Note

Synthesis of enzyme substrates*
I. Synthesis of 6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-mannose

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None of the (2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-mannoses have been found free in Nature nor, to the best of our knowledge, have they been isolated from the partial hydrolysis of a glycoprotein, glycolipid, or any other complex polysaccharide. The sequence (2-acetamido-2-deoxy-D-glucopyranosyl)-D-mannose commonly occurs in various types of glycoprotein, including fetuin[1], α_1-acid glycoprotein[2a-c], human chorionic gonadotropin[3] and ovalbumin[4a,b]. The availability of these disaccharides not only will facilitate the structural investigation of such complex carbohydrates by serving as reference compounds, but also provide model compounds for developing methods for their characterization based on mass and n.m.r. spectrometry. Furthermore, the investigation of the enzymes specific for a particular intersugar glycosidic bond involving 2-acetamido-2-deoxy-D-glucose would become possible. Highly specific enzymes of this type, such as (1→2)-α-L-fucosidase[5] and (1→2)-α-D-mannosidase[6], are already known. It may be pointed out that β-*N*-acetylglucosaminidases[7a-c] (E.C. 3.2.1.30) so far reported are specific for the sugar and β-D configuration, irrespective of the nature of the intersugar glycosidic bond.

In the work reported here, 2-methyl-(3,4,6-tri-*O*-acetyl-1′,2′-dideoxy-α-D-glucopyrano-[2′,1′:4,5]-2-oxazoline was condensed with 1,2,3,4-tetra-*O*-acetyl-β-D-mannopyranose. The resulting condensation product on deacylation yielded 6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-mannose. The anomeric configuration of the glycosidic linkage in the disaccharide was confirmed by its hydrolysis with β-*N*-acetylglucosaminidase.

RESULTS AND DISCUSSION

2-Methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glycopyrano)-(2′,1′:4,5)-2-oxazoline (**2**) has been shown to be an effective glycosylating agent[8,9]. The oxazoline **2** was

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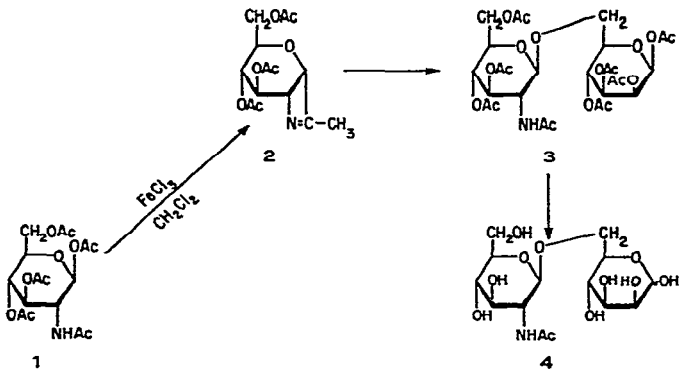

prepared from 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[10] by treatment with silver nitrate and collidine in acetone followed by the removal of collidine by chromatography on neutral alumina[11]. Pravdić, Inch, and Fletcher[12] obtained **2** by the action of acetic anhydride and anhydrous zinc chloride on 2-acetamido-2-deoxy-D-glucose. Recently, Bach and Fletcher[13] have modified their procedure to give **2** in 80% yield by treating 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-glucopyranose[14] (**1**) with anhydrous ferric chloride in dichloromethane.

In the present investigation, the pentacetate **1** prepared by the literature procedure[14] was converted into **2** by the modified procedure of Bach and Fletcher[13]. Condensation of **2** with 1,2,3,4-tetra-*O*-acetyl-β-D-mannose[15] was conducted in a refluxing 1:1 mixture of anhydrous toluene and nitromethane containing a catalytic amount of *p*-toluenesulfonic acid.

The protected disaccharide **3** thus obtained, on deacylation with barium methoxide in methanol[16], yielded 6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-mannose (**4**). The β-linkage of **4** was established by optical rotation, spectroscopically, and enzymically; it was hydrolyzed completely by the β-*N*-acetylglucosaminidase of *Aspergillus niger*[7a].

EXPERIMENTAL

General. — Melting points were determined with a Fisher–Johns apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer Model 141 Polarimeter with sodium light. N.m.r. spectra were recorded with a Varian T-60 spectrometer with tetramethylsilane as reference. I.r. spectra were taken in Nujol by using sodium chloride windows.

T.l.c. was performed on silica gel H and the spray reagent was potassium permanganate and sulfuric acid[17]. Paper chromatography was done on Whatman No. 3 and spots were detected with periodate followed by ammoniacal silver nitrate[18]. The microanalyses were performed by Galbraith Laboratories, Knoxville, Tennessee. The *A. niger* β-*N*-acetylglucosaminidase was purified as described earlier[7a].

*2-Methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-(1′,2′:4,5)-2-oxazo-*

line (2). — To a solution of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-glucopyranose[14] (1, 3.2 g) in dichloromethane (100 ml), anhydrous ferric chloride (1.6 g) was added. The reaction was allowed to proceed for 3 h at room temperature under anhydrous conditions. The reaction mixture was washed with water, dried (sodium sulfate), and evaporated to a colorless syrup[13]. The oxazoline 2 thus obtained was chromatographically indistinguishable from a sample prepared by the procedure of Zurabyan and coworkers[8,9]; ν_{max} 1742 (–OAc) and 1672 cm^{-1} (C=N).

6-O-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-1,2,3,4-tetra-O-acetyl-β-D-mannopyranose (3). — To a mixture of the oxazoline 2 (2.1 g) and 1,2,3,4-tetra-*O*-acetyl-β-D-mannose[15] (2.0 g) in a 1:1 mixture of anhydrous toluene and nitromethane (50 ml) was added *p*-toluenesulfonic acid (10 mg). The mixture was refluxed for 50 min at 110–120°. The light-brown mixture thus obtained was cooled, diluted with anhydrous chloroform (200 ml), washed with cold aqueous sodium hydrogen carbonate, and then water. The organic layer was dried (sodium sulfate), filtered, and evaporated under diminished pressure to a light-brown syrup (3.8 g) that crystallized from chloroform–ether to yield colorless crystals (1 g), $[\alpha]_D^{20}$ −26.3° (*c* 1, chloroform); m.p., 187–190°; homogeneous by t.l.c. (benzene–methanol, 9:1), R_F 0.53; ν_{max} 3400 (–NH), 1750 (–OAc), 1660 cm^{-1} (–CONH–); n.m.r. τ 7.84, 7.88, 7.94 and 8.01 (24 acetyl protons).

Anal. Calc. for $C_{28}H_{39}NO_{18}$: C, 49.63; H, 5.80; N, 2.07. Found: C, 49.44; H, 5.75; N, 2.04.

6-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-D-mannose (4). — A cold solution of 3 (0.8 g) in abs. methanol (100 ml) was treated with methanolic barium methoxide (1 ml, containing 0.038 g of BaO) and kept for 36 h at 4°. The mixture was neutralized with CO_2, filtered, and the filtrate evaporated to a syrup that with ethanol yielded a microcrystalline precipitate of 4 (0.5 g); $[\alpha]_D^{20}$ −8.8° (*c* 1, water), m.p., 128–130° (decomp.). The mother liquor from the crystallization of 3 was evaporated to dryness and deacetylated under similar conditions. The crude material thus obtained was purified by chromatography on a charcoal column (12 × 32 cm) previously washed thoroughly with water. Elution of the column with water (1 liter) followed by elution with 2 and 4% aqueous ethanol (200 and 400 ml, respectively) removed the monosaccharide contaminants. The disaccharide was eluted by 12% ethanol (400 ml); yield, 0.3 g.

The compound obtained either by crystallization or by charcoal-column chromatography showed a single spot by t.l.c. $R_{Mannose}$, 0.44 (*n*-propanol–ethyl acetate–water, 7:5:2); 0.775 (*n*-butanol–acetone–water, 4:5:3) and by paper chromatography; $R_{Mannose}$, 0.37 (*n*-butanol–ethanol–water, 41:19:11) and 0.53 (*n*-butanol–pyridine–water, 6:4:3); ν_{max} 3420–3320 (–OH), 2950 (–NH–), 1650, and 1560 (amide), 880 cm^{-1} (β-glycosidic configuration[19]); n.m.r. spectrum τ 7.93 (N–Ac), τ 4.8 (doublet, $J_{1,2}$ 8.5 Hz, H-1 (see refs. 14, and 20)

Anal. Calc. for $C_{14}H_{25}NO_{11}\cdot H_2O$): C, 41.86; H, 6.78; N, 3.49. Found: C, 41.91; H, 6.54; N, 3.28.

Action of β-N-acetylglucosaminidase on 4. — To a solution of the disaccharide in

0.05M sodium citrate buffer, pH 4.6 (100 μg in 80 μl), was added β-*N*-acetylglucosaminidase[7a] (10 μg in 20 μl of the same buffer), and the digest was incubated for 3 h. The hydrolyzate was freed of salt and protein by passing it through a column of mixed-bed resin, MB-3 (Mallinckrodt). The solution was concentrated to low volume. Examination by t.l.c. and paper chromatography indicated the release of mannose and 2-acetamido-2-deoxy-D-glucose.

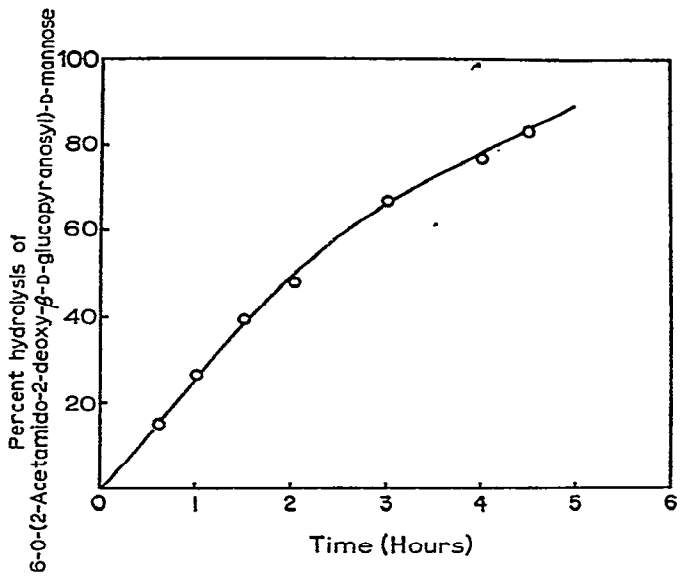

Fig. 1. Hydrolysis of 6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-mannose with β-*N*-acetylglucosaminidase.

In another experiment, a solution of the disaccharide **4** in the foregoing buffer (100 μg in 90 μl) was incubated with the enzyme (5 μg in 10 μl) at 37°. The aliquots of 10 μl were withdrawn at 0.5, 1, 1.5, 2, 3, 4, and 4.5 h time intervals. 2-Acetamido-2-deoxy-D-glucose thus released was estimated by the method of Reissig, Strominger and Leloir[21] (see Fig. 1). The corresponding hydrolysis at these times was 15, 26, 37, 46, 62, 75, and 82%, respectively.

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