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The use of glycosides of 6- and 8-acylamino-4-methylumbelliferone in studies of the specificity and properties of human lysosomal glycolipid hydrolases

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ABSTRACT

A series of 6- and 8-acylamino-4-methylumbelliferyl β-D-galactopyranosides, β-D-glucopyranosides, and α-L-fucopyranosides having various fatty acid residues were synthesized; 6- (**9**) and 8-hexadecanoylamino-4-methylumbelliferyl β-D-galactopyranoside (**10**) were shown to be substrates for human galactocerebrosidase. Analogs of **9** with shorter acyl residues (octanoyl and butanoyl) were substrates for another type of β-D-galactosidase, *i.e.*, G_{M1}-ganglioside-β-D-galactosidase. The specificity of various β-D-galactosidases for synthetic D-galactopyranosides, differing in the length and position of their acylamide residue, tested with enzyme preparations from patients with two types of glycolipidosis, Krabbe's disease (galactocerebrosidase deficiency) and G_{M1}-β-galactosidase deficiency), suggested that **9** is a specific substrate for galactocerebrosidase in biochemical tests for Krabbe's disease.

Fluorogenic 6-octanoyl- and 6-hexadecanoyl-amino-4-methylumbelliferyl β-D-glucopyranoside were much less readily hydrolyzed by both human and animal glucocerebrosidase than chromogenic 2-hexadecanoylamino-4-nitrophenyl β-D-glucopyranoside.

Comparison of the hydrolysis of 4-methylumbelliferyl α-L-fucopyranoside with that of 6-hexadecanoylamino-4-methylumbelliferyl α-L-fucopyranoside by multiple forms of human α-L-fucosidase showed that the enzyme is capable of hydrolyzing not only hydrophilic but also synthetic, lipid-like substrates.

INTRODUCTION

Glycolipid hydrolases are lysosomal enzymes that split sequentially the carbohydrate component of glycolipids[1,2]. Hereditary deficiencies of one of these enzymes result in glycolipidoses, which constitute the largest group of human lysosomal storage diseases[2–5].

In the present study, the substrate specificity of four glycolipid hydrolases, *i.e.*, galactocerebrosidase (β-D-galactosyl-*N*-acylsphingosine galactohydrolase, EC 3.2.1.46), G_{M1}-ganglioside-β-D-galactosidase (EC 3.2.1.23), glucocerebrosidase (β-D-glucosyl-*N*-acylsphingosine glucohydrolase, EC 3.2.1.45), and α-L-fucosidase (EC 3.2.1.51) was investigated. Deficiency of these enzymes results in the following glycolipidoses, respectively, Krabbe's disease[6], G_{M1}-gangliosidosis[7], Gaucher's disease[8], and fucosidosis[9], where L-fucoglycolipids accumulate along with L-fucosyloligosaccharides derived from glycoproteins[9,10].

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Galactocerebrosidase hydrolyzes galactocerebroside, monogalactosylglycerides, galactosylsphingosine, and lactosylceramide. The susceptible D-galactopyranosyl group in these substrates is either directly linked to the lipid component or is not futher than one monosaccharide residue (lactosylceramide) from this component. Galactocerebrosidase is not very active on the terminal D-galactosyl group of glycolipids having a longer carbohydrate chain, of glycoproteins, and of proteoglycans[2,3,6,11]. These compounds are substrates for G_{M1}-ganglioside-β-D-galactosidase[7]. However, there is some evidence that human G_{M1}-ganglioside-β-D-galactosidase may also cleave D-galactosyl groups from galactocerebroside and, to a significantly lesser degree, from galactosylsphingosine[12–14]. The natural substrate for glucocerebrosidase is glucocerebroside which is normally present in minor amounts in human spleen and liver, and is also formed by enzymic hydrolysis of gangliosides and other glycosphingolipids[4,8,15]. Besides glucocerebroside, the enzyme is able to split glucosylsphingosine and β-D-glucosides of steroids[16].

Radioactive or fluorescent labeling of the carbohydrate or lipid parts of natural substrates has been used for determining the enzymic activity[4,17–20], but the isolation and modification of natural substrates is a rather complicated process. The utilization of commercially available 4-methylumbelliferyl β-D-glucopyranoside and β-D-galactopyranoside is restricted[3,11,20,21], and chromogenic, synthetic, lipid substrates[22,23] do not provide a sensitive and specific determination[24,25].

Deficiency of α-L-fucosidase, which is known to catalyze the hydrolysis of oligosaccharides, glycopeptides, and glycoproteins[26,27], results in the accumulation of L-fucose-containing glycolipids in fucosidosis[9,28]. However, the use of glycolipids as substrates for α-L-fucosidases is restricted by their extremely low abundance in animal tissues. We describe herein the synthesis of potentially fluorogenic substrates for these enzymes in order to elucidate the significance of the length and position of the acylamide residue of the substrate for the catalytic action of the enzymes.

RESULTS AND DISCUSSION

6-Acylamino derivatives of 4-methylumbelliferone (7-hydroxycoumarin; 7-hydroxy-2*H*-1-benzopyran-2-one), *i.e.*, butanoyl (**3**), octanoyl (**4**), and hexadecanoyl (**5**), were obtained by acylation of 6-amino-4-methylumbelliferone (**1**) with the acyl chloride of butyric, caprylic, or palmitic acids, respectively. 8-Hexadecanoylamino-4-methylumbelliferone (**6**) was obtained by acylation of 8-amino-4-methylumbelliferone (**2**) with palmitoyl chloride[29]. The 1,2-*trans*-glycosides **7–12** were obtained by glycosylation of the respective aglycon by the Koenigs–Knorr method. The 1,2-*cis*-glycoside **13** was obtained by fusion of 1,2,3,4-tetra-*O*-acetyl-L-fucose with **6** in the presence of 4-toluenesulfonic acid[30]. The structure of the 6-acylamino-4-methylumbelliferone derivatives was confirmed by elemental analysis and ^{1}H-n.m.r. spectroscopy. The configuration of the 1,2-*trans*-glycoside bond was confirmed by the presence in the ^{1}H-n.m.r. spectrum of a doublet at δ 4.8–4.9 ($J_{1,2}$ 8.0–8.3 Hz). A doublet at δ 5.56 ($J_{1,2}$ 4.1 Hz) correspond to H-1^1 in the spectrum of the α-L-fucopyranoside **13**.

TABLE I

Constants of 6-and 8-acylamino derivatives of 4-methylumbelliferone (3–6) and 4-methylumbelliferyl glycopyranosides (7–13)

Comp.	*U.v. spectrum [λ_{max}, nm ($\varepsilon \times 10^{-3}$)]*		*Fluorescence spectrum[a] (nm)*			
	pH 7.0	*pH 10.5*	*pH7.0*		*pH 10.5*	
			λ_{ex}	λ_{em}	λ_{ex}	λ_{em}
3	341 (11.9)	385 (23.9)	342	420	380	456
4	340 (14.7)	385 (31.8)	342	420	380	456
5	340 (14.7)	385 (30.0)	342	415	382	450
6	320 (8.0)	369 (19.0)	330	430	370	480
7	338 (11.8)		338	420		
8	338 (12.1)		338	420		
9	320 (11.2)		312	413		
10	319 (13.3)		320	430		
11	338 (12.2)		338	420		
12	338 (11.2)		336	430		
13	336 (11.2)		336	415		

[a] Spectra were not corrected; λ_{ex}, maximum excitation; λ_{em}, maximum emission

U.v. and fluorescence spectra of synthesized glycosides and of aglycons at pH 7.0 (neutral form) and 10.5 (anion form) are shown in Table I. The fluorescence of aglycons **3–5** was similar to that described earlier for 4-methylumbelliferone. The fluorescence increased with increasing pH and reached its maximum at pH 10.5. The excitation and fluorescence emission maxima were observed to shift to a longer wavelength. Addition of excess alkali beyond pH 11.0 resulted in a disappearance of fluorescence, apparently due to the opening of the pyrone ring, which reclosed upon acidification[29]. The fluorescence quantum yields of the 6-substituted glycosides are practically equal (0.27–0.35) to those of the initial aglycons at pH 7.0. For the 8-substituted compounds **6** and **13**, the quantum yield is significantly lower[30] ($2.0–4.6 \times 10^{-3}$).

Specificity of β-D-galactocerebrosidase and G_{M1}-β-D-galactosidase. — The β-D-galactoside substrates **7–9** were hydrolyzed by enzyme preparations from various human organs and tissues to an extent directly proportional to the incubation time and amount of protein added (Fig. 1). The specific activities of the enzymes with respect to various substrates were determined by kinetic parameters obtained during the linear phase of the enzymic reaction.

Since the known types of glycolipidoses are monogenic diseases that develop as a result of the hereditary deficiency of a single enzyme or an activator protein[2,3], data on the enzyme deficiency in preparations with an identified pathology provide a reliable evaluation of the substrate specificity of glycolipid hydrolases without their preliminary purification. The results of the hydrolysis of synthetic substrates by enzyme preparations from organs and cells of healthy persons and patients with various types of glycolipidoses are given in Table II. Hexadecanoylaminogalactoside **9** was cleaved, to

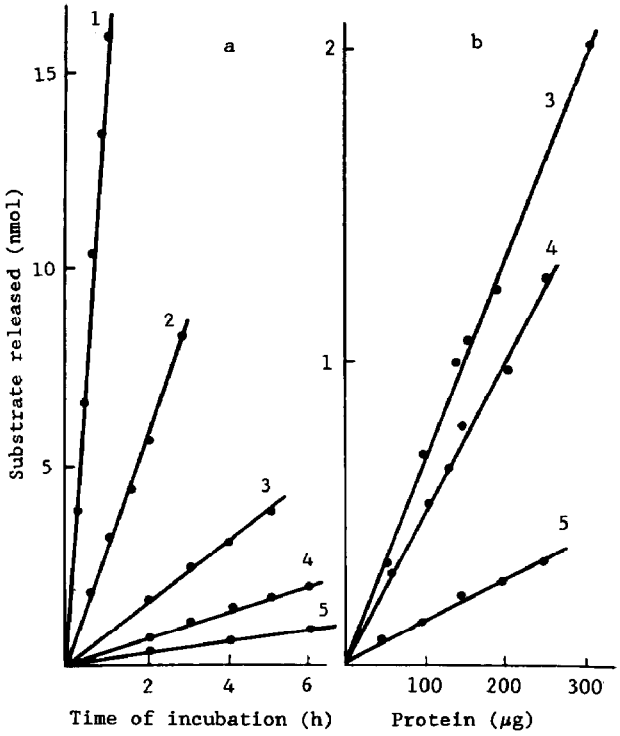
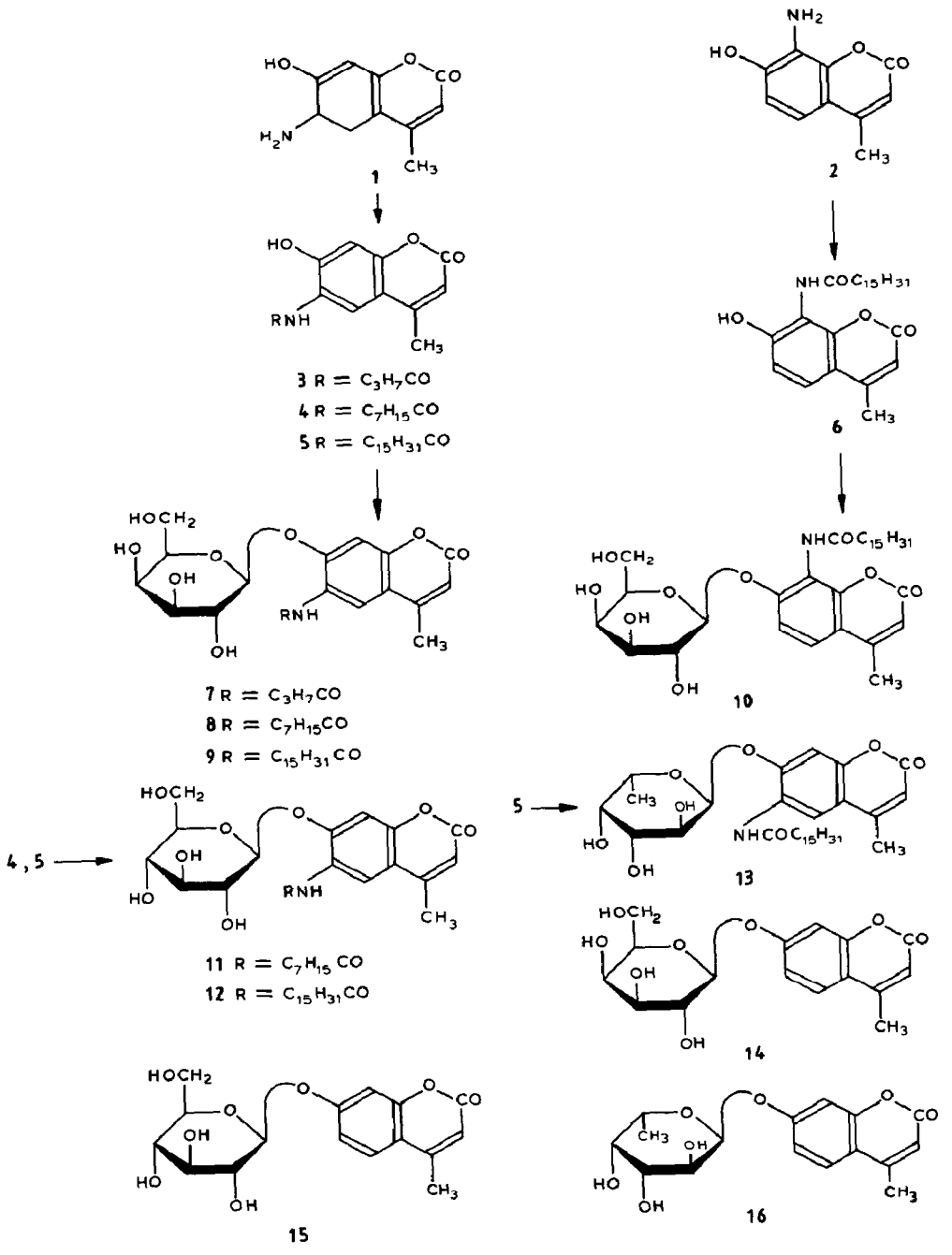
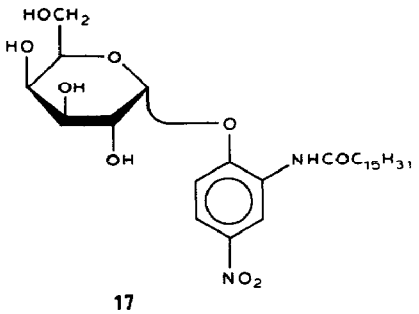


Fig. 1. Dependence of the enzyme hydrolysis of various substrates on: (a) Incubation time; and (b) amount of protein in the sample. (1) **7**; (2) **8**; (3) **10**; (4) **9**; and (5) **12**. Enzyme preparations: (1–4) Human leukocytes; (5) human placenta.

approximately the same extent, by preparations from healthy individuals and from patients with G_{M1}-gangliosidosis. In contrast, hydrolysis of substrates having shorter alkyl chains (**7**, **8**, and **14**) was sharply decreased in G_{M1}-gangliosidosis.

In Krabbe's disease, the enzymic cleavage of **9** by fibroblasts, leukocytes, and organs of the patients was significantly decreased, and hydrolysis of **7** and **8** was within the normal range. Thus, **9** is a substrate for D-galactocerebrosidase, and **7** and **8** are substrates for G_{M1}-β-D-galactosidase, indicating that the chain length of the fatty acid residue has a considerable influence on the activity of various types of β-D-galactosidase. Highly purified preparations of D-galactocerebrosidase from human liver and partially purified preparations from brain containing the monomeric (125 kDa) and hexameric (750 kDa) forms hydrolyzed compound **14** in addition to galactocerebroside, whereas a decrease in galactocerebrosidase activity for these two substrates was observed in a partially purified preparation from brain in Krabbe's disease[11]. Thus, galactocerebrosidase can split synthetic β-D-galactosides having different aglycons. Månsson and Svennerholm[31] showed that the highest activity of the enzyme was for *N*-nervonoylgalactosylsphingosine ($C_{24:1}$-cerebroside), whereas Parvathy *et al.*[32] observed the greatest activity for galactosylceramides having a saturated, medium-chain (C_{6-11}) fatty acids; cerebrosides having the longest fatty acid chains (C_{16-25}) were hydrolyzed 5–6 times less


1
2
3 R = C3H7CO
4 R = C7H15CO
5 R = C15H31CO
6
7 R = C3H7CO
8 R = C7H15CO
9 R = C15H31CO
10
11 R = C7H15 CO
12 R = C15H31CO
13
14
15
16
4, 5
5



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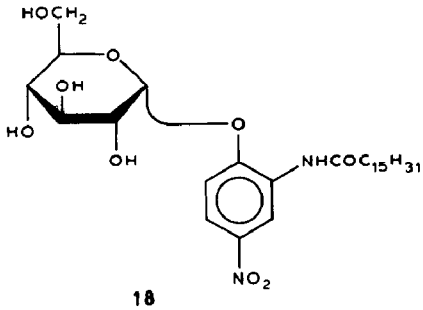

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rapidly. These data suggested two regions for the active site of galactocerebrosidase, one binding the hydrophilic D-galactosyl group and the other the hydrophobic lipid component.

Specific cleavage of compound **9** by D-galactocerebrosidase made possible the determination of this enzyme in normal individuals and to reveal its deficiency in Krabbe's disease in the presence of highly active G_{M1}-β-galactosidase. Comparison of the enzymic hydrolysis of **9** and of the isomer at C-8 (**10**) showed that the latter substrate is hydrolyzed 2–2.5 times more rapidly (see Fig. 1). The K_m values for both substrates were very similar (0.05mM), as measured in both leukocytes and fibroblasts, and are close to the K_m value observed with the natural substrate, galactocerebroside[18]. The value of V_{max} for **9** in leukocyte preparations was 1.37 and 2.9 nmol $mg^{-1}h^{-1}$ at a concentration of sodium taurocholate of 0.25 and 1.5%, respectively. The V_{max} for **10** was equal to 4.8 nmol $mg^{-1}h^{-1}$ for a concentration of 1.5% of sodium taurocholate.

Thus, the length of the acyl residue but also its position in the molecule is of considerable significance for the action of the enzyme. Unfortunately, a considerably

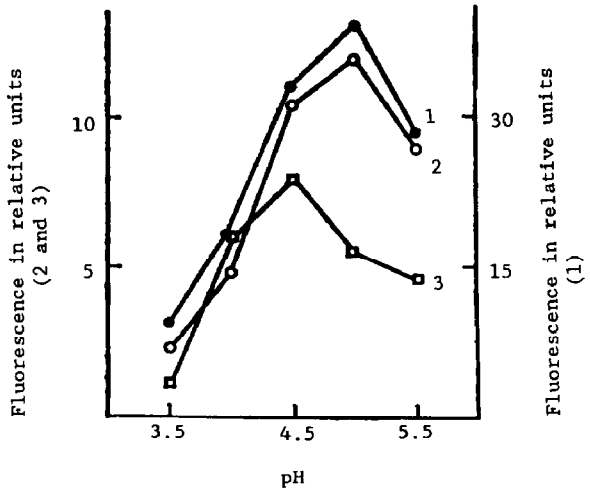

Fig. 2. Dependence of the rate of enzyme hydrolysis of substances on the pH and the percentage of sodium taurocholate in the sample: (1, -●-●-) **10**, leukocytes, and 1.5% sodium taurocholate; (2, -○-○-) **9**, leukocytes, and 1.5% sodium taurocholate; and (3, -□-□-) **9**, fibroblasts, and 0.25% sodium taurocholate.

TABLE II

Activity of β-D-galactocerebrosidase and G_{M1}-β-D-galactosidase in normal individuals and patients with G_{M1}-gangliosidosis and Krabbe's disease[a]

Substrate	*Normal*				*G_{M1}-Gangliosidosis*		*Krabbe's disease*		
	Fibroblasts	*Leukocytes*	*Liver*	*Brain*	*Fibroblasts*	*Leucoytes*	*Fibroblasts*	*Liver*	*Brain*
9	2.5 (1.0–3.5) n = 10	1.0 (0.6–2.0) n = 10	0.85	0.52	2.6 n = 1	0.9 (0.6–1.2) n = 4	0.06 (0.0–0.14) n = 3	0.03	0
8	26 (22–29) n = 3	12 (7.5–17) n = 17	20	3.3	7.3 n = 1	3.3 (3.0–3.7) n = 2	25 (19–33) n = 3	12	3.3
7	200 (120–370) n = 3	107 (36–180) n = 12	100	15	16.5 n = 1	7.2 (7.2–7.3) n = 2	220 (162–281) n = 3	94	17
14	235 (150–400) n = 10	180 (120–480) n = 10	349	38	6.7 n = 1	4.2 (2.3–5.4) n = 4	330 (220–440) n = 3	300	65

[a] nmol h^{-1} per 1 mg protein. The values in brackets are the ranges of activity in the samples from various individuals.

lower fluorescence quantum yield of **10** as compared to **9** restricts its use for the determination of galactocerebrosidase activity. The rate of cleavage of **9** and **10** was maximal at pH 4.5 in the presence of 0.25% of sodium taurocholate (fibroblasts) and at pH 5.0 in the presence of 1.5% of detergent (leukocytes) (Fig. 2). An activating effect of sodium taurocholate on the hydrolysis of **9** and **10** by enzyme preparations from leukocytes was observed with increasing detergent concentration (from 0.25 to 1.5%). A 1.5% concentration not only increased the specific activity of "normal" galactocerebrosidase but also minimized the difference between enzyme activities in the normal and in Krabbe's disease, because the residual activity of the enzyme was significantly increased. The reasons for this activation are not understood. The strong activating effect of sodium taurocholate on galactocerebrosidase from rat brain[33] and human leukocytes has already been described[34]. No hydrolysis of galactocerebroside was observed when the detergent was not included in the incubation medium, suggesting that it promotes the formation of optimal-size micelles which interact more efficiently with the enzyme. The efficient splitting of such amphipathic substrates as glycolipids requires *in vivo* a set of protein activators[35], one of which has been found for galactocerebrosidase[36]. Therefore, it cannot be ruled out that Krabbe's disease and other glycolipidoses can be due to a deficiency of the activator, and high concentrations of detergents can mask the difference in activities between normal individuals and Krabbe patients, thus complicating the identifications of the disease.

The use of compound **9** as a substrate for D-galactocerebrosidase significantly increases the sensitivity of the method, as compared to the use of the chromogenic substrate 2-hexadecanoylamino-4-nitrophenyl β-D-galactopyranoside[37] (**17**). Initial positive results were obtained in the diagnosis of two patients with Krabbe's disease, biochemically diagnosed[38] by use of **9**. Similarly, the use of **9** in the galactocerebrosidase assay of human leukocytes[39], as well as of chorion and in cell culture[40] of chorion-

TABLE III

Activity of β-D-glucosidase in various human and animal tissues[a]

Enzyme source	*Substrate*				
	15(−)[b]	**15**(+)[b]	**11**	**12**	**18**
Human placenta (villous chorion)	79.5	226	4.3	0.5	92.3
Human embryo fibroblasts	46.2	87		0	74.8
Human liver	28.5	11.5	0.36	0.02	2.6
Human spleen	3.5	3.8	0.07	0.01	4.0
Rabbit liver	973	371	10.1	2.1	35.6
Guinea pig spleen				1.75	31.8

[a] nmol h^{-1} per mg protein. [b] In the absence (−) or presence (+) of sodium taurocholate, respectively (see Experimental section).

derived cells, showed the high sensitivity of the method and indicated its potential for prenatal biochemical diagnosis of Krabbe's disease.

Specificity of β-D-glucocerebrosidase. — The enzymic hydrolysis of compound **12** as a function of enzyme concentration and incubation time is shown in Fig. 1, and Table III presents data on the β-D-glucosidase activity of various human and animal tissues for four different synthetic substrates. Placenta (villous chorion), in which the activity of many lysosomal glycosidases is shown[41], showed the highest activity out of all human tissues rested for hydrolysis of **15** and 2-hexadecanoylamino-4-nitrophenyl β-D-glucopyranoside (**18**). The activity with **12** was approximately 200 times lower than that obtained with the chromogenic analog **18**. The hydrolysis of **11** by enzyme preparations from human placenta was considerably lower than that of **15** and **18**.

Attempts to increase the rate of hydrolysis of **12** by addition of various amounts of dimethyl sulfoxide, which has been reported[42] to increase the solubility of the chromogenic substrate **18**, did not increase the solubility of **12** nor the rate of its hydrolysis. Increasing the sodium taurocholate concentration in the reaction mixture from 0.5 to 2.5% increased the rate of hydrolysis of **12** by 60 to 70%. An increase in concentration of Triton X-100 from 2 to 4mM did not increase the rate of hydrolysis of **12** by enzyme preparations from human placenta.

Attempts to use **12** in biochemical diagnostic tests of Gaucher's disease were unsuccessful. Although the β-D-glucosidase activity in the spleen of a patient with Gaucher's disease was 2.6 nmol $mg^{-1} h^{-1}$ with **18** as substrate (23% of normal, 11.5 nmol $mg^{-1} h^{-1}$), compound **12** did not reveal any differences between normal and diseased individuals, the activity in both cases being no more than 0.05 nmol $mg^{-1} h^{-1}$.

In order to compare the hydrolysis of **12** and **11** by acid or neutral forms of β-D-glucosodase, the two forms obtained from liver were separated by gel-filtration [21] on Sephadex G-200 (see Fig. 3). The identity of the enzymes was confirmed by the different effects of sodium taurocholate which activates the "acid" and inhibits the "neutral" form. Compounds **11** and **18**, as well as **18**, were hydrolyzed by the "acid" form, whereas the "neutral" β-D-glucosidase barely hydrolyzed these substrates.

Substitution of the chromogenic β-D-glucoside **18** for the fluorogenic analog **12** considerably decreased the rate of hydrolysis by both human and animal glucocerebrosidases. The use of **11**, with its shorter acyl residue, also gave no advantage over the chromogenic substrate **18**. The reasons for such a low rate of hydrolysis of the fluorogenic substrates are unclear. The activity of glucocerebrosidase from bovine spleen was also low in experiments that used 4-alkylumbelliferyl β-D-glucopyranosides with different lengths of the aliphatic chain[43] (C_7H_{15}, C_9H_{19}, and $C_{11}H_{23}$). Thus, for 4-nonylumbelliferyl β-D-glucoside, which was used later[44] for determination of intracellular activity of lysosomal glucosylceramidase, the activity of the enzyme did not exceed 1.12 nmol mg^{-1} of protein h^{-1}. This value is close to the specific activities of the enzyme preparations from guinea pig spleen or rabbit liver that we determined with **12** as substrate. The preferred substrates of human glucocerebrosidase from placenta, liver, and brain were β-D-glycosides having a C_{7-9}-alkyl chain. The rate of hydrolysis of C_{17}-derivatives was much less[45]. We assume that the aglycon centre of glucocerebrosi-

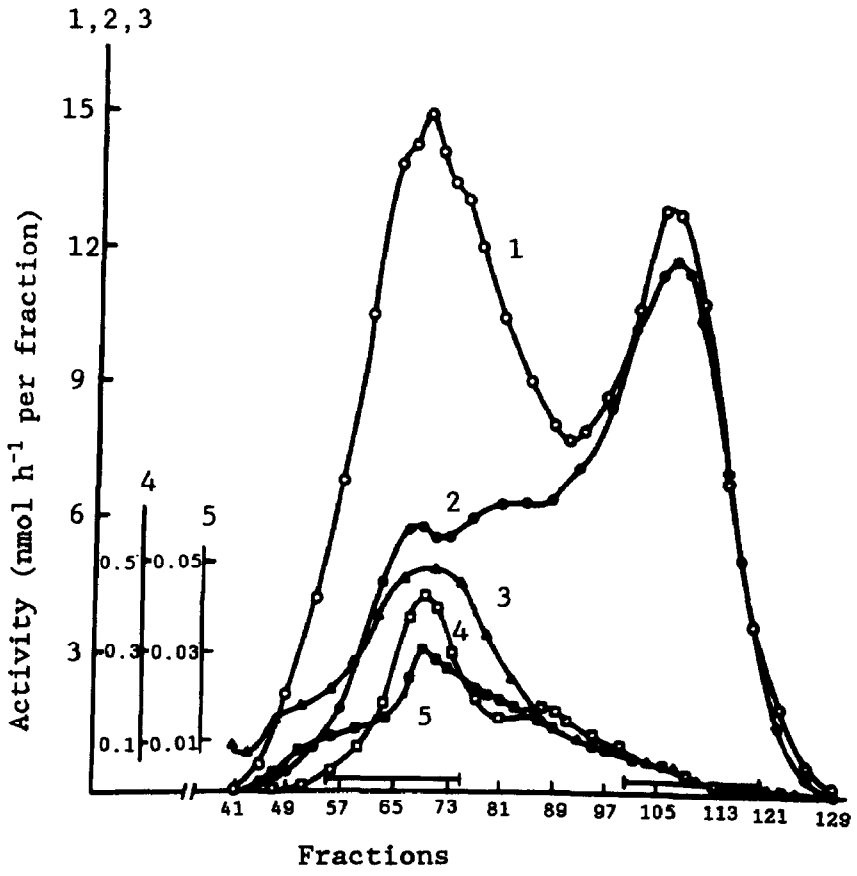

Fig. 3. Elution profile of the β-D-glucosidase activity in gel filtration of the enzyme preparation from human liver in a column (1.6 × 33.5 cm) of Sephadex G-200 eluted with 0.01M phosphate buffer, pH 5.8, containing 0.1M NaCl and 0.5% Triton X-100 at an elution rate of 7 mL/h, and a volume for each fraction of 0.5 mL, using various β-D-glucosides as substrates: (1, -○-○-) **15** (+); (2, -●-●-) **15** (−); (3, -▲-▲-) **18**; (4, -□-□-) **11**; (5, -■-■-) 12. The β-D-glucosidase activity was determined in the presence (+) or absence (−) of sodium taurocholate, respectively. The boundaries of grouping the fractions are designated as (|——|).

dase binding, which is supposed to have hydrophobic regions for both the fatty acid residue and the sphingosine fragment of glucosylceramide[46], is rather sensitive to differences in the structure of the chromogenic and fluorogenic substrates. Other sphingolipid hydrolases, specifically sphingomyelinase, that hydrolyzes 4-methylumbelliferone-based substrates at a considerably lower rate than their 4-nitrophenyl analogs have been reported[47]. It should also be noted that increasing the fatty acid acyl chain length of glucosylceramide from 1 to 24 carbon atoms had minor effects on the K_m but increased the V_{max} value up to 13-fold[48]. We concluded that introduction of a second lipid "tail" into the umbelliferyl glycoside molecule leads to a sharp increase in the rate of enzymic hydrolysis of such substrates by glucocerebrosidase.

Specificity of α-L-fucosidase. — Experiments with α-L-fucosidase showed that **13** is hydrolyzed by enzyme preparations from human kidney and liver. The rate of

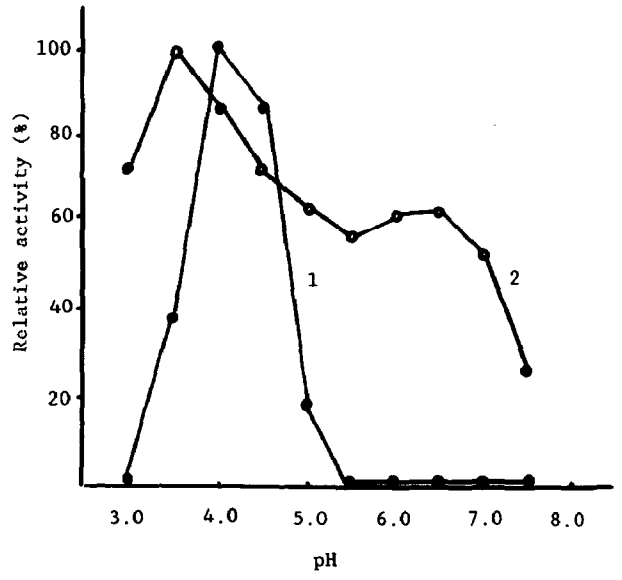

Fig. 4. pH Dependence of hydrolysis of **13** (1, -●-●-) and **16** (2, -○-○-) by an enzyme preparation of human α-L-fucosidase.

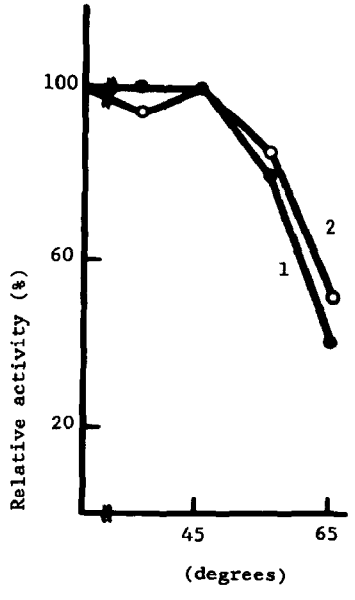

Fig. 5. Thermostability of human α-L-fucosidase with **13** (1, -●-●-) and **16** (2, -○-○-) as substrates. The enzyme solution was incubated for 20 min at various temperatures at pH 4, followed by assay of reducing power.

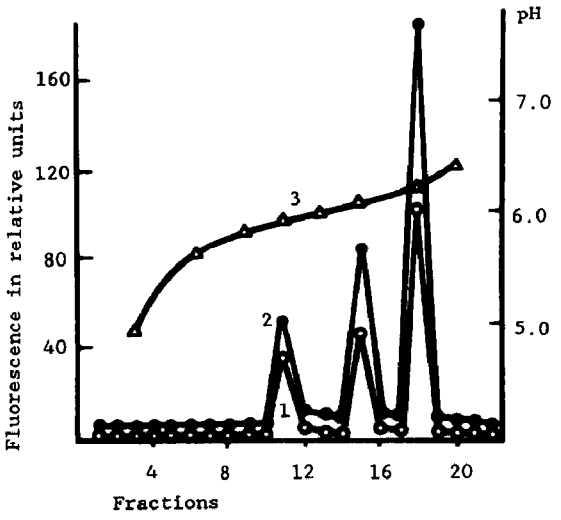

Fig. 6. Isoelectric focusing of human kidney α-L-fucosidase in a thin layer of poly(acrylamide) gel (pH 4-6): **13** (1, -○-○-); **16** (2, -●-●-); and pH profile (3, -△-△-).

reaction increased linearly for 15–20 min. Maximal hydrolysis of **13** was observed at pH 4.0 (Fig. 4), a value close to the pH optimum observed for hydrolysis of **16**. However, unlike **16**, the pH dependence profile of **13** hydrolysis was characterized by the virtual absence of enzyme activity at pH > 5.0. The K_m value for **13** was 0.13mM and was comparable to that for **16** usually used to determine α-L-fucosidase activity[27]. The α-L-fucosidase thermostability curves, determined by use of **13** and **16**, were also similar (Fig. 5).

As shown earlier, α-L-fucosidase is characterized by multiple forms differing in their rate of hydrolysis of various L-fucose-containing oligosaccharides[26,49–51]. As seen in Fig. 6, **13** was hydrolyzed by all forms of α-L-fucosidase capable of hydrolyzing **16**. The ratio of hydrolysis of these two substrates was the same for all three forms of α-L-fucosidase. The existence of a specific α-L-fucosidase acting on more complex synthetic or natural fucolipids cannot be ruled out. α-L-Fucosidase from octopus hepatopancreas showed significant differences in the kinetics of hydrolysis of **16** and L-fucose-containing G_{M1}-ganglioside[52]. Since the specificities of α-L-fucosidase from mollusks and mammals differ considerably[26], the issue of the specificity of action of human α-L-fucosidases on various natural L-fucoglycolipids still remains open. The first attempts in this direction showed that functionally different types of human pellet-associated and soluble α-L-fucosidases were both capable of hydrolyzing **16** and L-fucosyl-G_{M1} ganglioside[53].

EXPERIMENTAL

Materials. — Homogenates of leukocytes, and embryonal and postnatal human skin fibroblasts (strains 814, 822, and 1035), obtained from the Institute of Medical Genetics, U.S.S.R Academy of Medical Sciences, and grown in our laboratory by Drs.

I. D. Belyaeva and T. S. Ivleva, were used for the enzyme preparations of β-D-galactosidases and β-D-glucosidases. Cells from the U.S.A. Mutant Cells Bank, grown and maintained at the Cell Bank of the Institute of Medical Genetics, U.S.S.R. Academy of Medical Sciences (strain 953), were used as a reference of human skin fibroblasts with Krabbe's disease. Enzyme preparations from various organs of healthy persons and patients with Krabbe's disease[54] were from autopsy material. Leukocytes from patients with G_{M1}-gangliosidosis, kindly provided to us by Dr. I. V. Tsvetkova (Institute of Biological and Medical Chemistry, U.S.S.R. Academy of Medical Sciences), were also used in the experiments. Nonpurified (Soyuzkhimreaktiv, U.S.S.R.) and purified (Koch–Light) preparations of sodium taurocholate and oleic acid were used. Compound **18** was obtained by the method described by Gal *et al.*[23].

General methods. — Melting points were determined with a Boetius instrument. ^{1}H-N.m.r. spectra were recorded with a Bruker WH-250 spectrometer for solutions in CD_3OD and $(CD_3)_2SO$, and u.v. spectra with a Specord M-40 spectrophotometer for solutions in ethanol, and fluorescence spectra with a Hitachi MPF 4A spectrofluorometer for solutions in ethanol. T.l.c. was performed on Silufol 60F-254 plates (Czechoslovakia).

Enzyme preparations. — A partially purified enzyme preparation of α-L-fucosidase was obtained from human kidney and liver homogenates (autopsy material) according to the method of Beyer and assoc.[50,55], including precipitation with $(NH_4)_2SO_4$ (60% of saturation) and heating at 55° for 20 min. The α-L-fucosidase activity with **16** as substrate was determined by the previously described method[50,55]. The total leukocytic fraction was obtained according to Kuzmicheva and Wiederschain[56]. In order to obtain homogenates, cells were subjected to a three-fold freezing and thawing cycle in a minimal amount of distilled water, and then the suspension was homogenized in a Potter glass homogenizer with a Teflon pestle for 2–3 min. After removal of the cell debris by centrifugation, the homogenate was used as the β-D-galactosidase preparation.

Isoelectric focusing of the α-L-fucosidase preparation was carried out according to the method of Vesterberg[57]. To identify the α-L-fucosidase isoforms, the gel layer was cut into sections of 0.25 × 1.0 cm, which were kept overnight in 0.1–0.2M citrate–phosphate buffer, pH 4.0 (0.25 mL), to extract the enzyme. The extracts were used to determine the α-L-fucosidase activity with **13** and **16** as substrates[50,55].

Protein in the samples was determined by the Lowry method[58]. The values of K_m and V_{max} were calculated by the graphic method of Lineweaver–Burk[59].

β-D-Galactocerebrosidase assay. — In the galactocerebrosidase assay, the incubation mixture (total volume, 100 μL) contained the enzyme preparation (50 μL, 50–200 μg of protein) and the substrate mixture (50 μL, 50–70 nmol of respective substrate). The substrate mixture was prepared as follows. The substrate (1.2 mg) in 2:1 chloroform–methanol (2 mL), sodium taurocholate (10 mg) and a 1% solutiuon of oleic acid in hexane (0.06 mL) were mixed in a glass homogenizer. The mixture was evaporated to dryness in a flow of N_2 at 35–40°. The residue was suspended in 0.2M phosphate–citrate buffer, pH 4.5 (2 mL) and homogenized for several minutes with a Teflon pestle

and heating up to 50°. Later in our experiments the concentration of **9** was decreased to 8.5 nmol and the content of substrate mixture was modified[40].

β-D-Glucosidase assay. — The activity of β-D-glucosidase was determined by use of **15** (a) in the presence of 0.25% sodium taurocholate and 0.01% of oleic acid [**15**(+)], and (b) in their absence [**15**(−)]. The content of **15** in the incubation mixture (200 μL) was in both cases 444 nmol, and the pH of the medium (5.8) was maintained with 0.1M (on phosphate) citrate–phosphate buffer. In the determination of the β-D-glucosidase activity using **11**, **12** and **18**, the substrate mixtures were prepared as mentioned above for the respective D-galactosides. The content of the substrates in the incubation mixture (200 μL) was 62, 50, and 216 nmol, respectively.

β-D-Galactosidase assay. — The β-D-galactosidase assay using **14** was performed according to Kuzmichleva und Wiederschain[56].

α-L-Fucosidase assay. — In the α-L-fucosidase assay using **13**, the incubation mixture contained the substrate mixture (0.15 mL, 52 nmol) and the enzyme preparation (0.05 mL; 50 μg of protein). To prepare the substrate mixture, a 0.2% solution of **13** in 2:1 chloroform–methanol (0.2 mL) was added to 0.67% sodium taurocholate–0.04% oleic acid in 2:1 chloroform–methanol (2 mL); the solution was evaporated under a flow of N_2 and the residue dissolved in 0.1–0.2M citrate-phosphate buffer, pH 4.0 (2 mL). The enzymic reaction was stopped by addition of 5:2 ethanol–0.4M glycine buffer, pH 10.4. Fluorescence in the samples was determined with EF-3MA and BIAN-130 fluorometers (U.S.S.R.) and a Specord M-40 spectrophotometer (Carl Zeiss, Jena, Germany), equipped with an adaptor for measuring the fluorescence of the released aglycon relative to a standard solution in 2-propanol at pH 10.5; the required spectral range of the fluorescence was provided with a GVK 48 light filter ($\lambda > 455$ nm). Samples containing no enzyme preparations were used as controls.

6-Hexadecanoylamino-4-methylumbelliferone (**5**). — Palmitoyl chloride (35 mmol) was added to a solution of 6-amino-4-methylumbelliferone (**1**, 17.3 mmol) in abs. pyridine (90 mL), mixed for 3 h at 20°, and then acetonitrile (50 mL) was added. The precipitate was filtered off, suspended in ethanol (100 mL) and a 5% KOH in ethanol solution was added with stirring to pH 10–11 and then 5% HCl in ethanol solution to pH 5–6. The precipitate was filtered off, washed with ethanol and hexane; it crystallized from 2-propanol (yield 90%), m.p. 193–194°.

Anal. Calc. for $C_{26}H_{39}NO_4$: C, 72.7; H, 9.1. Found: C, 72.8; H, 9.2.

8-Hexadecanoylamino-4-methylumbelliferone (**6**). — This compound was synthesized from 8-amino-4-methylumbelliferone[29] (**2**), as described for **5**, in 83% yield, m.p. 135–136°.

Anal. Calc. for $C_{26}H_{39}NO_4$: C, 72.7; H, 9.1. Found: C, 73.0; H, 8.9.

6-Butanoylamino-4-methylumbelliferone (**3**). — Butyryl chloride (30 mmol) was added with stirring to a suspension of **1** (20 mmol) in dry pyridine (100 mL), and the mixture was kept for 24 h at 20°. It was diluted with ice-cold water (150 mL), and the precipitate was filtered off, washed with water, and dried in the presence of P_2O_5. Crystallization from ethanol gave **3** in 80% yield, m.p. 235–236°.

Anal. Calc. for $C_{14}H_{15}NO_4$: C, 64.4; H, 5.7. Found: C, 64.6; H, 5.7.

6-Octanoylamino-4-methylumbelliferone (**4**). — This compound was synthesized from 6-amino-4-methylumbelliferone (**1**) and octanoyl chloride in 85% yield, m.p. 182–184°.

Anal. Calc. for $C_{18}H_{23}NO_4$: C, 68.1; H, 7.2. Found: C, 67.9; H, 7.3.

*General method of obtaining 1,2-*trans*-glycosides* **7**, **8**, **9**, **10**, **11**, *and* **12**. — An equimolar amount of an 0.1M solution of sodium ethoxide in ethanol was added with stirring to a solution of *N*-acylamino derivative (**3**, **4**, **5**, or **6**; 10 mmol) in ethanol (10 mL) and the solvent was evaporated *in vacuo*. The residue was dried in the presence of KOH. The resulting sodium salt was suspended in abs. oxolane (40 mL) and a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide or 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide[60] (10 mmol) in dimethyl sulfoxide (15 mL; freshly distilled over KOH) was added. The mixture was boiled for 20 h and filtered. The filtrate was evaporated, and the residue diluted with water (200 mL) and extracted (5 ×) with ether, (20 mL). The extract was dried ($CaCl_2$) and filtered through a 2-cm layer of silica gel and the filtrate was evaporated. The yield of glycoside tetraacetates was 55–65%. Deacetylation of the glycoside tetraacetates was carried out in the presence of sodium methoxide[60] to give compounds **7**–**12** in 70–80% yield.

6-Butanoylamino-4-methylumbelliferyl β-D-galactopyranoside (**7**). — M.p. 252–254°, $[\alpha]_D^{20}$ – 40° (*c* 0.5, dimethyl sulfoxide); ^{1}H-n.m.r. (CD_3OD): δ 0.9 (t, 3 H, CH_3-acyl), 1.64 (m, 2 H, CH_2-acyl), 2.35 (t, 2 H, CH_2CO), 2.50 (s, 3 H, CH_3-4), 4.85 (d, 1 H, $J_{1,2}$ 7.65 Hz, H-1′), 6.12 (s, 1 H, H-3), 6.81 (s, 1 H, H-8), 8.17 (s, 1 H, H-5), and 9.35 (s, 1 H, HN).

Anal. Calc. for $C_{20}H_{25}NO_9$: C, 56.7; H, 5.9. Found: C, 56.2; H, 5.9.

6-Octanoylamino-4-methylumbelliferyl β-D-galactopyranoside (**8**). — M.p. 228–229°, $[\alpha[^{20}_D$ – 30.5° (*c* 0.5, dimethyl sulfoxide); ^{1}H-n.m.r. [$(CD_3)_2SO$]: δ 0.9 (t, 3 H, CH_3-acyl), 1.30 (s, 8 H, CH_2-acyl), 1.65 (m, 2 H, $CH_2\beta$-acyl), 2.38 (t, 2 H, CH_2CO), 2.50 (s, 3 H, CH_3-4), 4.85 (d, 1 H, $J_{1,2}$ 8.00 Hz, H-1′), 6.12 (s, 1 H, H-3), 6.81 (s, 1 H, H-8), 8.17 (s, 1 H, H-5), and 9.35 (s, 1 H, HN).

Anal. Calc. for $C_{28}H_{41}NO_9$: 62.8; H, 7.7. Found: C, 62.6; H, 7.5.

6-Hexadecanoylamino-4-methylumbelliferyl β-D-galactopyranoside (**9**). — M.p. 205–206°, $[\alpha]_D^{20}$ – 26° (*c* 0.5, dimethyl sulfoxide); ^{1}H-n.m.r. [$(CD_3)_2SO$]: δ 0.8 (t, 3 H, CH, CH_3-acyl), 1.25 (s, 24 H, CH_2-acyl), 1.65 (m, 2 H, $CH_2\beta$-acyl), 2.34 (t, 2 H, CH_2CO), 2.50 (s, 3 H, CH_3-4), 4.80 (d, 1 H, $J_{1,2}$ 8.00 Hz, H-1′), 6.12 (s, 1 H, H-3), 6.81 (s, 1 H, H-8), 8.17 (s, 1 H, H-5), and 9.35 (s, 1 H, HN).

Anal. Calc. for $C_{32}H_{49}NO_9$: C, 65.0; H, 8.3. Found: C, 64.7; H, 8.4.

8-Hexadecanoylamino-4-methylumbelliferyl β-D-galactopyranoside (**10**). — M.p. 186–187°, $[\alpha]_D^{20}$ – 27° (*c* 0.5, dimethyl sulfoxide); ^{1}H-n.m.r. [$(CD_3)_2SO$]: δ 0.75 (t, 3 H, CH_3-acyl), 1.17 (s, 24 H, CH_2-acyl), 1.55 (m, 2 H, $CH_2\beta$-acyl), 2.33 (t, 3 H, CH_3-4), 2.40 (t, 2 H, CH_2CO), 4.90 (d, 1 H, $J_{1,2}$ 8.30 Hz, H-1′), 6.17 (s, 1 H, H-3), 7.07 (d, 1H, $J_{6,5}$ 9,5 Hz, H-6), 7.53 (d, 1 H, $J_{5,6}$ 9,5 Hz, H-5), and 9.38 (s, 1 H, HN).

Anal. Calc. for $C_{32}H_{49}NO_9$: C, 65.0; H, 8.3. Found: C, 64.8; H, 8.1.

6-Octanoylamino-4-methylumbelliferyl β-D-glucopyranoside (**11**). — M.p. 223–225°, $[\alpha]_D^{20}$ – 38° (*c* 0.5, dimethyl sulfoxide); ^{1}H-n.m.r. [$(CD_3)_2SO$]: δ 4.75 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1′).

Anal. Calc. for $C_{28}H_{41}NO_9$: C, 62.8; H, 7.7. Found: C, 62.9; H, 7.8.

6-Hexadecanoylamino-4-methylumbelliferyl β-D-glucopyranoside (**12**). — M.p. 202–204°, $[\alpha]_D^{20} - 40°$ (*c* 0.5, dimethyl sulfoxide); ^{1}H-n.m.r. [$(CD_3)_2SO$]: δ 4.78 (d, 1H, $J_{1,2}$ 8.1 Hz, H-1′).

Anal. Calc. for $C_{32}H_{49}NO_9$: C, 65.0; H, 8.3. Found: C, 64.9; H, 8.3.

6-Hexadecanaylamino-4-methylumbelliferyl α-L-fucopyranoside (**13**). — A mixture of **6** (0.62 g, 1.44 mmol) and 1,2,3,4-tetra-*O*-acetyl-L-fucose[61] (0.48 g, 1.44 mmol) was blended with a 0.1% solution of 4-toluenesulfonic acid (8 mL) in ether. The solvent was evaporated and the residue fused *in vacuo* (2KPa) at 185–190° for 2 min. The mixture was cooled to 20° and extracted 7 times with ether (10 mL, each). The extract was filtered through a 2-cm layer of silica gel and evaporated. The residue was dissolved in chloroform (5 mL), chromatographed on a column of Silpearl UV-254 (Czechoslovakia), and eluted with chloroform. The resulting mixture of 6-hexadecanoylamino-4-methylumbelliferyl 2,3,4-tri-*O*-acetyl-α-L- and -β-L-fucopyranoside was separated by t.l.c. in 97:1:2 chloroform–2-butanone–methanol. The triacetate of the α-L-anomer (**13**) (0.36 g), m.p. 90–92°, was deacetylated by a 2% solution of NH_3 in methanol (15 mL) at 20° for 24 h. After evaporation of the solvent, the residue crystallized from ethanol to give **13** (40% yield), m.p. 185–187°.

Anal. Calc. for $C_{32}H_{49}NO_8$: C, 66.8; H, 8.5. Found: C, 67.0; H, 8.8.

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