Carbohydrate Research 346 (2011) 1592–1598

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

1-Deoxy-D-galactonojirimycins with dansyl capped N-substituents as β-galactosidase inhibitors and potential probes for G_{M1} gangliosidosis affected cell lines

Richard F. G. Fröhlich [a], Richard H. Furneaux [a], Don J. Mahuran [b], Robert Saf [e], Arnold E. Stütz [c,*], Michael B. Tropak [b], Jacqueline Wicki [d], Stephen G. Withers [d], Tanja M. Wrodnigg [c]

[a] Carbohydrate Chemistry Team, Industrial Research Limited, PO Box 31-310, 5040 Lower Hutt, New Zealand
[b] Department of Laboratory Medicine and Pathobiology, Sick Kids Hospital, 555 University Avenue, University of Toronto, Ont., Canada M5G 1X8
[c] Glycogroup, Institut für Organische Chemie, Technische Universität Graz, Stremayrgasse 9, A-8010 Graz, Austria
[d] Chemistry Department, University of British Columbia, 2036 Main Mall, Vancouver, BC, Canada V6T 1Z1
[e] Institute for Chemistry and Technology of Materials, Graz University of Technology, Stremayrgasse 9, A-8010 Graz, Austria

ARTICLE INFO

Article history:
Available online 24 May 2011

Dedicated to Professor Dr. András Lipták on the occasion of his 75th birthday

Keywords:
Iminoalditol
N-Alkylation
Galactosidase inhibitor
Molecular chaperone
GM1-gangliosidosis

ABSTRACT

Two simple and reliably accessible intermediates, *N*-carboxypentyl- and *N*-aminohexyl-1-deoxy-D-galactonojirimycin were employed for the synthesis of a set of terminally *N*-dansyl substituted derivatives. Reaction of the terminal carboxylic acid of *N*-carboxypentyl-1-deoxy-D-galactonojirimycin with *N*-dansyl-1,6-diaminohexane provided the chain-extended fluorescent derivative. Employing bis(6-dansylaminohexyl)amine, the corresponding branched di-*N*-dansyl compound was obtained. Partially protected *N*-aminohexyl-1-deoxy-D-galactonojirimycin served as intermediate for two additional chain-extended fluorescent 1-deoxy-D-galactonojirimycin (1-DGJ) derivatives featuring terminal dansyl groups in the *N*-alkyl substituent. These new compounds are strong inhibitors of D-galactosidases and may serve as leads en route to pharmacological chaperones for GM1-gangliosidosis.

1. Introduction

Over the past three decades, iminosugars in the widest sense and, in particular, iminoalditols as powerful inhibitors have been valuable tools for the investigation and characterization of glycoside hydrolases.[1] Due to the vital roles of these enzymes, their inhibitors exert notable biological activities including anti-viral and anti-metastatic properties as well as powerful effects on various metabolic disorders including type 2 Diabetes and certain forms of hereditary lysosomal disorders.[2–5] This group of over 40 metabolic diseases arises from mutations in specific genes that lead to deficiencies in enzymes involved in the lysosomal degradation of glycolipids and glycans.

Recently, these diseases have attracted considerable interest and considerable efforts have been made to provide novel therapeutic compounds which may relieve the various symptoms that arise from the inability of lysosomal glycosidases to degrade their respective substrates. These consequently accumulate in the cells and lead to irreversible damages of nerve tissue, bones as well as various other organs resulting in painful long term suffering of patients and their families.

The work of many groups has shown that iminosugars as well as amine-substituted carbasugars may be suitable therapeutic agents that function either by inhibiting upstream enzymes and, thus, reducing the production of metabolites that cannot be degraded rapidly enough by the respective enzyme mutant (substrate reduction therapy, SRT) or by helping the folding and transport of mutant enzymes to the lysosome (chaperone mediated therapy, CMT). The latter, in particular, would have quite a few attractive features: it was suggested that sub-inhibitory concentrations of active site specific molecules (pharmacological chaperones) could be exploited.[2] These small molecules bind to and stabilize mutant proteins such as lysosomal β-glucosidase, β-galactosidase or β-*N*-acetylhexosaminidase in their functional folded conformations and assist their exit from the endoplasmatic reticulum and subsequent transport to the lysosome. Mutant proteins that cannot obtain/retain their functional conformation are recognized as misfolded by the quality control machinery in the endoplasmatic reticulum and are eventually targeted for degradation.

Recent contributions by various leaders in the field seem to suggest that N-substituted iminoalditols and similarly functionalized structures bind better than their more polar parent compounds

* Corresponding author. Tel.: +43 316 873 8244; fax: +43 316 873 8740.
E-mail address: stuetz@tugraz.at (A.E. Stütz).

0008-6215
doi:10.1016/j.carres.2011.05.010

through stronger interactions with the aglycon binding site or with lipophilic pockets around the enzymes' active sites.

N-Butyl-1-DNJ (**1a**) is one of the best studied iminosugars in this context[5] (Fig. 1). Recently, unusual structures such as bicyclic nojirimycin derivatives **2**[6] and a lipophilic derivative of isofagomine, **3**,[7] have also been found to exert excellent activities. Other researchers such as Wong and his group[8] as well as Aerts and Overkleeft and their co-workers[9] have observed that large, lipophilic *N*-alkyl substituents, in particular adamantyl capped spacer arms (compounds **4**), greatly improve the interaction between iminoalditol derivatives and the lysosomal β-glucosidase.

For treatment of GM1-gangliosidosis, which is characterized by deficient activity of lysosomal β-galactosidase, N-modified derivatives of 1-deoxy-D-galactonojirimycin (**5**) as well as of 1-deoxynojirimycin (**1**) have been investigated as candidates for substrate reduction therapy in a mouse model.[10]

One of the current benchmark molecules for chaperone mediated therapy (CMT) is NOEV (*N*-octyl-*epi*-valieneamine) **6**, a carbasugar.[11]

These guiding results, in line with our interest in fluorescently tagged glycosidase inhibitors for diagnostic purposes,[12,13] as well as encouraging biochemical results with one of our dansylated compounds,[14] have led us to prepare a range of N-modified 1,5-iminoglucitols from partially protected L-*arabino*-hexos-5-ulose (**7**), amongst them compounds **8**, **9** as well as **13** and **15**.

2. Results and discussion

Based on previous results with a fluorescent iminoalditol-lysine hybrid,[14] compounds reported here were designed to provide insight into the contribution of simple linear and branched spacer arms featuring various degrees of lipophilicity and polarity. Three differently structured amides and one tertiary amine were prepared and their physico-chemical behaviour and biological activities were investigated.

We have found *N*-aminohexyl as well as *N*-carboxypentyl derivatives very versatile and convenient intermediates en route to the desired inhibitors addressed in this and previous studies.

Based on experimental results in the D-*gluco* series,[15] compounds **8** and **9** were prepared following a reliable route, this time starting from *N*-carboxypentyl-1-deoxy-D-galactonojirimycin (**5c**), which, in turn, had been accessed by reaction of 3,4-isopropylidene-L-*arabino*-hexos-5-ulose[16] (**7**) with methyl 6-aminohexanoate under reductive amination conditions and subsequent acidic cleavage of the isopropylidene acetal to give **5b**, followed by saponification of the methyl ester with NaOH in water/dioxane (Scheme 1).

Reaction of intermediate **5c** with *N*-dansyl-1,6-diaminohexane[15] **A** (prepared in four simple steps from 6-aminohexanol) under standard peptide coupling conditions employing *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) in Et_3N/DMF gave iminoalditol **8** as a yellow-green fluorescent syrup (Scheme 2). Similarly, compound **9** was prepared by reacting di-(*N*-dansyl-6-amino)hexylamine **B**[15] with intermediate **5c** (TBTU, Et_3N, DMF).

Starting from known[16] *N*-(6-amino)hexyl-3,4-*O*-isopropylidene-1-deoxy-D-galactonojirimycin (**10**), the desired fluorescent chain extension was introduced in good yield by reaction of mesylate **C**[15], to provide partially protected compound **11** (47%, Scheme 3). As a side product, tertiary amine **12** (9%) was obtained and deprotected to provide di-dansyl substituted iminosugar **13** (quant.). N-Acylation of the secondary amine **11** with octanoic chloride led to lipophilic intermediate **14** (71%) which upon acid treatment was converted into inhibitor **15** (quant.; Scheme 4).

In a general screen with three standard galactosidases (Table 1), compound **8** was found to be a powerful inhibitor of the GH1 β-glucosidase from *Agrobacterium* sp. (K_i = 0.45 μM; Parent compound **5**: K_i = 12 μM) as well as the GH2 *Escherichia coli* lacZ β-galactosidase (K_i = 0.27 μM) and inhibited the GH27 α-galactosidase from green coffee beans (GCB) with K_i = 3.4 μM. Compound **9** had a similar inhibitory profile with K_i 0.63 μM (*Agrobacterium* sp.), K_i = 0.29 μM (*E. coli*) and K_i = 2.6 μM (GCB). Compound **13** was better by a factor of two with the two β-galactosidases (K_i = 0.23 μM and 0.12 μM, resp.) and about fivefold more active (K_i = 0.53 μM) with the α-specific enzyme. Interestingly, inhibitor **15** showed impressive β-galactosidase inhibition (K_i 28 nM and 47 nM,

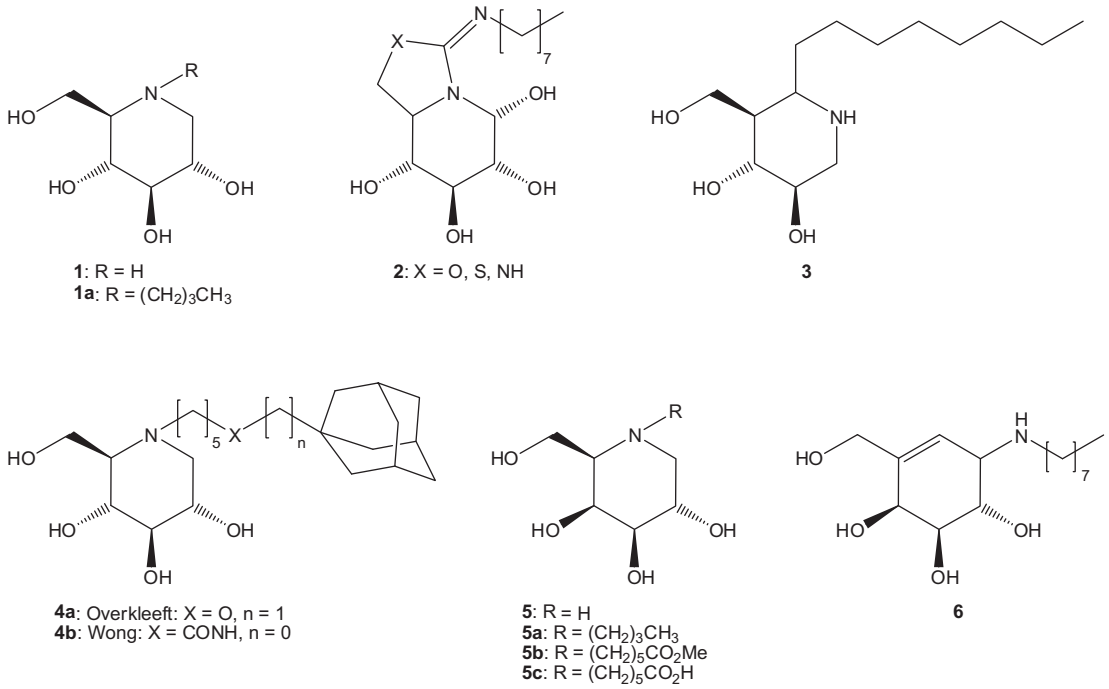
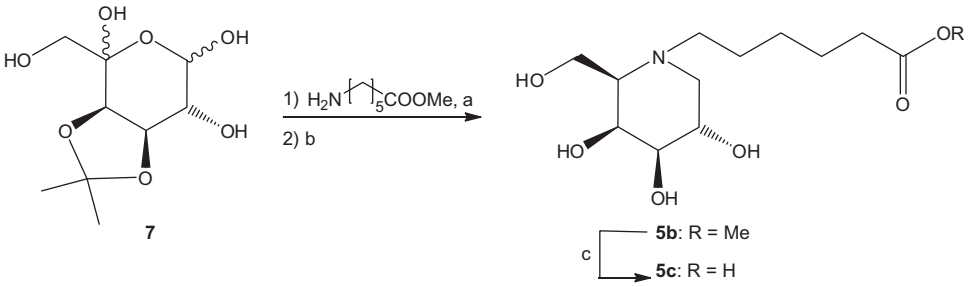
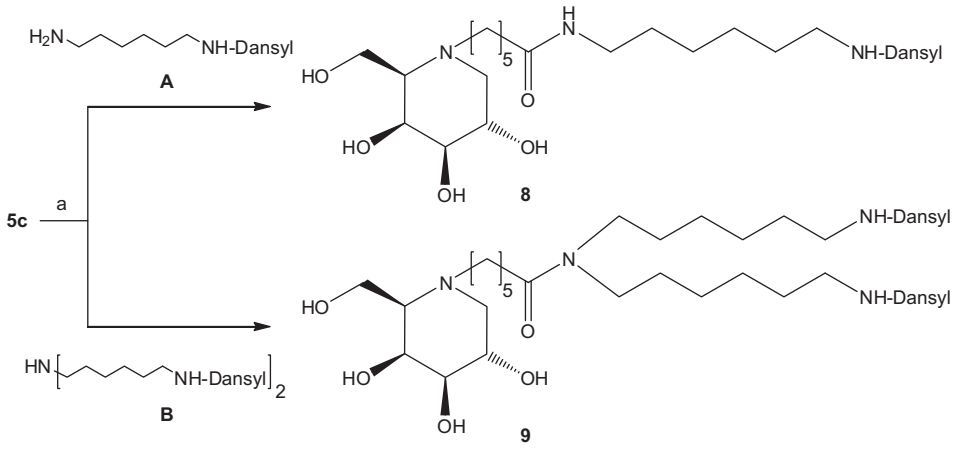


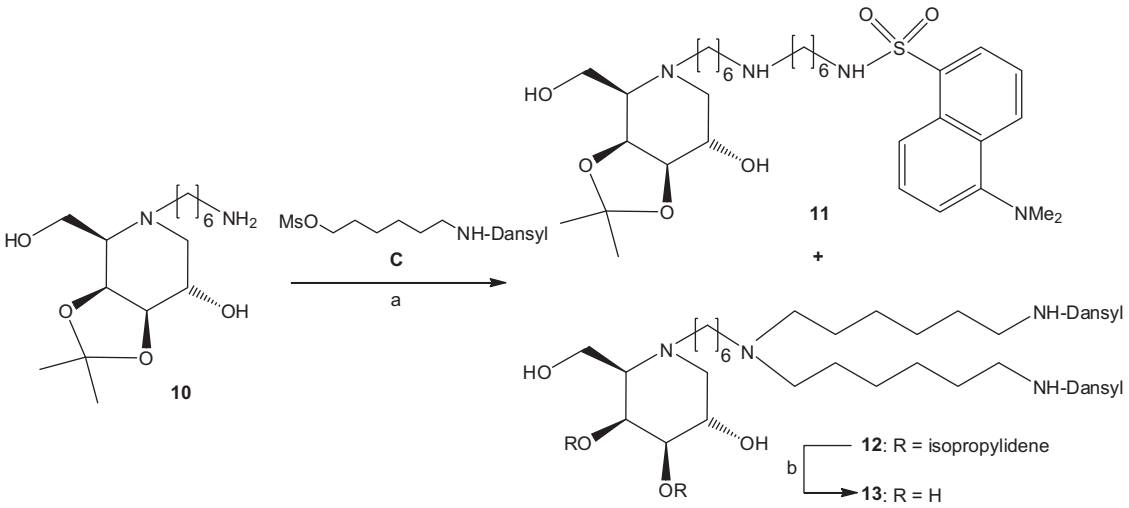
Figure 1. Glucosidase and galactosidase inhibitors as potential therapeutic principles for lysosomal deficiencies of D-glucosidase and D-galactosidase, respectively.



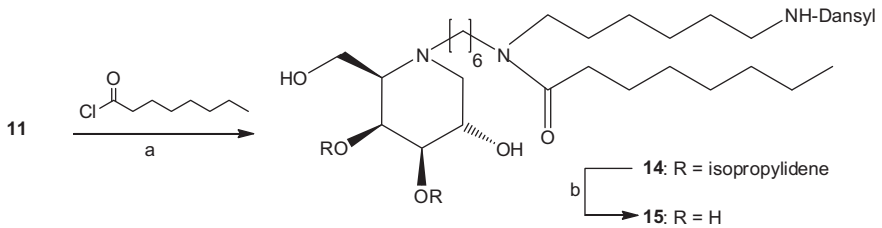
Scheme 1. Reagents: (a) H_2, Pd/C, MeOH; (b) HCl, MeOH, 23% over all; (c) NaOH, dioxane/water, quant.



Scheme 2. Preparation of inhibitors **8** (86%) and **9** (87%). Reagents: (a) TBTU, Et_3N, DMF.



Scheme 3. Preparation of intermediates **11** (47%) and **12** (9%). Reagents: (a) Na_2CO_3, DMF; (b) aq HCl, quant.



Scheme 4. Route to inhibitor **15**. Reagents: (a) pyridine, CH_2Cl_2, 71%; (b) aq HCl, quant.

Table 1
K_i-values (μM) of compounds with ABG = β-glucosidase/β-galactosidase from *Agrobacterium* sp.; *E. coli* = β-galactosidase from *E. coli*, GCB = α-galactosidase from green coffee beans

Enzyme	Compounds				
	5	**8**	**9**	**13**	**15**
ABG (K_i)	12	0.45	0.63	0.23	0.028
E. coli (K_i)	12.5	0.27	0.29	0.12	0.047
GCB (K_i)	0.0016	3.4	2.6	0.53	0.02
HLβG (IC_{50})	69	6.2	0.85	2.7	1.8
HLαG (IC_{50})	0.27	104	41	81	24

HLβG = human lysosomal β-galactosidase (pH 4.5), HLαG = human lysosomal α-galactosidase (pH 4.5); IC_{50} (μM) for compounds with human lysosomal β- as well as α-galactosidase.

respectively) and also functioned as a potent inhibitor of the green coffee bean α-galactosidase (K_i 0.20 μM).

With regard to human lysosomal β-galactosidase, compounds **8**, **9**, **13** and **15** functioned as low micromolar inhibitors (IC_{50} = 6.2 μM, 0.85 μM, 2.7 μM and 1.8 μM, respectively). In contrast, compounds **8**, **9**, **13** and **15** acted as weak inhibitors of lysosomal α-galactosidase, displaying IC_{50} more than 50-fold greater relative to the values obtained for lysosomal β-galactosidase (104 μM, 41 μM, 81 μM and 24 μM, respectively).

Intracellular β-galactosidase and β-*N*-acetylhexosaminidase was determined using MU based substrates following lysis of treated cells. Activity was normalized to DMSO treated cells.

A feline fibroblast line bearing the p.Arg483Pro mutation in β-galactosidase[17] was used to examine the chaperoning efficacy of each of the four compounds (Fig. 2A and B). Relative to DMSO treated cells, compounds **8**, **9**, and **13** maximally increased intracellular β-galactosidase activity 1.7 (258 μM), 1.5 (2.1 μM) and 1.8 (3.0 μM) fold. With the exception of compound **8**, β-galactosidase and β-hexosaminidase activity relative to DMSO treated cells declined at concentrations between 5 and 20 μM, indicative of inhibitory effects by compounds **9**, **13**, and **15** at these levels. Compound **8** was exceptional in that it had a large range over which it enhanced β-galactosidase activity. This may be due to the fact that it is a slightly weaker inhibitor and, thus, causes a later onset of inhibitory effects compared to **9**, **13** and **15**. No undesired inhibitory effect was exerted on lysosomal *N*-acetyl-β-hexosaminidase (Fig. 2B).

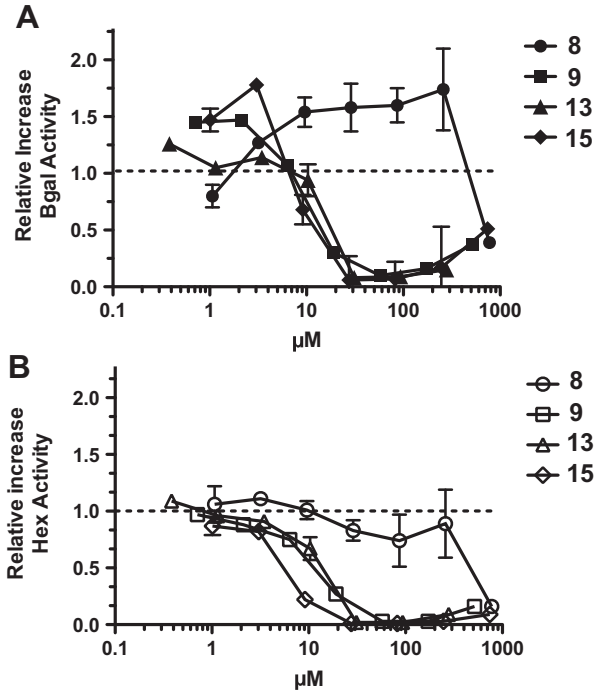


Figure 2. (A) Intracellular β-galactosidase following treatment of feline fibroblasts expressing the β-galactosidase p.Arg483Pro mutation. Fibroblasts were grown in the presence of escalating dose of compounds **8**, **9**, **13** and **15** for 5 days. (B) Effects of compounds on lysosomal *N*-acetylhexosaminidase.

When human fibroblasts expressing wild type β-galactosidase were treated overnight with different concentrations of the compounds and subsequently examined using fluorescence microscopy a punctate staining pattern was observed (Fig. 3). One interpretation consistent with this staining pattern is that compounds were found in the lysosome, either due to the hydrophobic nature of the compounds or by way of their association with β-galactosidase. These results not only demonstrate the enzyme enhancement efficacy of the compounds but are also suggestive of the utility of these derivatives as novel intracellular organellar probes of the lysosome.

3. Experimental

3.1. General methods

Optical rotations were measured on a Perkin Elmer 341 polarimeter at the wavelength of 589 nm and a path length of 10 cm at 20 °C. NMR spectra were recorded on a Varian INOVA 500 operating at 599.82 MHz (^{1}H), and at 125.894 MHz (^{13}C) or on a Bruker Ultrashield spectrometer at 300.36 and 75.53 MHz, respectively. $CDCl_3$ was employed for protected compounds and MeOH-d_4 or D_2O for unprotected inhibitors. Chemical shifts are listed in delta employing residual, non-deuterated solvent as the internal standard. The signals of the *N*-dansyl aromatic moieties were found in the expected regions and are not listed explicitly. MALDI-TOF Mass Spectrometry was performed on a Micromass TofSpec 2E Time-of-Flight Mass Spectrometer. Analytical TLC was performed on precoated aluminum plates Silica Gel 60 F254 (E. Merck 5554), detected with UV light (254 nm), 10% vanillin/sulfuric acid as well as ceric ammonium molybdate (100 g ammonium molybdate/8 g ceric sulfate in 1 L 10% H_2SO_4) and heated on a hotplate. Preparative TLC was performed on precoated glass plates Silica Gel 60 F254, 0.5 mm (E. Merck 5744). For column chromatography Silica Gel 60 (230–400 mesh, E. Merck 9385) was used.

3.2. Kinetic studies

3.2.1. *Agrobacterium* sp. β-galactosidase/-glucosidase

The purification and assay of *Agrobacterium* sp. β-galactosidase/-glucosidase was done as described.[18] Kinetic studies were performed at 37 °C in pH 7.0 sodium phosphate buffer (50 mM) containing 0.1% bovine serum albumin, using 7.2×10^{-5} mg/mL enzyme. Approximate values of K_i were determined using a fixed concentration of substrate, 4-nitrophenyl β-D-glucopyranoside (0.11 mM = 1.5 × K_m) and inhibitor concentrations ranging from 0.2 times to 5 times the K_i value ultimately determined. A horizontal line drawn through $1/V_{max}$ in a Dixon plot of this data ($1/V$ vs [I]) intersects the experimental line at an inhibitor concentration equal to $-K_i$. Full K_i determinations where required, were performed using the same range of inhibitor concentrations while also varying substrate (4-nitrophenyl glucoside) concentrations from approximately 0.015–0.6 mM. Data were analysed by direct fit to the Michaelis Menten equation describing reaction in the presence of inhibitors using the program GRAFIT.

3.2.2. *E. coli* β-galactosidase

Sigma: kinetic studies for *E. coli* β-galactosidase were performed at 37 °C in pH 7.0 sodium phosphate buffer (50 mM), using sufficient enzyme such less than 10% of the total substrate was converted to product during the observation period of approx 5 min.

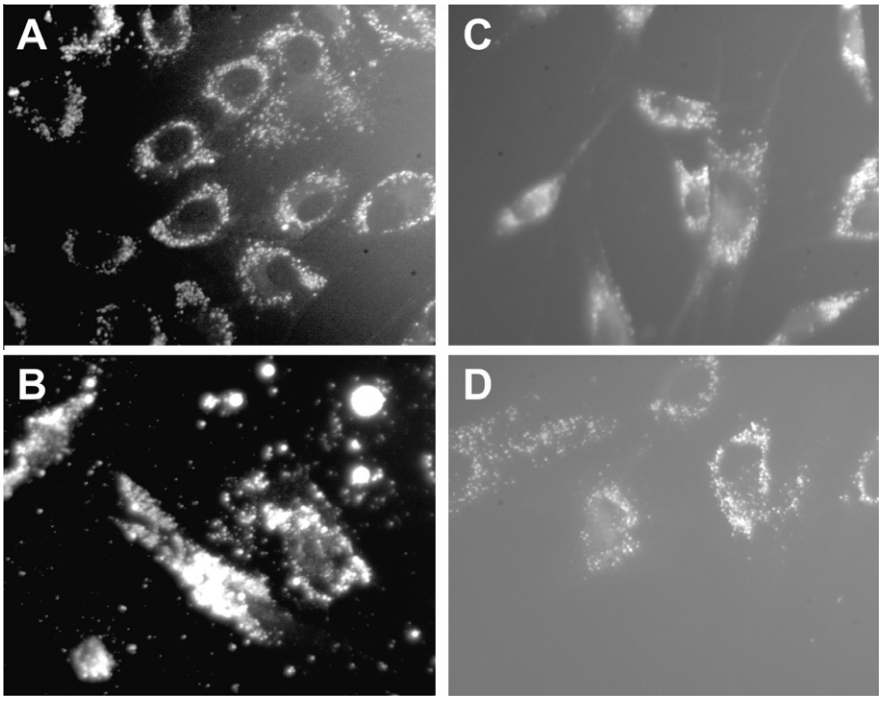


Figure 3. Intracellular staining of human fibroblasts with fluorescent compounds **8** (258 μM), **9** (177 μM), **13** (3.4 μM) and **15** (82 μM) (A–D, respectively). Fibroblasts were treated with the compounds overnight. Fluorescent images were obtained using FITC filter. All images were exposed using the same settings.

Approximate values of K_i were determined using a fixed concentration of substrate, 2-nitrophenyl β-D-galactopyranoside (0.15 mM = 1.5 × K_m) and inhibitor concentrations ranging from 0.2 times to 5 times the K_i value ultimately determined.

3.2.3. Green coffee bean α-galactosidase

Sigma: kinetic studies for green coffee bean α-galactosidase were performed at 37 °C in pH 6.5 sodium phosphate buffer (50 mM), using sufficient enzyme that less than 10% of the total substrate was converted to product during the observation period of approx 5 minutes. Approximate values of K_i were determined using a fixed concentration of substrate, 4-nitrophenyl α-D-galactopyranoside (0.7 mM = 1.5 × K_m) and inhibitor concentrations ranging from 0.2 times to 5 times the K_i value ultimately determined.

3.2.4. Human lysosomal α- and β-galactosidase

The inhibitory activity of compounds **8**, **9**, **13** and **15** was also evaluated using a lysosomal enzyme enriched conA-fraction from human placenta as the enzyme source.[19] 4-Nitrophenyl β-D-galactopyranoside (6.3 mM) or 4-nitrophenyl α-D-galactopyranoside (17 mM) were employed as substrates. Reactions were performed at 37 °C in pH 4.5 citrate phosphate buffer (100 mM) as described previously.[20] IC_{50} values (μM) were extracted from the enzyme activity curves in the presence of increasing concentrations of the inhibitor using non-linear regression as implemented within Graphpad Prism 5.

3.2.5. Intracellular β-galactosidase activity

GM1 gangliosidosis fibroblasts derived from an affected cat were treated with escalating doses of compounds **8**, **9**, **13** and **15** (dissolved in DMSO) for 5 days. Intracellular β-galactosidase activity was measured using the fluorogenic substrate 4-methylumbelliferyl β-D-galactopyranoside as previously described.[20]

3.2.6. Intracellular staining

Human fibroblasts expressing wild type lysosomal β-galactosidase were treated overnight with increasing doses of compounds **8**, **9**, **13** and **15** (dissolved in DMSO) diluted in alpha-MEM growth media containing 10% fetal calf serum. Intracellular fluorescence arising from the compounds was visualized using the FITC filter on a Leica microscope with a DFC300F digital camera.

3.3. *N*-Methoxycarbonylpentyl-1,5-dideoxy-1,5-imino-D-galactitol (5b)

To a solution of 3,4-isopropylidene-L-*arabino*-hexos-5-ulose[16] (**7**, 580 mg, 2.46 mmol) in MeOH (30 mL), methyl 6-aminocaproate (410 mg, 2.82 mmol, 1.15 equiv) and $Pd(OH)_2$ (20%, 100 mg) were added and the mixture was stirred under an atmosphere of hydrogen at ambient pressure for 48 h. The catalyst was removed by filtration and washed with MeOH. To the combined filtrate and washings, a 5% methanolic solution of HCl (freshly prepared by addition of acetyl chloride to MeOH) was added and the reaction was kept at ambient temp for 24 h. The solvent was removed under reduced pressure and the remaining residue was chromatographed on silica gel ($CHCl_3$/MeOH/concd NH_4OH 30:10:1 v/v/v) to give known compound **5b** (165 mg, 23%) as a colourless syrup. $[\alpha]_D^{20}$ −14.3 (*c* 1.0, MeOH); ^{1}H NMR (500 MHz, MeOH-d_4): δ = 4.01 (br s, 1H, H-4), 3.88–3.78 (m, 3H, H-2, H-6a, H-6b), 3.65 (s, 3H, OMe), 3.29–3.23 (m, 1H, H-3), 3.05 (dd, 1H, $J_{5,6}$ = 4.3 Hz, $J_{6,6'}$ = 11.0 Hz, H-1eq), 2.87–2.76 (m, 1H, H-1′a), 2.69–2.55 (m, 2H, H-1′b, H-5), 2.34 (t, 2H, J = 7.4 Hz, H-5′), 2.25 (m, 1H, H-1ax), 1.69–1.60 (m, 2H), 1.60–1.50 (m, 2H), 1.37–1.35 (m, 2H); ^{13}C NMR (125 MHz, MeOH-d_4): δ = 175.8 (C-6′), 76.8, 72.0, 68.5, 65.4, 62.1, 57.4 (C-1, C-2, C-3, C-4, C-5, C-6), 53.9 (C-1′), 52.0 (OMe), 34.6, 27.9, 25.8, 24.6 (C-2′, C-3′, C-4′, C-5′). MS: Calcd for $[C_{13}H_{25}NO_6H]$: m/z 292.1760 $[M+H]^+$; Found: $[M+H]^+$ 292.1743.

3.4. *N*-(Dansylamino)hexylaminocarbonylpentyl-1,5-dideoxy-1,5-imino-D-galactitol (8)

To an aqueous solution (3 mL) of *N*-methoxycarbonylpentyl-1-deoxy-D-galactonojirimycin (**5b**, 150 mg, 0.51 mmol), 0.5 M aqueous NaOH (2.0 mL, 1.0 mmol) was added and the mixture was kept

at ambient temp. for 90 min. The mixture was brought to pH 5 by addition of aqueous HCl (5%) and the solvent was removed under reduced pressure to give crude **5c**. Mono-*N*-dansyl-1,6-diaminohexane[15] (**A**, 200 mg, 0.57 mmol) in dichloromethane (5 mL) was added to this crude **5c** and the solvent was again removed under reduced pressure. The residue was dissolved in DMF (4.5 mL), Et_3N (125 μL, 0.9 mmol) and TBTU (96%, 130 mg, 0.40 mmol) were added. After it completed conversion (TLC, ca. 90 min), the mixture was concentrated under reduced pressure. Chromatography of the residue ($CHCl_3$/MeOH/concd NH_4OH = 10:4:1 v/v/v) gave compound **8** (267 mg, 86%) as a pale yellow syrup. $[\alpha]_D^{20}$ −7.7 (*c* 2.7, MeOH); ^{1}H NMR (500 MHz, MeOH-d_4): δ = 3.99 (br s, 1H, H-4), 3.87–3.76 (m, 3H, H-2, H-3, H-6a), 3.24 (dd, 1H, *J* = 2.5 Hz, *J* = 9.1 Hz, H-6b), 3.04–2.95 (m, 3H, incl. H-1eq), 2.92 (br s 1H, H-5), 2.88–2.77 (m, 8H), 2.77–2.67 (m, 1H, H-1′a), 2.57–2.49 (m, 1H, H-1′b), 2.46–2.39 (m, 1H, H-5), 2.20–2.10 (m, 3H, incl. H-1ax), 1.65–1.55 (m, 2H), 1.55–1.45 (m, 2H), 1.34–1.20 (m, 6H), 1.15–1.01 (m, 4H); ^{13}C NMR (125 MHz, MeOH-d_4): δ = 175.9 (C-6′), 77.0, 72.1, 68.8, 65.2, 62.2, 57.8 (C-1, C-2, C-3, C-4, C-5, C-6), 53.8, 45.8, 43.7, 40.1 (C-1′, C-1″, C-6″, NMe_2), 37.0 (C-5′), 30.4, 30.1, 28.0, 27.2, 27.0, 26.9, 24.7. MS: Calcd for [$C_{30}H_{48}N_4O_7SH$]: *m*/*z* 609.3322 [M+H]$^+$; Found: [M+H]$^+$ 609.3340.

3.5. *N*-Bis(dansylaminohexyl)aminocarbonylpentyl-1,5-dideoxy-1,5-imino-D-galactitol (9)

To an aqueous solution (1.5 mL) of *N*-methoxycarbonylpentyl-1-deoxynojirimycin (**5b**, 32 mg, 0.11 mmol), 0.5 M aqueous NaOH (0.30 mL, 0.15 mmol) was added and the mixture was kept at ambient temp for 60 min. The mixture was brought to pH 5 by addition of aqueous HCl (5%) and the solvent was removed under reduced pressure. Bis(6-dansylamino)hexylamine[15] (**B**, 100 mg, 0.15 mmol) in dichloromethane (3 mL) was added to the residue and the solvent was again removed under reduced pressure. The residue was dissolved in DMF (1.5 mL), Et_3N (30 μL, 0.22 mmol) and TBTU (96%, 39 mg, 0.12 mmol) were added. After it completed conversion (TLC, ca. 10 min), the mixture was concentrated under reduced pressure and the residue was chromatographed ($CHCl_3$/MeOH/concd NH_4OH, 50:10:1 v/v/v) providing compound **9** (90 mg, 87%) as a fluorescent yellow syrup. $[\alpha]_D^{20}$ −6.0 (*c* 2.8, MeOH); ^{1}H NMR (500 MHz, MeOH-d_4): δ = 3.99 (m, 1H, H-4), 3.87–3.77 (m, 3H, H-2, H-3, H-6a), 3.64–3.57 (m, 2H), 3.24 (dd, 1H, *J* = 2.9 Hz, *J* = 9.2 Hz, H-6b), 3.10–2.97 (m, 5H, incl. H-1eq), 2.89–2.66 (m, 16H), 2.59–2.49 (m, 1H, H-1′a), 2.49–2.38 (m, 1H, H-1′b), 2.27–2.11 (m, 3H, incl. H-1ax), 1.63–1.43 (m, 4H), 1.34–1.14 (m, 14H), 1.14–1.04 (m, 4H), 1.03–0.93 (m, 4H); ^{13}C NMR (125 MHz, MeOH-d_4): δ = 174.8 (C-6′), 78.3, 75.9, 71.0, 67.7, 64.0, 61.1 (C-1, C-2, C-3, C-4, C-5, C-6), 57.1, 52.7, 48.7, 46.5, 45.7, 44.8 (C-1′, 2 C-1″, 2 C-6″, 2 NMe_2), 42.6, 42.5, 32.7, 29.3, 29.2, 28.6, 27.2, 27.1, 26.1, 26.0, 25.4, 23.7. Caused by the nature of the amide bond, some signals of carbons of the X″ sections appear at slightly different parts per million values. MS: Calcd for [$C_{48}H_{72}N_6O_9S_2H$]: *m*/*z* 941.4880 [M+H]$^+$; Found: [M+H]$^+$ 941.4921.

3.6. *N*-(Dansylaminohexylamino)hexyl-3,4-*O*-isopropylidene-1,5-dideoxy-1,5-imino-D-galactitol (11) and *N*-bis(dansylaminohexyl)aminohexyl-3,4-*O*-isopropylidene-1,5-dideoxy-1,5-imino-D-galactitol (12)

To a solution of known[16] compound **10** (128 mg, 0.42 mmol) and mesylate **C**[15] (185 mg, 0.43 mmol) in dry *N*,*N*-dimethylformamide, sodium carbonate (271 mg, 2.54 mmol) was added and the mixture was stirred at 60 °C for 44 h when TLC ($CHCl_3$/MeOH/NH_4OH concd = 12:4:1 v/v/v) indicated complete conversion of the starting material. The mixture was concentrated under reduced pressure and the remaining residue was chromatographed ($CHCl_3$/MeOH/concd NH_4OH = 40:10:1 v/v/v) to give syrupy secondary amine **11** (127 mg, 47%) and less polar tertiary amine **12** (38 mg, 9%).

Compound **11**: $[\alpha]_D^{20}$ −3.7 (*c* 1.9, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.27 (m, 1H, H-4), 3.91 (dd, 1H, *J* = 3.6 Hz, *J* = 11.8 Hz, H-6a), 3.86–3.78 (m, 3H, H-2, H-3, H-6b), 2.94 (m, 1H), 2.89–2.82 (m, 8H), 2.72 (m, 1H, H-1eq), 2.64–2.46 (m, 6H), 2.08 (m, 1H, H-1ax), 1.56–1.20 (m, 18H), 1.19–1.08 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 109.9 (isopropylidene), 81.0, 70.6, 61.8, 60.8, 54.9, 52.9 (C-1, C-2, C-3, C-4, C-5, C-6), 49.8, 49.6, 45.7, 43.4 (C-1′, C-6′, C-1″, C-6″, NMe_2), 29.7, 29.5, 29.3, 28.7, 27.5, 27.4, 26.8, 26.7, 26.4, 24.4. MS: Calcd for [$C_{33}H_{54}N_4O_6SH$]: *m*/*z* 635.3842 [M+H]$^+$; Found: [M+H]$^+$ 635.3897.

Compound **12**: $[\alpha]_D^{20}$ −2.8 (*c* 1.9, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.29 (m, 1H, H-4), 3.92 (dd, 1H, *J* = 5.1 Hz, *J* = 11.9 Hz, H-6a), 3.89–3.81 (m, 3H, H-2, H-3, H-6b), 2.96 (m, 1H, H-1eq), 2.90–2.81 (m, 16H), 2.73 (m, 1H, H-1′a), 2.64 (m, 1H, H-5), 2.55 (m, 1H, H-1′b), 2.32–2.21 (m, 6H), 2.18–2.07 (m, 2H, incl. H-1ax), 1.52 (s, 3H), 1.49–1.30 (m, 12H), 1.30–1.05 (m, 14H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 110.1 (isopropylidene), 80.9, 77.8, 70.9, 61.7, 60.6, 54.7 (C-1, C-2, C-3, C-4, C-5, C-6), 54.0 (2 C-6″), 52.9 (C-1′), 45.8 (2 NMe_2), 43.6 [N–(CH_2–)$_3$], 29.8, 28.6, 27.5, 27.4, 27.1, 26.8, 26.75 (3 C), 26.7 (3 C), 24.4 (2 C). MS: Calcd for [$C_{51}H_{78}N_6O_8S_2H$]: *m*/*z* 967.5401 [M+H]$^+$; Found: [M+H]$^+$ 967.5460.

3.7. *N*-Bis(dansylaminohexyl)aminohexyl-1,5-dideoxy-1,5-imino-D-galactitol (13)

To compound **12** (36 mg, 0.037 mmol), aqueous HCl (6%, 2 mL) was added and the mixture was stirred at ambient temp for 3 h. Removal of the solvent under reduced pressure provided a slightly yellow glass which was triturated with dichloromethane and again dried under reduced pressure to provide inhibitor **13** as the tetrahydrochloride (40 mg, 100%). $[\alpha]_D^{20}$ +2.3 (*c* 2.0, MeOH); ^{1}H NMR (500 MHz, D_2O) δ = 4.10 (br s, 1H, H-4), 3.87 (ddd, 1H, $J_{1eq,2}$ = 5 Hz, $J_{1ax,2} = J_{2,3}$ = 10.6 Hz, H-2), 3.77 (m, 2H, H-6a, H-6b), 3.40 (m, 2H), 3.35–3.20 (m, 14H), 3.15–2.95 (m, 3H), 2.86–2.80 (m, 7H), 2.80–2.55 (m, 4H), 1.70–0.38 (m, 24H). MS: Calcd for [$C_{48}H_{74}N_6O_8S_2H$]: *m*/*z* 927.5088 [M+H]$^+$; Found: [M+H]$^+$ 927.5034.

3.8. *N*-[(Dansylaminohexyl)-*N*-octanoylamino]hexyl-3,4-*O*-isopropylidene-1,5-dideoxy-1,5-imino-D-galactitol (14)

To a solution of compound **11** (123 mg, 0.194 mmol) in dichloromethane (6 mL) containing pyridine (25 μL, 0.31 mmol), a solution of octanoic chloride (37 μL, 0.21 mmol) in dichloromethane (3 mL) was added at 0 °C over 30 min. The reaction was then quenched with MeOH (1 mL) and the mixture was concentrated under reduced pressure. The residue was purified on silica gel ($CHCl_3$/MeOH/concd NH_4OH = 150:10:1 v/v/v) to provide compound **14** (105 mg, 71%) as a faintly yellow syrup. $[\alpha]_D^{20}$ −3.5 (*c* 1.4, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.29 (br s, 1H, H-4), 3.92 (m, 1H, H-2), 3.88–3.81 (m, 3H, H-3, H-6a, H-6b), 3.29–3.11 (m, 3H), 3.08 (dd, 1H, *J* = 7.4 Hz, *J* = 8 Hz), 2.98 (m, 1H), 2.90–2.81 (m, 8H), 2.74 (m, 1H, H-1′a), 2.63 (m, 1H, H-5), 2.54 (m, 1H, H-1′b), 2.23 (t, 1H, *J* = 7.5 Hz), 2.19 (t, 1H, *J* = 7.5 Hz), 2.08 (m, 1H, H-1ax), 1.65–1.05 (m, 32H), 0.88–0.82 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.2/173.1, 110.0/109.9 (isopropylidene), 81.0/80.9, 77.8/77.7, 70.9/70.8, 61.8/61.7, 60.9/60.8, 54.7, 52.8/52.7, 48.2/48.1 (C-1, C-2, C-3, C-4, C-5, C-6, C-1′, C-6′), 45.9 (C-1″), 45.8 (NMe_3), 43.4/43.3 (C-6″), 33.5/33.4, 32.1, 29.9, 29.8/29.7, 29.5, 29.2, 28.7/28.6, 27.7/27.6, 27.5/27.2, 27.0/26.9, 26.7, 26.5/26.4, 25.9/25.8, 23.0, 14.4. Due to the amide bond, double signals are observed for several carbon atoms. MS: Calcd for [$C_{41}H_{68}$ N_4O_7SH]: *m*/*z* 761.4887 [M+H]$^+$; Found: [M+H]$^+$ 761.4835.

3.9. *N*-[(Dansylaminohexyl)-*N*-octanoylamino]hexyl-1,5-dideoxy-1,5-imino-D-galactitol (15)

To compound **14** (105 mg, 0.138 mmol), HCl (6% in water, 3 mL) was added and the mixture was stirred at ambient temp for 2 h. The solvent was removed under reduced pressure to provide inhibitor **15** as the di-hydrochloride (110 mg, pract. quant.) $[\alpha]_D^{20}$ +3.5 (*c* 1.25, MeOH); ^{1}H NMR (500 MHz, D_2O) δ = 4.03 (m, 1H), 3.90 (m, 1H, H-2), 3.75 (m, 2H, H-6a, H-6b), 3.40 (m, 1H, H-3), 3.35–3.3.18 (m, 6H), 3.13–2.85 (m, 6H), 2.73–2.59 (m, 3H), 2.89–2.79 (m, 2H), 2.10 (m, 1H, H-1ax), 2.00 (m, 2H), 1.60–1.43 (m, 2H), 1.37–0.67 (m, 24H), 0.83–0.75 (m, 3H); ^{13}C NMR (125 MHz, D_2O): δ = 175.1, 73.3, 70.5, 65.1, 64.7/64.6, 59.9/59.8, 54.0, 53.8, 48.7, 47.8 (2 C), 45.9, 43.2, 33.4, 32.1, 32.0, 29.7, 29.4, 29.0/29.3, 28.9, 27.6, 26.4/26.3, 26.1/26.0, 25.9/25.8, 23.0/22.9, 22.4, 14.5/14.4. MS: Calcd for $[C_{38}H_{64}N_4O_7SH]$: *m*/*z* 721.4574 $[M+H]^+$; Found: $[M+H]^+$ 721.4576.

Acknowledgments

Financial support by the Austrian *Fonds zur Förderung der Wissenschaftlichen Forschung* (FWF), Vienna, (Project P18998-N17) as well as by the Canadian Institutes of Health Research are gratefully acknowledged.

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