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4-METHYLUMBELLIFERYL 2-ACETAMIDO-2-DEOXY-α-D-GLUCOPYRANOSIDE, A FLUOROGENIC SUBSTRATE FOR *N*-ACETYL-α-D-GLUCOSAMINIDASE

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ABSTRACT

Condensation of dimeric 3,4,6-tri-*O*-acetyl-2-deoxy-2-nitroso-α-D-glucopyranosyl chloride with 4-methylumbelliferone gave crystalline 4-methylumbelliferyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-oximino-α-D-*arabino*-hexopyranoside. Acetylation of this adduct, reduction of the resulting crude *O*-acetyloxime with borane in oxolane, and acetylation gave the 3,4,6-tri-*O*-acetyl derivative of 4-methylumbelliferyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**1**). A new sensitive assay of *N*-acetyl-α-D-glucosaminidase (EC 3.2.1.50) is made possible by fluorometric measurement of 4-methylumbelliferone liberated by enzymic hydrolysis of glycoside **1**. Such assays are illustrated by results obtained with enzyme preparations from pig liver and human-blood serum.

INTRODUCTION

N-Acetyl-α-D-glucosaminidase (EC 3.2.1.50) is a glycohydrolase of widespread occurrence in the animal kingdom[1–3]. The catalytic properties of the mammalian enzyme have been studied[4], and an apparently homogeneous preparation from urine has been reported[5]. In cells, the enzyme is localized in lysosomes[4]. Its participation in human catabolism of heparan sulfate is demonstrated by excessive accumulation of this polysaccharide in the Sanfilippo B syndrome, a disease characterized by genetic deficiency of the enzyme[6]. Although synthetic phenyl[1,2] and *p*-nitrophenyl[4] 2-acetamido-2-deoxy-α-D-glucopyranosides have served as convenient test substrates for *N*-acetyl-α-D-glucosaminidase, more sensitive substrates are desirable because of sparse occurrence of this enzyme. Glycosides of 4-methylumbelliferone have been popular substrates for other hydrolases (*e.g.*, refs. 7 and 8), because of easy fluorometric measurement of this substituted phenol, when liberated by enzymic hydrolysis in minute amounts. The synthesis of 4-methylumbelliferyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**1**), and its application as a test substrate for *N*-acetyl-α-D-glucosaminidase are described herein.

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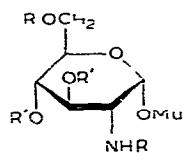

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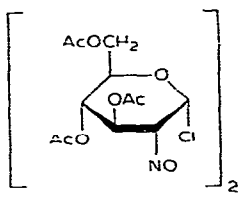

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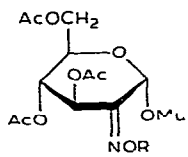

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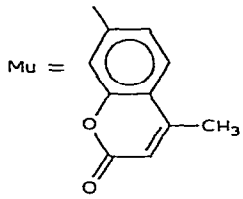

RESULTS AND DISCUSSION

Syntheses of aryl 2-acetamido-2-deoxy-α-D-glucopyranosides have so far been accomplished by direct routes only for the phenyl derivative[2,9]. For example, application of the Koenigs–Knorr[10] or Helferich[9] condensations to *p*-nitrophenol under any useful range of reaction conditions gave only the β-D-glycoside. Although the *o*- and *p*-nitrophenyl α-D-glycosides have been prepared by nitration[9], this route is clearly not general. The present work exploits the condensation of phenols or alcohols with dimeric 3,4,6-tri-*O*-acetyl-2-deoxy-2-nitroso-α-D-glucopyranosyl chloride (**4**), a route developed by Lemieux *et al.*[11,12]. For condensations of **4** in the examples given by these authors, the nonparticipating character of the 2-nitroso group permitted formation of an α-D-glycoside as the principal product; subsequent reduction of the nitroso to an amino group was accomplished stereospecifically.

Following condensation of the nitrosochlorosugar **4** with 4-methylumbelliferone, the desired 4-methylumbelliferyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-oximino-α-D-*arabino*-hexopyranoside (**5**) was isolated by crystallization in 18% yield. Based on the model reactions of Lemieux *et al.*[12] and on present trials, reduction of the oximino to an amino group by borane in oxolane was undertaken *via* the syrupy *O*-acetyloxime **6**. The free amine **2** produced was acetylated without prior isolation to give the crystalline *N*-acetyl derivative **3** in a yield of ~30%, based on **5**. *O*-Deacetylation (75% yield) gave the desired glycoside **1**.

In the foregoing sequence of reactions, reduction of **6** was expected[12] to lead to **3** having the D-*gluco* configuration, as indicated. However, the product isolated might conceivably have been the epimeric 2-acetamido-2-deoxy-D-mannopyranoside. Thus, the final product **1** was hydrolyzed with acid, and the amino sugar liberated, after *N*-acetylation, was shown by paper chromatography to be 2-acetamido-2-deoxy-D-glucose. Also, the initial condensation might have given the β-D, rather than the expected α-D anomer **5**. This possibility was eliminated by comparing the physical constants of the presumed tetraacetyl derivative **3** with those of the anomeric 4-methylumbelliferyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside, for which values of $[\alpha]_D$ −18.9° and −19.2° (chloroform) have been reported[8]. From this comparison, it was clear that **3** as well as **1**, **2**, **5**, and **6** were α-D-glycosides.

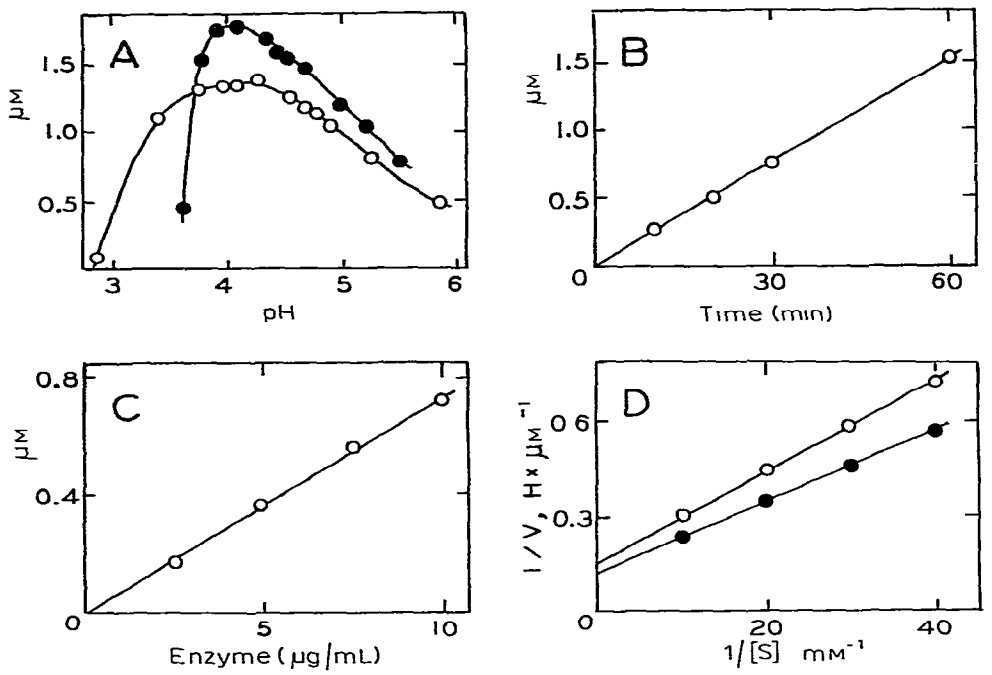

Fig. 1. Effect of variables on the assay of *N*-α-acetyl-D-glucosaminidase, partially purified from pig liver, with the 4-methylumbelliferyl glycoside **1** as substrate. Except as noted, 0.1mM **1** in 50 μL of sodium citrate buffer (ionic strength 0.05 and pH 4.3), in the presence (●) or absence (○) of 0.13M sodium chloride was incubated for 30 min at 25° with enzyme concentrations of 20 (A), 10 (B), or 50 mg/L (D): A, variation of pH; B, variation of incubation time; C, variation of enzyme concentration; and D, variation of substrate concentration.

The use of the new glycoside **1** as a test substrate for *N*-acetyl-α-D-glucosaminidase is illustrated by the results shown in Fig. 1 for a partially purified enzyme preparation from pig liver. At the ionic strength of 0.05, the region of pH values for substantial enzymic activity is broad, with an optimum at about pH 4.3. Addition of 0.13M sodium chloride shifted the optimum to pH 4.0 and stimulated the activity. For the standard conditions of assay adopted, product formation increased linearly with incubation time or enzyme concentration. A value of K_m of 0.100mM was calculated from the Lineweaver–Burk plot for the ionic strength of 0.05.

As mentioned in an earlier report[4], enzymic activity is significantly stimulated by addition of an electrolyte. At pH 4.3, addition of sodium chloride gave the following velocities relative to the velocity (1.00) observed for digests containing only buffer of ionic strength 0.05:

Sodium chloride (M)	0.13	0.23	0.45	0.95
Velocity	1.24	1.46	0.92	0.05

For digests containing 0.13M sodium chloride, the value of 0.101mM for K_m was calculated from the data shown in Fig. 1D, indistinguishable from K_m observed in the absence of salt. The salt effect is, thus, attributable exclusively to an increase of maximal velocity.

The efficiency and sensitivity of assays with the fluorogenic substrate **1** may be compared to those with the *p*-nitrophenyl glycoside by use of data obtained for the pig liver enzyme. For the *p*-nitrophenyl glycoside, K_m of 0.30mM had previously been measured[4] at 37° and pH 4.8. From this value and present comparative assays, the maximal velocity for the new substrate **1** at 25° and pH 4.3 was ~75% of that for the *p*-nitrophenyl glycoside at 37° and pH 4.8. Although the two substrates thus have similar efficiency, the fluorogenic substrate is far more sensitive. Under the conditions described, the amount of any given enzyme preparation required for the fluorometric assay is ~1/20 of that required for the colorimetric assay for similar incubation interval and accuracy. Because the values for substrate and reagent blanks are comparatively small, the advantage can be pursued further in case of need, without too much loss of accuracy.

Since the improved sensitivity of this method may be advantageous for the study of some clinical problems (*e.g.*, see refs. 6 and 13), a preliminary examination of *N*-acetyl-α-D-glucosaminidase in human-blood serum was undertaken with the substrate **1**. The effects of variables on assays are illustrated in Fig. 2. The pH optimum is similar to that for the pig liver enzyme, pH 4.3. The value of K_m, calculated from the measurements in Fig. 2D, is 47μM. Under the standard conditions presently used, an assay value of 94 mU/L was found for the sample of blood serum in the fluorometric assay. Data in the literature for human plasma permit calculation of the value 300 mU/L for assays with the *p*-nitrophenyl glycoside substrate[14].

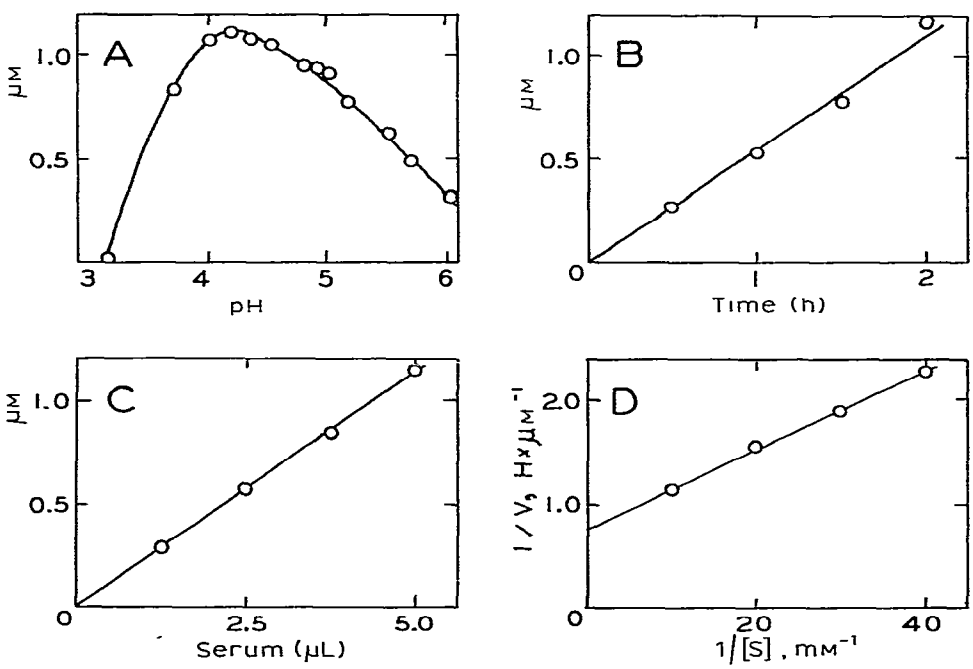

Fig. 2. Effect of variables on the assay of *N*-acetyl-α-D-glucosaminidase in human-blood serum. Except as noted, 5 μL of serum was present in each 50-μL digest, the incubation time was 2 h, and other conditions were as described in the legend to Fig. 1: A, variation of pH; B, variation of incubation time; C, variation of serum concentration; and D, variation of substrate concentration.

In view of the high concentration of protein in the serum digests and the earlier observation of an endogenous inhibitor in crude tissue-extracts[4], it was necessary to demonstrate absence of interference from this source. That such inhibition effects cannot be important under the present circumstances was indicated by the linear increase in product formation with amount of serum added (Fig. 2C). Moreover, assays of purified pig-liver enzyme and of serum, separately and in mixture, gave additive results. The kinetics observed with dialyzed serum are identical to those shown in Fig. 2 for untreated serum, if allowance is made for dilution during dialysis.

EXPERIMENTAL

Materials and general methods. — 3,4,6-Tri-*O*-acetyl-2-deoxy-2-nitroso-α-D-glucopyranosyl chloride dimer was purchased from Raylo Chemicals Ltd., Edmonton, Alberta (Canada). *p*-Nitrophenyl 2-acetamido-2-deoxy-α-D-glucopyranoside was prepared as described[9]. Borane–oxolane was obtained as a M solution of borane in oxolane from Aldrich Chemical Co., Inc. (Milwaukee, WI 53233). Oxolane was distilled in the presence of lithium aluminum hydride before use. *N,N*-Dimethylformamide was dried and distilled in the presence of phosphorus pentaoxide and stored over Linde molecular sieve 4A. Reagent-grade chloroform containing ethanol as preservative was used as a solvent for polarimetry, where indicated Silica Gel used for column chromatography was Grade 923 (100–200 mesh) from the Davison Chemical Division of W. R. Grace and Co. (Baltimore, MD 21203).

Melting points, determined by the capillary method, are uncorrected. Optical rotation was measured with a Perkin–Elmer Model 141 polarimeter for solutions in 1-dm cells. Elementary analyses were performed by Micro-Tech Labs. (Skokie, IL 60076). Paper chromatograms on Whatman No. 1 paper impregnated with borate buffer[14] were developed by downward irrigation with 6:4:3 (v/v) 1-butanol–pyridine–water and stained with a silver reagent[16].

4-Methylumbelliferyl 3,4,6-tri-O-acetyl-2-deoxy-2-oximino-α-D-arabinohexopyranoside (**5**). — A solution of 4-methylumbelliferone (1.59 g) and nitrosochloro sugar **4** (2.03 g) in dry *N,N*-dimethylformamide (10 mL) was stored for 5 days at room temperature. The light-brown mixture was diluted with chloroform (100 mL), washed three times with 50-mL portions of cold 0.1M sodium hydroxide, washed five times with 10% sodium sulfate solution, and dried (sodium sulfate). Evaporation of the solvent *in vacuo* and crystallization of the residue from ethanol–water, with seeding, gave the pure oximino glycoside **6** (0.50 g, 18%), m.p. 172–173°, $[\alpha]_D^{25}$ +100.6° (*c* 0.54, chloroform).

Anal. Calc. for $C_{22}H_{23}NO_{11}$: C, 55.54; H, 4.86; N, 2.93. Found: C, 55.31; H, 4.99; N, 2.84.

Seed crystals were obtained from an earlier reaction mixture after chromatography on silica gel with 2:1 (v/v) ethyl acetate–hexane as eluent. When operations were conducted on a five-fold scale, yields were greatly diminished.

4-Methylumbelliferyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyrano-

side (3). — The oximino glycoside **5** (1.58 g) was dissolved in a mixture of acetic anhydride (4 mL) and pyridine (20 mL). After 24 h at room temperature, the solvents were removed *in vacuo*, leaving the amorphous *O*-acetyloxime **6** (1.93 g). To a stirred solution of this residue in dry oxolane (10 mL), maintained at −5° in an argon atmosphere, was added M borane in oxolane (20 mL) dropwise over a period of 5 min. The mixture was kept for 24 h at room temperature, methanol was added to destroy the excess of borane, and the solvents were removed *in vacuo* The residue, presumably the amine **2**, was acetylated at room temperature with acetic anhydride (8 mL) and pyridine (20 mL). After 24 h, the solvents were removed *in vacuo*. The residue crystallized from ethanol to give **3** (0.51 g, 30%), m.p. 192–192.5° (dec.), $[\alpha]_D^{25}$ +195.5° (*c* 0.65, chloroform).

Anal. Calc. for $C_{24}H_{27}NO_{11}$: C, 57.02; H, 5.38; N, 2.77. Found: C, 56 91; H, 5.48; N, 2.63.

4-Methylumbelliferyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**1**). — For *O*-deacetylation, a solution of **3** (0.25 g) in chloroform (2 mL) was boiled briefly on a hot-plate to expel moisture, supplemented with dry methanol (1 mL) and, when the solution boiled again, with 3M sodium methoxide (10 μL). After being boiled for 3 min longer, the solution, when allowed to cool, rapidly deposited colorless, fluffy needles of **1** (0.14 g, 75%), m.p. 224.5–225°, $[\alpha]_D^{25}$ +257° (*c* 0.1, water).

Anal. Calc. for $C_{18}H_{21}NO_8$: C, 56.99; H, 5.58; N, 3.69. Found: C, 56.87; H, 5.66; N, 3.59.

To verify the amino sugar component, a sample (5 mg) of **1** was heated for 22 h at 100° in a sealed tube with 4M hydrochloric acid (0.5 mL). The solution was evaporated to dryness over sodium hydroxide in a vacuum desiccator. The residue, dissolved in M potassium hydrogencarbonate (0.4 mL) and methanol (0.4 mL), was treated for 24 h with acetic anhydride (10 μL) at room temperature. Cations were removed by passage through Dowex 50 (H^+) cation-exchange resin and washing with water. The pooled effluent was concentrated to a volume of 1 mL. Paper chromatography of this solution revealed a single spot, which migrated identically to a standard of 2-acetamido-2-deoxy-D-glucose (R_{GlcNAc} 1.00; 2-acetamido-2-deoxy-D-mannose showed R_{GlcNAc} 0.65 in this system).

Enzyme assays. — Digests (2 mL) with mM *p*-nitrophenyl 2-acetamido-2-deoxy-α-D-glucopyranoside in 50mM sodium citrate buffer of pH 4.8 (ionic strength ~0.10) were incubated for 2 h at 37°, as previously described[4]. Generally, assays with 4-methylumbelliferyl 2-acetamido-2-deoxy-α-D-glucopyranoside (*cf.* refs. 8 and 17) were performed at a substrate concentration of 0.1mM in sodium citrate buffer (50 μL) having ionic strength 0.05 and pH 4.3, and containing bovine-serum albumin (0.5 mg/mL). After incubation for 30 min (liver enzyme) or 120 min (serum) at 25.0°, 10mM sodium glycinate buffer (pH 10.1, 3.50 mL) was added to each digest, control, μM 4-methylumbelliferone standard, and reagent blank. The fluorescence was measured without delay in a Turner Model 110 filter fluorometer with 360-nm primary and 480-nm secondary filters (Wratten 2A plus No. 48). In either assay, one unit (U) of enzyme liberated 1 μmol of substituted phenol per min under the

assay conditions specified. The pH values reported are in all cases those measured at 25° in complete digests. In the experiments with blood serum, the pH values observed before addition of the serum sample were some 0.2 pH unit lower than those for the complete digests.

Sources of enzyme. — Human-blood serum was used without purification. Blood freshly drawn from a normal, adult male was permitted to clot, and then centrifuged. A portion of the supernatant serum was reserved, and the remainder was dialyzed overnight against mM sodium phosphate buffer (pH 7.3) Both samples were stored at −18°. Pig liver *N*-acetyl-α-D-glucosaminidase was partially purified. A homogenate of frozen-fresh tissue in an equal volume of water was centrifuged. The supernatant extract was fractionated with ammonium sulfate. The fraction collected at 0.20–0.42 saturation was subjected to gel chromatography on Sephadex G-200. The most active fractions were pooled, concentrated by precipitation with ammonium sulfate, dialyzed against 10mM sodium citrate buffer (pH 6), and stored at −18°. This preparation had a specific activity of 6.5 mU/mg of protein with the *p*-nitrophenyl glycoside substrate, and 2.26 mU/mg of protein with the 4-methylumbelliferyl glycoside **1**.

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