Acetyl-β-D-glucosaminidase Activity toward VI. The enzyme specimen and the analytical procedure were the same as that of the previous report.[1]

Two mM of VI in a pH 4.5-citrate buffer were incubated with the enzyme (concentration from 0.5 ppm to 50 ppm) at 37°C for 24 hr. No *p*-nitrophenol liberated from VI could be detected.

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5) G. Avigad, *Carbohyd. Res.*, **11**, 119 (1969).