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A Novel Chromogenic Substrate for Assaying Glucocerebrosidase Activity (39546)

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The recently synthesized phosphorylcholine derivative of 2-hexadecanoylamino-4-nitrophenol which structurally resembles the naturally occurring glycolipid, sphingomyelin, was employed as a substrate for the catabolic enzyme sphingomyelinase (sphingomyelin phosphodiesterase, EC 3.1.3.12) (1, 2). Displaying a sufficient specificity, this synthetic substrate analog was successfully used in the enzymatic diagnosis of Niemann-Pick disease, a familial metabolic disorder characterized by a deficiency of sphingomyelinase (3).

Based on this successful application, it was of interest to investigate the properties of β-D-glucopyranoside and β-D-galactopyranoside derivatives of 2-hexadecanoylamino-4-nitrophenol. These two glycosides are analogs of glucocerebroside and galactocerebroside. These glycolipids accumulate in two metabolic disorders, Gaucher's disease and Krabbe's disease, due to deficiencies in the enzymes that normally degrade them, glucocerebroside-β-glucosidase (4) and galactocerebroside-β-galactosidase, respectively (5).

With the synthesis of 2-hexadecanoylamino-4-nitrophenyl-β-D-galactoside, the first synthetic substrate for galactocerebrosidase with the required specificity was developed. It was shown that this galactoside was readily hydrolyzed by tissue extracts such that its breakdown reflected the respective levels of galactocerebroside-β-D-galactosidase in the presence of other β-galactosidases. Recently, it was used in the clinical diagnosis and carrier detection of Krabbe's disease (6).

The present study describes the synthesis and the enzymatic hydrolysis of 2-hexadecanoylamino-4-nitrophenyl-β-D-glucopyranoside and compares its properties to other substrates of glucocerebrosidase.

Materials and methods. 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide was purchased from Sigma Chemical Co. Cutscum was obtained from Fisher Chemical Co. Sodium taurocholate was purchased from Difco or Sigma Chemical Co. Silica gel G plates were supplied by Analtech, Inc. The compounds on the layer were visualized by charring with ammonium bisulfate according to Gal (7). Optical rotations were measured with a Perkin-Elmer 141 polarimeter at the 589-nm sodium line. Infrared spectra were obtained with a Perkin-Elmer 621 infrared grating spectrophotometer on KBr disks (1.0 mg of sample/300 mg of KBr). Visible absorption was measured on a Beckman ACTA MVI spectrophotometer. Melting points were taken on a Thomas-Hoover melting point apparatus and are corrected.

Enzymatic hydrolysis. Routine assays of β-glucosidase activity with 3 m*M* hexadecanoylamino-nitrophenyl-glucopyranoside were incubated in 50 m*M* citrate-phosphate buffer, pH 6.0, with 0.84% Cutscum and tissue extract in a final volume of 0.2 ml. Following incubation at 37° for 1 hr, the reaction was terminated by the addition of 0.1 ml of 1.0 *M* glycine-NaOH, pH 10.5. The suspension was clarified by the addition of 1.0 ml of ethanol followed by centrifugation at 2000*g* for 10 min. The clear supernatant was read at the absorption maximum of 415 nm. The extinction coefficient of the hydrolyzed product, the sodium salt of 2′-hydroxy-5′-nitrohexadecananilide, at this wavelength was determined to be 15,000. Glucocerebrosidase (8), 4-methylumbelliferyl-β-glucosidase (9) and *para*-nitrophenyl-β-glucosidase (10) were assayed as previously described.

Human spleen homogenates were prepared as 20% extracts (w/v) of previously frozen tissue in 20 m*M* citrate-phosphate buffer, pH 6.0, with 0.2% Cutscum and 1% sodium taurocholate. Following homogenization in a Waring Blendor, the suspension was centrifuged at 49,000*g* for 15 min and

the clear supernatant was used in the above assays.

The synthesis of 2-hexadecanoylamino-4-nitrophenyl-β-D-glucoside [3]. (Fig. 1).

2-Hexadecanoylamino-4-nitrophenyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside [2]. The commercially available 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide [1] was purified by washing an etheral solution of this compound (15×, v/w) three times with 1 *N* sodium bicarbonate. After drying for 24 hr over anhydrous calcium chloride, the solvent was removed. The residue was recrystallized from (15×, v/w) of isopropyl ether; mp, 88–89°.

The bromide, [4] 8.22 g (20 mmoles), and the sodium salt of 2′-hydroxy-5′-nitrohexadecananilide (1, 2), 8.28 g (20 mmoles), were dissolved in 200 ml of acetone. After 6 hr at 25° the solution was refluxed for 24 hr and filtered from sodium bromide. The yellowish filtrate containing about 6% unreacted sodium salt was acidified with 0.2 ml of 6 *N* hydrochloric acid and evaporated. The residue was partially dissolved in 200 ml of ether and filtered from the starting material. The precipitates were washed with 20 ml of ether and the volume of the filtrate was reduced to about 50 ml and again filtered. The ether was evaporated and the residue was recrystallized from 500 ml of ethanol. Yield of [2], 9.4 g (65%); mp, 94–95°; $[\alpha]_D^{25} = -57.5°$ (*c* 1.0 in acetone). On thin-layer chromatography in chloroform–methanol (99:1) it had $R_f = 0.49$ (acetobromoglucose had $R_f = 0.65$; compound [1] had $R_f = 0.22$).

Anal. Calcd for $C_{36}H_{54}N_2O_{13}$ (722.84): C, 59.82; H, 7.53; N, 3.87. Found: C, 59.51; H, 7.60; N, 3.94.

The above described reaction was repeated with 50% excess of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide. The yield could not be improved.

2-Hexadecanoylamino-4-nitrophenyl-β-D-glucopyranoside [3]. To 2-hexadecanoylamino-4-nitrophenyl-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside [2], 7.22 g (10 mmoles), in 250 ml of distilled tetrahydrofuran was added 6 ml of a 0.5 *N* solution of sodium methylate in methanol (3 mmoles). The solution was kept for 1 day at room temperature. It was then stirred with 10 g of Dowex 50W-X8 (100–200 mesh) ion-exchange resin for 1 hr and filtered. The resin was washed with an additional 20 ml of tetrahydrofuran. The solvent was evaporated and the residue was recrystallized from 125 ml of methanol. Yield, 4.5 g (81%); mp, 148–149°. One gram of this product was recrystallized from 100 ml of methanol to which about 10 μl of 1 *N* hydrochloric acid was added (for discoloration). The yield was 700 mg; mp, 152–153°; $[\alpha]_D^{25} = -59.3°$ (*c* 1.0 in acetone). The infrared spectrum displayed bands at 3400 (O–H stretch), 2905, 2850 (alkane), 1670 (amide), 1520,1335 (nitro), 1200 (phenol, weak), and 1070 cm^{-1} (primary alcohol). Thin-layer chromatography was carried out in chloroform–methanol–water (50:10:1); the compound had $R_f = 0.4$.

Anal. Calcd for $C_{28}H_{46}N_2O_9$ (554.69): C, 60.63; H, 8.36; N, 5.05. Found: C, 60.43; H, 8.35; N, 4.93.

A 2 m*M* solution of [3] in acetone buffer (1:1) solution showed less than 3% decomposition during 24 hr (25°) between pH 2 and 10. (potassium carbonate, borate, and hydroxide and potassium chloride–hydrochloric acid buffers, concentration: 5 m*M*). When [3] was heated with 2 *N* sodium hydroxide–acetone (1:1) for 2 hr at 100°, the sodium salt of 2′-hydroxy-5′-nitrohexadecananilide was formed displaying λ_{max} at 415 nm (ϵ=15,000) (2).

Results and discussion. The 2-hexadecanoylamino-4-nitrophenyl-β-D-glucoside was

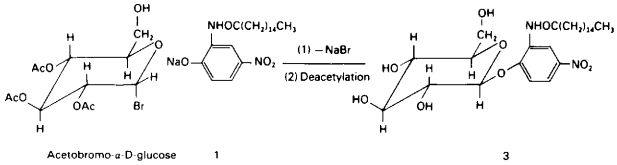

FIG. 1. Formulae and flow diagram for the synthesis of 2-hexadecanoylamino-4-nitrophenyl-β-D-glucopyranoside.

hydrolyzed by crude human spleen extracts or by highly purified human placental glucocerebrosidase (8) at a pH optimum of 6.0. The kinetic characterization of this hydrolysis is summarized in Table I. This catalyzed hydrolysis is specific for human β-glucosidase since bitter almond β-glucosidase will not hydrolyze the glucoside (Table II). In contrast two other synthetic glucosides, 4-methylumbelliferyl-β-D-glucoside and *para*-nitrophenyl-β-D-glucoside which have been employed for the assay of glucocerebrosidase, lack this specificity since they are actively hydrolyzed by the plant enzyme (Table II). With this newly synthesized β-glucoside we can detect and quantitate the deficiency of glucocerebrosidase in Gaucher spleen (Table III). Studies are underway to investigate the action of leukocyte and cultured fibroblast extracts on this substrate in order to test its application further in the clinical diagnosis and carrier detection of Gaucher's disease.

2-Hexadecanoylamino-4-nitrophenyl-β-D-glucoside represents a newly synthesized glycoside which can be used in the assay of glucocerebrosidase. Although not as active as the natural substrate, it can be employed more readily because of the easily detectable chromogenic product formed as a result of its enzymatic breakdown. Its specificity over other synthetic β-glucosides favors its use in the diagnosis of Gaucher's disease.

TABLE I. KINETIC ANALYSIS OF 2-HECADECANOYLAMINO-4-NITROPHENYL-β-D-GLUCOPYRANOSIDE AND GLUCOCEREBROSIDE HYDROLYSIS BY PURIFIED HUMAN PLACENTAL GLUCOCEREBROSIDASE.

Parameter	Synthetic glucoside	Glucocerebroside
K_m (mM)	2.74	0.065
Turnover number (μmoles/hr/mg protein)	16	1000
pH optimum	6.0	6.4

TABLE II. HYDROLYSIS OF β-GLUCOSIDES BY HUMAN AND PLANT β-GLUCOSIDASE.

Substrate	Placental glucocerebrosidase (μmoles hydrolyzed/hr/mg protein)	Bitter almond β-glucosidase (μmoles hydrolyzed/hr/mg protein)
2-Hexadecanoylamino-4-nitrophenyl-β-D-glucoside	16	0
β-D-Glucosylceramide	400	0
4-Methylumbelliferyl-β-D-glucoside	20	15
para-Nitrophenyl-β-D-glucoside	40	25

TABLE III. HYDROLYSIS OF 2-HEXADECANOYLAMINO-4-NITROPHENYL-β-D-GLUCOSIDE (HNG) BY NORMAL AND GAUCHER SPLEEN EXTRACTS.

Spleen sample	Glucocerebrosidase[a] (nmoles/mg of protein/hr)	(% of control)	HNGase[b] (nmoles/mg of protein/hr)	(% of control)
Normal	43.0	–	0.72	–
Normal	41.0	–	0.75	–
Gaucher	3.8	9.0	0.12	16.3
Gaucher	3.8	9.0	0.05	6.8
Gaucher	4.0	13.5	0.11	14.9
Gaucher	3.9	9.2	0.07	9.5

[a] Assays performed with 20 μl (0.20 mg of protein) of spleen homogenate.

[b] Assays performed with 100 μl (1.0 mg of protein) of spleen homogenate.

Summary. 2-Hexadecanoylamino-4-nitrophenyl-β-D-glucopyranoside was synthesized. This compound structurally resembles glucocerebroside and is a specific substrate for glucocerebrosidase. This specificity offers advantages over other artificial substrates and the compound can readily be used in the detection of enzyme deficiency in extracts of spleens of patients with Gaucher's disease.

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