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Research Paper

Combinatorial library of five-membered iminocyclitol and the inhibitory activities against glyco-enzymes

Chikako Saotome [a], Chi-Huey Wong [b], Osamu Kanie [a,*]

[a] *Mitsubishi Kagaku Institute of Life Sciences (MITILS), Minami ooya 11, Machida-shi, Tokyo 194-8511, Japan*
[b] *Department of Chemistry, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, USA*

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Abstract

Background: Oligosaccharide processing enzymes are important classes of catalysts involved in synthesizing specific oligosaccharide structures on proteins and sphingolipids. Development of specific inhibitors of such enzymes is of current interest as these inhibitors may be used to control cellular functions. Five-membered iminocyclitols have been shown to be potent inhibitors of such enzymes. Since a rational design and synthesis of inhibitors is often extremely difficult due to the limited information regarding the structure of the active site, we carried out a combinatorial library approach.

Results: To create diversity, we decided to use an aldehyde group of a protected iminocyclitol for reductive amination and the Strecker reaction. After transformation of the nitrile group introduced by the Strecker reaction into an amine and amide and complete deprotection, a small library of five-membered iminocyclitols consisting of 27 compounds was synthesized. A series of compounds obtained by reductive amination was first screened as potential inhibitors of glycosidases and glycosyltransferases. Among them, compounds carrying a C_{10}-alkyl group showed marked enhancement of inhibitory activity against α-mannosidase at 10 μM concentration when compared with its parent compound and deoxymannojirimycin. Furthermore, compounds having the phenylethyl group showed an extremely strong inhibitory effect against α-galactosaminidase at a K_i value of 29.4 nM. Compounds with an aminomethyl and amide group at the *C*-1′ position of these two molecules showed a decrease in inhibitory activities.

Conclusions: A combinatorial approach based on five-membered iminocyclitols with a *galacto*-configuration was exploited. The potential usefulness of the library as a source of inhibitors of glycoenzymes is clearly shown in this study.

Keywords: Combinatorial library; Glycosidase; Glycosyltransferase; Iminocyclitol; Inhibitor

1. Introduction

Enzymes to synthesize and process oligosaccharides such as glycosyltransferases and glycosidases are important classes of catalysts involved in the assembly of specific oligosaccharide structures on proteins [1,2] and sphingolipids [3]. Development of specific inhibitors of such enzymes is of current interest as these inhibitors may be used to control cellular functions. Enzymatic hydrolysis of a glycosidic bond generally takes place via general acid and base catalysis that requires two critical residues, a proton donor and a nucleophile [4–6]. A distorted half-chair-like transition state leading to a carboxonium ion is considered to be involved in the reaction (Fig. 1). Five-membered iminocyclitols carrying hydroxyl groups with specific orientation to mimic the shape and charge of the transition state of the reacting sugar moiety have been shown to be potent inhibitors of such enzymes [7–12]. Since a cation-like transition state is expected to be involved in both the glycosyltransferase- and glycosidase-catalyzed reactions, five- and six-membered iminocyclitols can be used as core components for the development of transition-state analog inhibitors of both families of enzymes [7–19].

During the course of our investigation toward this goal, we synthesized a series of five-membered iminocyclitols and have shown that some of them possess strong and/or interesting inhibitory activities against glycosyltransferases and glycosidases [11,12]. Since a rational design and

* Corresponding author.
E-mail address: kokanee@libra.ls.m-kagaku.co.jp (O. Kanie).

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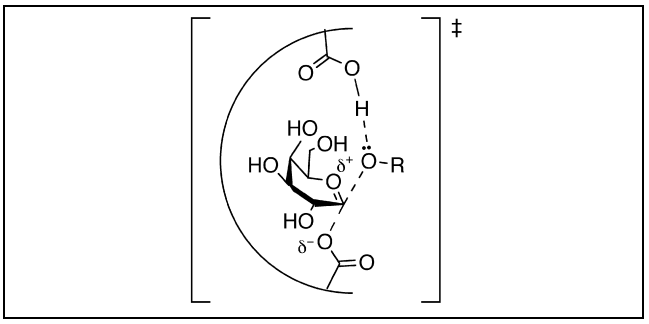


Fig. 1. Diagram of the transition state of the enzymatic hydrolysis reaction of the glycoside. The hydrolysis reaction involves a distorted half-chair conformation in the transition state.

synthesis to afford desired inhibition is often extremely difficult due to the limited information regarding the structure of the active site, we considered another potential approach based on a combinatorial library derived from a five-membered iminocyclitol. Despite the potential usefulness of the library strategy, there is a chance that none of a series of compounds is inhibitory. Therefore, we decided to focus first on creating a small, but diverse, group of compounds to test as potential inhibitors of glycoenzymes. Here, we report the synthesis of a five-membered iminocyclitol library of *galacto*-configuration and subsequent evaluation of their inhibition against various glycosylhydrolases and glycosyltransfer enzymes such as α-glucosidase (Glc-ase; EC 3.2.1.20), α-mannosidase (Man-ase, EC 3.2.1.24), α-galactosidase (Gal-ase, EC 3.2.1.22) and β-Gal-ase (EC 3.2.1.23), α-GalNAc-ase (EC 3.2.1.49), β-1,4-galactosyltransferase (β-1,4-GalT-ase; EC 2.4.1.22) and α-1,3-galactosyltransferase (α-1,3-GalT-ase; EC 2.4.1.90).

2. Results and discussion

2.1. Synthesis of a combinatorial library

In this study, we selected a five-membered iminocyclitol with a 2(*R*), 3(*R*), 4(*S*), 5(*R*)-configuration which is referred to as either the '*galacto*'-form (**1a**) or the '*manno*'-form (**1b**) depending on the conformation of the pyrrolidine ring (Fig. 2). The five-membered ring system is known to be conformationally more flexible than the six-membered system and exists as an equilibrium of various conformers at physiological conditions, thus both conformers (**1a** and **1b**) may have to be considered as structural candidates for the inhibition study on glycoenzymes. This five-membered system can be considered advantageous as a lead structure for the combinatorial library, where further specificity and affinity can be developed by incorporation of additional functionalities in structure **2**. We considered that these conformers (**2a** and **2b**) may be treated as 'conformational diversity factors' in the library because different conformers are thought to interact with the corresponding enzyme families regarding their binding specificities (Fig. 3).

Few approaches have been reported regarding carbohydrate-related libraries targeting glycoenzymes so far [20–25]. Approaches based on transition state inhibitors [21–24] are our particular interest. Several possibilities exist regarding how diversity is generated, i.e. linear type (involving iterative reactions of doubly functionalized synthetic units), scaffold type (involving reactions of a template with multiple functional groups) and cascade type (a reaction product gives an additional function for the next reaction) [26]. Considering that a series of molecules of our interest have a low molecular weight and should fit in the primary catalytic site of the target enzyme, which covers approximately 100 Å^2 of the protein surface according to the available crystallographic data of related enzymes [27–31], we thought that the added functionalities in the created molecule should not be scattered but rather condensed close to the core unit, an iminocyclitol in this study. Based on these considerations, we took the third strategy for our library synthesis.

To create diversity based on compound **1**, we decided to use the aldehyde group at the *C*-1′ position. The decision was based on our previous observation that *N*-alkylation of the ring nitrogen often resulted in decreased inhibitory activity at the molecular level [11,12], although increased hydrophobicity is advantageous for penetrating the cell membrane [32–34]. Aldehyde **3** was thus synthesized as reported in our previous paper and used to create a library [12]. First, we designed and utilized imines formed by reaction of **3** and amines for various reaction conditions including reductive amination, Strecker condensation, Ugi-type reaction and Mannich reaction as well as subsequent introduction of polyethylene glycol by acylation of

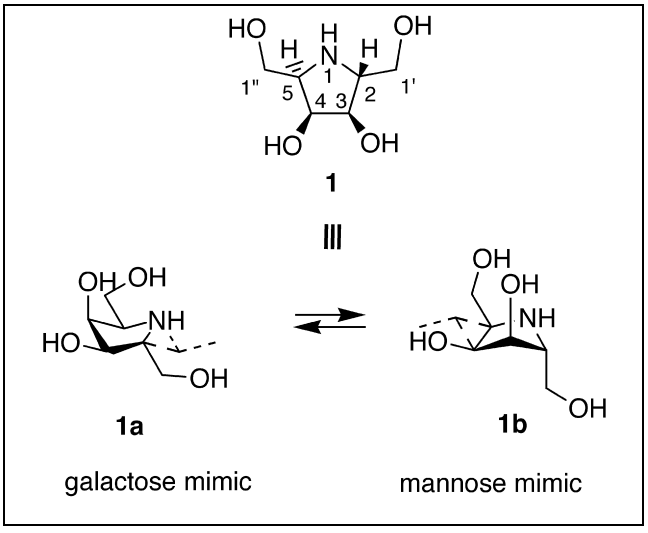
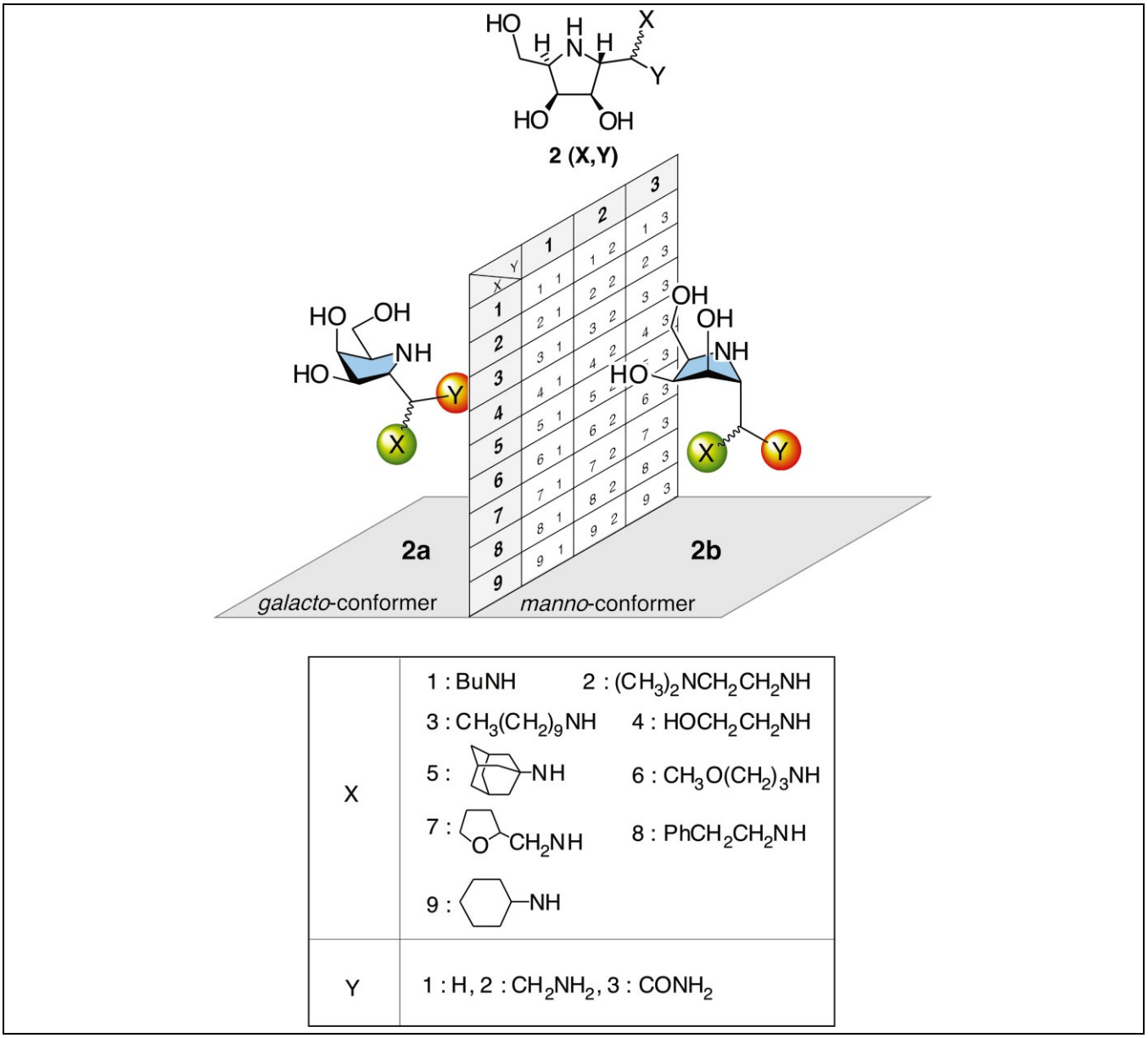


Fig. 2. Five-membered iminosugar **1** and two possible conformers. Compound **1** can be considered either a galactose or a mannose mimic. Equilibrium exists among conformers. Thus, five-membered iminocyclitols are considered advantageous because 'one compound' can be a candidate to inhibit different enzymes with different stereospecificities.



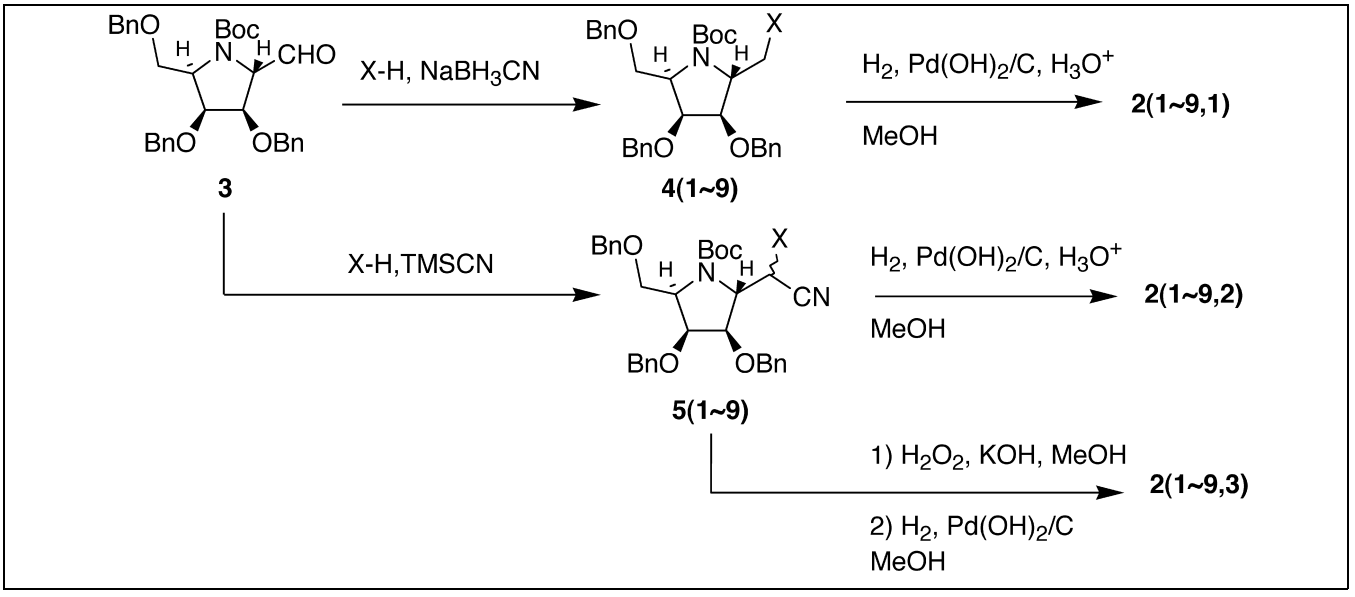
X	1 : BuNH 2 : $(CH_3)_2NCH_2CH_2NH$ 3 : $CH_3(CH_2)_9NH$ 4 : $HOCH_2CH_2NH$ 5 : 6 : $CH_3O(CH_2)_3NH$ 7 : 8 : $PhCH_2CH_2NH$ 9 :
Y	1 : H, 2 : CH_2NH_2, 3 : $CONH_2$

Fig. 3. Diagram of a combinatorial library. At the *C*-1′ position a diversity was introduced (X,Y). The series of compounds **2**(**X**,**Y**) share a five-membered iminosugar which may exist in equilibrium with the *galacto*-conformer **2a** and the *manno*-conformer **2b**.

the products to enhance the diversity and to ease the 'fish-out' process of the condensation product from a reaction mixture. However, as we examined these reactions, we found that some of the reactions did not give satisfactory results. It was suggested that this was due to the severe steric problems of *t*-butylcarbonyl (Boc), benzyl (Bn) and X of amines around the reaction site. Reactions which were found to be applicable in the synthesis were reductive amination and the Strecker reaction without subsequent acylation. Thus, we could not gain a large diversity in the iminocyclitol-based library but alternatively considered including transformation reactions of a nitrile group into amine, amide and carboxylic acid (Ys). The selected X and Y groups are shown in Fig. 3.

At first, reductive amination reactions of **3** using a variety of amines (X-H) were carried out. Reduction of the imine proceeded smoothly under standard conditions with sodium cyanoborohydride to give compounds **4**(**1**–**9**), which were deprotected in one step by hydrogenolysis under acidic conditions to afford compounds **2**(**1**–**9**,**1**) in good yields after purification using a Sep-Pak CM cartridge. The individual conversion rate of the reductive amination reaction was estimated by densitographic analysis of the TLC plate, which indicated that compound **3** was transformed into **4** in a range of 42–98% yield.

Next, the Strecker reaction was carried out. Trimethylsilylcyanide (TMSCN) was used in the presence of catalytic $ZnCl_2$ after imine formation with nine amines [35] (Scheme 1, Fig. 3). It was found that reactions did not proceed without Lewis acid. No significant stereoselectiv-



Scheme 1. Synthesis of a library consisting of **2(X,Y)**. Reductive amination and Strecker reaction of aldehyde **3** gave **4(X)** and **5(X)**. The nitrile group of compounds **5(X)** was further transformed to amine- and amide-functional groups for Y. Deprotection of these compounds afforded **2(X,Y)**.

ity was observed in any case, which suggested that Lewis acid directly activated the imine intermediate but not through other functional groups since auxiliary effects could not be observed. The condensation products were subjected to the following reactions without isolation. The conversion rate of the aldehyde **3** into **5** was in the range of 57–97% according to densitography. The mixtures were subjected to various reaction conditions such as hydrolysis and reduction to transform the nitrile group into a carboxylate, amide or amine. Reduction of **5(1–9)** to convert amine **2(1–9,2)** was achieved under acidic hydrogenolysis conditions, which removed the Boc and Bn groups at the same time. When we attempted to hydrolyze the nitrile group to obtain a carboxylic acid and amide (Y), both acidic and basic hydrolytic conditions gave complex mixtures. Thus, **5(1–9)** were converted oxidatively into amides, which were then hydrogenolyzed to **2(1–9,3)**. Further transformation of the intermediate to carboxylate failed under these conditions, even after raising the temperature.

Having a series of compounds in hand, we selected enzymes to be examined using compounds **2(1–9,1)** as potential inhibitors. α-Gal-ase, β-Gal-ase and α-GalNAc-ase were chosen as the counterparts of the *galacto*-conformer **2a(X,Y)** (Fig. 3). Two glycosyltransferases, β-1,4-GalT-ase and α-1,3-GalT-ase, were also included because compound **1** was an inhibitor of β-1,4-GalT-ase against UDP-Gal with a K_i value of 27 μM [12]. In the same manner, α-Man-ase was selected for analysis of the *manno*-conformer **2b(X,Y)**. α-Glc-ase was also added to verify other factors.

2.2. Screening of the library against glycoenzymes

In order to assay the inhibitory activities of the library compounds against α-1,3-galactosyltransferase [36–39], the required substrate and the product were first synthesized enzymatically. 4-Methylumbelliferyl (4-MU-) LacNAc (**6**) was first synthesized according to our previous report [40] and was used as the substrate. Formation of 4-MU-Gal-α(1→3)-Gal-β(1→4)-GlcNAc (**7**) during the reaction of **6** and UDP-Gal in the presence of the transferase was confirmed using capillary electrophoresis. Although we did not isolate **7**, we carried out MALDI-TOF mass spectral analysis of the reaction mixture after Sep-Pak C18 to confirm the presence of **6** and **7**.

To examine the potential of the strategy and each member of the library as an inhibitor of glycosidases and glycosyltransferases, we carried out preliminary inhibition screening assays at a fixed concentration of potential inhibitors. The suitable concentration in each enzyme reaction was determined by an initial inhibition test using a representative compound in a library and/or known inhibitor of the enzyme at various concentrations. The inhibitory activity was shown as a percentage at the concentration determined. Each enzyme reaction was performed in a well of a titer plate and the inhibitory activity against the corresponding substrates was estimated by absorbance at 405 nm for *p*-nitrophenol in the case of glycosidases. Common inhibitors were used for the generality of the result when they were available.

For the inhibition assay of transferases, we used the corresponding substrate 4-MU-glycosides such as 4-MU-GlcNAc, 4-MU-LacNAc (**6**) and 4-MU-Gal-α(1→3)-Gal-β(1→4)-GlcNAc (**7**). Inhibition reactions were carried out in microcentrifuge tubes for 5 min and the inhibitory activities were determined as the conversion rate relative to the acceptor substrate using capillary electrophoresis at 214 nm.

As shown in Fig. 4, a series of compounds in the library showed broad inhibitory spectra for the enzymes tested except for α-1,3-GalT-ase for which the graph was omitted as no inhibition was observed. Depending on the enzyme, some compounds showed a dramatic enhancement of inhibition compared to the parent compound **1**. Such examples can be found for α-Glc-ase (A), α-Man-ase (B), α-GalNAc-ase (D) and β-Gal-ase (E, F). For the inhibition of α-Glc-ase with an inhibitor concentration of 100 μM, some were found to have activity twice as potent as **1**

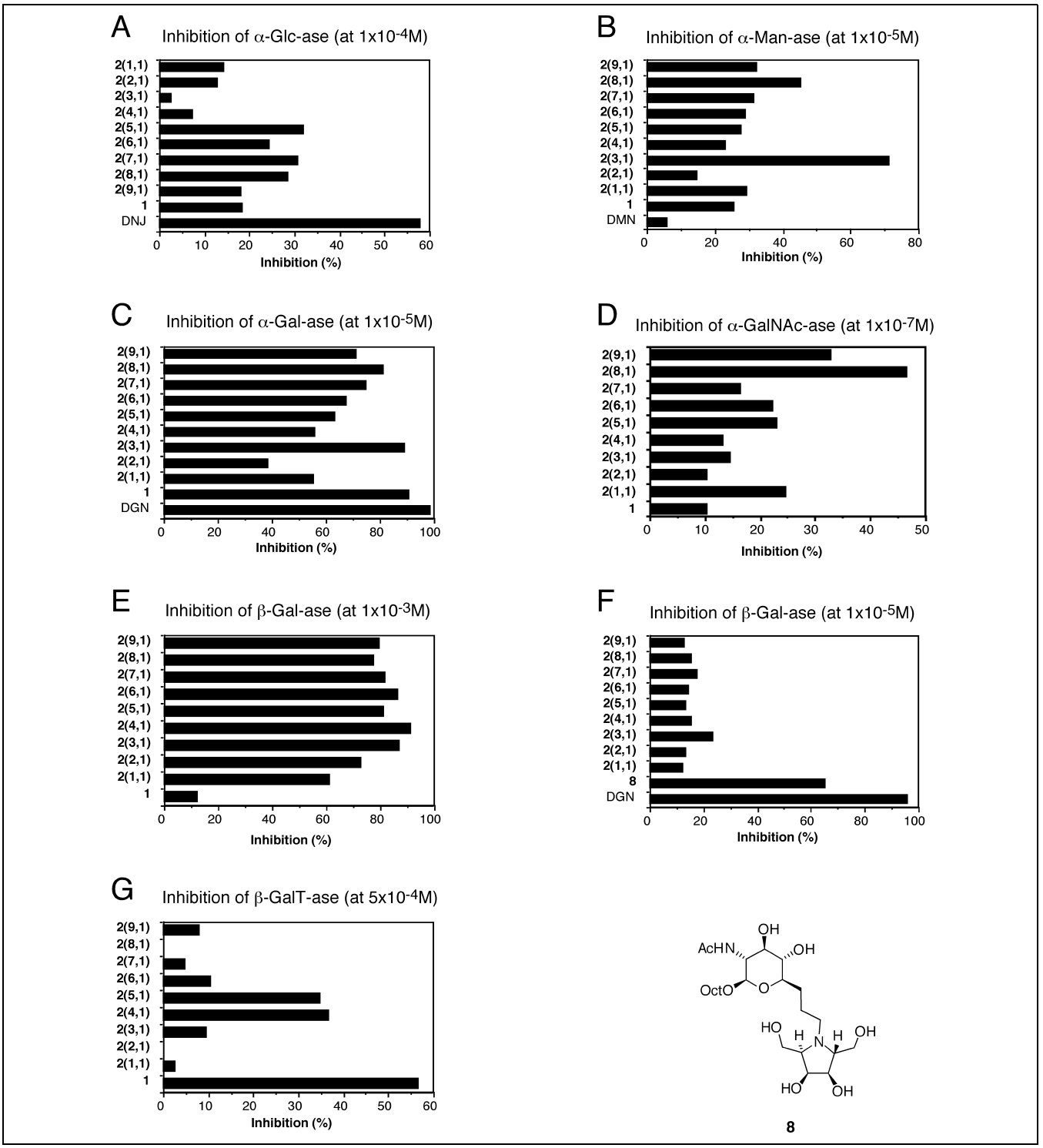


Fig. 4. Inhibitory activities of compounds **2(X,1)** against various glyco-enzymes. (A) Inhibition of α-Glc-ase at an inhibitor concentration of 1×10^{-4} M; (B) α-Man-ase at 1×10^{-5} M; (C) α-Gal-ase at 1×10^{-5} M; (D) α-GalNAc-ase at 1×10^{-7} M; (E, F) β-Gal-ase at 1×10^{-3} M and at 1×10^{-5} M, respectively; (G) β-1,4-GalT-ase at 5×10^{-4} M. The synthesis of compounds **1** and **8** has been reported in [12].

although none showed comparable inhibition to deoxynojirimycin (DNJ). It was found that addition of a hydrophobic group far from the iminosugar is preferred in the case of α-Man-ase, where compound **2**(**3**,**1**) with a C_{10}-alkyl group showed 276% and 1120% increases in inhibition at 10 μM concentration against compound **1** ($K_i = 27$ μM) [12] and deoxymannojirimycin (DMN, $K_i = 43$ μM) [12], respectively. For the inhibition of α-GalNAc-ase, only the siastatin B analog has been reported to be an inhibitor with an IC_{50} value in the μM range [41]. Strikingly, five of the eight compounds were found to be more than twice as potent compared to their parent compound (**1**) in the nM order. Among them, compound **2**(**8**,**1**), having a phenethyl group, showed a 467% increase in activity. Further kinetic analysis of **2**(**8**,**1**) revealed $K_i = 29.4$ nM whereas the K_m value of α-PNP-GalNAc was 0.65 mM. In the case of β-Gal-ase, all the compounds tested were found to be much better inhibitors of the enzyme compared to **1** ($K_i = 1.0$ mM) [12], but they only exhibited weak inhibition at the μM range as shown in Fig. 4F. Also, it was suggested that the substitution at the *C*-1′ position was not important for the binding since there was no significant difference in activity among the series of compounds. It is worth noting that some compounds were inhibitory against β-GalT-ase despite the fact that none inhibited α-GalT-ase even though these two enzymes share UDP-Gal as the donor substrate and our compounds are thought to mimic the donor transition state. This result may reflect the required geometry of cationic species in the enzyme catalytic site of these enzymes: one is an inverting and the other is a retaining enzyme regarding the configuration of the transferred galactosyl residue.

Compounds shown to have strong inhibitory effects such as compounds **2**(**3**,**1**) and **2**(**8**,**1**) were selected as leads for further evaluations to see the effects of the Y group (Fig. 5). Compounds **2**(**3**,**2**) and **2**(**3**,**3**) lost almost all inhibitory activity against α-Man-ase at the concentration used for the assay of compound **2**(**3**,**1**). Compounds **2**(**8**,**2**) and **2**(**8**,**3**) inhibited α-GalNAc-ase with 52.8% and 50.5% of the inhibitory effect of **2**(**8**,**1**), respectively. Although there is an important question to be answered – whether one of the diastereoisomers formed during the Strecker reaction had superior inhibitory activity over the other – we did not isolate the diastereomers based on the considerations below. The aromatic ring is important for the enhancement of affinity. Similar inhibition modes are expected for both isomers because the major component in the structure is the same. The binding site therefore is considered to be the same. Considering that no obvious effect of Y (Y: 2 or 3) was observed even though the electron-withdrawing potential of each group was reversed, the newly introduced secondary amino group had no effect on the binding affinity. Further, aminomethyl and amide substitutions had no effect. In this case, the only considerable factor that can affect the inhibitory activity is steric effects. If, for example, one of the isomers of compound **2**(**8**,**2**) was the only component responsible for the observed inhibitory activity, the minor isomer should have ca. 132% enhanced inhibitory activity over **2**(**8**,**1**) since an almost 3:2 ratio of diastereomers was formed during the Strecker reaction. Although we cannot discount the possibility, it is highly unlikely that this is the case because linkages between *C*-1 and the aromatic ring (five bonds) can rotate while fixing the iminosugar component and aromatic ring. Therefore, even if one isomer accounts for all the inhibitory activity, the effect is still too small and thus we ceased experiments on this compound.

Finally, completely different inhibitory spectra were observed for the enzymes tested, which indicates that the selectivity can be controlled by the substituent group at the *C*-1′ position of the same core unit. In addition, these compounds showed inhibitory activities against enzymes whose substrate specificities are for both the *galacto*- and *manno*-series. This observation suggests that the pyrrolidine ring compounds can be treated as conformational mixtures in a library, which may enhance the utility of such compounds.

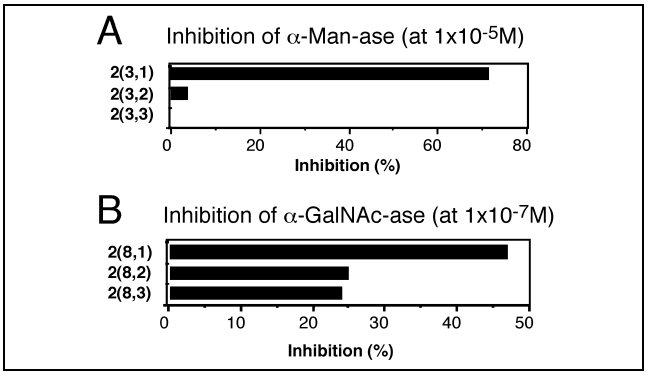


Fig. 5. Inhibitory activities of compounds **2**(**3**,**Y**) and **2**(**8**,**Y**) against α-Man-ase and α-GalNAc-ase. Two series of compounds **2**(**3**,**Y**) and **2**(**8**,**Y**) were selected to evaluate the effects of the Y group. (A) Inhibition of α-Man-ase at 1×10^{-5} M. (B) α-GalNAc-ase at 1×10^{-7} M.

3. Significance

A potential combinatorial approach based on five-membered iminocyclitol with a 2(*R*), 3(*R*), 4(*S*), 5(*R*)-configuration was exploited using reductive amination and the Strecker condensation reaction. The novel idea of a 'conformational diversity factor' in library design was proposed because different conformers are thought to bind to the corresponding enzymes with different substrate specificities.

During the investigation, difficulties associated with the bulky protecting groups near the reaction site were encountered, which limited the scope of reactions. However, the usefulness of the library as a source of inhibitors of glycoenzymes was clearly shown. Thus, the library can be screened to find small molecules that modulate cellular

functions since α-Glc-ase, α-Man-ase and β-GalT-ase are deeply involved in the glycan processing of glycoproteins [1–3] together with the involvement of α-GalNAc-ase activity in immunodeficiency [42].

4. Materials and methods

4.1. Materials

The following enzymes were purchased as indicated: α-glucosidase (α-Glc-ase; α-glucoside glucohydrolase; EC 3.2.1.20), *Saccharomyces* sp. (ToYoBo); α-mannosidase (α-Man-ase, α-D-mannoside mannohydrolase; EC 3.2.1.24), jack bean (Sigma); α-galactosidase (α-Gal-ase, α-D-galactoside galactohydrolase; EC 3.2.1.22), green coffee bean (Sigma); β-galactosidase (β-Gal-ase, β-D-galactoside galactohydrolase; EC 3.2.1.23), *Aspergillus oryzae* (Sigma); α-GalNAc-ase (2-acetamido-2-deoxy-α-D-galactoside acetamidodeoxygalactohydrolase; EC 3.2.1.49) from chicken liver (Sigma); β-1,4-galactosyltransferase (β-1,4-GalT-ase; EC 2.4.1.22), bovine milk (Sigma); α-1,3-galactosyltransferase (α-1,3-GalT-ase; EC 2.4.1.90), porcine, recombinant, *Escherichia coli* (Calbiochem). 4-Methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside (4-MU-GlcNAc) was from Wako Pure Chemicals. UDP-galactose and uridine 5′-diphosphate (UDP) were from Sigma. Cacodylic acid sodium salt, HEPES [2-{4-(2-hydroxyethyl)-1-piperazinyl}ethanesulfonic acid], MES [2-(*N*-morpholino)ethanesulfonic acid], $MnCl_2$, sodium tetraborate, and potassium hydroxide were from Nakalai Tesque Inc. Double deionized water was prepared from a Milli-Q system (Millipore Corp.). Sep-Pak cartridges were from Waters Corp. Millex-GV syringe filters (0.22 mm × 4 mm i.d.) were purchased from Nihon Millipore Ltd.

4.2. General methods for chemical synthesis

Dried solvents were used for all reactions. Solutions were evaporated under reduced pressure at a bath temperature not exceeding 50°C. Column chromatography was performed on silica gel (Merck Kieselgel 60) or Iatro Beads (60 μm) (Dia-Iatron Laboratories) when specified. Gel permeation chromatography was performed using Bio Gel P-2. TLC spots were visualized using 7% 12-molybdo(VI)phosphoric acid *n*-hydrate in EtOH. A JEOL EX-270 spectrometer was used to obtain nuclear magnetic resonance (NMR) spectra at 25°C. ^{1}H NMR (270 MHz) spectra were recorded in $CDCl_3$ or D_2O using Me_4Si (δ 0.00) or DOH (δ 4.80) as the internal standard. ^{13}C NMR (67.5 MHz) spectra were recorded in $CDCl_3$ or D_2O using Me_4Si (δ 0.0), $CDCl_3$ (δ 77.0), or CD_3CN (δ 118.2) as the internal standard. Only partial assignments were reported. MALDI-TOF mass spectra were recorded on an Applied BioSystems Voyager with 2,5-dihydroxybenzoic acid as matrix. Color development of TLC was determined using the ATTO Densitograph software library Lane Analyzer (ATTO Corp.).

4.3. General methods for glycosyltransfer reactions and analyses

Enzyme reactions were performed in a total volume of 250 μl in microtubes. The reaction mixture contained 0.1 M cacodylate buffer (pH 7), 10 mM $MnCl_2$, 0.1 mM UDP-Gal, 0.2 mM 4-MU-glycoside, 0.5 mM inhibitor, and 20 mU/ml galactosyltransferase. After incubation for 5 min at 37°C, the reaction was terminated by the addition of 50 μl of 0.1 M borate and heating at 80°C for 10 min. The resulting mixture was finally filtered with Millex GV filter to remove the precipitate.

Kinetic analysis was performed on a Waters Quanta 4000E capillary electrophoresis system, which was equipped with a fused silica capillary of i.d. 75 μm and length as specified. Samples were loaded by means of hydrostatic pressure at 10 cm height for 10 s. Detection was carried out by on-column measurement of UV absorption at 214 nm at 7.5 cm from the cathode. Pherograms were recorded on a Millennium 2010 system (Waters Corp.).

*4.4. Typical procedure for reductive amination of compound **3***

A solution of aldehyde **3** (9 mg, 0.017 mmol) in toluene (300 μl), phenethylamine (4.4 μl, 0.035 mmol) was added at 0°C and stirred at room temperature for 2 h, then 1 M solution of $NaBH_3CN$ in THF (51.0 μl, 3 equiv.) was added at 0°C and stirred at room temperature overnight. The mixture was diluted with EtOAc, washed with saturated $NaHCO_3$ and dried over $MgSO_4$. Conversion yields of reductive amination were estimated by densitographic analyses of TLC, where spots were visualized by molybdophosphate reagent. Only UV-positive spots corresponding to the protected iminocyclitol were analyzed. Yields (%): **4**(**1**), 98.1; **4**(**2**), 42.4; **4**(**3**), 80.3; **4**(**4**), 57.5; **4**(**5**), 80.9; **4**(**6**), 61.0; **4**(**7**), 54.9; **4**(**8**), 79.4; **4**(**9**), 80.3. After removal of the solvent, the residue was dissolved in MeOH (0.8 ml) and 1 M HCl (0.2 ml) and stirred with a catalytic amount of 20% $Pd(OH)_2$ on C under H_2 atmosphere at room temperature for 2 days. The crude material, obtained after removal of the catalyst and solvent, was dissolved in H_2O (1 ml) and stirred with Dowex 1X8 (OH form) at room temperature for 30 min. After filtration and removal of the solvent, the residue was dissolved in H_2O and applied onto a Waters Sep-Pak Plus CM cartridge, which was pretreated with 1 M HCl (10 ml) and water (20 ml). The cartridge was washed with H_2O (20 ml), after which elution with 10% NH_3 (10 ml), filtration through a Millex GV filter, and lyophilization gave **2**(**8**,**1**) (2.5 mg, 53% from **3**). In parallel reactions, **2**(**1**,**1**) (55%), **2**(**2**,**1**) (65%), **2**(**3**,**1**) (10%), **2**(**4**,**1**) (80%), **2**(**5**,**1**) (36%), **2**(**6**,**1**) (74%), **2**(**7**,**1**) (63%), **2**(**9**,**1**) (29%) were obtained. Selected physical data of these compounds are listed below.

2(**1**,**1**): ^{1}H NMR (D_2O): δ 4.20 (t, 1H, J = 3.8 Hz), 3.98 (dd, 1H, J = 4.0, 8.4 Hz), 3.84 (dd, 1H, J = 6.6, 11.2 Hz), 3.69 (dd, 1H, J = 4.7, 11.2 Hz), 3.34 (dd, 1H, J = 3.5, 6.6 Hz), 0.96 (t, 3H, J = 7.3 Hz, CH_3); MALDI-TOF MS calcd. for $C_{10}H_{22}N_2O_3$: 218; found: *m*/*z* 219 $(M+H)^+$. **2**(**2**,**1**): ^{1}H NMR (D_2O): δ 4.19 (t, 1H, J = 3.9 Hz), 3.91 (dd, 1H, J = 4.4, 8.6 Hz), 3.84 (dd, 1H, J = 6.5, 10.9 Hz), 3.67 (dd, 1H, J = 6.6, 11.2 Hz), 2.66 (s, 6H, $N(CH_3)_2$). **2**(**3**,**1**): ^{1}H NMR (D_2O): δ 4.19 (t, 1H, J = 3.5 Hz), 3.97 (dd, 1H, J = 3.9, 7.8 Hz), 0.89 (t, 3H, J = 6.7 Hz, CH_3); MALDI-TOF MS calcd. for $C_{16}H_{34}N_2O_3$: 302; found: *m*/*z* 303 $(M+H)^+$. **2**(**4**,**1**): ^{1}H NMR (D_2O): δ 4.19 (t, 1H, J = 3.8 Hz), 3.96 (dd, 1H, J = 4.3, 8.5 Hz); MALDI-TOF MS calcd. for $C_8H_{18}N_2O_4$: 206; found: *m*/*z* 207 $(M+H)^+$. **2**(**5**,**1**): ^{1}H NMR (D_2O): δ 4.30 (t, 1H, J = 3.5 Hz), 4.19 (dd, 1H, J = 3.5, 8.6 Hz); MALDI-TOF MS calcd. for $C_{16}H_{28}N_2O_3$: 296; found: *m*/*z* 297 $(M+H)^+$. **2**(**6**,**1**); ^{1}H NMR (D_2O): δ 4.19 (t, 1H, J = 3.8 Hz), 3.94 (dd, 1H, J = 4.2, 8.3 Hz), 3.82 (dd, 1H, J = 6.8, 11.3 Hz), 3.67 (dd, 1H, J = 6.2, 11.3 Hz), 3.58 (t, 2H, J = 6.3 Hz), 3.38 (s, 3H, OCH_3), 2.93 (dd, 1H, J = 4.7, 12.2 Hz), 2.81 (t, 2H, J = 7.2 Hz), 1.85 (quintet, 2H, J = 6.8 Hz); MALDI-TOF MS calcd. for

$C_{10}H_{22}N_2O_4$: 234; found: *m/z* 235 $(M+H)^+$. **2(7,1)**: ^1H NMR (D_2O): δ 4.24 (t, 1H, $J=3.6$ Hz), 4.19 (t, 1H, $J=3.7$ Hz), 4.03 (dd, 1H, $J=4.1$, 8.6 Hz); MALDI-TOF MS calcd. for $C_{11}H_{22}N_2O_4$: 246; found: *m/z* 247 $(M+H)^+$. **2(8,1)**: ^1H NMR (D_2O): δ 7.43–7.35 (m, 5H), 4.16 (t, 1H, $J=4.0$ Hz), 3.90 (dd, 1H, $J=3.9$, 8.2 Hz), 3.80 (dd, 1H, $J=6.9$, 11.1 Hz); MALDI-TOF MS calcd. for $C_{14}H_{22}N_2O_3$: 266; found: *m/z* 267 $(M+H)^+$. **2(9,1)**; ^1H NMR (D_2O): δ 4.18 (t, 1H, $J=3.4$ Hz), 3.96 (dd, 1H, $J=4.4$, 8.3 Hz), 3.82 (dd, 1H, $J=6.6$, 10.8 Hz), 3.67 (dd, 1H, $J=6.6$, 10.5 Hz); MALDI-TOF MS calcd. for $C_{12}H_{24}N_2O_3$: 244; found: *m/z* 245 $(M+H)^+$.

4.5. Typical procedure for Strecker reaction of compound 3

To a solution of aldehyde **3** (24 mg, 0.045 mmol) in toluene (700 µl), phenethylamine (6.9 µl, 0.054 mmol) was added at 0°C and stirred at room temperature for 2 h, then TMSCN (18.1 µl, 0.14 mmol) and 0.5 M solution of zinc chloride in THF (9.0 µl, 0.1 equiv.) was added at 0°C and stirred at room temperature overnight. The mixture was diluted with EtOAc, washed with saturated $NaHCO_3$, and dried over $MgSO_4$. After removal of the solvent, the crude material of nitrile **5(8)** (29 mg, 96.0%*p* (6:4)) was obtained, where %*p* stands for purity of the product in a crude mixture as estimated by densitographic analysis of TLC, and the following number in parentheses indicates the ratio of diastereoisomers. Those without the ratio indicate that the stereoisomers had the same R_f values. Spots were visualized by molybdophosphate reagent and only UV-positive spots corresponding to protected iminocyclitol were analyzed. In parallel reactions, **5(1)** (43 mg, 81.9%*p* (1:1), from **3** (39 mg, 0.074 mmol)), **5(2)** (26 mg, 58.2%*p*, from **3** (27 mg, 0.052 mmol)), **5(3)** (27 mg, 91.2%*p* (6:4), from **3** (28 mg, 0.053 mmol)), **5(4)** (29 mg, 67.8%*p*, from **3** (27 mg, 0.051 mmol)), **5(5)** (30 mg, 70.6%*p*, from **3** (24 mg, 0.046 mmol)), **5(6)** (27 mg, 56.9%*p* (6:4), from **3** (25 mg, 0.046 mmol)), **5(7)** (26 mg, 87.5%*p* (6:4), from **3** (26 mg, 0.048 mmol)) and **5(9)** (30 mg, 57.0%*p*, from **3** (26 mg, 0.050 mmol)) were obtained. These compounds were used for the next reaction without further purification. Selected physical data are shown below.

5(1): ^1H NMR ($CDCl_3$): δ 3.62 (d, 1H, $J=8.4$ Hz, CHCN), 1.43 (s, 9H, $C(CH_3)_3$), 0.91 (t, 3H, $J=7.0$ Hz, CH_3); ^{13}C NMR ($CDCl_3$): 119.35 and 118.65 (CN), 80.99 ($C(CH_3)_3$), 28.29 ($C(CH_3)_3$), 13.84 (CH_3); MALDI-TOF MS calcd. for $C_{37}H_{47}N_3O_5$: 613; found: *m/z* 614 $(M+H)^+$. **5(2)**: ^1H NMR ($CDCl_3$): δ 3.63 (d, 1H, $J=9.0$ Hz, CHCN), 2.22 (s, 6H), 1.44 (s, 9H); MALDI-TOF MS calcd. for $C_{37}H_{48}N_4O_5$: 628; found: *m/z* 629 $(M+H)^+$. **5(3)**: ^1H NMR ($CDCl_3$): δ 4.12 (t, 1H, $J=8.5$ Hz), 3.63 (d, 1H, $J=8.5$ Hz, CHCN), 1.43 (s, 9H), 0.88 (t, 3H, $J=6.4$ Hz); MALDI-TOF MS calcd. for $C_{43}H_{59}N_3O_5$: 697; found: *m/z* 698 $(M+H)^+$, 720 $(M+Na)^+$, 736 $(M+K)^+$. **5(4)**: ^1H NMR ($CDCl_3$): δ 1.44 (s, 9H); MALDI-TOF MS calcd. for $C_{35}H_{43}N_3O_6$: 601; found: *m/z* 602 $(M+H)^+$, 624 $(M+Na)^+$. **5(5)**: ^1H NMR ($CDCl_3$): δ 3.64 (d, 1H, $J=8.1$ Hz, CHCN), 1.46 (s, 9H); MALDI-TOF MS calcd. for $C_{43}H_{53}N_3O_5$: 691; found: *m/z* 692 $(M+H)^+$. **5(6)**: ^1H NMR ($CDCl_3$): δ 3.63 (d, 1H, $J=8.3$ Hz, CHCN), 3.31 (s, 3H), 1.44 (s, 9H). **5(7)**: ^1H NMR ($CDCl_3$): δ 3.63 (d, 1H, $J=7.5$ Hz, CHCN), 1.43 (s, 9H); MALDI-TOF MS calcd. for $C_{38}H_{47}N_3O_6$: 641; found: *m/z* 642 $(M+H)^+$. **5(8)**: ^1H NMR ($CDCl_3$): δ 4.26 (dt, $J=2.5$, 7.0 Hz, 1H), 3.94 (d, 2H, $J=4.1$ Hz), 3.61 (t, 1H, $J=6.5$ Hz), 1.44 (s, 9H); ^{13}C NMR ($CDCl_3$); 118.58 (CN), 81.00 ($C(CH_3)_3$), 28.14 ($C(CH_3)_3$); MALDI-TOF MS calcd. for $C_{41}H_{47}N_3O_5$: 661; found: *m/z* 662 $(M+H)^+$. **5(9)**: ^1H NMR ($CDCl_3$): δ 4.14 (t, 1H, $J=8.3$ Hz), 3.98 (d, 2H, $J=4.8$ Hz), 3.63 (d, 1H, $J=6.6$ Hz, CHCN), 1.45 (s, 9H); MALDI-TOF MS calcd. for $C_{39}H_{49}N_3O_5$: 639; found *m/z* 640 $(M+H)^+$.

4.6. Typical transformation reaction of nitrile to amino group: synthesis of compound 2(8,2)

To a solution of crude compound **5(8)** (8 mg, corresponding to **2** (7 mg, 0.013 mmol)) dissolved in MeOH (0.8 ml) and 1 M HCl (0.2 ml) a catalytic amount of 20% $Pd(OH)_2$ on C was added. The reaction mixture was stirred under H_2 atmosphere at room temperature for 4 days. The crude material, obtained after removal of the catalyst and solvent, was dissolved in H_2O (1 ml) and stirred with Dowex 1X8 (OH form) at room temperature for 30 min. After filtration and removal of the solvent, the residue was dissolved in H_2O and applied onto a Waters Sep-Pak Plus CM cartridge pretreated with 1 M HCl (10 ml) and water (20 ml). The cartridge was washed with H_2O (20 ml), after which elution with 10% NH_3 (10 ml), filtration through a Millex GV filter, and lyophilization gave **2(8,2)** (2.3 mg, 58% from **3**). In parallel reactions were obtained **2(1,2)** (51%), **2(2,2)** (41%), **2(3,2)** (8%), **2(4,2)** (41%), **2(5,2)** (32%), **2(6,2)** (31%), **2(7,2)** (27%), **2(9,2)** (12%). Selected physical data are listed below.

2(1,2): ^1H NMR (D_2O): δ 0.90 (t, 3H, $J=6.5$ Hz, CH_3). **2(2,2)**: ^1H NMR (D_2O): δ 1.95 (s, 6H); **2(3,2)**: MALDI-TOF MS calcd. for $C_{17}H_{37}N_3O_5$: 331; found: *m/z* 332 $(M+H)^+$. **2(4,2)**: ^1H NMR (D_2O): δ 3.75 (t, $J=5.6$ Hz), 3.00 (t, $J=5.6$ Hz); MALDI-TOF MS calcd. for $C_9H_{21}N_3O_4$: 235; found: *m/z* 236 $(M+H)^+$, 274 $(M+K)^+$. **2(5,2)**: MALDI-TOF MS calcd. For $C_{17}H_{31}N_3O_3$: 325; found: *m/z* 326 (M+H). **2(8,2)**: MALDI-TOF MS calcd. for $C_{15}H_{25}N_3O_3$: 295; found: *m/z* 296 $(M+H)^+$. **2(9,2)**: MALDI-TOF MS calcd. for $C_{13}H_{27}N_3O_3$: 273; found: *m/z* 274 $(M+H)^+$, 312 $(M+K)^+$.

4.7. Typical transformation reaction of nitrile to amide group: synthesis of compound 2(1,3)

To a solution of crude compound **5(1)** (8 mg, corresponding to **3** (10 mg, 0.018 mmol)) in MeOH (0.8 ml) and 6 M KOH (0.2 ml) was added 30% H_2O_2 (17 µl, 0.15 mmol). The reaction mixture was stirred at 50°C for 2 h. After cooling, the reaction mixture was poured into ice-water and extracted with CH_2Cl_2, and dried over $MgSO_4$. To this residue dissolved in MeOH (0.8 ml) and 1 M HCl (0.2 ml) a catalytic amount of 20% $Pd(OH)_2$ on C was added. The reaction mixture was stirred under H_2 atmosphere at room temperature for 3 days. The crude material, obtained after removal of the catalyst and solvent, was dissolved in H_2O (1 ml) and stirred with Dowex 1X8 (OH form) at room temperature for 30 min. After filtration and removal of the solvent, the residue was dissolved in H_2O and applied onto a Waters Sep-Pak Plus CM cartridge pretreated with 1 M HCl (10 ml) and water (20 ml). The cartridge was washed with H_2O (20 ml), after which elution with 10% NH_3 (10 ml), filtration through a Millex GV filter, and lyophilization gave **2(1,3)** (1.4 mg, 28% from **3**). In parallel reactions were obtained **2(2,3)** (42%), **2(3,3)** (15%), **2(4,3)** (33%), **2(5,3)** (17%), **2(6,3)** (28%), **2(7,3)** (12%), **2(8,3)** (27%), **2(9,3)** (26%). Selected physical data of these compounds are listed below.

2(1,3): ^1H NMR (D_2O): δ 3.34 (t, $J=6.5$ Hz), 0.92 (t, $J=6.2$

Hz); MALDI-TOF MS calcd. for $C_{11}H_{23}N_3O_4$: 261; found: *m/z* 262 $(M+H)^+$; before hydrogenolysis: MALDI-TOF MS calcd. for $C_{37}H_{49}N_3O_6$: 631; found: *m/z* 632 $(M+H)^+$. **2**(**2**,**3**): MALDI-TOF MS calcd. for $C_{11}H_{24}N_4O_4$: 276; found: *m/z* 277 $(M+H)^+$; before hydrogenolysis: MALDI-TOF MS calcd. for $C_{37}H_{50}N_4O_6$: 646; found: *m/z* 647 $(M+H)^+$, 685 $(M+K)^+$. **2**(**3**,**3**): ^{1}H NMR (D_2O): δ 0.89 (t, 3H, $J=6.0$ Hz); MALDI-TOF MS calcd. for $C_{17}H_{35}N_3O_4$: 345; found: *m/z* 346 $(M+H)^+$. **2**(**4**,**3**): ^{1}H NMR (D_2O): δ 3.00 (t, $J=5.2$ Hz); MALDI-TOF MS calcd. for $C_9H_{19}N_3O_5$: 249; found: *m/z* 250 $(M+H)^+$; before hydrogenolysis: MALDI-TOF MS calcd. for $C_{35}H_{45}N_3O_7$: 619; found: *m/z* 620 $(M+H)^+$, 642 $(M+Na)^+$. **2**(**5**,**3**): MALDI-TOF MS calcd. for $C_{17}H_{29}N_3O_4$: 339; found: *m/z* 340 $(M+H)^+$; before hydrogenolysis: MALDI-TOF MS calcd. for $C_{43}H_{55}N_3O_6$: 709; found: *m/z* 710 $(M+H)^+$. **2**(**6**,**3**): ^{1}H NMR (D_2O): δ 3.54 (t, $J=6.3$ Hz), 3.35 (s, 3H); MALDI-TOF MS calcd. for $C_{11}H_{23}N_3O_5$: 277; found: *m/z* 278 $(M+H)^+$. **2**(**7**,**3**): MALDI-TOF MS calcd. for $C_{12}H_{23}N_3O_5$: 289; found: *m/z* 290 $(M+H)^+$; before hydrogenolysis: MALDI-TOF MS calcd. for $C_{38}H_{49}N_3O_7$: 659; found: *m/z* 660 $(M+H)^+$. **2**(**8**,**3**): MALDI-TOF MS calcd. for $C_{15}H_{23}N_3O_4$: 309; found: *m/z* 310 $(M+H)^+$, 322 $(M+Na)^+$; before hydrogenolysis: ^{1}H NMR ($CDCl_3$): δ 6.58, 6.46, 5.39, 5.22 (br. s, $CONH_2$); ^{13}C NMR ($CDCl_3$) δ 175.20 and 174.68 ($CONH_2$), 155.38 (NCOO), 28.38 (CH_3); MALDI-TOF MS calcd. for $C_{41}H_{49}N_3O_6$: 679; found: *m/z* 680 $(M+H)^+$. **2**(**9**,**3**): MALDI-TOF MS calcd. for $C_{13}H_{25}N_3O_4$: 287; found: *m/z* 288 $(M+H)^+$; before hydrogenolysis: MALDI-TOF MS calcd. for $C_{39}H_{51}N_3O_6$: 657; found: *m/z* 658 $(M+H)^+$.

4.8. Screening of library against glycosidases

Incubations were performed in microtiter plate wells (50 μl/well). Reaction mixtures contained suitable buffer (40 mM phosphate buffer (pH 7) for α-glucosidase, α-galactosidase and β-galactosidase, 20 mM acetate buffer (pH 5) for α-mannosidase, 40 mM citrate buffer (pH 4) for α-galactosaminidase), 1 mM PNP-glycoside, various amounts of inhibitors (1 nM–1 mM) and glycosidase (0.4 U/ml α-glucosidase, 0.2 U/ml α-galactosidase, 1 U/ml β-galactosidase, 0.2 U/ml α-mannosidase, 0.6 U/ml α-galactosaminidase). In the case of compound **2**(**3**,**Y**), a stock 5 mM solution was first prepared by dissolving the compound in dimethyl sulfoxide (DMSO) and dilution with H_2O (H_2O:DMSO = 6:1 (v/v)), and aliquots of the solution were used for each reaction. After incubation at room temperature for 5 min, the reaction was terminated by addition of 50 μl of 0.2 M sodium carbonate. Each experiment was carried out in duplicate. The inhibitory results of a series of compounds in the library are shown in Fig. 4A–F.

4.9. Assay of α-1,3-GalT-ase

Incubations were performed at pH 6.5 in the presence of α-1,3-galactosyltransferase for varying times (1–15 min) for kinetic analysis. Inhibitory assays of iminosugars against α-1,3-GalT-ase were carried out under the same conditions as kinetic analysis for 5 min in the presence of 0.5 mM iminosugars. None of these iminosugars exhibited inhibitory activity against α-1,3-GalT-ase.

Electrophoresis was performed at 15 kV using a 60 cm fused silica capillary. The migration times for acceptor 4-MU-LacNAc (**6**) and product 4-MU-Gal-α(1→3)-Gal-β(1→4)-GlcNAc (**7**) were 9.3 and 10.0 min, respectively. Mass spectrometric analysis of the reaction mixture after Sep-Pak C18 gave satisfactory data. MALDI-TOF MS: compound **6**: calcd. for $C_{24}H_{31}NO_{13}$: 541; found: *m/z* 564 $(M+Na)^+$; **7**: calcd. for $C_{30}H_{41}NO_{18}$: 703; found: *m/z* 726 $(M+Na)^+$.

4.10. Screening of library against β-1.4-GalT-ase

Incubations were performed at pH 7.0 in the presence of β-1,4-GalT-ase for varying times (1–15 min) for kinetic analysis. Inhibitory assays of iminosugars were carried out under the same conditions as kinetic analysis for 5 min in the presence of 0.5 mM iminosugar. The inhibitory result is shown in Fig. 4G.

Electrophoresis was performed at 20 kV using a 49 cm fused silica capillary. 50 mM sodium borate was used as the electrolyte. The migration times for acceptor 4-MU-GlcNAc and product 4-MU-LacNAc (**6**) were 2.4 and 2.6 min, respectively.

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