Glycoconj J (2009) 26:495–510
DOI 10.1007/s10719-008-9199-4

C_1-/C_2-aromatic-imino-glyco-conjugates: experimental and computational studies of binding, inhibition and docking aspects towards glycosidases isolated from soybean and jack bean

Amit Kumar · Nitin K. Singhal · Balaji Ramanujam · Atanu Mitra · Nagender R. Rameshwaram · Siva K. Nadimpalli · Chebrolu P. Rao

Received: 24 July 2008 / Revised: 24 September 2008 / Accepted: 1 October 2008 / Published online: 25 October 2008
© Springer Science + Business Media, LLC 2008

Abstract Several C_1-imino conjugates of D-galactose, D-lactose and D-ribose, where the nitrogen center was substituted by the salicylidene or naphthylidene, were synthesized and characterized. Similar C_2-imino conjugates of D-glucose have also been synthesized. All the glyco-imino-conjugates, which are transition state analogues, exhibited 100% inhibition of the activity towards glycosidases extracted from soybean and jack bean meal. Among these, a galactosyl-napthyl-imine-conjugate (**1c**) showed 50% inhibition of the activity of pure α-mannosidase from jack bean at 22±2.5 μM, and a ribosyl-naphthyl-imine-conjugate (**3c**) showed at 31±5.5 μM and hence these conjugates are potent inhibitors of glycosidases. The kinetic studies suggested non-competitive inhibition by these conjugates. The studies are also suggestive of the involvement of aromatic, imine and carbohydrate moieties of the glyco-imino- conjugates in the effective inhibition. The binding of glyco-imino-conjugate has been established by extensive studies carried out using fluorescence emission and isothermal titration calorimetry. The conformational changes resulted in the enzyme upon interaction of these derivatives has been established by studying the fluorescence quench of the enzyme by KI as well as from the secondary structural changes noticed in CD spectra. All these studies revealed the difference in the binding strengths of the naphthylidene *vs.* salicylidene as well as galactosyl *vs.* lactosyl moieties present in these conjugates. The differential inhibition of these glyco-conjugates has been addressed by quantifying the specific interactions present between the glyco-conjugates and the enzyme by using rigid docking studies.

Keywords C1-/C2-aromatic-imino-glyco-conjugates · Glycosidase inhibition · Pure α-mannosidase · Rigid docking · Fluorescence quenching · Glycosidases from soybean and jack bean

Electronic supplementary material The online version of this article (doi:10.1007/s10719-008-9199-4) contains supplementary material, which is available to authorized users.

A. Kumar · N. K. Singhal · B. Ramanujam · A. Mitra · C. P. Rao (✉)
Bioinorganic Laboratory, Department of Chemistry, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India
e-mail: cprao@iitb.ac.in

N. R. Rameshwaram · S. K. Nadimpalli
Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India

Introduction

Glycosidases are involved in the biosynthesis of oligosaccharide chains from their parental polysaccharides and also in the quality control mechanisms in the endoplasmic reticulum (ER) of N-linked glycoproteins [1, 3]. Therefore, the inhibition of glycosidases can have profound effect on quality control, maturation, transport and secretion of glycoproteins, and can alter cell–cell or cell–virus recognition processes. Indeed, this is the basis for

the potential use of glycosidase inhibitors for viral infection, cancer and genetic disorders. Following the discovery of the classical glycosidase inhibitor, *viz.*, nojirimycin from the cultured broth of the *Streptomyces* species [4], a number of inhibitors have been isolated from plants, fungi and bacteria. Most of the literature reports on glycosidase inhibition studies were concerned with the carbohydrate mimics of simple as well as amino-, imino-cyclitols and their derivatives including those of nojirimycin [5, 15], and not the direct carbohydrate derivatives. In a recent paper that reports the study of about 29 C_3-amino sugars of α-D-altopyranosides and α-D-glucopyranosides, only one derivative showed an IC_{50} value of 70 μM against β-galactosidase from bovine liver [16, 17]. Thus the use of carbohydrate derivatives as glycosidase inhibitors has been scarce in the literature and to our knowledge no C_1-/C_2-modified derivatives were employed for such studies. Therefore, the synthesis, characterization and property evaluation of carbohydrate-based molecules as transition state analogues of glycosidase inhibitors are some ever-challenging tasks to chemists. In the present study we explore the potential of C_1-modified carbohydrate derivatives of D-galactose, D-lactose and D-ribose, and C_2-modified derivatives of D-glucose, and not the cyclitols, as transition state analogues on the inhibition of glycosidase enzyme activities isolated from soybean and jack bean. The binding of these derivatives has been studied based on fluorescence and isothermal titration calorimetry, and the conformational changes have been addressed based on fluorescence and circular dichroism studies. The interactions of the glycoconjugates have been modeled by rigid docking studies using rice α-galactosidase and human α-mannosidase and the results obtained have been found to correlate very well with their inhibition data.

Experimental section

Materials

Seeds of soybean were purchased from local markets and ground into fine powder. Jack bean meal was purchased from Hi Media chemicals. Both were defatted using acetone (A.R. grade) and the resulting powder was air dried and used as starting material. Seralose-6B, α-D-mannopyranoside, α-D-galactopyranoside, β-*N*-acetylglucosaminide, divinyl sulfone, dialysis membrane, D-galactose, D-mannose, Tris–HCl, phenyl sepharose gel, 2-hydroxy-1-naphthaldehyde were purchased from Sigma Aldrich Chemical Co., USA. All other chemicals used in the present study were purchased from reputed local firms.

Synthesis and characterization of glyco-conjugates

Both the synthesis and characterizations details for all the key glycoconjugates were given in this section and those reported earlier by us were referred accordingly. ^{1}H NMR spectra for these are given in the supporting information (SI 01, Electronic supplementary material).

1b

Compound **1b** has been synthesized by adopting the procedure given earlier [18, 19]. Yield: (5.2 g, 85%); Melting Point: 139°C; FT-IR (KBr, cm^{-1}): 3,394 (b) ($\nu_{O–H}$) and 2,938 (S) & 2,935 (S) ($\nu_{N–H}$) and 2,920 (S) ($\nu_{C–H}$), 1,631 (S) ($\delta_{CH=N}$); ^{1}H NMR (DMSO-d_6, ppm): 3.2–3.8 (m, 5H, C2-H, C3-H, C4-H & C6-H_2), 4.4 (d, H, $^3J_{C1H–C2H}$, 8.239 Hz C1-H), 4.5–5.1 (d, 3H, C2-OH, C3-OH, & C4-OH), 4.7 (t, H, C6-OH), 6.9–7.6 (m, 4H, Ar-OH), 8.3 (s, H, CH=N), 13.3 (s, H, Ar-H); FAB-MS: *m*/*z* 284 ([M + H]$^+$, 100%); Anal. calc. for [$C_{13}H_{17}O_6N$]: C, 55.14; H, 6.00; N, 4.95 found C, 55.08; H, 6.12; N, 5.10.

1c

Compound **1c** has been synthesized by adopting the procedure given earlier [18, 19]. Yield: (4.01 g, 70%); Melting Point: 142°C; FT-IR (KBr, cm^{-1}): 3,320 (b) ($\nu_{O–H/N–H}$), 2,940 (S), 2,975 (S) and 2,910 (S) ($\nu_{C–H}$), 1,660 (S) ($\nu_{CH=N}$); ^{1}H NMR (DMSO-d_6, ppm): 3.1–3.6 (m. 5H, C2-H, C3-H, C4-H, C6-H_2), 3.72–3.75 (m, H, C5-H), 4.4–5.4 (m, 4H, C2-OH, C3-OH, C4-OH, C6-OH), 4.6 (d, H, $^3J_{C1H–C2H}$ 11.02 Hz, C1-H), 6.8–7.8 (m, 6H, Ar-H), 8.1 (s, H, CH=N), 14.2 (s, H, Phenol-OH); FAB-MS: *m*/*z*=334 ([M + H]$^+$, 70%), 333 ([M]$^+$, 40%); Anal. calc. for [$C_{17}H_{21}O_6N$]: C, 61.24; H, 5.75; N, 4.20 found C, 61.22; H, 6.00; N, 3.94.

2b

To a suspension of **2a** (6.86 g, 20 mmol) in 45 ml ethanol, 2-hydroxy-1-napthaldehyde (2.2 g, 20.96 mmol) was added and then the reaction mixture was allowed to reflux for 6 h. During the course of reflux, yellow solid was formed. The reaction mixture was allowed to cool at room temperature and was left as such overnight. Some more solid was formed which was then separated by filtration and washed with a small portion of ethanol and then with petroleum ether. Yield, (4.5 g, 76%); Melting Point: 137°C; FT-IR (KBr, cm^{-1}): 3,392 (b) ($\nu_{O–H/N–H}$), 2,901 (S), 2,706 (S) and 2,516 (S) ($\nu_{C–H}$), 1,632 (S) ($\nu_{CH=N}$); ^{1}H NMR (DMSO-d_6, ppm): 4.0–5.2 (m, 4H, C2-OH, C3-OH, C4-OH, C6-OH), 5.7 (d, H, $^3J_{C1H–C2H}$ 11.02 Hz, C1-H), 6.7–8.2 (m, 6H, Ar-H), 9.2 (s, H, CH=N), 14.2 (s, H,

Phenol-OH). ESI-MS m/z=496 ($[M + H]^+$, 100%); Anal. calc. for $[C_{19}H_{27}O_{11}N]$: C, 51.23; H, 6.11; N, 3.14 found C, 51.27; H, 6.18; N, 3.12.

2c

To a suspension of **2a** (6.86 g, 20 mmol) in 45 ml ethanol, salicylaldehyde (2.2 ml, 20.96 mmol) was added and the reaction mixture was allowed to reflux for 6 h. During the course of reflux, yellow solid was formed. The reaction mixture was allowed to cool at room temperature and was left as such overnight. Some more solid was formed which was then separated by filtration and washed with a small portion of ethanol and then with petroleum ether. The filtrate was concentrated to dryness and dichloromethane as added to dissolve the pasty mass. To that, petroleum ether was added to result in a second crop of solid. Yield, (3.2 g, 68%); Melting Point: 149°C; FT-IR (KBr, cm^{-1}): 3,449 (b) ($\nu_{O-/N-}$), 3,000 (S), 2,897 (S) and 2,873 (S) (ν_{C-H}), 1,626 (S) ($\nu_{CH=N}$); ^{1}H NMR (DMSO-d_6, ppm): 4.0–5.2 (m, 3H, C2-OH, C3-OH, & C4-OH), 5.5 (s, H, C1-H), 6.8–7.6 (4H, Ar-H), 8.7 (s, H, CH=N), 13.1 (s, H, Phenol-OH); ESI-MS m/z=446 ($[M + H]^+$, 100%); Anal. calc. for $[C_{23}H_{29}O_{11}N]$: C, 55.75; H, 5.90; N, 2.83 found C, 55.77; H, 5.96; N, 2.86.

3c

This has been synthesized by adopting the procedure given earlier [18, 19]. Yield: (7.8 g, 78%), Melting Point: ~185°C; FT-IR (KBr, cm^{-1}): 1,626 (s) ($\nu_{C=N}$), 3,384 (b) & 3,242 (b), 3,562 (s) (ph. O–H) (ν_{O-H}), 2,933 (m) & 2,879 (m) (ν_{C-H}); ^{1}H NMR (DMSO-d_6, ppm): 3.2–3.8 (m, 5H, C2-H, C3-H, C4-H, C5-H), 5.0–5.3 (m, 3H, C2-OH, C3-OH, & C4-OH), 5.0 (s, H, C1-H), 6.6–8.0 (m, 6H, Ar-H), 9.0 (d, H, CH=N), 13.40–13.46 (m, H, Phenol-OH); FAB-MS: m/z 304 ($[M + H]^+$, 70%), 303 ($[M]^+$ 30%); Anal. calc. for $[C_{16}H_{17}O_4N \cdot H_2O]$: C, 63.36; H, 5.65; N, 4.62; found C, 63.42; H, 5.56; N, 4.86.

4b

This has been synthesized starting from **4a** by adopting the procedure given for **1b**. Yield: (81%); Melting Point: 132°C; FT-IR (KBr, cm^{-1}): 1,632 (s) ($\nu_{C=N}$), 3,395 (b), 3,052 (b) (ph. O–H) (ν_{O-H}), 2,936 (m) & 2,734 (m) (ν_{C-H}); ^{1}H NMR (DMSO-d_6, ppm): 2.5 (q, 6H, DMSO peak), 3.2–3.8 (m, 5H, C2-H, C3-H, C4-H, C5-H), 4.5–4.9 (4d, 4H, C1-OH, C3-OH, C4-OH & C6-OH), 5.1 (d, H, $^3J_{C1H-C2H}$, 5.5 Hz C1-H), 6.2–7.6 (2d, 2t, 4H, Ar-OH), 8.6 (s, H, CH=N), 13.2 (s, H, Ar-OH). ESI-MS m/z=284 ($[M + H]^+$, 100%); Anal. calc. for $[C_{13}H_{17}O_6N]$: C, 55.12; H, 6.05; N, 4.94; found C, 55.14; H, 6.06; N, 4.98.

4c

This has been synthesized starting from **4a** by adopting the procedure given for **1c**. Yield: (89%); Melting point: 157°C; FT-IR (KBr, cm^{-1}): 1,637 (s) ($\nu_{C=N}$), 3,475 (b), 3,029 (b) (ph O–H) (ν_{O-H}), 2,961 (m) & 2,866 (m) (ν_{C-H}); ^{1}H NMR (DMSO-d_6, ppm): 2.5 (q, 6H, DMSO peak), 3.1–3.7 (m. 5H, C2-H, C3-H, C-4H, C5-H & C6-H), 4.5–5.4 (m, 4H, C1-OH, C3-OH, C-4OH, C6-OH), 5.6 (d, H, $^3J_{C1H-C2H}$ 5.2 Hz, C1-H), 6.6–8.1 (6H, Ar-H), 8.9 (d, H, CH=N), 13.6 (t, H, Aromatic-OH). ESI-MS m/z=334 ($[M + H]^+$, 100%); Anal. calc. for $[C_{17}H_{19}O_6N]$: C, 61.25; H, 5.75; N, 4.20; found C, 61.34; H, 5.86; N, 4.28.

Preparation of enzyme fractions

The enzymes were isolated from soybean and jack bean meal as given in this section. All the operations were carried out at 4°C. TBS was prepared from 25 mM Tris–HCl and 1.5 mM NaCl at pH 7.4 and was used further. The defatted powder (60 g) was extracted with 150 ml of TBS buffer containing 5 mM $MnCl_2$ and 5 mM $CaCl_2$ for about 15 h, and the extract was clarified by centrifugation at 12,000 rpm for 20 min. Ammonium sulfate was added to the supernatant to 80% saturation and the suspension was centrifuged at 12,000 rpm for 20 min. The pellet was re-dissolved in minimum amount of TBS buffer containing 5 mM $MnCl_2$ and 5 mM $CaCl_2$ and was dialysed extensively (three times with a 2 liters of buffer each time) against TBS buffer and was centrifuged at 12,000 rpm for 20 min. The supernatant was passed through the seralose–mannose gel in case of jack bean and seralose–galactose gel in case of soybean, where the corresponding affinity columns were prepared as reported in the literature [20]. The unbound fraction from the affinity gels containing the enzyme activities, were separately pooled and 1.5 M ammonium sulfate was added and passed through two separate phenyl Sepharose gels (one for jack bean extracts and the other for soybean extracts) equilibrated with 50 mM Tris–HCl buffer at pH 7.4 containing 1.5 M ammonium sulfate (buffer A). The column was washed with buffer A. Elution of the enzyme was done with buffer A devoid of ammonium sulfate. The eluates containing the glycosidase activities were pooled and dialyzed against 50 mM NaOAc buffer of pH 5.0. This is a mixture of three enzymes, *viz.*, α-galactosidase, α-mannosidase and β-hexosaminidase. These were analyzed by SDS-PAGE under reducing conditions and proteins were detected by Coomassie blue as well as by silver staining. Concentration of the protein was measured both by absorbance and by Lowry method [21]. Each enzyme was assayed using the respective substrate (α-D-galactopyranoside, α-D-mannopyranoside, and β-*N*-acetylglucosaminide) in the presence of the other two enzymes [22] (SI 02, Electronic supplementary material).

Purification of α-mannosidase from jack bean

The partially purified enzyme possessing α-galactosidase, α-mannosidase and β-hexosaminidase was further purified by adopting the literature protocol [23] with marginal modifications. The fractions eluted from the phenyl sepharose chromatography were dialyzed in 50 mM acetate buffer pH 5.0. The dialyzed fraction was treated with pyridine using 0.2 by volume and incubated at 37°C for 20 min. Pyridine treated fraction was showing only α-mannosidase activity. Proteins precipitated with the pyridine were removed by centrifugation at 15,000 rpm. The supernatant containing the α-mannosidase activity was subjected to 80% ammonium sulfate saturation. The precipitated fraction was dialyzed in 50 mM acetate buffer pH 5.0 and loads on the S-100 matrix for gel filtration chromatography to remove the unwanted proteins. Fractions showing the α-mannosidase activity were pooled and used for the further studies.

Preparation of bulk solutions and stability of the conjugates

The calculated amount of the glyco-conjugate (inhibitor) was dissolved in 20–40 μl of dimethyl sulfoxide (DMSO) and made up to 1 ml by using sodium acetate buffer (pH 5.0) to give a bulk solution of 10 mM. The stability of the glyco-imino-conjugates has been followed by measuring the absorption and fluorescence spectra for 24 h and found no changes suggesting that there is no degradation of the conjugates take place in this medium. These conjugates exhibited same chromatographic behaviour on TLC whether these were spotted from buffer solution or from the organic solvent, indicating that the conjugates do not degrade under the present experimental conditions. However, precipitation occurs when concentrated buffer solutions of conjugates were left for long time. Hence, freshly prepared bulk solutions were always used for fluorescence, CD, ITC as well as for inhibition assay and kinetics studies. The bulk solution of the potassium iodide (KI) used in the fluorescence titration was 2.0 M in sodium acetate buffer.

Enzyme inhibition assay

Enzyme inhibition studies were carried out with incubating 50 μl (1 mg/ml) of enzyme with increasing amounts of the inhibitor (*i.e.*; from 0 to 2 mM final conc.) at 37°C for 20 min. Fixed amount of the corresponding substrate (*i.e.*; 50 μl of 5 mg/ml) was added and final volume was made up to 250 μl with 50 mM NaOAc Buffer, pH 5.0. This mixture was incubated at 37°C for 20 min. The reaction was stopped with the addition of 250 μl of 0.2 M Na_2CO_3 buffer pH 11. Absorbance was recorded at 405 nm for the release of *p*-nitrophenolate ion for the enzyme activity. The activity without the inhibitor was considered to be 100% and the remaining activities at each concentration of inhibitor were determined w.r.t. this value.

Studies of enzyme kinetics

For the kinetic studies 100 μl (of 1 mg/ml) of enzyme was incubated with a fixed amount of inhibitor (*i.e.*; 42 μM and 83 μM for all studies) at 37°C for 20 min. Increasing amount of corresponding enzyme's substrate (from 14 μM to 553 μM in case of soy bean galactosidase; from 122 μM to 1,220 μM in case of hexosaminidase from soybean and jack bean and 111 μM to 1,770 μM in case of the mannosidase of the jack bean) was added. Volume was made up to 250 μl with the 50 mM NaOAc Buffer pH 5.0. This mixture was incubated at 37°C for 20 min. The reaction was stopped with the addition of 350 μl of 0.2 M Na_2CO_3 buffer pH 11. Each point has been taken in triplicate to minimize the experimental error. Absorbance was recorded at 405 nm for the release of *p*-nitrophenolate ion for the enzyme activity. The rates were calculated by using standard curve for *p*-nitrophenolate ion.

Fluorescence studies

All the fluorescence titrations were carried out on a Perkin Elmer LS 55 at 280 nm excitation wave length using 1 cm quartz cells. All the experiments were repeated for at least three times or more and found that the results were reproducible. The solutions were excited at 280 nm wave length and the emission spectra were recorded in the range 290 to 450 nm.

Isothermal titration calorimetry (ITC)

All the ITC studies were carried out in acetate buffer using MicroCal VP-ITC system.

Results and discussions

Simple glycoses were appropriately modified either at C_1- or at C_2- in order that the resultant derivatives are useful for binding as well as for inhibiting the glycosidases isolated from soybean and jack bean as reported in this paper.

Glycoconjugates

Simple glycoses used for derivatizations were D-galactose (**1**), D-lactose (**2**) and D-ribose (**3**) in order to modify at C_1 and D-glucosamine (**4a**) in order to modify at C_2. Upon derivatization, these resulted in the formation of either C_1-

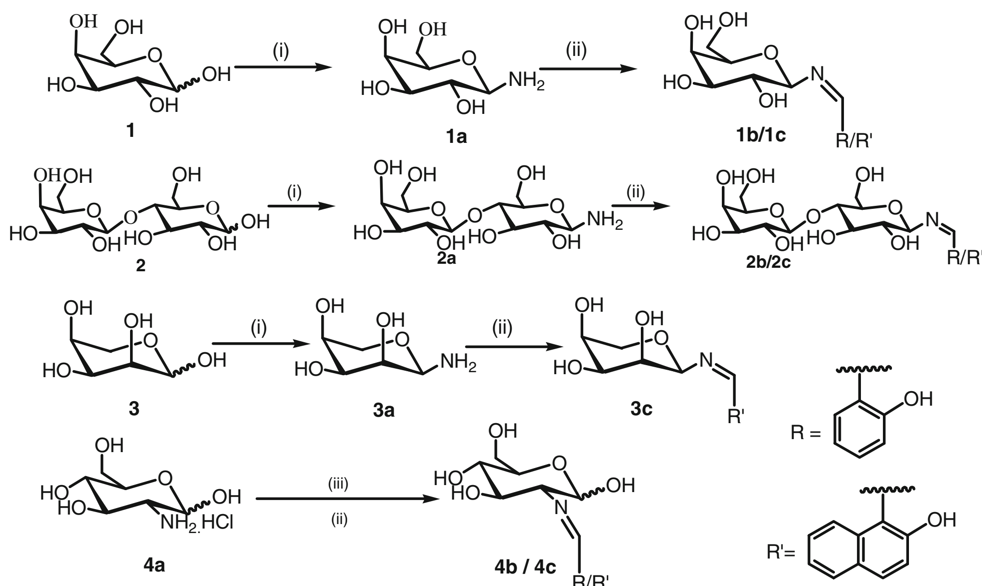
NH_2 (**1a**, **2a** and **3a**) or C_1-N=C(H)(Ar), where 'Ar' refers to the aromatic moiety arising from salicylidene (**1b**, **2b**) or naphthylidene (**1c**, **2c**, **3c**). The corresponding imino-conjugates were synthesized in two steps, while the first step deals with the C_1-amination using ammonia leading to C_1-NH_2, the second step deals with the condensation of C_1-NH_2 moiety with an appropriate aldehyde to yield the corresponding C_1-imino-conjugate {C_1-N=C(H)(Ar)} as shown in Scheme 1. On the other hand the C_2-imino-conjugates of D-glucose (**4b** and **4c**) were synthesized by a single step condensation reaction between **4a** and the corresponding aldehyde (Scheme 1). The products were characterized thoroughly by analytical and spectral techniques. All the synthesis and characterization details are given in the experimental section (SI 01). Selective recognition of **1c** and its amino-counter parts towards Cu^{2+} as well as amino acids have been reported by us recently [18, 19].

Glycosidases

Glycosidases were extracted, isolated, and partially purified by ion exchange hydrophobic chromatography in our lab from soybean and jack bean meal as described in the experimental. The enzymes were isolated more than four times in different batches (SI 02, Electronic supplementary material). Partially purified extracts contain α-galactosidase (G), α-mannosidase (M) and β-hexosaminidase (H) to different proportions as analyzed for their individual enzyme activities using 4-nitrophenyl-α-D-galactopyranoside, 4-nitrophenyl-α-D-mannopyranoside and 4-nitrophenyl-*N*-acetyl-β-glucosaminide respectively. The ratio of the enzymes, α-galactosidase: β-hexosaminidase: α-mannosidase is 4:4:2 in soybean and 2:4:4 in jack bean, respectively. While the levels of β-hexosaminidase is same in both the seeds, α-mannosidase is high in jack bean where as the α-galactosidase is high in soybean. Among the attempts made to separate the individual enzymes from the partially purified preparations, only the α-mannosidase could be separated from jack bean. Therefore all the studies reported in the present paper were carried out with pure α-mannosidase from jack bean (Pure M) as well as with the partially purified fractions (jack bean and soybean). These fractions are amenable for studies since the individual enzyme activities are very specific towards the substrate. Inhibition of glycosidases by the synthetic glycoconjugates has been assayed in presence of the corresponding glycoside analogue that releases *p*-nitrophenolate ion. The activity has been quantitatively monitored by measuring the absorbance at 405 nm. The less the OD_{405}, the more the inhibition is.

Inhibition by glycoconjugates

Percent of inhibition was calculated using the absorbance data obtained from the enzyme assay studies carried out in the presence of these conjugates using appropriate controls as given in the experimental section. The inhibition data exhibited by simple glycoses, their C_1-/C_2-NH_2 and C_1-/C_2-N=C(H)(Ar) derivatives (glyco-imino-conjugates) have been shown for all the three enzymes obtained from soybean and jack bean and representative results are given in Fig. 1 (SI 03, Electronic supplementary material). Gross examination of this data indicate two sets of inhibition trends, while one set shows 100% inhibition, the second set shows only a little or marginal inhibition. While the glyco-imino-conjugates are the ones which gives the curves under the first category, the simple glycoses and C_1-/C_2-NH_2 derivatives gives those under the second. Among the first category, while the naphthylidene-conjugates (**1c**, **2c**, **3c**



Scheme 1 Synthesis of C_1-/C_2-glyco-conjugates of D-galactose, **1** (**1a**, **1b** and **1c**), D-lactose, **2** (**2a**, **2b** and **2c**), D-ribose, **3** (**3a** and **3c**) and C_2-glucosylamine, **4a** (**4b** and **4c**): (*i*) NH_3/CH_3OH/4°C; (*ii*) C_2H_5OH/R-CHO or R′-CHO; (*iii*) N$(CH_2CH_3)_3$

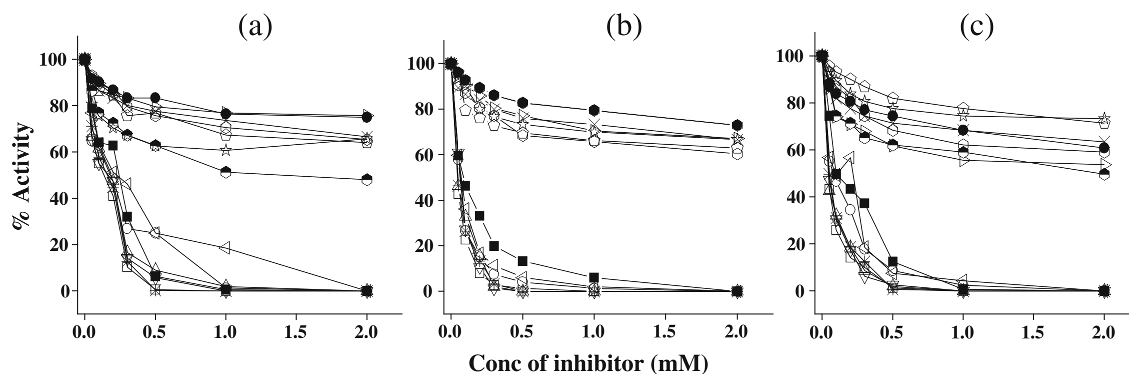


Fig. 1 Inhibition of glycosidase activity obtained from soybean meal by different glyco-conjugates in terms of % activity: **a** α-galactosidase, **b** α-mannosidase and **c** β-hexosaminidase □ **1c**, ○ **1b**, △ **3c**, ▽ **2c**, ◁ **2**, ▷ **1a**, ⬡ **3a**, ⬠ **2a**, ⬢ **1**, ☆ **3**, × **2**, ✳ **4c**, ■ **4b**, ● **4a**

and **4c**) exhibit 100% inhibition around 0.3 to 0.5 mM, the salicylidene-conjugates (**1b**, **2b** and **4b**) exhibit 100% inhibition only in the range, 1.0 to 2.0 mM. However, the molecules of the second set, *viz.*, **1**, **2**, **3**, **1a**, **2a**, **3a** and **4a**, exhibit inhibitions of 20 to 30% only at 0.5 mM and 30 to 40% only at 2 mM. Further examination of the curves under the second set reveal an inhibition trend, *viz.*, **4a**<**1**, **2**, **3** ≤ **1a**, **2a**, **3a**, indicating that among these only C_1-NH_2 shows considerable inhibition. It is not easily possible to get 100% inhibition with simple glycoses, however, some of the glycoses are able to inhibit some enzymes at around 50 or 100 mM or higher concentration (*viz.*, **1** or **2** *vs.* galactosidase, and mannose *vs.* mannosidase) (SI 03, Electronic supplementary material).

The general trend observed with the inhibition is, glyco-imino-conjugates >>> glyco-amines ≥ simple glycoses. Thus only the C_1-/C_2-glyco-imino-conjugates are capable of bringing 100% inhibition of the glycosidase activity of soybean and jack bean and not the others, indicating that both the imine- and aromatic moiety present in the derivative contributes immensely to the inhibition. The 0.5 mM concentration of control molecules, *viz.*, 2-naphthol, 1-naphthaldehyde and 2-hydroxy-1-naphthaldehyde exhibited inhibition of 5–25%, 0–20% and 30–40% (w.r.t. the enzymes of soybean and jack bean, SI 03, Electronic supplementary material) respectively. The data seem to suggest that both the 2-OH and 1-CHO groups are simultaneously required on the naphthyl moiety in order to bring considerable inhibition, indicating that the presence of both these groups seem to direct the molecule for a better binding with the enzyme. Therefore, the comparison of the inhibition data of the C_1-/C_2-glyco-imino-conjugates with that of the control molecules clearly suggests that all the three components, *viz.*, carbohydrate, imine and aromatic moiety, are required together to bring 100% inhibition of the glycosidase activity. Further, the data obtained in case of controls (**1**, **2**, **3** and **4a**) in comparison with the others indicate that the carbohydrate moiety present in these conjugates can contribute to 10–30% towards inhibition. The extent of glycosidase inhibition has not been greatly altered even in the presence of 20 mM glycose (SI 03, Electronic supplementary material). This means that the introduction of aromatic imine at C_1-/C_2-position can substantially enhance the glycosidase inhibition capacity of the glyco-conjugates. It is indeed noted in the literature that the introduction of $-NH_2$ group on some glycoses enhances the enzyme inhibition [18, 19]. The reference compound, 1-β-D-glucosylamine, showed 50% inhibition only at 1200 μM against apricot β-glucosidase in the literature [4].

Concentration at which a 50% inhibition of enzymes is brought (IC_{50}) by C_1-/C_2-glyco-imino-conjugates has been given in Fig. 2 (Table 1). In effect, the enzymes of soybean

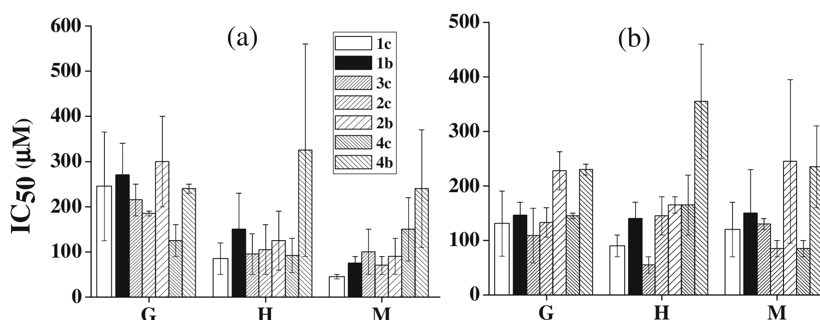


Fig. 2 Histogram indicating the concentrations of the glyco-imino-conjugates in μM at 50% inhibition of glycosidases (*G* galactosidases; *H* hexosaminidases and *M* mannosidases) (IC_{50}): **a** from soybean and **b** from jack bean

Table 1 IC_{50} values in μM for the inhibition of enzymes by glyco-imino-conjugates

	Soybean			Jack bean			
	G	H	M	G	H	M	Pure M
1c	245±120	85±35	45±5	131±60	90±20	120±50	22±2.5
2c	185±5	105±55	70±20	133±27	145±35	85±15	23±2.5
3c	215±35	95±45	100±50	109±50	55±15	130±10	31±5.5
4c	125±35	92±38	150±70	145±5	165±55	85±15	29±3.5
1b	270±70	150±80	75±15	146±24	140±30	150±80	51±3.5
2b	300±100	125±65	90±40	228±35	165±15	245±15	50±2.5
4b	240±10	325±38	240±70	230±10	355±55	235±75	99±6.5

Errors in the IC_{50} values have been calculated by repeating the inhibition assay for at least four times

are inhibited relatively at lower concentrations of glyco-imino-conjugates as compared to those of the jack bean. The IC_{50} (μM) values from both the sources generally follows a trend, α-galactosidase > β-hexosaminidase ≥ α-mannosidase. Thus there is a differential inhibition of these enzymes by different glyco-imino-conjugates, and with respect to C_1-imino-conjugates the α-mannosidase and β-hexosaminidase are more susceptible for inhibition than that of α-galactosidase at least by a factor two or more.

While it is the α-mannosidase that can be inhibited at lower concentration in case of soybean, *viz.*, 45±5 μM by the galactosyl-naphthyl-imino-conjugate, **1c**, (average over all the glyco-imino-conjugates is 88±33 μM), it is the β-hexosaminidase in case of jackbean, *viz.*, 55±15 μM by the ribosyl-naphthyl-imino-conjugate, **3c** (average over all the glyco-imino-conjugates is 127±28 μM). In the literature, the jack bean α-mannosidase was shown to be inhibited by 50% at 350 μM by imino-cyclitol glyco-mimics [10], and this concentration is much higher than that observed in the present study. Other conjugates, in the present study, whose IC_{50} values are in the vicinity of 100 μM include, **1b**, **2b**, **2c** and **3c** (α-mannosidase from soybean), **1c**, **2c**, **3c** and **4c** (β-hexosaminidase from soybean), **2c** and **4c** (α-mannosidase from jack bean) and **1c** and **3c** (β-hexosaminidase from jack bean). Thus the C_1-/C_2-imino-conjugates were found to be very effective in inhibiting the glycosidases from soybean and jack bean. Among the imino-conjugates, the naphthylidene derivatives (**1c**, **2c**, **3c** and **4c**) show higher inhibition at lower concentration as compared to their counter-salicylidene-conjugates (**1b**, **2b** and **4b**). Comparison of the inhibition data reported in the literature in case of C_3-amino sugars [16, 17] with that of the present study clearly suggest that the C_1-/C_2-imino-conjugates are certainly effective glycosidase inhibitors at the same or even at lower concentrations. While it is only one among the twenty nine C_3-amino sugars studied was found to be effective inhibitor of β-galactosidase from bovine liver, several of C_1-/C_2-imino-conjugates were shown to be effective inhibitors of glycosidases in the present study.

Similar to that observed in case of the partially purified enzyme, even the pure α-mannosidase was inhibited mainly by the glyco-imino-conjugates and not by simple glycoses or their C_1-/C_2-NH_2 derivatives (Fig. 3a). Corresponding IC_{50} values in μM for these are, 22–23(**1c**, **2c**)<29–31 (**3c**, **4c**)< 50–51 (**1b**, **2b**)<99 (**4b**), indicating that the napthylidene-conjugates are effective inhibitors by a factor of two, when compared to the corresponding salicylidene-conjugates. The IC_{50} value of any of these glyco-imino-conjugates is at least three to four fold lower for pure α-mannosidase, when compared to the partially purified enzyme (Fig. 3b). Thus, the extent of inhibition exhibited by the conjugates reported in this paper is far superior over the controls as well as that reported in the literature and hence these can be potent inhibitors of glycosidases.

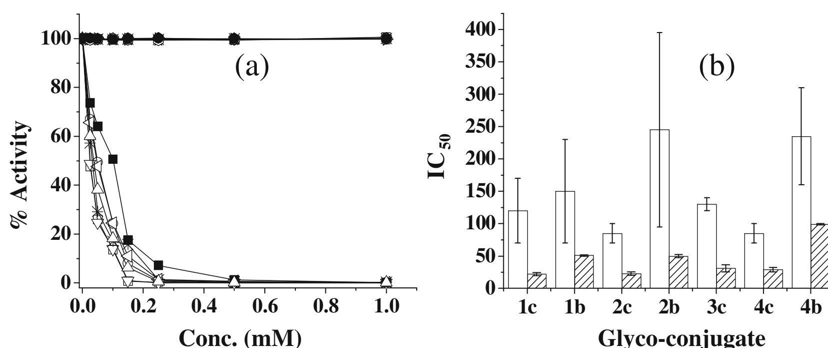


Fig. 3 **a** Inhibition of pure α-mannosidase from jack bean by glyco-conjugates (*symbols* have same meaning as in Fig. 1 and ⊞ 4. **b** Comparison of IC_{50} values by glyco-conjugates between the partially purified α-mannosidase (*unfilled columns*) and the purified α-mannosidase (*crossed lined columns*). *Error bars* are based on three different measurements

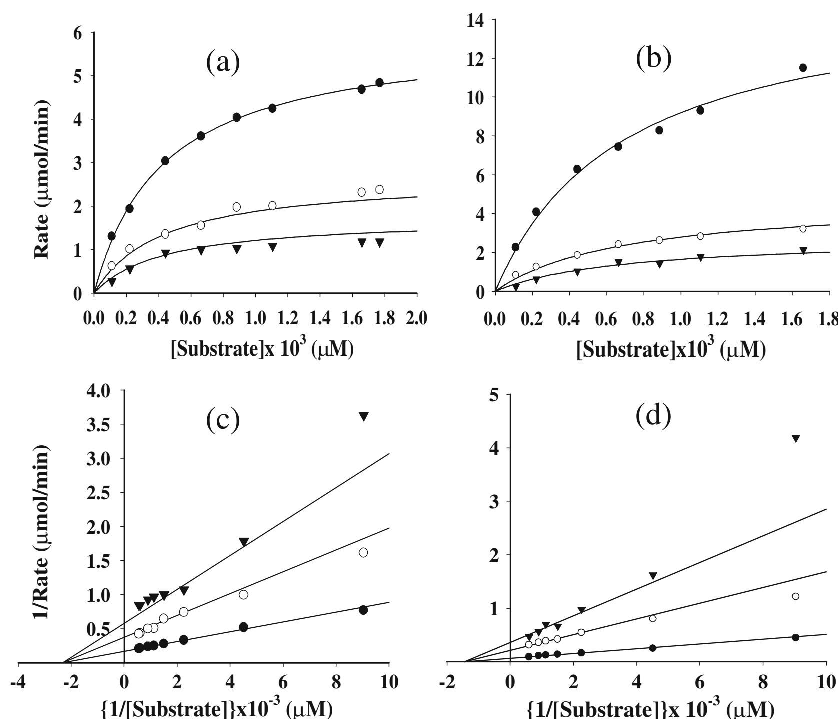


Fig. 4 Michaelis–Menten plots for the titration of **1c** with: **a** partially purified α-mannosidase (Jack bean); **b** pure α-mannosidase (Jack bean). Lineweaver–Burk plots given under (**c**) and (**d**) corresponds to those given under (**a**) and (**b**) respectively. The *symbols* present in (**a**) and (**c**) means ▼ 83 μM, ○ 42 μM and ● 0 μM, whereas in (**b**) and (**d**) means ▼ 20.0 μM, ○ 10 μM and ● 0 μM

Kinetics of inhibition

Kinetics of inhibition has been studied using all the seven C_1-/C_2-glyco-imino-inhibitors (**1b**, **1c**, **2b**, **2c**, **3c**, **4b** and **4c**). The Michaelis–Menten plots suggested non-competitive inhibition of the enzyme with all these conjugates (Fig. 4). The K_i values were derived based on Lineweaver–Burk plots as given in Table 2 (SI 04, Electronic supplementary material). This data clearly suggests at least 10 fold lower K_i values in case of naphthalydene-conjugates as compared to the salicylidene-ones. This means that higher the size of the aromatic moiety, the lesser the K_i is. The ribosyl derivative (**3c**) exhibit K_i values which are twice to that of galactosyl (**1c**) or lactosyl (**2c**), suggesting the involvement of the carbohydrate moiety in the inhibition and further reflects on the variation in the carbohydrate.

Table 2 K_i (in μM) values for the inhibition of glycosidases by the aromatic-imino-glyco-conjugates

Inhibitor	Soybean		Jack bean		Jack bean
	G	H	H	M	Pure M
1c	29±1	30±6	27±1	30±4	5±0.5
2c	28±3	32±3	34±4	31±3	6±0.5
3c	64±5	66±1	69±4	60±2	10±1
4c	26±1	38±3	28±4	35±2	6±0.5
1b	288±10	266±14	279±19	258±3	46±4
2b	276±2	333±35	285±9	299±18	53±5
4b	226±12	371±29	291±48	332±39	56±5

Similar studies carried out using the pure fraction of α-mannosidase (jack bean) yielded K_i values which are at least five to seven fold lower than that obtained in case of the partially purified enzyme for a given glyco-conjugate (Table 2). Even in this case, the salicylidene-conjugates exhibited an order of magnitude higher K_i compared to the naphthylidene-conjugates. The K_i obtained in the present case (naphthylidene-conjugates, 5 to 10 μM; salicylidene-conjugates, 46 to 56 μM) are much lower than those reported in case of triazole derivatives (lowest K_i is 120 μM) [17].

Fluorescence binding

In the fluorescence titration of enzymes obtained from soybean and jack bean with simple glycoses and their glyco-conjugates, intrinsic fluorescence emission of tryptophans was measured by exciting at 280 nm. No considerable change was observed in the fluorescence emission intensity in case of simple glycoses and glyco-amines, however, the C_1-/C_2-imino-conjugates showed asymptotic decrease in the intensity as a function of their concentration (Fig. 5). The results suggest that the jack bean enzyme is susceptible for higher fluorescence quenching in general by the naphthylidene derivatives as compared to the soybean enzyme. Thus the fluorescence quenching follows a trend, *viz.*, glycoses<glyco-C_1-NH_2<<<glyco-salicylidene-conjugates<glyco-naphthylidene-conjugates (Fig. 5, SI 05, Electronic supplementary material), which is in line with the trend observed for inhibition.

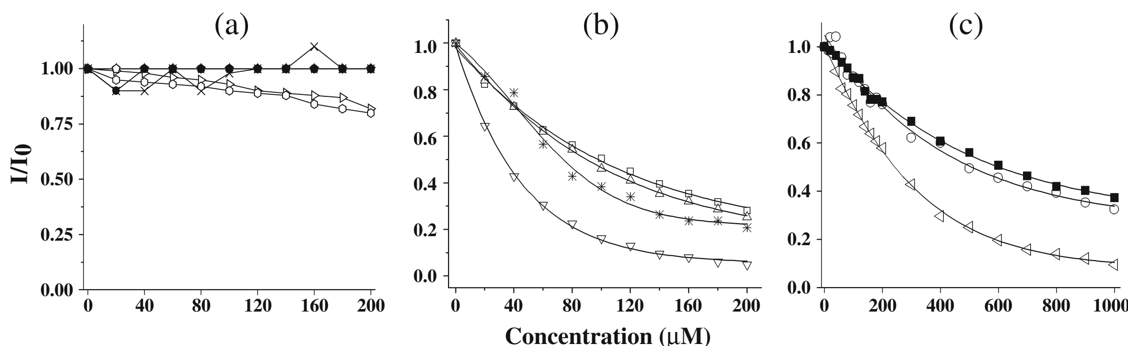


Fig. 5 Relative fluorescence intensity plots (I/I_0) in the titration of partially purified jack bean enzymes by glyco-conjugates: **a** simple glycoses and glyco-C_1-NH_2; **b** naphthylidene-conjugates, and **c** salicylidene-conjugates. *Symbols* carry the same meaning as in Fig. 1

While the naphthylidene-conjugates exhibit an almost complete quenching of the fluorescence intensity at 335 nm in case of soybean as well as jack bean enzyme, the salicylidene-conjugates, *viz*., **1b, 2b** and **4b** shows an altogether different behaviour (SI 05, Electronic supplementary material). Similar to that observed in case of naphthylidene-conjugates, even in case of the salicylidene-conjugates, the jack bean enzyme exhibits higher fluorescence quenching than that of the soybean enzyme. While the 2-naphthol has no affect on the fluorescence intensity, that of 1-naphthaldehyde shows (SI 06) a behavior parallel to that of the salicylidene-conjugates, suggesting that both the hydrophobic moiety as well as the imine function are required for quenching. Some of the naphthylidene-conjugates are able to bring I/I_0 to 0.2 within 60–100 μM and that of the salicylidene-conjugates brings at 600–800 μM. This suggests that the napthylidene-conjugates bind more effectively than their salicylidene-counter parts. This can be further understood from the concentrations of the conjugates required to bring a 50% fluorescence quenching (CF_{50}) as shown in Fig. 6. The CF_{50} for naphthylidene-conjugates was found to be in the range 20–120 μM and that of the salicylidene-conjugates were in the range 200–700 μM. Thus the difference observed in the fluorescence quenching between naphthylidene- *vs.* salicylidene-conjugates is parallel to that observed in their inhibitory capacities. This suggests that the inhibition is affected by binding.

The fluorescence decay pattern as well as the trends observed in quenching the pure fraction of α-mannosidase is similar to that observed with the partially purified enzyme and this follows, *viz.*, (**1c**, **3c**)>(**2b**, **2c** and **4c**)> (**1b**, **4b**)>>>(**1**, **2**, **3**, **1a**, **2a**, **3a**, **4a**) (Fig. 7). This trend is same as that observed for the inhibition. The quenching behaviour observed with the salicylidene-conjugates can be explained as arising from at least two types of tryptophan emissions, one centered at 334 and the other centered at 360 nm. Fluorescence intensity of the first peak diminishes upon titration and that of the second one does not (Fig. 8). Thus the binding and inhibition behaviour of pure enzyme as well as its partially purified are similar. Presence of more than one different type of Trp was reported in a number of glycosidases in the literature [24–26].

In order to analyze the fluorescence quenching of enzyme by the inhibitor, the Stern Volmer equation,

$$\frac{F_0}{F} = 1 + K_Q\tau_0[Q] = 1 + K_{SV}[Q],$$

Where F_0 and F are the fluorescence intensities in the absence and in the presence of the inhibitor, $[Q]$ is the inhibitor concentration and K_{sv} ($=K_Q\tau_0$) is the Stern Volmer quenching constant (K_Q is the bimolecular quenching rate constant and τ_0 is the average life time of the tryptophans in the absence of the quencher), and has been used to obtain the data given in Table 3.

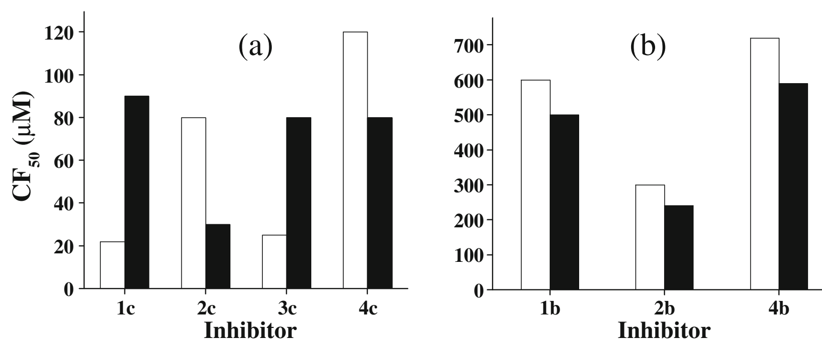


Fig. 6 Concentration of the glyco-imino-conjugates when the fluorescence intensity is decreased by 50% (CF_{50}): **a** naphthylidene derivatives; **b** salicylidene derivatives. *Unfilled columns* are for soybean and *filled columns* are for jack bean

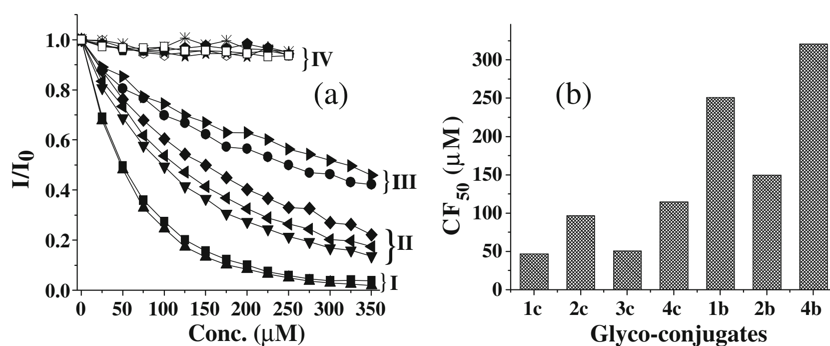


Fig. 7 Titration of pure α-mannosidase from jack bean: **a** Plots of relative fluorescence intensity (I/I_0) vs. concentration of glyco-conjugates; **b** histogram showing the concentration of the glycoconjugate, when fluorescence intensity was quenched to 50% (CF_{50}). The groups in (**a**) are (I) ■ **1c**, ▲ **3c**; (II) ▼ **2c**, ◄ **4c**, ◆ **2b**; (III) ● **1b**, ► **4b**; (IV) ⬡ **1**, ★ **2**, ⬟ **3**, ✳ **1a**, □ **2a**

The fluorescence quenching studies are suggestive of the fact that the higher the hydrophobic portion present on the nitrogen center in the C_1-glyco-conjugate, the better the association and perhaps bring higher conformational changes. Fluorescence quenching efficiency is higher in case of the enzymes from jack bean when compared to that from soybean. Among all the conjugates studied, those of C_2-, *viz.*, **4b** and **4c** exhibited least effect on the fluorescence quenching. In effect, the naphthyl-conjugates, in general, are efficient quenchers as compared to the salicilyl ones and that of the C_1-glyco-conjugates are better quenchers than the C_2-glyco-conjugates.

The extent of exposure of the Trp moieties in presence of naphthylidene- and salicylidene-conjugates has been further established by carrying out potassium iodide (KI) quenching experiments. The data clearly suggested that the Trp residues are certainly more exposed with naphthylidene-conjugates as compared to the salicylidene-ones (Fig. 9a and b) indicating a higher conformation change in the presence of the former.

The extent of quenching of fluorescence by the glyco-conjugates has been further checked by carrying out the same experiment in the presence of 20 mM simple glycose as shown in Fig. 10. No significant change was observed indicating that the presence of simple glycoses does not affect the binding of the glyco-imino-conjugate which means that the glycoconjugates indeed can replace the simple glycoses. Thus the fluorescence studies also provided sufficient evidence for the differential binding between the naphthylidene *vs.* salicylidene, C_1- *vs.* C_2-conjugation in addition to jack bean *vs.* soybean enzyme activity and its implication on the buried *vs.* exposed tryptophan residues.

CD studies

Conformational changes brought in the enzyme upon binding the conjugates have also been revealed from circular dichroism studies. The enzyme from the jack bean has been titrated with different glyco-imino-conjugates, *viz.*, **1c**, **1b**, **2c** and **2b** and the data showed considerable changes in the ellipticity as well as in the band position (red shifted from 224 nm to 237 nm) (Fig. 11, SI 07, Electronic supplementary material). Thus, the changes observed in the CD spectra are clearly suggestive of secondary structural changes in the enzyme upon binding by the inhibitor. This is true with the pure fraction of α-mannosidase as well as the partially purified one. These results are in conformity with the findings from the fluorescence that Trp residues are indeed exposed more upon glyco-conjugate binding. Also true was that the secondary structural changes observed in CD were more with the naphthylidene-conjugates as compared to their salicylidene-counter parts, a conclusion that has already been arrived at based on the inhibition data as well as the fluorescence data.

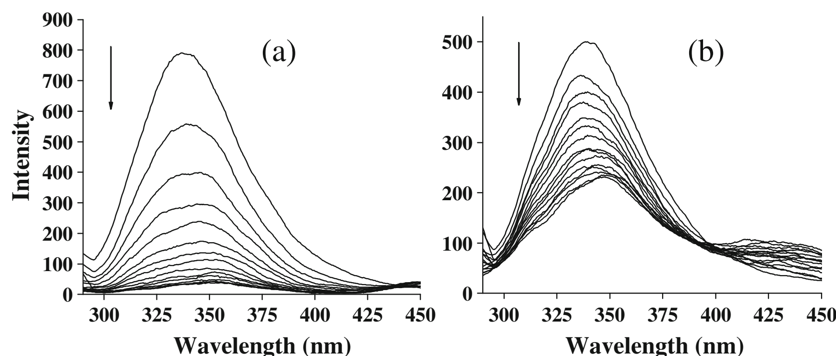


Fig. 8 Fluorescence spectral traces in the titration of pure α-mannosidase by glyco-imino-conjugates: **1c** (**a**); **1b** (**b**)

Table 3 K_{sv} values for the binding of glyco-imino-conjugates as obtained from the fluorescence studies

	K_{sv} (M^{-1})		
	Soybean	Jack bean	
	Partially pure	Partially pure	Pure M
1c	4.01×10^5	1.62×10^5	2.40×10^4
2c	1.11×10^5	1.44×10^5	1.04×10^4
3c	2.01×10^5	2.81×10^5	1.30×10^4
4c	1.90×10^4	2.04×10^4	1.32×10^4
1b	1.74×10^3	3.12×10^4	3.10×10^3
2b	1.92×10^4	5.07×10^4	7.60×10^3
4b	1.41×10^3	2.22×10^3	2.39×10^3

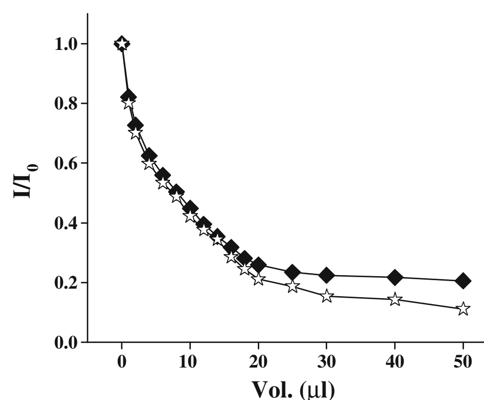


Fig. 10 Plots of relative fluorescence intensity of the enzyme with **1c** in the absence (♦) and in the presence (☆) of 20 mM added galactose

Binding by isothermal titration calorimetry (ITC)

The binding of glyco-imino conjugates were further confirmed based on ITC studies carried out using the enzyme obtained from jack bean meal (Fig. 12). The ITC data obtained in case of napthylidene-conjugate (**1c**) can be fitted to a reasonable binding curve with K_{ass} of 196± 12 M^{-1}. However, the data obtained in case of **1b** could not be fitted to any reasonable binding, suggesting that the salicylidene-conjugate, **1b** has considerably weak binding with the enzyme. The K_{ass} for lactosyl-imine conjugate are 90±6 and 34±5 M^{-1} for **2c** and **2b** respectively, indicating that binding with salicylidene-conjugate is weaker than the naphthylidene. The low K_{ass} further reveal that the interaction of glyco-imino-conjugates is weak and primarily hydrophobic in nature.

Computational docking studies

In order to understand the nature of binding of these glyco-conjugates with the glycosidases, computational docking studies were performed using a web-based docking server PARDOCK [27]. These were performed with Rice-α-galactosidase [28] (crystallized with α-galactose, R-α-Gal, PDB ID: 1UAS) and Human class I α-1,2-mannosidase [29] (crystallized with kifunensine, H-α-Man, PBD ID: 1FO3), since no crystal structure is available for the enzymes isolated from the soybean and/or jack bean meal. Binding of **1c** with both these enzymes can be seen from Fig. 13 as obtained from PARDOCK and the corresponding results were reported in this section (SI 08, Electronic supplementary material). The catalytic residues of R-α-Gal are Asp130 & 185, and that of H-α-Man are Glu330 and Asp463. The residues interacting with the glyco-conjugates have been analyzed with Accelrys "DS Visualizer" software.

Amino acid interactions with the glyco-conjugates (Fig. 14) and the corresponding binding affinities (Fig. 15) were given for both the R-α-Gal as well as for H-α-Man. Overall about 29 amino acids, *viz.*, Asp51, 52, 130, 185, 188, 214 and 216, Glu219, Cys53, 101, 132, 162, Lys128, Tyr20, 93, 129, Thr100, Ser102, 160, Asn14, 17, Arg181, Trp16, 164, Leu161, 245, Ile186, Ala187, Met217, in case of R-α-Gal, and about 27 amino acids, *viz.*, Glu330, 467, 599, 602, 657, 663, 689, Asp463, Lys471, Tyr667, Thr532, 688, Ser464, Arg334, 461, 597, Trp284, Leu525, 595, Ile333, Ala396, 460, 690, Pro598, Phe329, 528, 659 in case of H-α-Man were found to interact with the glyco-conjugates. Careful examination (Fig. 14a) of this reveals

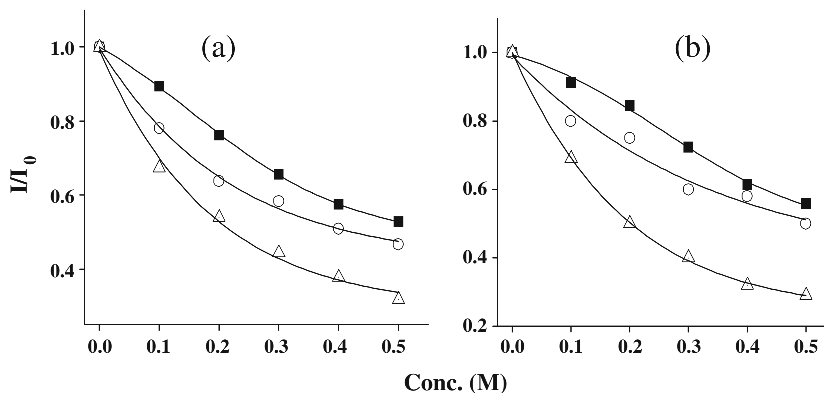


Fig. 9 Relative fluorescence intensity plots for the KI titration: **a** galactosyl-imino-conjugates; **b** lactosyl-imino-conjugates {■=only enzyme, ○=enzyme+ (**1b**/**2b**) and Δ=enzyme+ (**1c**/**2c**)}

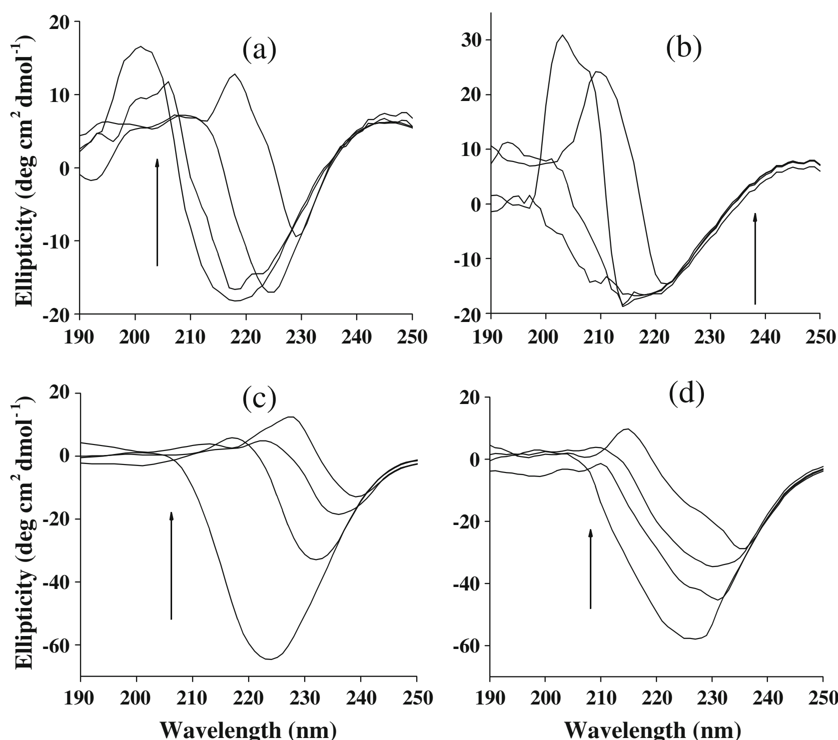


Fig. 11 CD spectral traces obtained during the titration of the enzyme from jack bean with varying concentrations of the glyco-imino-conjugates (inhibitors): **1c** (**a**); **1b** (**b**); **1c** (**c**) and **1b** (**d**). (**a**) and (**b**) with the Pure M, and (**c**) and (**d**) are with the partially purified enzyme

that while >75% polar amino acids interact in case of R-α-Gal, it is only about 60% in case of H-α-Man, though in both the cases there are eight carboxylic based (*viz.*, Asp or Glu) amino acids involved in the interactions. If the catalytic center in H-α-Man is less polar than that in R-α-Gal, it is reasonable to expect that IC_{50} value to be lower with the former. Indeed we noticed that the IC_{50} values (μM) of glyco-conjugates are considerably lower with the pure α-mannosidase (**1c**, 22±2.5; **2c**, 23±2.5; **3c**, 31±5.5; **4c**, 29±3.5) when compared to the partially purified one (**1c**, 120±50; **2c**, 85±15; **3c**, 130±10; **4c**, 85±15).

The amino acid residues interacting with the glyco-conjugates have been analyzed in both the cases and the corresponding data has been reported in Fig. 14b. Significant number of interactions has been exhibited between the glycoconjugate and the enzyme mainly through ten different amino acids in case of R-α-Gal and about eight different amino acids in case of H-α-Man. In case of R-α-Gal, 90% of the interactions were extended by three sets of amino acids where each of the set exhibits about 30%. These sets are, Asp, {Cys + Trp} and {Tyr + Arg + Met + Lys}. On the other hand, in case of H-α-Man, about 95% of the interactions were extended by Glu (28%), {Asp + Phe + Arg + Ser} (55%) and {Ala + Ile} (12%).

Binding affinity analysis obtained from the docking studies shows that simple glycoses and their amine derivatives exhibit a less favourable binding when compared to their corresponding naphthylidene or salicylidene-imino-conjugates. This is also being reflected in the computed binding affinity by showing a value of about 0.5 kcal/mol higher stabilization in case of H-α-Man when compared to R-α-Gal for a given C_1-glyco-naphthylidene-conjugate (Fig. 15). In effect, it is the α-mannosidase that is being inhibited more by these glyco-conjugates than the α-

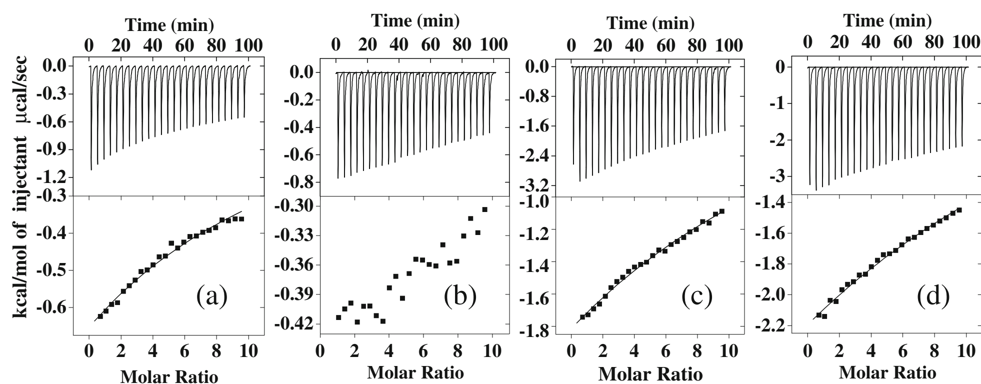


Fig. 12 ITC profile and best fit of the interaction of glyco-derivatives with jack bean enzyme in 50 mM NaOAc buffer at pH 5.0. *Top*: raw data obtained from 25 injections of 10 μl each of 5 mM solution of the glyco-imine derivative and 0.0149 mM of the enzyme. *Bottom*: the integrated curve showing the experimental points (■) and the best fit (–). **a** for **1c**; **b** for **1b**; **c** for **2c** and **d** for **2b**

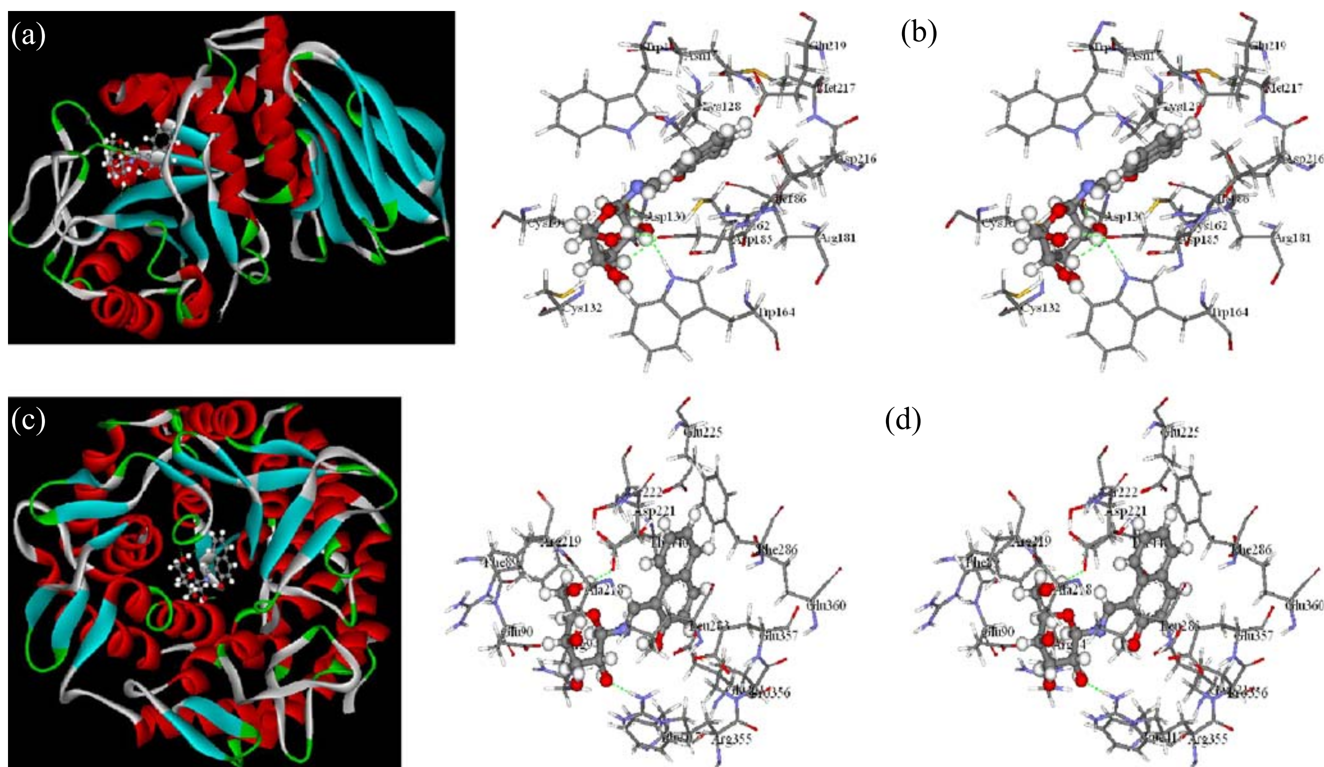


Fig. 13 Docking of **1c** as obtained from PARDOCK: **a** with rice α-galactosidase; **b** stereoview of the interacting region in (**a**); **c** with human α-mannosidase; **d** stereoview of the interacting region in (**c**)

galactosidase, a result that was already supported by the inhibition studies as reported in this paper.

On an average, about 14 amino acids interact with a glyco-conjugate in case of R-α-Gal, while all the imino-conjugates always exceed this number in their interaction. Even in case of H-α-Man the number of amino acids interacting with the imino-glyco-conjugates are always higher than the average one. Among these, are the naphthylidene-imino-conjugates, which interact with more number of amino acids than their counter salicylidene-imino-conjugates. Same thing is true even with the binding affinities, *viz.*, imino-conjugates exhibit higher binding affinity and among these the naphthylidene ones show higher by about 15% than their salicylidene ones. All these observations support the experimentally obtained inhibition data (IC_{50}) as well as the K_i values, where in these are lower with the naphthylidene conjugates as compared to the salicylidene ones.

The interactions noticed between the carbohydrate moiety and the enzyme were analyzed from the point of view of the glyco-moiety and the corresponding data is shown in the form of histogram in Fig. 16a. Galactosyl-conjugates exhibit higher number of interactions with the galactosidase and the lactosyl-conjugates (possessing glu-

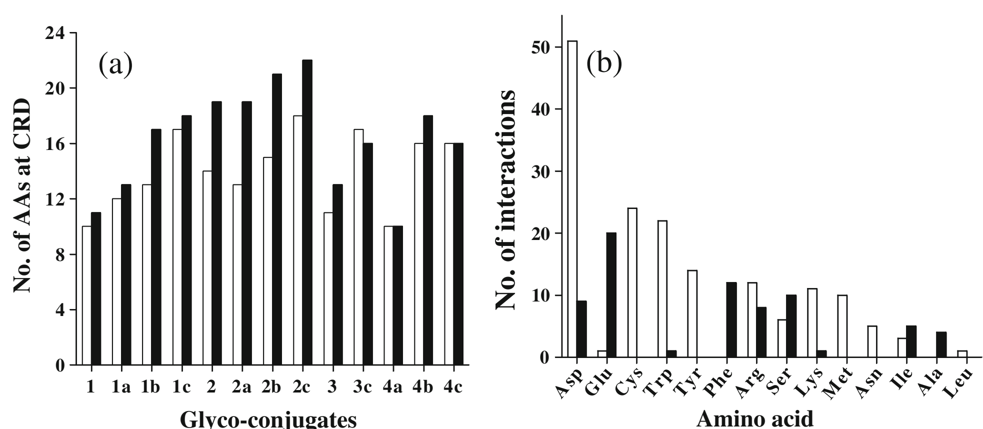


Fig. 14 Interaction of the amino acid residues present at the active center with glyco-conjugates: **a** Number of amino acids interacting with the glyco-conjugate; **b** interactions extended by each type of amino acid (*unfilled columns* are from R-α-Gal and *filled* are from H-α-Man)

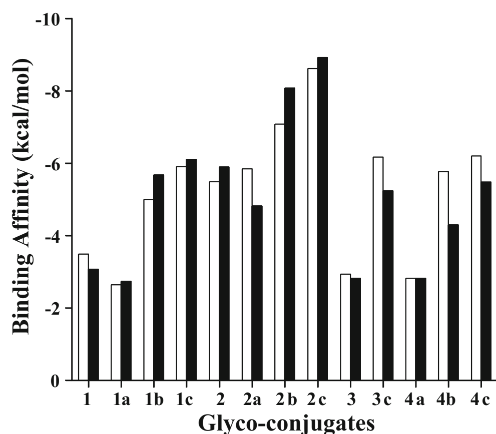


Fig. 15 Binding affinity (BA, kcal/mol) of glyco-conjugates (*unfilled columns* are from R-α-Gal and *filled* are from H-α-Man)

cosyl moiety) exhibit higher number of interactions with the mannosidase enzyme suggesting that the carbohydrate portion of the glycoconjugate is appropriately recognized by the corresponding enzyme. On an average, the stabilization energy of van der Waals together with the hydrophobic interactions is increased by 12 to 15% on going from simple glycoses to glyco-imino-conjugates (Fig. 16b) indicating that the additional aromatic moiety is being felt in the interactions.

Thus the computational docking studies clearly suggested the involvement of the carbohydrate moiety, imine moiety and aromatic portions in the interaction with the enzyme. Thus the docking results support the experimental data obtained on inhibition as well as binding studies.

Conclusions and correlations

All the glyco-naphthylidene-conjugates were found to be very effective in bringing the activity of enzymes to 50% at quite low concentrations. Of these, the most effective were the C_1-conjugates. While **1c** potentially inhibit α-mannosidase from soybean, the **3c** inhibits the activity of α-galactosidase and β-hexosaminidase from jack bean meal. The potent inhibition activity noticed in case of these glyco-imino-conjugates can be attributed to their structural mimicking of the transition state analogue of the enzyme as shown in Fig. 17.

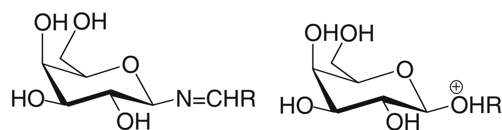


Fig. 17 Comparison of the structure of C_1-imino-conjugates reported here with the transition state analogue

The IC_{50} values (in μM) observed for glyco-imino-naphthylidene-conjugates with the pure fraction of α-mannosidase (**1c**, 22±2.5; **2c**, 23±2.5; **3c**, 31±5.5; **4c**, 29±3.5) are at least 15 times lower than that reported in literature for the inhibition of α-mannosidase by imino-cyclitol glycomimics (350 μM) [10]. These conjugates are also effective at IC_{50} values lower than the literature one, *viz.*, **1c**, 120±50; **2c**, 85±15; **3c**, 130±10; **4c**, 85±15, even with the partially purified enzyme fraction. While it was only one among the twenty nine C_3-amino sugars studied in the literature [16] was effective in inhibiting the activity of β-galactosidase from bovine liver, almost all the C_1-imino-conjugates reported in the present study have shown effective glycosidase inhibition. Even the salicylidene-conjugates exhibited IC_{50} values (**1b**, 51±1; **2b**, 50±3.5; **4b**, 99±6.5) lower than the literature ones. The comparison of the inhibition data of the control molecules with that of the C_1-/C_2-N=C(H)(Ar) conjugates clearly suggests that all the three components, *viz.*, carbohydrate, imine and aromatic moiety, are required together to bring 100% inhibition. The binding of these conjugates to the enzyme was found to be non-competitive

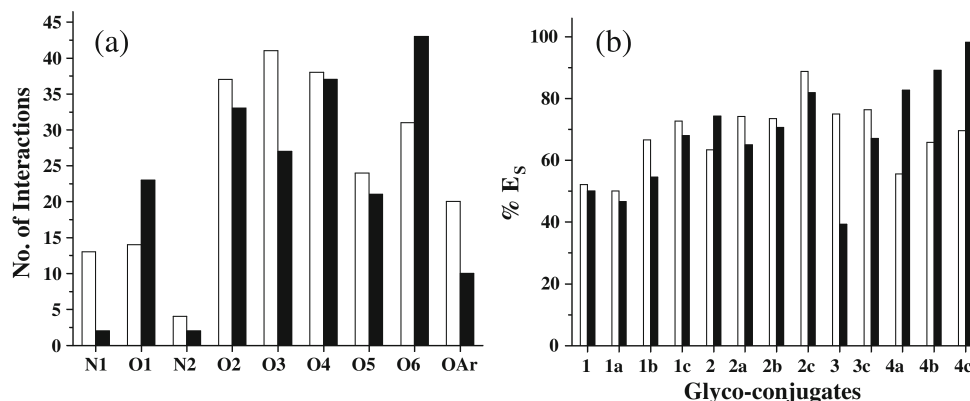


Fig. 16 Histogram indicating the number of interactions exhibited by an atom or group of the glyco-conjugate with the enzyme (*filled columns* are from H-α-man and *unfilled columns* are from R-α-Gal) as obtained from docking studies. *O1* to *O6* refers to the hydroxyl groups attached to C1 and C6 of the glycose-portion. *N1* and *N2* correspond to the nitrogen centers present on the C1 and C2 respectively. '*OAr*' refers to the –OH moiety attached to the aromatic ring

inhibition. The kinetics data reveal that there is a factor of 10 increase in K_i on going from naphthylidene to salicylidene and a factor of two on going from galactosyl/lactosyl to ribosyl derivatives, suggesting the influence of both the aromatic as well as the carbohydrate moieties.

The fluorescence titration studies carried out using C_1-imino-conjugates not only revealed their binding to the enzymes but also the difference that exists between the salicylidene and naphthylidene moieties. This seems to indicate the preference of the glycoconjugates to bind through hydrophobic interactions and hence the naphthylidene conjugates bring larger conformational changes. While the salicylidene-conjugates yield a weak or no binding isotherm, those of the naphthylidene does yield binding isotherm with moderate K_{ass} values, indicating the involvement of the hydrophobic binding site for the derivative. Based on the studies reported in this paper, it seems possible to visualize that the inhibitor molecule uses its hydrophobic (predominantly) or hydrophilic (carbohydrate portion to some extent) or both the moieties for binding. The conformational changes that occur upon binding were demonstrated based on KI fluorescence quenching experiments as well as circular dichroism studies.

Even the computational docking studies revealed that the glyco-imino-conjugates exhibit more favourable binding than simple glycoses and/or their C_1-NH_2 derivatives. Among these, the naphthylidene conjugates certainly show higher number of interactions as well as higher binding affinity as compared to their salicylidene-counter parts. While the galactosyl-conjugates exhibit higher number of interactions with the galactosidase, the lactosyl possessing glucosyl moiety exhibit higher number of interactions with the mannosidase enzyme suggesting that the carbohydrate portion of the glyco-conjugate is appropriately recognized by the corresponding enzyme. A 12 to 15% increase in the stabilization energy was noticed with the van der Waals plus hydrophobic energy in presence of the aromatic-imino conjugates. All the docking results, including 0.5 kcal/mol additional stabilization energy, observed with the H-α-Man supports the experimentally obtained inhibition data (IC_{50}) as well as the K_i values.

The crystal structures of the inhibitor bound enzymes including rice α-galactosidase and human α-mannosidase clearly indicates the presence of Trp containing hydrophobic clefts in close proximity to the carbohydrate bound region in the catalytic domain [28, 30–36]. These observations noted from the substrate bound glycosidase structures seem to support that carefully modified carbohydrate derivative upon incorporation of some hydrophobic moiety either at C_1- or C_2- could certainly enhance the binding as well as the inhibition of the glycosidase activity. The present work indeed follows this strategy in developing the glycosidase inhibitors. Thus from our studies it is evident that the C_1- and or C_2-imino-conjugates of simple carbohydrates are potential inhibitors of glycosidases through non-competitive inhibition. Our on-going thrust is to crystallize these complexes and study their 3-d structures.

Acknowledgements CPR acknowledges the financial support from DST, CSIR, DAE-BRNS. AK and AM are grateful to CSIR, New Delhi for research fellowships. We thank Prof. B.J. Rao for providing us access to the ITC.

References

1. Hirsch, C., Bolm, D.I., Polegh, H.L.: A role for N-glycanase in the cytosolic turnover of glycoproteins. EMBO J. **22**, 1036–1046 (2003). doi:10.1093/emboj/cdg107
2. Movsichoff, F., Castro, O.A., Parodi, A.: Characterization of schizosaccharo-myces pombe ER α-mannosidase: A reevaluation of the role of the enzyme on ER-associated degradation. J. Mol. Biol. Cell **16**, 4714–4724 (2005). doi:10.1091/mbc.E05-03-0246
3. Tokunaga, F., Brostrom, C., Koide, T., Arvan, P.: Endoplasmic reticulum (ER)-associated degradation of misfolded n-linked glycoproteins is suppressed upon inhibition of ER mannosidase I. J. Biol. Chem. **275**, 40757–40764 (2000). doi:10.1074/jbc.M001073200
4. Niwa, T., Tsuruoka, T., Goi, H., Kodama, Y., Itoh, J., Inouye, S., Yamada, Y., Niida, T., Nobe, M., Ogawa, Y.: Novel glycosidase inhibitors, nojirimycin b and d-mannonic-δ-lactam isolation, structure determination and biological property. J. Antibiot. **37**, 1579–1586 (1984)
5. Leroy, E., Reymond, J.L.: Anomer-selective inhibition of glycosidases using aminocyclopentanols. Org. Lett. **1**, 775–777 (1999). doi:10.1021/ol990754 m
6. Boss, O., Leroy, E., Blaser, A., Reymond, J.L.: Synthesis and evaluation of aminocyclopentitol inhibitors of β-glucosidases. Org. Lett. **2**, 151–154 (2000). doi:10.1021/ol991252b
7. Kleban, M., Hilgers, P., Greul, J.N., Kugler, R.D., Li, J., Picasso, S., Vogel, P., Jager, V.: Syntheses via isoxazolines, part 25. Amino (hydroxymethyl)cyclo-pentanetriols, an emerging class of potent glycosidase inhibitors—Part I: synthesis and evaluation of β-d-pyranoside analogues in the *manno*, *gluco*, *galacto*, and GlcNAc series. ChemBioChem **2**, 365–368 (2001). doi:10.1002/1439-7633(20010504)2:5<365::AID-CBIC365>3.0.CO;2-M
8. Greul, J.N., Kleban, M., Schneider, B., Picasso, S., Jager, V.: Amino-(hydroxymethyl)cyclopentanetriols, an emerging class of potent glycosidase inhibitors—Part II: synthesis, evaluation, and optimization of β-d-galactopyranoside analogues. ChemBioChem **2**, 368–370 (2001). doi:10.1002/1439-7633(20010504)2:5<368::AID-CBIC368>3.0.CO;2-A
9. Saotome, C., Wong, C.H., Kanie, O.: Combinatorial library of five-membered iminocyclitol and the inhibitory activities against glyco-enzymes. Chem. Biol. **8**, 1061–1070 (2001). doi:10.1016/S1074-5521(01)00074-6
10. Lemaire, S.G., Popowycz, F., Garcia, E.R., Asenjo, A.T.C., Robina, I., Vogel, P.: An efficient combinatorial method for the discovery of glycosidase inhibitors. ChemBioChem **3**, 466–470 (2002). doi:10.1002/1439-7633(20020503)3:5<466::AID-CBIC466>3.0.CO;2-D
11. Wu, C.Y., Chang, C.F., Chen, J.S.Y., Wong, C.H., Lin, C.H.: Rapid diversity-oriented synthesis in microtiter plates for in situ screening: discovery of potent and selective α-fucosidase inhibitors. Angew. Chem. Int. Ed. **42**, 4661–4664 (2003). doi:10.1002/anie.200351823

12. Liang, P.H., Cheng, W.C., Lee, Y.L., Yu, H.P., Wu, Y.T., Lin, Y.L., Wong, C.H.: Novel five-membered iminocyclitol derivatives as selective and potent glycosidase inhibitors: new structures for antivirals and osteoarthritis. ChemBioChem **7**, 165–173 (2006). doi:10.1002/cbic.200500321
13. Bordier, A., Compain, P., Martin, O.R., Ikeda, K., Asano, N.: First stereocontrolled synthesis and biological evaluation of 1,6-dideoxy-L-nojirimycin. Tetrahedron **14**, 47–51 (2003). doi:10.1016/S0957-4166(02)00752-8
14. Markad, S.D., Karanjule, N.S., Sharma, T., Sabharwal, S.G., Dhavale, D.D.: Synthesis and evaluation of glycosidase inhibitory activity of N-butyl 1-deoxy-d-gluco-homonojirimycin and N-butyl 1-deoxy-l-ido-homonojirimycin. Bioorg. Med. Chem. **14**, 5535–5539 (2006). doi:10.1016/j.bmc.2006.04.027
15. Tatsuta, K.: Total synthesis and chemical design of useful glycosidase inhibitors. Pure Appl. Chem. **68**, 1341–1346 (1984). doi:10.1351/pac199668061341
16. Maxwell, V.L., Evinson, E.L., Emmerson, D.P.G., Jenkins, P.R.: Synthesis, glycosidase activity and X-ray crystallography of 3-amino-sugars. Org. Biomol. Chem. **4**, 2724–2732 (2006). doi:10.1039/b605916c
17. Rossi, L.L., Basu, A.: Glycosidase inhibition by 1-glycosyl-4-phenyl triazoles. Bioorg. Med. Chem. Lett. **15**, 3596–3599 (2005). doi:10.1016/j.bmcl.2005.05.081
18. Singhal, N.K., Ramanujam, B., Mariappandar, V., Rao, C.P.: Carbohydrate-based switch-on molecular sensor for Cu(II) in buffer: absorption and fluorescence study of the selective recognition of Cu(II) ions by galactosyl derivatives in HEPES buffer. Org. Lett. **8**, 3525–3528 (2006). doi:10.1021/ol061274f
19. Ahuja, R., Singhal, N.K., Ramanujam, B., Ravikumar, M., Rao, C.P.: Experimental and computational studies of the recognition of amino acids by galactosyl-imine and -amine derivatives: an attempt to understand the lectin–carbohydrate interactions. J. Org. Chem. **72**, 3430–3442 (2007). doi:10.1021/jo0700979
20. Kumar, N.S., Rao, D.R.: The nature of lectins from *Dolichos lablab*. J. Biosci. **10**, 95–109 (1986). doi:10.1007/BF02702844
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1951)
22. Papet, M.D., Delay, D., Monsigny, M., Delmotte, F.: Characterization of two galactosidases extracted from wheat germ with a hydroalcoholic solvent. Biochimie **74**, 53–56 (1992). doi:10.1016/0300-9084(92)90183-F
23. Snaith, S.M., Levvy, G.A.: Purification and properties of α-d-mannosidase from jack-bean meal. Biochem. J. **110**, 663–670 (1968)
24. Bismuto, E., Nucci, R., Rossi, M., Irace, G.: Structural and dynamic aspects of β-glycosidase from mesophilic and thermophilic bacteria by multitryptophanyl emission decay studies. Proteins **35**, 163–172 (1999). doi:10.1002/(SICI)1097-0134(19990501)35:2<163::AID-PROT3>3.0.CO;2-8
25. McAllister, K.A., Marrone, L., Clarke, A.J.: The role of tryptophan residues in substrate binding to catalytic domains A and B of xylanase C from *Fibrobacter succinogenes* S85. Biochim. Biophys. Acta. **1480**, 342–352 (2000)
26. Golubev, A.M., Nagem, R.A.P., Neto, J.R.B., Neustroev, K.N., Eneyskaya, E.V., Kulminskaya, A.A., Shabalin, K.A., Savel'ev, A.N., Polikarpov, I.: Crystal structure of α-galactosidase from *Trichoderma reesei* and its complex with galactose: implications for catalytic mechanism. J. Mol. Biol. **339**, 413–422 (2004). doi:10.1016/j.jmb.2004.03.062
27. Gupta, A., Gandhimathi, A., Sharma, P., Jayaram, B.: ParDOCK: an all atom energy based Monte Carlo docking protocol for protein–ligand complexes. Protein Pept. Lett. **14**, 632–646 (2007). doi:10.2174/092986607781483831
28. Fujimoto, Z., Kaneko, S., Momma, M., Kobayashi, H., Mizuno, H.: Crystal structure of rice α-galactosidase complexed with D-galactose. J. Biol. Chem. **278**, 20313–20318 (2003). doi:10.1074/jbc.M302292200
29. Vallee, F., Karaveg, K., Herscovics, A., Moremen, K.W., Howell, P.L.: Structural basis for catalysis and inhibition of N-glycan processing class I-α-1,2-mannosidases. J. Biol. Chem. **275**, 41287–41298 (2000). doi:10.1074/jbc.M006927200
30. Wakarchuk, W.W., Campbell, R.L., Sung, W.L., Davoodi, J., Yaguchi, M.: Mutational and crystallographic analyses of the active site residues of the *Bacillus circulans* xylanase. Protein Sci. **3**, 467–475 (1994)
31. Garman, S.C., Hannick, L., Zhu, A., Garboczi, D.N.: The 1.9 Å Structure of α-N-acetylgalactosaminidase: molecular basis of glycosidase deficiency diseases. Structure **10**, 425–434 (2002). doi:10.1016/S0969-2126(02)00726-8
32. Czjzek, M., Cicek, M., Zamboni, V.R., Burmeister, W.P., Bevan, D.R., Henrissat, B., Esen, A.: Crystal structure of a monocotyledon (maize ZMGlu1) β-glucosidase and a model of its complex with p-nitrophenyl β-d-thioglucoside. Biochem. J. **354**, 37–46 (2001). doi:10.1042/0264-6021:3540037
33. Czjzek, M., Alberto, F., Jordi, E., Bernard, H.: Crystal structure of inactivated *Thermotoga maritima* invertase in complex with the trisaccharide substrate raffinose. Biochem. J. **395**, 457–462 (2006). doi:10.1042/BJ20051936
34. Wang, Y., Xiong, J.P., Xia, Z.X.: Crystal structure of trichosanthin–NADPH complex at 1.7 Å resolution reveals active-site architecture. Nat. Struct. Biol. **1**, 695–700 (1994). doi:10.1038/nsb0394-145
35. Prive, G.G., Ahn, V.E., Faull, K.F., Whitelegge, J.P., Fluharty, A. L.: Crystal structure of saposin B reveals a dimeric shell for lipid binding. Proc. Natl. Acad. Sci. USA **100**, 38–43 (2003). doi:10.1073/pnas.0136947100
36. Premkumar, L., Sawkar, A.R., Adamsky, S.B., Toker, L., Silman, I., Kelly, J.W., Futerman, A.H., Sussman, J.L.: X-ray structure of human acid-β-glucosidase covalently bound to conduritol-B-epoxide. J. Biol. Chem. **280**, 23815–23819 (2005). doi:10.1074/jbc.M502799200