DOI: 10.1002/cbic.200900025

Synthesis and Biological Characterisation of Novel *N*-Alkyl-Deoxynojirimycin α-Glucosidase Inhibitors

Amy J. Rawlings,[a, b] Hannah Lomas,[a] Adam W. Pilling,[a, b] Marvin J.-R. Lee,[a] Dominic S. Alonzi,[a] J. S. Shane Rountree,[a, b] Sarah F. Jenkinson,[b] George W. J. Fleet,[b] Raymond A. Dwek,[a] John H. Jones,[b] and Terry D. Butters*[a]

The N-alkylated deoxynojirimycin compound, *N*-(6′-(4″-azido-2″-nitrophenylamino)hexyl)-1-deoxynojirimycin (**6**) was synthesised as a potential photoaffinity probe for endoplasmic reticulum (ER) α-glucosidases I and II. Surprisingly this compound was a highly potent inhibitor of α-glucosidase I (IC_{50}, 17 nM) in an in vitro assay and proved equally effective at inhibiting cellular ER glucosidases, as determined by a free oligosaccharide (FOS) analysis. A modest library of compounds was synthesised to obtain structure–activity information by variation of the N-alkyl chain length and modifications to the azido-nitrophenyl group. All of these compounds failed to improve on the efficacy of compound **6**, but most showed greater enzyme inhibitory potency than *N*-butyl-deoxynojirimycin (*N*B-DNJ), a pharmacological agent that has been evaluated for the treatment of several viruses for which infectivity is dependent on host cell glycosylation.

Introduction

1-Deoxynojirimycin (DNJ) imino sugars have demonstrated considerable therapeutic potential for the correction of protein-misfolding disorders, including those associated with lysosomal storage disease.[1–4] By contrast, a strategy that promotes glycoprotein misfolding in the endoplasmic reticulum (ER) has utility for reducing viral infectivity, especially as caused by HIV and hepatitis.[5,6] DNJ is an inhibitor of ER α-glucosidases I and II, and the recognition site for the natural oligosaccharide substrate, $Glc_3Man_9GlcNAc_2$, of α-glucosidase I has been reported.[7] The presumed mechanism for α-glucosidase inhibition by DNJ and its analogues is that protonation of the imino sugar nitrogen allows mimicry of the charge on the proposed oxocarbonium ion transition state formed during hydrolysis.[7]

The *N*-butyl analogue of DNJ is a better inhibitor of ER α-glucosidases than DNJ in cultured HepG2 cells.[8] However, the inhibitory activity of *N*B-DNJ in cells is poor relative to the in vitro activity with purified enzyme, requiring a concentration 2000 times greater than the K_i value.[9] It has been suggested that this could be due to low accessibility of the compound to the ER lumen or a loss of inhibitory activity from deprotonation of the compound ($pK_a = 6.6$)[10] in the ER (pH 7.1).[11,12]

One goal of our studies was to understand the principles that govern imino sugar entry to the ER and potential binding partners. To this end a number of DNJ derivatives that could be utilised as photolabile affinity probes in cells were synthesised. Surprisingly, some of these compounds were shown to be more potent in inhibiting ER α-glucosidases than both DNJ and *N*-butyl-DNJ. Further chemical synthesis allowed some structure–activity relationships to be elucidated. Inhibitors were biologically tested with two criteria in mind. Firstly, the in vitro potential against purified ER α-glucosidases I and II was determined. Secondly, cellular bioactivity was evaluated by using a free oligosaccharide (FOS) analysis, recently developed in our laboratory.[13] The latter is the far more interesting and salient assay as it considers the access of the compounds to the ER and quantifies the amount of inhibition produced.

Results and Discussion

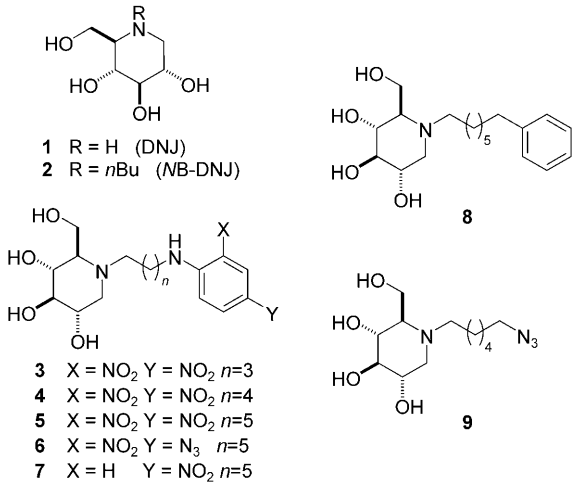
The target molecules for the photoaffinity labelling study were derived from DNJ. Suitable precursors for photoaffinity labelling were chosen to be chemically inert; there should be no possibility of rearrangement in the reactive species and they must have suitable absorption maxima. The photolabile compounds were therefore chosen as 4-azido-2-nitophenyl, 2,4-dinitrophenyl, 4-nitrophenyl and an unsubstituted phenyl group—readily synthesised compounds.[14,15] To probe the effect of a nonaromatic group, an alkyl azide was also investigated. The photolabile group was attached to the iminosugar by using an alkyl linker of between four and six carbons in length.

It was envisaged that the target compounds could be accessed by a reductive amination reaction of the iminosugar with an aldehyde, to which the photolabile moiety was already attached. In order to minimise side reactions, such as hemiami-

[a] *A. J. Rawlings, H. Lomas, Dr. A. W. Pilling, M. J.-R. Lee, Dr. D. S. Alonzi, Dr. J. S. S. Rountree, Prof. Dr. R. A. Dwek, Dr. T. D. Butters*
Oxford Glycobiology Institute, Department of Biochemistry
University of Oxford, South Parks Road, Oxford OX1 3QU (UK)
Fax: (+44) 1865-275216
E-mail: terry.butters@bioch.ox.ac.uk

[b] *A. J. Rawlings, Dr. A. W. Pilling, Dr. J. S. S. Rountree, Dr. S. F. Jenkinson, Prof. Dr. G. W. J. Fleet, Dr. J. H. Jones*
Chemistry Research Laboratory, Department of Chemistry
University of Oxford, Mansfield Road, Oxford OX1 3TA (UK)

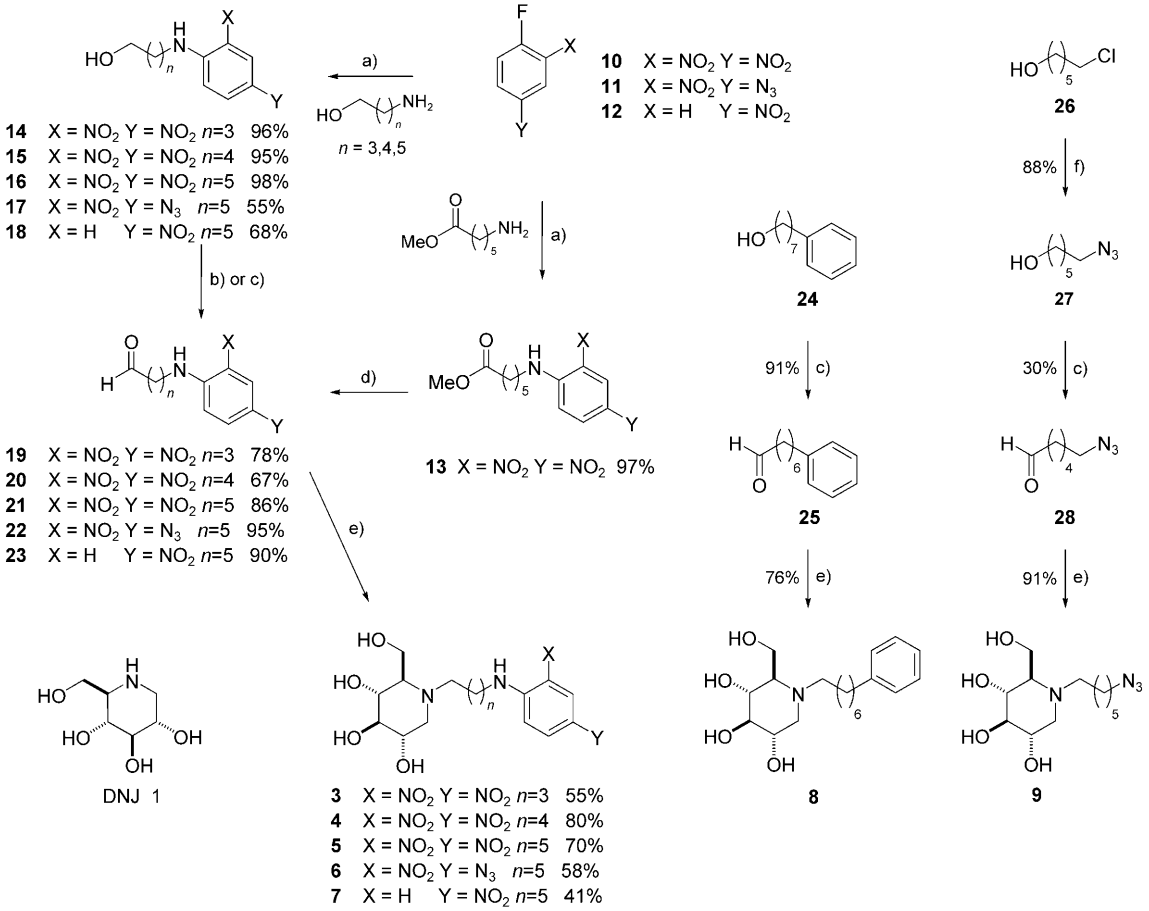
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900025.



nal formation, a masked aldehyde was used, and the photolabile moiety was introduced prior to the unmasking of the aldehyde functionality.

Two possible pathways to the desired aldehydes were proposed (Scheme 1); either via methyl ester **13** or via alcohols **14**–**18**. Direct S_NAr displacement of the aromatic fluorine of **10** by methyl 6-aminohexanoate gave the ester derivative **13** in 97% yield. The methyl ester functionality was then partially reduced to the aldehyde by using diisobutylaluminium hydride (DIBALH) to give **21** in 63% yield. The route via the alcohol derivatives **14**–**18** was found to be generally more reliable; in this case, the aromatic fluoride was displaced by an aminoalcohol to give the alcohol derivatives in good to excellent yields (**14**: 96%, **15**: 95%, **16**: 98%, **17**: 55%, **18**: 68%). The free hydroxy group was then oxidised to the aldehyde by using either Dess–Martin periodinane (**19**: 78%, **20**: 67%, **21**: 86%, **22**: 95%) or the Swern reaction (**23**: 90%). Reductive amination with DNJ in the presence of sodium cyanoborohydride and acetic acid in methanol proceeded smoothly in most cases to give the desired target molecules in good yield (**3**: 55%, **4**: 80%, **5**: 70%, **6**: 58%, **7**: 41%). The unsubstituted aromatic target compound **8** and the azido compound **9** were synthesised in a similar manner (Scheme 1).

Biological testing of compounds was carried out by first considering the in vitro potential of these compounds against α-glucosidases I and II using a previously reported HPLC method[13] (Table 1). A representative HPLC analysis of labelled oligosaccharide digestion products following glucosidase I incubation in the presence or absence of inhibitor **6** is shown in Figure 1. Compounds **6** and **5** were both shown to be significantly more potent than *N*B-DNJ **2** against both α-glucosida-



Scheme 1. Reagents and conditions: a) Et_3N, 1,4-dioxane, RT (85 °C for **18** and **13**); b) Dess–Martin periodinane, DCM, RT; c) $(COCl)_2$, DMSO, DCM, Et_3N, −60 °C; d) DIBALH, DCM, −78 °C; e) DNJ, $NaCNBH_3$, AcOH, MeOH, RT; f) NaN_3, DMF, 50 °C.

Table 1. In vitro inhibition of purified rat liver α-glucosidase I and II with $Glc_{1-3}Man_5GlcNAc_1$-2AA-labelled substrate.[a]

Enzyme	α-Glucosidase I	α-Glucosidase II	α-Glucosidase II
Substrate	$Glc_3Man_5GlcNAc_1$	$Glc_2Man_5GlcNAc_1$	$Glc_1Man_5GlcNAc_1$
Compound	IC_{50} [μM]	IC_{50} [μM]	IC_{50} [μM]
2	0.68 ± 0.05	10.8 ± 0.50	53.0 ± 6.6
6	0.017 ± 0.001	0.30 ± 0.10	0.83 ± 0.18
5	0.108 ± 0.02	6.9 ± 3.40	1.90 ± 0.40
17	NI[b]	NI[b]	NI[b]

[a] Enzyme was incubated with substrate and inhibitor before NP-HPLC analysis of the reaction products and estimation of the amount of hydrolysis (see Figure 1). The IC_{50} values for novel α-glucosidase inhibitors are compared to *N*B-DNJ **2**. [b] NI = non-inhibitory at the highest concentration tested (200 μM).

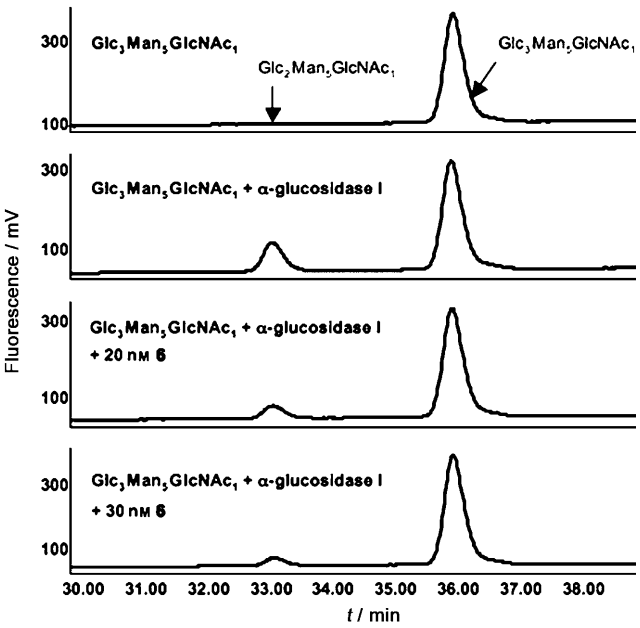


Figure 1. HPLC analysis of α-glucosidase I hydrolysis of $Glc_3Man_5GlcNAc_1$ oligosaccharide. Glucosidase enzyme was incubated with 2-AA-labelled oligosaccharide, as described in the text, in the absence of inhibitor or in the presence of 20 or 30 nM **6**. Hydrolysis in the presence of a range of concentrations of **6** was evaluated by measuring the peak areas of substrate and hydrolysis product ($Glc_2Man_5GlcNAc_1$) and used to calculate the concentration of **6** at which hydrolysis was inhibited by 50% (IC_{50} value).

se I and II. The enhancement of inhibitory activity (40 times greater than *N*B-DNJ) for **6** reveals this compound to be the most potent inhibitor of α-glucosidase I described. A previously synthesised photolabile probe, 4-(*p*-azidosalicylamido)butyl-5-amido-pentyl-1-DNJ, showed no improvement in in vitro glucosidase inhibition when compared to N-alkylated DNJ.[16] Also, **17** was shown to have no inhibitory effect, thus demonstrating that DNJ was a necessary substituent to enable inhibition (Table 1).

The in vitro data were matched by a cellular FOS analysis. A typical HPLC profile of the free oligosaccharide separation following inhibitor **6** treatment of HL60 cells is shown in Figure 2. The level of α-glucosidase I and II inhibition was determined by the amount of $Glc_3Man_5GlcNAc_1$ and $Glc_1Man_4GlcNAc_1$, respectively, produced in the cytosol as a result of endoplasmic reticulum-associated protein degradation (ERAD). These FOS species are the major glucosylated FOS species produced in response to α-glucosidase inhibition in the ER as a result of glycoprotein retrotranslocation via an ERAD pathway and the actions of PNGase and cytosolic α-mannosidase to release and truncate the oligosaccharide, respectively.[17] The level of glucosylated FOS was higher with **6**. Compound **5** also generated more glucosylated FOS than *N*B-DNJ. Comparison of the series of compounds **3**–**5** reveals a requirement for a minimum of six carbon atoms as a linker. Further experiments are in progress to evaluate the effects of extended chains on inhibitory potency. Previously we have shown that increases in N-alkyl chain length (4 to 18 carbon atoms) had a weak and nonpredictive structure–activity effect on glucosidase activity in vitro,[18] thus indicating the importance of the phenyl group in contributing to binding.

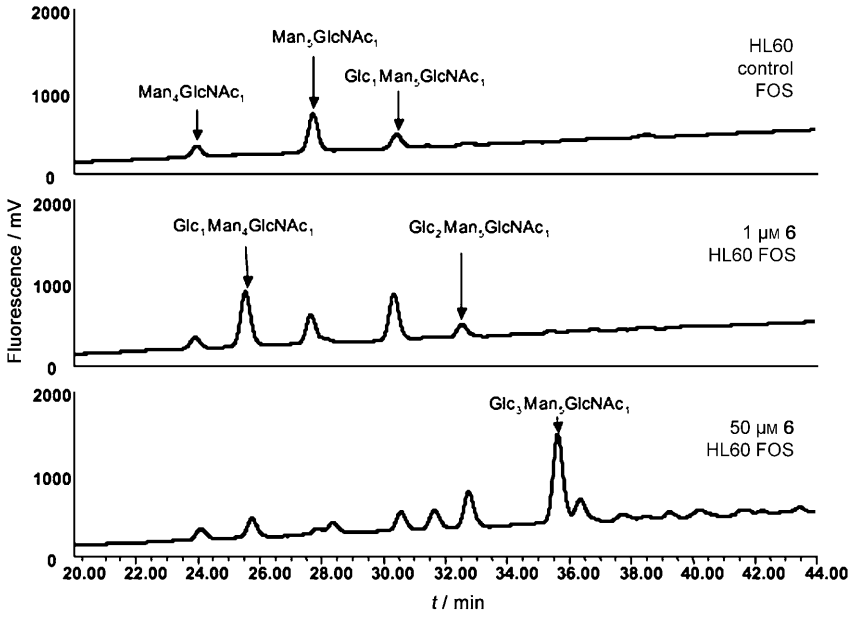


Figure 2. HPLC analysis of 2-AA-labelled free oligosaccharides (FOS). HL60 cells were grown in the absence or presence of 1 or 50 μM **6** for 24 h before isolating FOS, 2-AA labelling and separation by NP-HPLC. The labelled oligosaccharide peaks were identified following a comparison to a dextran ladder and authentic standards in addition to an enzymatic structural analysis. Peak areas for the relevant FOS species were calculated in replicate analyses to generate the data shown in Table 2.

Comparison of FOS produced at 1 μM attempts to focus on the selective inhibition of α-glucosidase II, since no triglucosylated FOS was produced by any of the inhibitors at this concentration (Table 2). Consequently, the level of $Glc_1Man_4GlcNAc_1$ (glucose unit GU = 5.31) was monitored, since this species is only produced following inhibition of α-glucosidase II and is not present in untreated control cells.[13] Compounds **8** and **6** were excellent α-glucosidase inhibitors; this again demonstrated the value of the six-carbon chain linker and the aromatic group. It is important to note that **17** had no inhibitory effect at any concentration tested (up to 100 μM).

Table 2. Free oligosaccharides (FOS) analysis of HL60 cells treated with 1 or 50 μM α-glucosidase inhibitor for 24 h.[a]

Inhibitor	$Glc_1Man_4GlcNAc_1$ 1 μM for 24 h	$Glc_3Man_5GlcNAc_1$ 50 μM for 24 h
2	0.00	0.25 ± 0.01
3	0.00	0.00
4	0.24 ± 0.01	0.00
5	1.55 ± 0.08	1.53 ± 0.07
6	2.83 ± 0.14	6.58 ± 0.33
7	1.05 ± 0.05	3.49 ± 0.16
8	3.62 ± 0.20	6.01 ± 0.30
9	0.29 ± 0.02	1.19 ± 0.06
17	0.00	0.00

[a] Cytosolic $Glc_1Man_4GlcNAc_1$ (GU = 5.31) produced following α-glucosidase II inhibition in cells (relative to $Man_4GlcNAc_1$) with 1 μM inhibitor for 24 h. Cytosolic $Glc_3Man_5GlcNAc_1$ (GU = 8.29) produced following α-glucosidase I inhibition in cells (relative to $Man_4GlcNAc_1$) with 50 μM inhibitor for 24 h. $Man_4GlcNAc_1$ is a lysosomal-derived FOS species that remains constant at the concentration of inhibitor administered in this study.[13] Standard deviations from replicate experiments are shown. See Figure 2 for the HPLC profile.

The FOS analysis at 50 μM allowed a comparison of the inhibition of α-glucosidase I at a noncytotoxic level for all the DNJ analogues. The FOS species produced following inhibition of α-glucosidase I, $Glc_3Man_5GlcNAc_1$, was analysed (GU = 8.29). The results showed a 25-fold increase in the amount of $Glc_3Man_5GlcNAc_1$ produced by **6** compared to *N*B-DNJ at 50 μM (Table 2).

The FOS assay provides a more meaningful analysis of the effects of cellular enzyme inhibition and reveals that, for **6** and **5**, the potency was not due to greater uptake or ER penetration. The contribution of the aryl nitro and azido moieties to inhibitor binding energy would allow further modifications to improve potency, particularly since **8** is almost as potent as **6** in the cellular assay at 50 μM (Table 2). However, when the same analysis was performed at 10 μM, **6** led to a four- to five-fold increase in $Glc_3Man_5GlcNAc_1$ over **8**, thus revealing a concentration-dependent mode of glucosidase inhibition that requires critical evaluation for comparative analysis of inhibitory potential. Interestingly, when the alkyl spacer chain on **8** was truncated, inhibitory potency decreased (D.S.A. et al., unpublished results).

Conclusions

In the design of a photoaffinity probe for cellular glucosidases, potent inhibition of ER-α-glucosidases I and II by compound **6** was achieved. The in vitro inhibition of α-glucosidase I (IC_{50}, 17 nM) measured by using oligosaccharide substrates is a significant improvement on previously reported imino sugars[4] and is the best inhibitor of this enzyme reported to date. The potent in vitro inhibition was matched by the inhibition of ER-glucosidases in cells and therefore has potential therapeutic value as an antiviral strategy.[5]

Experimental Section

Synthesis–general experimental: ^{1}H NMR spectra were recorded on a Bruker DPX 400 (400 MHz) or a Bruker AV 500 (500 MHz) spectrometer and were calibrated according to the chemical shift of the deuterated solvent. ^{13}C NMR spectra were recorded on a Bruker DQX 400 (100 MHz) or a Bruker AV 500 (125.8 MHz) and were calibrated according to the chemical shift of the deuterated solvent. Chemical shifts (δ) are quoted in ppm and coupling constants (J) in Hz. Spectra were fully assigned by using COSY, DEPT and HMQC. Infrared spectra were recorded on a Bruker Tensor 27 FTIR spectrophotometer by using thin films on NaCl or Ge plates as stated, and peaks are given in cm^{-1}. Only characteristic peaks are quoted. Low-resolution mass spectra (LRMS) were recorded on a Fissions Platform (ESI) spectrometer or a Micromass VG Autospec 500 OAT (Cl(NH_3)) spectrometer. High-resolution mass spectra (HRMS) were recorded on a Micromass VG Autospec 500 OAT (Cl-(NH_3)) spectrometer or a Micromass GCT (FI) spectrometer. Optical rotations were measured on a Perkin–Elmer 241 polarimeter with a path length of 1 dm; concentrations (c) are quoted in g per 100 mL. Elemental analyses were performed by the microanalysis service of the Inorganic Chemistry Laboratory (Oxford). Melting points (m.p.) were measured on a Kofler hot-block apparatus and are uncorrected. Thin-layer chromatography (TLC) was carried out on aluminium sheets coated with $60F_{254}$ silica from Merck, the plates were visualised under ultraviolet light and were developed by staining with 6% phosphomolybdic acid (*w*/*v*) in ethanol with subsequent heating. Flash column chromatography was carried out with Sorbsil C60 40/60 silica. Purification of **3**–**9** was carried out with Waters Sep-Pak Cartridges packed with reversed-phase sorbent C18. Solvents (HPLC grade) and commercially available reagents were used as supplied. For Swern oxidations, dichloromethane was dried over calcium hydride and distilled. Triethylamine was dried with sodium hydroxide solution and distilled from 2% phenyl isocyanate. Dimethylsulfoxide was purchased anhydrous from Fluka.

Detailed chemical syntheses of the compounds shown in Scheme 1 can be found in the Supporting Information.

Biological assays

Materials: Tissue culture media were from Gibco/Invitrogen or Sigma. AnalaR and HPLC-grade solvents were from VWR International (Lutterworth, UK). All other reagents were from Sigma. Water was Milli-QTM grade.

Inhibitors: Synthesised as described in the Supporting Information, the inhibitors were:
N-(4′-(2″,4″-Dinitrophenylamino)butyl)-1-deoxynojirimycin (**3**)
N-(5′-(2″,4″-Dinitrophenylamino)pentyl)-1-deoxynojirimycin (**4**)
N-(6′-(2″,4″-Dinitrophenylamino)hexyl)-1-deoxynojirimycin (**5**)

N-(6′-(4″-Azido-2″-nitrophenylamino)hexyl)-1-deoxynojirimycin (**6**)
N-(6′-(4″-Nitrophenylamino)hexyl)-1-deoxynojirimycin (**7**)
N-(7′-Phenylheptyl)-1-deoxynojirimycin (**8**)
N-(6′-Azidohexyl)-1-deoxynojirimycin (**9**)

In vitro glucosidase inhibition: 2-AA-Labelled free oligosaccharides were isolated and purified as substrates for either α-glucosidase I or α-glucosidase II (both purified from rat liver).[13] The labelled substrates $Glc_1Man_5GlcNAc_1$, $Glc_2Man_5GlcNAc_1$ and $Glc_3Man_5GlcNAc_1$ were added to separate 1.5 mL centrifuge tubes with varying concentrations of imino sugar and dried under vacuum. Normal-phase HPLC analysis of the 2-AA-labelled oligosaccharide and degradation products following enzyme hydrolysis was performed as described below. Peak area measurement was used to determine the amount of hydrolysis. Inhibitors were added at various concentrations (0–200 μM) to determine the IC_{50} values.[13]

Cell culture: HL60 cells were cultured in RPMI media containing 10% foetal calf serum, 2 mM L-glutamine and 1% penicillin-streptomycin (Invitrogen).

Isolation of free oligosaccharides (FOS) from cells: Cells were cultured to high density (1×10^7 cells mL^{-1}) before the medium was replaced with fresh medium containing inhibitor at varying concentrations, and the cells were seeded at a lower density so as to achieve a high density at the end of the incubation period. Following cell culture, the medium was removed, and the cells were washed three times with PBS by centrifugation. Washed cells were stored at −20 °C for a short time before thawing and Dounce homogenisation in water. The conditions for extraction of FOS were optimised to maximise recovery of FOS. Essentially, the homogenate was desalted and deproteinated by passage through a mixed-bed ion-exchange column [0.2 mL AG50W-X12 (H^+, 100–200 mesh) over 0.4 mL AG3-X4 (OH^-, 100–200 mesh)], pre-equilibrated with water (5×1 mL). The homogenate was added and collected with water washes (4×1 mL). The extracted purified FOS were then dried under vacuum.

Carbohydrate fluorescent labelling: The FOS were labelled with anthranilic acid, as described in the literature.[13] Briefly, anthranilic acid (30 mg mL^{-1}) was dissolved in a solution of sodium acetate trihydrate (4%, *w*/*v*) and boric acid (2% *w*/*v*) in methanol (see the Supporting Information for a detailed protocol).

Purification of fluorescently labelled FOS: Labelled oligosaccharides in Tris/HCl buffer (50 mM, pH 7.2) were purified through Concanavalin A (ConA)–Sepharose 4B column (100 μL packed resin). The column was pre-equilibrated with water (2×1 mL) followed by $MgCl_2$ (1 mM), $CaCl_2$ (1 mM) and $MnCl_2$ (1 mM) in water (1 mL) and finally Tris/HCl buffer (2×1 mL, 50 mM, pH 7.2). The sample was added and washed with Tris/HCl buffer (2×1 mL, 50 mM, pH 7.2). The bound FOS were then eluted with hot (70 °C) methyl α-D-mannopyranoside (2×1 mL, 0.5 M) in Tris/HCl buffer (50 mM, pH 7.2).

Carbohydrate analysis by normal-phase HPLC: ConA–Sepharose-purified 2-AA-labelled oligosaccharides were separated by NP-HPLC on a 4.6×250 mm TSK gel Amide-80 column (Anachem, Luton, UK) with slight modifications to the published method.[13]

Acknowledgements

The Glycobiology Institute is thanked for its support.

Keywords: glycosylation · imino sugars · nitrophenyl · oligosaccharides · photolabile groups

[1] P. Alfonso, S. Pampin, J. Estrada, J. C. Rodriguez-Rey, P. Giraldo, J. Sancho, M. Pocovi, *Blood Cells Mol. Dis.* **2005**, *35*, 268.
[2] T. D. Butters, *Expert Opin. Pharmacother.* **2007**, *8*, 427.
[3] C. Norez, S. Noel, M. Wilke, M. Bijvelds, H. Jorna, P. Melin, H. DeJonge, F. Becq, *FEBS Lett.* **2006**, *580*, 2081.
[4] L. Yu, K. Ikeda, A. Kato, I. Adachi, G. Godin, P. Compain, O. Martin, N. Asano, *Bioorg. Med. Chem.* **2006**, *14*, 7736.
[5] R. A. Dwek, T. D. Butters, F. M. Platt, N. Zitzmann, *Nat. Rev. Drug Discovery* **2002**, *1*, 65.
[6] N. Zitzmann, T. Block, A. Methta, P. Rudd, D. Burton, I. Wilson, F. Platt, T. Butters, R. A. Dwek, *Adv. Exp. Med. Biol.* **2005**, *564*, 1.
[7] T. D. Butters, L. A. G. M. van den Broek, G. W. J. Fleet, T. M. Krulle, M. R. Wormald, R. A. Dwek, F. M. Platt, *Tetrahedron: Asymmetry* **2000**, *11*, 113.
[8] A. Tan, L. van den Broek, S. van Boeckel, H. Ploegh, J. Bolscher, *J. Biol. Chem.* **1991**, *266*, 14504.
[9] L. A. van den Broek, M. W. Kat-van den Nieuwenhof, T. D. Butters, C A. van Boeckel, *J. Pharm. Pharmacol.* **1996**, *48*, 172.
[10] B. Brumshtein, H. M. Greenblatt, T. D. Butters, Y. Shaaltiel, D. Aviezer, I. Silman, A. H. Futerman, J. L. Sussman, *J. Biol. Chem.* **2007**, *282*, 29052.
[11] H. R. Mellor, D. C. A. Neville, D. J. Harvey, F. M. Platt, R. A. Dwek, T. D. Butters, *Biochem. J.* **2004**, *381*, 861.
[12] H. R. Mellor, D. C. A. Neville, D. J. Harvey, F. M. Platt, R. A. Dwek, T. D. Butters, *Biochem. J.* **2004**, *381*, 867.
[13] D. S. Alonzi, D. C. Neville, R. H. Lachmann, R. A. Dwek, T. D. Butters, *Biochem. J.* **2008**, *409*, 571.
[14] G. W. Fleet, J. R. Knowles, R. R. Porter, *Biochem. J.* **1972**, *128*, 499.
[15] G. W. J. Fleet, R. R. Porter, J. R. Knowles, *Nature* **1969**, *224*, 511.
[16] A. V. Romaniouk, A. Silva, J. Feng, I. K. Vijay, *Glycobiology* **2004**, *14*, 301.
[17] R. G. Spiro, *Cell. Mol. Life Sci.* **2004**, *61*, 1025.
[18] H. R. Mellor, J. Nolan, L. Pickering, M. R. Wormald, F. M. Platt, R. A. Dwek, G. W. Fleet, T. D. Butters, *Biochem. J.* **2002**, *366*, 225.

Received: January 16, 2009
Published online on March 17, 2009

