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Fluorescent-tagged sp^2-iminosugars with potent β-glucosidase inhibitory activity

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ABSTRACT

New fluorescently-labelled sp^2-iminosugars based on the 5*N*,6*S*-[*N*′-(4-aminobutyl)iminomethylidene]-6-thionojirimycin skeleton have been synthesized as photoprobes to monitor glycosidase binding. Dansyl, dapoxyl and coumarin fluorophores were appended to the terminal amino group at the N′-substituent by either sulfonamide or amide bridging reaction. All the conjugates behaved as strong (low micromolar to nanomolar) and selective inhibitors of β-glucosidases (almonds and bovine liver) and naringinase, in agreement with the inhibition pattern previously encountered for related iso(thio)urea-type bicyclic sp^2-iminosugars. The presence of the fluorescent probe allows real-time and continuous monitoring of β-glucosidase inhibition by fluorescence resonance energy transfer (FRET), taking advantage of the intrinsic tryptophan-associated fluorescence of the protein.

1. Introduction

Iminosugar glycosidase inhibitors such as 1-deoxynojirimycin (**1**; DNJ) and 2,5-dideoxy-2,5-imino-D-mannitol (**2**; DMDP) are important tools in glycobiology, helping both the unravelling of the glycosidases action mechanism and the development of new pharmaceuticals,[1] for example, in the treatment of diabetes type II symptoms[2] and in hereditary enzyme deficiency diseases.[3] However, most representatives of this family of alkaloids exhibit broad inhibitory profiles within a given configurational substrate pattern, inhibiting simultaneously several α- and β-glycosidases, which represents a serious limitation for clinical applications.[4] With the aim of improving the enzyme selectivity we developed a new family of glycomimetics in which the endocyclic sp^3-hybridized amine-type nitrogen, characteristic for iminosugars, has been replaced by a pseudoamide-type nitrogen atom having a high sp^2-hybridisation character (sp^2-iminosugars).[5] These compounds feature an extremely strong anomeric effect that results in very high chemical and configurational stabilities even for reducing derivatives, which is notably different from the case of classical iminosugars.[6,7] Most remarkably, the affinity towards α- or β-glycosidases as well as towards specific isoenzymes within a series can be finely tuned by the incorporation of substituents that provide additional interactions with protein regions at the vicinity of the active site.[8] For instance, the cyclic carbamate nojirimycin derivative **3** was a potent and selective inhibitor of the neutral α-glucosidase II of the endoplasmic reticulum (yeast), being inactive against β-glucosidases. Derivatives of **3** incorporating N-substituents at the pseudoanomeric (C-1) position have shown significant antiproliferative activity in human breast carcinoma cells.[9] In stark contrast to this, the *N*′-octyl isothiourea analogue **4** was a selective inhibitor of β-glucosidases, including the human lysosomal β-glucocerebrosidase (β-Glu) and shows great promise as a chemical chaperone for the treatment of Gaucher disease.[10] The chaperone activity was assumed to rely on the specific binding of **4** to the active site of misfolded β-Glu mutants at the endoplasmic reticulum thereby facilitating trafficking to the lysosome,[11] although no direct proof could be obtained. In order to investigate the mechanisms involved in cell internalization and organelle distribution more deeply, the development of fluorescent-tagged sp^2-iminosugars seemed very appealing (Fig. 1).

Previous work has shown that the introduction of fluorescent probes such as dansyl residues onto lateral chains in classical iminosugars, like **1** and **2**, results in compounds that may exhibit similar or even enhanced glycosidase inhibitory activity (e.g., **5** and **6**).[12,13] The requisite for these channels is that the presence of a lipophilic substituent at the functionalized position in the iminosugar core is tolerated, which is the case for the nitrogen atom in **1** or the primary positions in **2**. The utility of the fluorescently-labelled iminosugar inhibitors for the construction of

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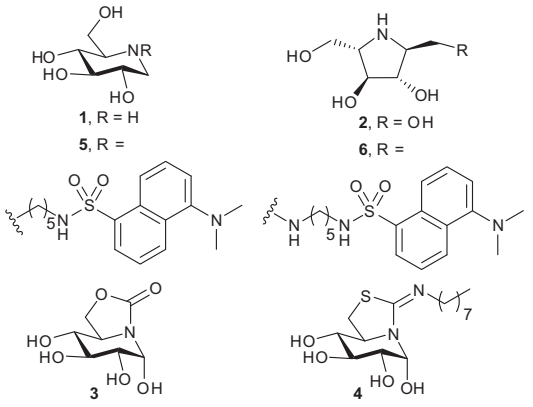


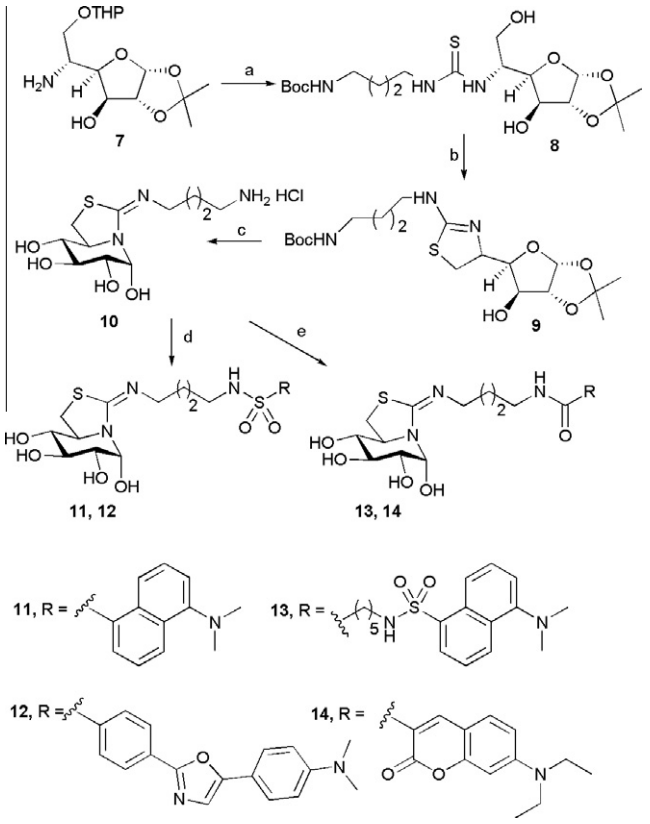
Figure 1. Structures of DNJ (1), DMDP (**2**), the bicyclic nojirimycin analogues **3** and **4** and the fluorescent-tagged DNJ and DMDP derivatives **5** and **6**.

sensors to detect glycosidase binding[14] and as chemical chaperones[15] has been demonstrated, illustrating the potential of the approach in glycobiology. Taking into consideration that the hydrophobic chain in the sp^2-iminosugar **4** is critical for the inhibition of β-glucosidases, it was conceived that related derivatives having appended fluorescent moieties at this region of the molecule might maintain the biological activity profile while incorporating interesting chemo-optical properties. To test this hypothesis we have now synthesized dansyl-, coumarin- and dapoxyl-labelled analogues of **4** and evaluated their inhibitory activity against a panel of commercial glycosidases. The feasibility of fluorescence resonance energy transfer (FRET)[16] as a protocol for the real-time and continuous monitoring of glycosidase inhibition by fluorescently-labelled sp^2-iminosugars is also demonstrated.

2. Results and discussion

2.1. Synthesis

In our molecular design of fluorescent sp^2-iminosugar glycosidase inhibitors we took advantage of the information previously obtained from X-ray and thermodynamic studies of **4** and other structurally related bicyclic nojirimycin glycomimetics in complex with the β-glucosidase from *Thermotoga maritime* (*Tm*GH1),[17] an enzyme that belongs to family GH1 in the CaZy classification.[18] The octyl chain at the exocyclic nitrogen in the enzyme–inhibitor complex was found to occupy a hydrophobic pocket at the entrance of the active site in all cases, keeping substantial mobility. A similar situation was encountered for a related sp^2-iminosugar in complex with recombinant human β-glucocerebrosidase.[19] It was then inferred that structural modifications at this portion of the molecule would not affect the extensive hydrogen bond network involving the hydroxyl groups and would be well tolerated as far as its hydrophobic nature is conserved. Shortening the alkyl chain from octyl to butyl was considered to guarantee the necessary flexibility for an optimal induced fit at the active site of β-glucosidases without unduly extending the length of the substituent after appending the fluorophore, which could result in steric clashes or insufficient solubility in aqueous media. Coupling of commercially available activated fluorescent probes to an amine-functionalized scaffold was selected as the conjugation method of choice since it represents one of the most efficient and popular strategies for the preparation of fluorescent-tagged conjugates.[20] Moreover, this approach is well-suited for convergent schemes in which the fluorescent moiety is incorporated at the very last step. Following these considerations the 5*N*,6*S*-(4′-aminobutyliminomethylidene)-6-thionojirimycin derivative **10** (Scheme 1) was conceived as the key pivotal intermediate for the preparation of the title compounds.



Scheme 1. Reagents and conditions: (a) (1) SCN(CH$_2$)$_4$NHBoc, Et$_3$N, pyridine, rt, 18 h; (2) TsOH, (1:1) CH$_2$Cl$_2$–MeOH, rt, 2 h, 70% (global); (b) MsCl, pyridine, −20 °C→rt, 7 h, 78%; (c) (1) 90% TFA–H$_2$O, rt, 30 min; (2) Amberlite IRA-68 (OH$^-$); (3) HCl 1 N, 82%; (d) ClSO$_2$R, DMF, Et$_3$N, 0 °C, 4 h, 54–98%; (e) RCOOH, TBTU, DMF, Et$_3$N, rt, 1 h, 54–56%.

The synthesis of **10** started from known 5-amino-5-deoxy-6-*O*-tetrahydropyranyl-1,2-*O*-isopropylidene-α-D-glucofuranose **7**.[21] The Boc-protected butylamino spacer was first incorporated by thiourea coupling reaction of **7** with 4-(*tert*-butoxycarbonylamino)butyl isothiocyanate. Selective hydrolysis of the tetrahydropyranyl group was achieved by treatment of the crude adduct with *p*-toluenesulfonic acid to give the *vic*-hydroxythiourea **8** (70% yield). Activation of the primary hydroxyl by methanesulfonylation (mesylation) with mesyl chloride proceeded with spontaneous nucleophilic displacement of the resulting mesylate ester by the thiocarbonyl sulfur atom, affording the 2-aminothiazoline pseudo-*C*-nucleoside derivative **9**. Further rearrangement of the furanose ring into a piperidine derivative relies in the ability of the nitrogen atom of the cyclic isothiourea functionality to participate in intramolecular nucleophilic addition reactions to the masked aldehyde group of the monosaccharide in the open chain form. Thus, simultaneous acid-promoted hydrolysis of the acetal and Boc protecting groups in **9** using TFA–water followed by elimination of the acid by coevaporation with water and final neutralization with basic ion-exchange resin led to the required sp^2-iminosugar, characterized as the corresponding hydrochloride salt **10**, in 82% yield (Scheme 1).

For the final assembly of the target fluorescently-tagged bicyclic nojirimycin derivatives two different types of coupling reactions were considered, namely sulfonamide and amide coupling. Reaction of **10** with dansyl and dapoxyl chloride in the presence of Et$_3$N afforded the corresponding sulfonamide conjugates **11** and **12** in 98% and 54% yield, respectively. The synthesis of the amide-linked conjugates **13** and **14**, was performed in 53–56% yield by reaction of **10** with 6-dansylaminohexanoic acid[10] and

7-diethylaminocoumarin-3-carboxylic acid using TBTU as coupling reagent (Scheme 1).

The ^{1}H NMR spectra of the final compounds showed signals in the aromatic region compatible with the presence of the fluorescent tags in the molecules. The coupling constant values between vicinal protons at the piperidine ring were in agreement with the $^4C_1$ conformation and the axial orientation of the pseudoanomeric hydroxyl group ($J_{1,2}$ = 3.4–3.5 Hz) fitting the anomeric effect, a distinctive feature of sp^2-iminosugars. In the ^{13}C NMR spectra, the high field shift of the pseudoanomeric carbon resonance (76.3–77.2 ppm) further confirmed the aminoketalic bicyclic structure.

2.2. Inhibitory activities

The fluorescent sp^2-iminosugars **11**–**14** were assayed against a panel of selected commercial glycosidases including α-glucosidase (yeast), β-glucosidase (almonds), β-glucosidase (bovine liver, cytosolic), α-mannosidase (Jack bean), β-mannosidase (*Helix pomatia*), trehalase (pig kidney), amyloglucosidase (*Aspergillus niger*), naringinase (β-glucosidase/α-rhamnosidase; *Penicillium decumbens*),[22,23] α-galactosidase (green coffee beans) and isomaltase (yeast). The non-fluorescent precursor **10** and the reference compound **4** were also included in our study for comparative purposes (Table 1). According to previous results on related sp^2-iminosugars, the presence of the N′-substituent results in a higher affinity for β- as compared to α-glucosidases, in spite of the presence of an axially-oriented pseudoanomeric hydroxyl group that is stereocomplementary to the aglycon in the natural α-glucosides. Thus, no or weak inhibition of yeast α-glucosidase, isomaltase, trehalase or amyloglucosidase was observed in all cases. In contrast, **11**–**14** behaved as strong competitive inhibitors of both almond and bovine (cytosolic) β-glucosidases, the inhibition potency being about 2 orders of magnitude higher for the first enzyme (K_i = 0.36–0.55 μM vs K_i = 29–32 μM). The presence of the fluorophores notably increased the activity as compared to the precursor derivative **10**, but was much less influenced by the nature of the linking functionality, amide or sulfonamide. A strong inhibition of the β-glucosidase activity of naringinase was also detected (K_i = 0.072–1.2 μM).

It has been previously observed for compound **4** that although the α-anomer, was the only species detected by NMR in water solution, as determined by the anomeric effect, mutarotation may occur in the presence of β-glucosidase. Thus, **4** was encountered in the β-orientation in the active site of *Tm*GH1, thereby matching the enzyme anomeric specificity.[17] It has been also argued that compound **4** and other derivatives sharing the bicyclic isothiourea skeleton, which are protonated at pH 7.0–7.3 to an extend of at least 50% (pK_a 7.0–7.7), might interact preferentially with a doubly deprotonated catalytic apparatus of the β-glucosidases.[17] Moreover, molecular modelling indicated that the presence of the hydrophobic substituent results in unfavourable contacts with amino acids at the vicinity of the catalytic site in the case of α-glucosidases. The situation is probably very similar for compounds **10**–**14**, leading to high β-anomeric selectivity.

As expected from the configurational pattern of the assayed compounds, analogous to that of D-glucose, no or week inhibition of enzymes acting on substrates other than glucosides was normally observed. The coumarin derivative **14** is an exception to this rule, with significant inhibitory potency towards β-mannosidase (K_i = 11 μM). In general, the presence of the aromatic fluorescent moiety resulted in an increased selectivity towards almond β-glucosidase as compared with the bovine cytosolic enzyme. The ensemble of results stresses the utmost importance of pseudoaglyconic interactions in the binding affinity of glycomimetics to different glycosidases and the potential they offer for modulation of the inhibition potency and selectivity.

Table 1
K_i values (μM) for **4**, **10**, **11**–**14** against several glycosidases[a]

Enzyme	4	10	11	12	13	14
α-Glucosidase (baker's yeast)	ni[b]	ni	246	714	451	313
β-Glucosidase (almond), pH 7.3	0.76	12.8	0.36	0.55	0.50	0.45
β-Glucosidase (bovine liver)	3.7	ni	29	23	32	32
α-Mannosidase (Jack beans)	ni	ni	ni	235	407	199
β-Mannosidase (*Helix pomatia*)	nd	ni	387	522	186	11
Trehalase (pig kidney)	13.4	116	293	ni	179	28.2
Amyloglucosidase (*Aspergillus niger*)	nd	ni	ni	ni	ni	167
Naringinase (*Penicillium decumbens*)	0.23	1.8	0.21	1.2	0.18	0.072

[a] Inhibition was competitive in all cases. No inhibition was observed for any compound at 2 mM concentration on, green coffee bean α-galactosidase or yeast isomaltase.
[b] ni, no inhibition observed at 2 mM concentration of the inhibitor.

2.3. Characterization of glycosidase binding by fluorescent resonance energy transfer (FRET)

The above results demonstrate that the incorporation of fluorescent tags at the N′-substituent in the 5*N*,6*S*-(*N*′-alkyliminomethylidene)-6-thionojirimycin framework is compatible with powerful and selective inhibitory activity towards β-glucosidases. In principle, this allows the employment of fluorescence spectroscopy methods for conducting binding studies. To check this possibility, the dansyl conjugate **11** was chosen on the basis of its comparatively lower synthetic cost and favourable characteristics as chemical chaperone for different mutant human lysosomal β-Glu related to Gaucher disease (data not shown). The fluorescence spectrum of almond β-glucosidase (1.25 μM) was first recorded at 37 °C in phosphate buffer (pH 7.3) for a wavelength range between 320 and 520 nm, showing the characteristic emission for the first excited singlet state of tryptophan at 339 nm. The dansyl fluorophore is an appropriate energy acceptor for this emission; consequently, binding of the dansyl labelled inhibitor **11** to the active site of the enzyme suppresses the tryptophan-related protein fluorescence highly efficiently. In a typical titration experiment, the addition of aliquots of a solution of the dansyl labelled inhibitor **11** (50 μM) to a solution of β-glucosidase, while maintaining constant the concentration of the latter, resulted in a gradual decrease of the intensity of the band at 339 nm and the corresponding increase in the emission band for the dansyl group at 515 nm (Fig. 2). Any of these parameters can be used to build the corresponding binding isotherm. Least-square fitting of the experimental points was compatible with a 1:1 binding stoichiometry, discarding contributions from unspecific adsorption of the fluorescent moiety at the protein surface. A dissociation constant (K_d) of 0.20 ± 0.03 μM (Fig. 3) was obtained from the binding curve, in good agreement with the K_i value (0.36 μM) determined from competition experiments using *p*-nitrophenyl β-D-glucopyranoside as substrate. A virtually identical K_d value, was obtained by a reverse titration experiment using a constant concentration of **11** (1.25 μM) and increasing concentrations of almond β-glucosidase (see Supplementary data).

3. Summary and conclusions

The results here collected illustrate the suitability of the synthetic strategy disclosed for bicyclic sp^2-iminosugars having a 5*N*,6*S*-(*N*′-alkyliminomethylidene)-6-thionojirimycin skeleton for the preparation of fluorescent-tagged glycosidase inhibitors through a convergent conjugation scheme that implies sulfonamide or amide-coupling using commercially available fluorescent reagents. In general, the use of a tetramethylene spacer has been shown to preserve the configurational selectivity of the fluorescent

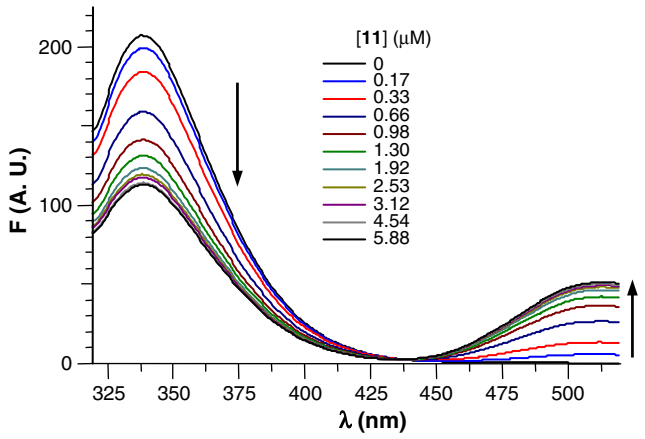


Figure 2. Fluorescence emission spectra of almond β-glucosidase (1.25 μM) in the presence of increasing concentrations of the dansyl conjugate **11**. The band at 339 nm corresponds to the tryptophan-associated fluorescence, which is quenched by the dansyl fluorophore following inhibitor–enzyme complex formation, whereas the band at 515 nm is ascribed to the dansyl-associated emission.

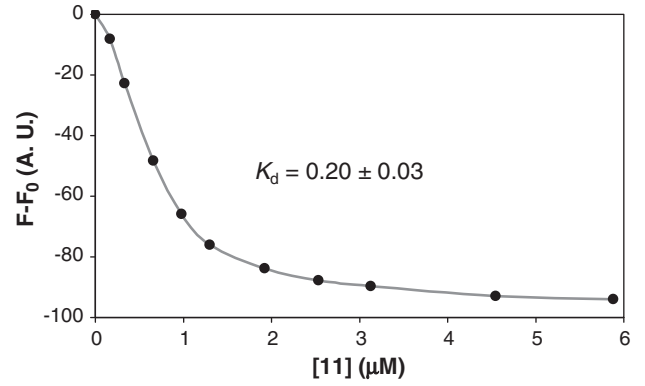


Figure 3. Binding isotherm plot for titration of almond β-glucosidase (1.25 μM) with the fluorescent sp^2-iminosugar inhibitor **11**.

conjugates as determined by the hydroxylation pattern at the piperidine ring, complementary of that of D-glucose, as well as the anomeric β- versus α-glucosidase selectivity. A notable increase in the selectivity towards almond β-glucosidase was observed when considering the pair of β-glucosidase isoenzymes from almonds and bovine liver (cytosolic) in comparison with the *N*′-octyl derivative **4**, which must be ascribed to the presence of the aromatic rings of the fluorophores. The chemo-optical properties of the new compounds allows direct determination of the binding parameters to glycosidases by exploiting the transfer of energy between the first excited singlet state of tryptophan groups of the protein and the fluorophore moiety of the inhibitor, as illustrated for the particular case of the dansyl conjugate **11** and the enzyme from almonds. Most interestingly, the incorporation of the fluorescent tag is compatible with strong binding to normal β-glucocerebrosidase as well as Gaucher disease-associated mutants and allows investigation of important aspects related to the use of sp^2-iminosugars as chemical chaperones such as cellular uptake pathways and intracellular distribution. Results in that direction will published in due curse.

4. Experimental

4.1. General methods

Reagents and solvents were purchased from commercial sources and used without further purification. Optical rotations were measured with a JASCO P-2000 polarimeter, using a sodium lamp (λ = 589 nm) at 22 °C in 1 cm or 1 dm tubes. NMR experiments were performed at 300 (75.5) and 500 (125.7) MHz using Bruker DMX300 and DRX500, and Varian INOVA 500 spectrometers. 1-D TOCSY as well as 2-D COSY and HMQC experiments were carried out to assist in signal assignment. In the FABMS spectra, the primary beam consisted of Xe atoms with maximum energy of 8 keV. The samples were dissolved in *m*-nitrobenzyl alcohol or thioglycerol as the matrices and the positive ions were separated and accelerated over a potential of 7 keV. NaI was added as cationizing agent. Thin-layer chromatography was performed on E. Merck precoated TLC plates, silica gel 30F-245, with visualization by UV light and by caring with 10% H_2SO_4 or 0.2% w/v cerium(IV) suphate-5% ammonium molybdate in 2 M H_2SO_4 or 0.1% ninhydrin in EtOH. Column chromatography was performed on Chromagel (SdS silica 60 AC.C 70–200 μm). Elemental analyses were performed at the Servicio de Microanálisis del Instituto de Investigaciones Químicas de Sevilla, Spain. Compound **7** was prepared by hydrogenation of 5-azido-5-deoxy-1,2-*O*-isopropylidene-6-*O*-tetrahydropyranyl-α-D-glucofuranose (see hereinafter), obtained at its turn from commercial 6,3-D-glucuronolactone, following the procedure already reported.[21]

4.1.1. Inhibition studies

Inhibition constant (K_i) values were determined by spectrophotometrically measuring the residual hydrolytic activities of the glycosidases against the respective *o*- (for β-glucosidase from bovine liver) or *p*-nitrophenyl α- or β-D-glycopyranoside (for other glycosidases) or α,α′-trehalose (for trehalase) in the presence of compounds **4**, **10–14**. Each assay was performed in phosphate buffer or phosphate–citrate buffer (for α- or β-mannosidase and amyloglucosidase) at the optimal pH for the enzymes. The reactions were initiated by addition of enzyme to a solution of the substrate in the absence or presence of various concentrations of inhibitor. The mixture was incubated for 10–30 min at 37 °C or 55 °C (for amyloglucosidase) and the reaction was quenched by addition of 1 M Na_2CO_3 or a solution of GLC-Trinder (Sigma, for trehalase). Reaction times were appropriate to obtain 10–20% conversion of the substrate in order to achieve linear rates. The absorbance of the resulting mixture was determined at 405 nm or 505 nm (for trehalase). Approximate values of K_i were determined using a fixed concentration of substrate (around the K_M value for the different glycosidases) and various concentrations of inhibitor. Full K_i determinations and enzyme inhibition mode were determined from the slope of Lineweaver–Burk plots and double reciprocal analysis. Representative examples of the Lineweaver–Burk plots, with typical profile for competitive inhibition mode, are shown in Supplementary data.

4.1.2. Binding studies by fluorescence spectroscopy

Quenching of the intrinsic tryptophan fluorescence of β-glucosidase from almonds by titration with **11** was studied by fluorescence resonance energy transfer (FRET) using a F-2500 Hitachi spectrofluorophotometer. Fluorescence spectra were recorded in conventional 1-cm quartz cuvettes at 37 ± 0.1 °C between 320 and 520 nm, using 2.5 mm excitation and emission slits. Excitation wavelength (λ_{ex}) was 280 nm. In a typical titration experiment a 1.25 μM stock solution of the enzyme was prepared in 0.05 M potassium phosphate buffer, pH 7.3, a 1.5 mL aliquot was transferred to the titration cuvette and the initial fluorescence spectra was recorded. Then a solution of inhibitor **11** (50 μM) in the stock solution of enzyme was prepared and portion-wise added to the quartz cell via microsyringe. The additions were continued until no change in fluorescence intensity were observed ([**11**] ~ 6 μM). A spectrum was recorded after each addition and the fluorescence intensity at 339 nm (tryptophan fluorescence decrease) and

515 nm (dansyl fluorescence increase) obtained at 10–15 different enzyme–inhibitor ratios were then plotted against inhibitor concentration. Iterative least-squares fitting of the experimental binding isotherm to a 1:1 binding model allowed estimation of K_d within a ±15% estimated error.[24] Dissociation constant was also determined by reversed fluorescence titration of β-glucosidase from almonds with **11** at constant concentration. In this case the emission was recorded between 400 and 600 nm. A 1.0 μM stock solution of sp^2-iminosugar **11** in 0.05 M potassium phosphate buffer, pH 7.3, was prepared and the initial spectrum was recorded. The enzyme was dissolved in the stock solution (containing the inhibitor) at a final concentration of 5 μM, and added stepwise to the cuvette under stirring until no changes in fluorescence intensity were observed ([enzyme] ~1.9 μM). Fluorescence intensity at 515 nm was measured after each addition and data points were plotted versus enzyme concentration. Least-square fitting to a 1:1 binding model furnished a K_d value that did not significantly differ from the direct titration procedure.[24]

4.2. 5-[*N*′-(4-*tert*-Butoxycarbonylaminobutyl)thioureido]-5-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (8)

A solution of 5-azido-5-deoxy-1,2-*O*-isopropylidene-6-*O*-tetrahydropyranyl-α-D-glucofuranose[21] (700 mg, 2.13 mmol) in MeOH (12 mL) was hydrogenated at atmospheric pressure for 1 h using 10% Pd/C (234 mg) as catalyst. The suspension was filtered through Celite and concentrated to give 5-amino-5-deoxy-1,2-*O*-isopropylidene-6-*O*-tetrahydropyranyl-α-D-glucofuranose[21] (**7**) as a hygroscopic material that was used in the next step without purification. To a solution of **7** thus obtained in pyridine (12 mL), Et_3N (1.6 mL, 11.7 mmol) and 4-(*tert*-butoxycarbonylamino)butyl isothiocyanate (433 mg, 2 mmol) were added and the mixture was stirred at room temperature for 18 h. Then, the solvent was removed under reduced pressure and the residue coevaporated several times with toluene. The syrup was dissolved in CH_2Cl_2–MeOH (1:1, 42 mL) and *p*-toluenesulfonic acid (69 mg, 0.16 mmol) was added. The reaction mixture was stirred for 2 h at room temperature, and then diluted with CH_2Cl_2 (15 mL), washed with saturated aqueous $NaHCO_3$ (2 × 15 mL), dried ($MgSO_4$), filtered, and concentrated. The resulting residue was purified by column chromatography using 30:1→15:1 CH_2Cl_2–MeOH as eluent to obtain **8** (629 mg, 70%). $[\alpha]_D$ +45.5 (*c* 1.0 in CH_2Cl_2); R_f 0.33 (15:1 CH_2Cl_2–MeOH); ν_{max} cm^{-1} 3342, 2933, 1682, 1549, 1367, 1254, 1165, 1075; λ_{max} (CH_2Cl_2) nm 247 (ε_{mM} 12.3); δ_H (300 MHz, $CDCl_3$, 313 K) δ_H 6.96 (br s, 1H, N′H), 6.72 (br d, 1H, $J_{NH,5}$ = 7.8 Hz, NH), 5.92 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 5.07 (br s, 1H, OH), 4.82 (br s, 1H, NH), 4.58 (d, 1H, H-2), 4.54 (m, 1H, H-5), 4.19 (d, 1H, $J_{3,4}$ = 1.9 Hz, H-3), 4.09 (dd, 1H, $J_{4,5}$ = 9.8 Hz, H-4), 4.03 (dd, 1H, $J_{6a,6b}$ = 11.3 Hz, $J_{5,6a}$ = 3.1 Hz, H-6a), 3.80 (dd, 1H, $J_{5,6b}$ = 3.0 Hz, H-6b), 3.47 (m, 2H, CH_2NHCS), 3.11 (m, 2H, CH_2NHCO), 1.60 (m, 4H, CH_2), 1.49, 1.31 (2 s, 6H, CMe_2), 1.49 (s, 9H, CMe_3); δ_C (75.5 MHz, $CDCl_3$, 313 K) δ 181.8 (CS), 156.7 (CO), 111.6 (CMe_2), 104.9 (C-1), 84.7 (C-2), 79.8 (CMe_3), 79.7 (C-4), 73.8 (C-3), 62.4 (C-6), 53.7 (C-5), 44.1 (CH_2NHCS), 40.2 (CH_2NHCO), 28.4 (CMe_3), 27.4 (CH_2), 26.7, 26.0 (CMe_2), 25.7 (CH_2); *m/z* (FAB) 472 ([M+Na]$^+$, 100%), 450 (15). (Found: C, 50.78; H, 7.93; N, 9.28; S, 6.96. $C_{19}H_{35}N_3O_7S$ requires C, 50.76; H, 7.85; N, 9.35, S, 7.13).

4.3. (4*R*)-2-(4-*tert*-Butoxycarbonylamino)butylamino-4-[(4′*R*)-1′,2′-*O*-isopropylidene-β-L-threofuranos-4′-yl]-2-thiazoline (9)

To a solution of the corresponding thioureido derivative **8** (514 mg, 1.14 mmol) in anhydrous pyridine (17 mL) at −20 °C under Ar, methanesulfonic chloride (110 μL, 1.42 mmol, 1.2 equiv) was added. The reaction mixture was stirred for 7 h and allowed to warm to room temperature. Then, ice-water (30 mL) was added and the solution was extracted with CH_2Cl_2 (3 × 20 mL). The combined extracts were washed with iced saturated aqueous $NaHCO_3$ (25 mL), dried ($MgSO_4$), filtered, and concentrated. The resulting residue was purified by column chromatography using 20:1→10:1 CH_2Cl_2–MeOH as eluent to obtain **9** (384 mg, 78%). $[\alpha]_D$ −7.3 (*c* 1.0 in CH_2Cl_2); R_f 0.46 (7:1 CH_2Cl_2–MeOH); ν_{max} 3372, 2934, 1696, 1521, 1367, 1251, 1166, 1075 cm^{-1}; δ_H (300 MHz, $CDCl_3$) 5.93 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 4.79 (br s, 1H, NH), 4.54 (d, 1H, H-2), 4.42 (m, 1H, H-5), 4.25 (br s, 2H, OH, NH), 4.23 (d, 1H, $J_{3,4}$ = 2.5 Hz, H-3), 4.03 (dd, 1H, $J_{4,5}$ = 8.4 Hz, H-4), 3.51 (dd, 1H, $J_{6a,6b}$ = 10.9 Hz, $J_{5,6a}$ = 7.4 Hz, H-6a), 3.39 (dd, 1H, $J_{5,6b}$ = 4.9 Hz, H-6b), 3.24 (m, 2H, CH_2N), 3.11 (m, 2H, CH_2NHCO), 1.53 (m, 4H, CH_2), 1.48, 1.30 (2 s, 6H, CMe_2), 1.33 (s, 9H, CMe_3); δ_C (75.5 MHz, $CDCl_3$) 164.6 (CN), 156.1 (CO), 111.5 (CMe_2), 105.1 (C-1), 85.2 (C-2), 81.6 (C-4), 79.3 (CMe_3), 74.9 (C-3), 69.8 (C-5), 44.9 (CH_2N), 40.0 (CH_2NHCO), 37.4 (C-6), 28.4 (CMe_3), 27.3, 26.8 (CH_2), 26.8, 26.1 (CMe_2); *m/z* (FAB) 454 ([M+Na]$^+$, 50%), 432 (100). (Found: C, 52.66; H, 7.65; N, 9.48; S, 7.19. $C_{19}H_{33}N_3O_6S$ requires C, 52.88; H, 7.71; N, 9.74; S, 7.43).

4.4. 5-*N*,6-*S*-[*N*′-(4-Amino)butyliminomethylidene]-6-thionojirimycin hydrochloride (10)

The corresponding 2-amino-2-thiazoline precursor **9** (340 mg, 0.79 mmol) was treated with TFA–H_2O (9:1, 3.5 mL) for 30 min, concentrated under reduced pressure, coevaporated several times with water, neutralized with Amberlite IRA-68 (OH^-) ion-exchange resin, and subjected to column chromatography using 10:1:1→6:3:1 CH_3CN–H_2O–NH_4OH as eluent to obtain the isothiourea **10** as the corresponding hydrochloride (258 mg, 82%). α–β ratio 1:0.1 (H-1 integration); $[\alpha]_D$ −11.3 (*c* 1.0 in H_2O); R_f 0.22 (6:3:1 CH_3CN–H_2O–NH_4OH). α anomer: δ_H (500 MHz, D_2O) 5.61 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1), 4.32 (m, 1H, H-5), 3.76 (m, 2H, H-3α, H-6a), 3.62 (dd, 1H, $J_{2,3}$ = 9.5 Hz, H-2), 3.56 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.6 Hz, H-4), 3.46 (m, 3H, H-6b, CH_2N), 3.00 (t, 2H, $^3J_{H,H}$ = 7.1 Hz, CH_2NH_2), 1.73 (m, 4H, CH_2); δ_C (125.7 MHz, D_2O) 175.7 (CN), 79.0 (C-1), 75.7 (C-4), 74.2 (C-3), 73.1 (C-2), 66.0 (C-5), 50.6 (CH_2N), 41.5 (CH_2NH_2), 33.9 (C-6), 27.7, 26.5 (CH_2). β anomer: δ_H (500 MHz, D_2O) 5.02 (d, 1H, $J_{1,2}$ = 8.2 Hz, H-1), 4.08 (m, 1H, H-5), 3.76 (m, 1H, H-3), 3.70 (dd, 1H, $J_{6a,6b}$ = 11.6 Hz, $J_{5,6a}$ = 7.6 Hz, H-6a), 3.60 (m, 1H, H-2), 3.56 (m, 1H, H-4), 3.46 (m, 3H, H-6b, CH_2N), 3.00 (m, 2H, CH_2NH_2), 1.73 (m, 4H, CH_2); δ_C (125.7 MHz, D_2O) 175.7 (CN), 87.4 (C-1), 77.1 (C-4), 75.8 (C-3), 72.8 (C-2), 69.1 (C-5), 50.5 (CH_2N), 41.5 (CH_2NH_2), 32.5 (C-6), 27.9, 26.5 (CH_2); *m/z* (FAB) 314 ([M+Na−HCl]$^+$, 40%), 292 (90). (Found: C, 39.95; H, 6.47; N, 12.49; S, 9.41. $C_{11}H_{22}ClN_3O_4S$ requires C, 40.30; H, 6.76; N, 12.82; S, 9.78).

4.5. 5-*N*,6-*S*-[*N*′-(4-Dansylamino)butyliminomethylidene]-6-thionojirimycin (11)

To a solution of **10** (68 mg, 0.21 mmol) in anhydrous DMF (15 mL) at 0 °C under Ar, triethylamine (64 μL, 1 equiv) and 5-dimethylaminonaphtalene-1-sulfonyl chloride (61.8 mg, 1.1 equiv) were added. The reaction mixture was stirred for 4 h and the solvent was removed under reduce pressure. The resulting residue was purified by column chromatography using 90:10:1→60:10:1 CH_2Cl_2–MeOH–H_2O to obtain **11** (107 mg, 98%). $[\alpha]_D$ −7.4 (*c* 0.7 in MeOH); R_f 0.63 (40:10:1 CH_2Cl_2–MeOH–H_2O); δ_H (500 MHz, CD_3CN) 8.52 (d, 1H, dansyl), 8.24 (d, 1H, dansyl), 8.15 (d, 1H, dansyl), 7.60 (m, 2H, dansyl), 7.28 (d, 1H, dansyl), 5.48 (d, 1H, $J_{1,2}$ = 3.4 Hz, H-1), 3.79 (m, 1H, H-5), 3.62 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 3.46 (dd, 1H, $J_{6a,6b}$ = 11.2 Hz, $J_{5,6a}$ = 4.4 Hz, H-6a), 3.40 (dd, 1H, H-2), 3.30 (t, 1H, $J_{4,5}$ = 9.3 Hz, H-4), 3.10 (dd, 1H, $J_{5,6b}$ = 7.3 Hz, H-6b), 3.06 (t, 2H, $^3J_{H,H}$ = 10.5 Hz, CH_2N), 3.00 (m, 2H, CH_2NH), 2.83 (br s, 6H, dansyl), 1.44 (m, 2H, CH_2), 1.34 (m, 2H, CH_2); δ_C (125.7 MHz, CD_3CN) 163.1 (CN), 152.0, 135.4, 130.2–129.5, 129.2, 128.4, 123.7, 119.1, 115.5 (dansyl), 76.3 (C-1), 74.3

(C-4), 72.8 (C-3), 71.5 (C-6), 61.1 (C-5), 54.5 (CH_2N), 44.9 (dansyl), 42.5 (CH_2NH), 31.2 (C-6), 26.8, 26.7 (CH_2); *m/z* (FAB) 547.1661 $[M+Na]^+$. $C_{23}H_{32}N_4O_6NaS_2$ requires 547.1661. (Found: C, 52.34; H, 6.10; N, 10.47; S, 11.89. $C_{23}H_{32}N_4O_6S_2$ requires C, 52.65; H, 6.15; N, 10.68; S, 12.22).

4.6. 5-*N*,6-*S*-[*N*′-(4-Dapoxylsulfonylamino)butyliminomethylidene]-6-thionojirimycin (12)

To a solution of **10** (32.2 mg, 0.11 mmol) in anhydrous DMF (10 mL) at 0 °C under Ar, triethylamine (15 μL, 1 equiv) and dapoxylsulfonyl chloride (39.9 mg, 1 equiv) were added. The reaction mixture was stirred for 4 h and the solvent was removed under reduce pressure. The resulting residue was purified by column chromatography using 100:10:0.5→70:10:0.5 CH_2Cl_2–MeOH–NH_4OH as eluent to obtain **12** (37 mg, 54%). $[\alpha]_D$ −14.7 (*c* 0.3 in MeOH); R_f 0.40 (40:10:1 CH_2Cl_2–MeOH–NH_4OH); δ_H (500 MHz, CD_3CN) 8.20 (d, 2H, $J_{2',3'}$ = 8.8 Hz, H-2′), 7.90 (d, 2H, H-3′), 7.65 (d, 2H, $J_{9',10'}$ = 8.0 Hz, H-9′), 7.61 (s, 1H, H-7′), 6.80 (d, 2H, H-10′), 5.57 (br s, 1H, OH), 5.31 (d, 1H, $J_{1,2}$ = 3.4 Hz, H-1), 5.18, 4.78, 4.68 (3 br s, 3H, OH), 3.47 (m, 1H, H-5), 3.43 (m, 1H, H-3), 3.27 (dd, 1H, $J_{6a,6b}$ = 10.7 Hz, $J_{5,6a}$ = 6.4 Hz, H-6a), 3.12 (dd, 1H, $J_{2,3}$ = 9.3 Hz, H-2), 3.01 (t, 1H, $J_{4,5}$ = 9.3 Hz, H-4), 2.96 (s, 6H, CH_3N), 2.95 (m, 2H, CH_2N), 2.87 (dd, 1H, $J_{5,6b}$ = 8.8 Hz, H-6b), 2.76 (t, 2H, $^3J_{H,H}$ = 6.4 Hz, CH_2N), 1.40 (m, 4H, CH_2); δ_C (125.7 MHz, CD_3CN) 168.6 (CN), 158.2 (CN), 157.3 (CN), 153.4 (C-6′), 151.2 (C-11′), 142.0 (C-1′), 130.8 (C-4′), 128.1 (C-2′), 126.8 (C-3′), 126.2 (C-7′), 122.1 (C-9′), 115.3 (C-8′), 112.8 (C-10′), 77.2 (C-1), 75.1 (C-4), 73.9 (C-3), 72.5 (C-2), 59.6 (C-5), 54.4 (CH_2N), 43.2 (CH_3N), 40.8 (CH_2NH), 31.0 (C-1), 28.7, 27.7 (CH_2). *m/z* (HRFAB) 640.1909 $[M+Na]^+$. $C_{28}H_{35}N_5O_7NaS_2$ requires 640.1876.

4.7. 5-*N*,6-*S*-[*N*′-[4-(6-Dansylaminohexanamido)butyl]iminomethylidene]-6-thionojirimycin (13)

To a solution of dansyl chloride (742 mg, 2.75 mmol) in CH_2Cl_2 (25 mL), triethylamine (0.8 mL, 2.1 equiv) and methyl 6-aminohexanoate hydrochloride (500 mg, 1.0 equiv) were added and the mixture was stirred at room temperature for 18 h. The mixture was partitioned between CH_2Cl_2 and 6% aqueous HCl and the organic layer was washed with saturated aqueous $NaHCO_3$ until neutral pH, dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The residue was purified by column chromatography using cyclohexane→1:3 EtOAc–cyclohexane. To a 10% solution of the resulting residue in dioxane–H_2O 1:1 (v/v), aqueous NaOH (10%) was added dropwise until pH 10 was reached and the mixture was stirred at room temperature for 24 h. The solution was neutralised with Amberlite IRA-120 (H^+) ion-exchange resin, filtered, and concentrated under reduced pressure to give the free acid[10] (81%). To a 10% solution of this compound in anhydrous DMF, compound **10**, TBTU, and triethylamine (1 equiv) were added and the reaction mixture was stirred at room temperature for 1 h. The solvent was removed under reduce pressure and the residue was purified by column chromatography using 100:10:0.5→50:10:0.5 CH_2Cl_2–MeOH–NH_4OH as eluent to obtain **13** (24 mg, 56%). $[\alpha]_D$ −1.7 (*c* 1.2 in MeOH); R_f 0.29 (70:10:0.5 CH_2Cl_2–MeOH–NH_4OH); δ_H (500 MHz, CD_3CN) 8.54 (d, 1H, $J_{3',4'}$ = 8.8 Hz, H-4′), 8.27 (d, 1H, $J_{8',9'}$ = 8.8 Hz, H-9′), 8.16 (d, 1H, $J_{2',3'}$ = 7.3 Hz, H-2′), 7.60 (m, 2H, H-3′, H-8′), 7.27 (d, 1H, $J_{7',8'}$ = 7.8 Hz, H-7′), 5.51 (d, 1H, $J_{1,2}$ = 3.4 Hz, H-1), 3.88 (m, 1H, H-5), 3.60 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.52 (dd, 1H, $J_{6a,6b}$ = 10.7 Hz, $J_{5,6a}$ = 7.1 Hz, H-6a), 3.40 (dd, 1H, H-2), 3.31 (t, 1H, $J_{4,5}$ = 9.8 Hz, H-4), 3.23 (m, 3H, H-6b, CH_2N), 3.13 (m, 2H, CH_2NH), 2.80 (m, 10H, CH_2NH, CH_3), 1.58 (m, 2H, CH_2), 1.49 (m, 2H, CH_2), 1.33 (m, 4H, CH_2), 1.13 (m, 2H, CH_2); δ_C (125.7 MHz, CD_3CN) 162.4 (CN), 160.0 (CO), 152.1 (C-6′), 135.7 (C-1′), 130.2–129.6 (C-4′, C-5′, C-10′), 129.2 (C-2′), 128.3 (C-8′), 123.7 (C-3′), 119.2 (C-9′), 115.4 (C-7′), 76.5 (C-1), 74.3 (C-4), 73.0 (C-3), 71.6 (C-2), 61.6 (C-5), 52.0 (CH_2N), 44.9 (CH_3N), 42.7, 35.6 (CH_2NH), 35.7 (C-6), 28.9, 26.9, 26.6, 25.8, 25.0 (CH_2). *m/z* (HRFAB) 660.2503 $[M+Na]^+$. $C_{29}H_{43}N_5O_7NaS_2$ requires 660.2502. (Found: C, 53.39; H, 6.67; N, 10.71; S, 9.72. $C_{29}H_{43}N_5O_7S_2$ requires C, 54.61; H, 6.80; N, 10.98; S, 10.05).

4.8. 5-*N*,6-*S*-[*N*′-[4-(7′-Diethylaminocoumarin-3′-ylcarboxamido)butyl]iminomethylidene]-6-thionojirimycin (14)

To a solution of **10** (50.2 mg, 0.17 mmol) in anhydrous DMF (14 mL), 7-diethylaminocoumarin-3-carboxylic acid (45.1 mg, 1 equiv), TBTU (55.4 mg, 1 equiv) and triethylamine (24 μL, 1 equiv) were added. The reaction was stirred for 1 h, the solvent was removed under reduce pressure, and the residue was purified by column chromatography using 100:10:0.5→70:10:0.5 CH_2Cl_2–MeOH–NH_4OH as eluent to obtain **14** (36.6 mg, 53%). $[\alpha]_D$ −6.4 (*c* 0.6 in MeOH); R_f 0.62 (70:10:0.5 CH_2Cl_2–MeOH–NH_4OH); δ_H (500 MHz, MeOD) 8.50 (br s, 1H, H-4′), 7.44 (d, 1H, $J_{8',9'}$ = 8.8 Hz, H-9′), 6.71 (d, 1H, H-8′), 6.45 (br s, 1H, H-6′), 5,50 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 4.39 (m, 1H, H-5), 3.62 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.53 (m, 1H, H-6a), 3.42 (m, 2H, CH_2CH_3), 3.35 (m, 4H, H-2, H-4, CH_2N), 3.28 (m, 3H, H-6b, CH_2NH), 3.11 (m, 2H, CH_2CH_3), 1.62 (m, 4H, CH_2), 1.21 (t, 3H $^3J_{H,H}$ = 7.3 Hz, CH_3), 1.13 (t, 3H, $^3J_{H,H}$ = 6.8 Hz, CH_3); δ_C (125.7 MHz, MeOD) 163.5 (CN), 163.5 (CO ester), 162.9 (CO amide), 158.0 (C-3′), 153.5 (C-10′), 148.1 (C-7′), 131.5 (C-4′), 110.5–96.1 (C-8′, C-9′, C-6′, C-5′), 77.2 (C-1), 74.3 (C-4), 73.0 (C-3), 71.8 (C-2), 62.5 (C-5), 52.0 (CH_2N), 44.8 (CH_2CH_3), 38.8 (CH_2NH), 31.2 (C-6), 26.8, 26.7 (CH_2), 11.5, 8.0 (CH_3); *m/z* (HRFAB) 557.2042 $[M+Na]^+$. $C_{25}H_{34}N_4O_7NaS$ requires 557.2046. (Found: C, 55.91; H, 6.28; N, 10.22; S, 5.64. $C_{25}H_{34}N_4O_7S$ requires C, 56.16; H, 6.41; N, 10.48; S, 6.00.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.003.

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