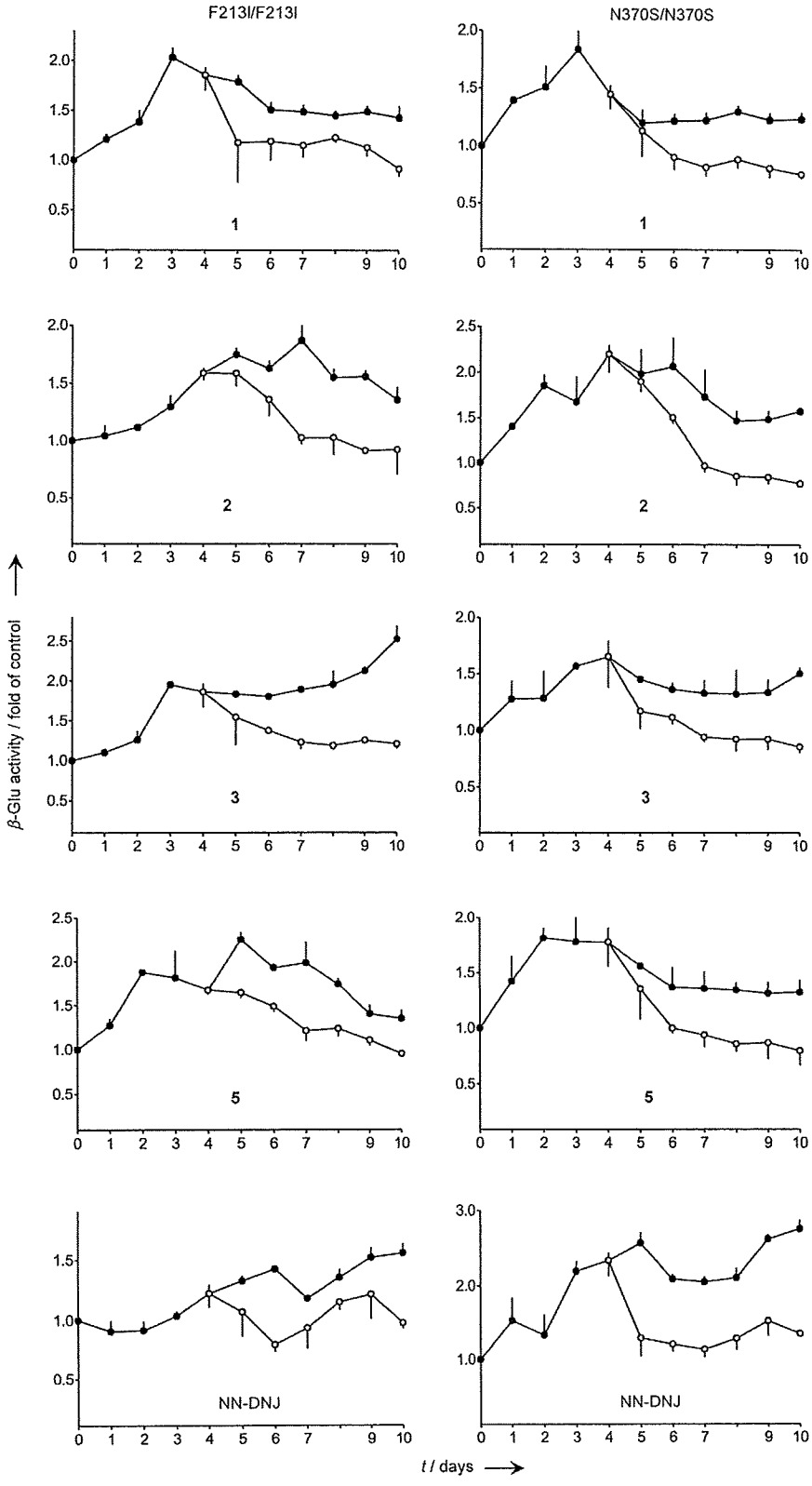

Figure 2. Chaperone activities of bicyclic NJ (1–3) and GNJ derivatives (5) and NN-DNJ on mutant β-Glu in fibroblasts (time course). Cells were cultured in the presence of the optimal concentration of each compound (30 μM for 1–3 and 5; 1 μM for NN-DNJ) of the corresponding bicyclic nojirimycin for up to 10 days (●). A subset of cells was cultured with chaperones for four days, washed and further cultured without the drug for six days (○). β-Glu activity in cells was determined at the indicated time in triplicate.

cell line for any of the assayed compounds. In the normal H37 cells, β-Glu activity seemed to increase slightly in the presence of compounds **1–3** and **5** during the first few days, then it dropped back to the basal level (see Supporting Information, Figure 2S). No change in the presence of NN-DNJ was observed.

To evaluate the cytotoxicity of compounds **1–3** and **5**, we cultured normal and F213I/F213I, N370S/N370S and L444P/L444P mutant human fibroblasts in the presence of various concentrations of each sp^2 iminosugar (0, 0.3, 1, 3, 10 and 30 μM) for four days, and then assayed the cell viabilities. NN-DNJ was included in this study for comparative purposes. The results showed that the viabilities of all four fibroblast types were unchanged after incubation with the inhibitors, even at the maximum concentration of 30 μM.

Discussion

Chemical chaperone therapy is a promising approach for the treatment of lysosomal storage disorders because of its potential for simple oral administration, penetration of the blood–brain barrier and low cost. Chemical chaperone activity in Gaucher disease (GD) mutants has been reported for several *N*-substituted derivatives of the carbasugar β-valienamine[21,46] and of the iminosugar 1-deoxynojirimycin (DNJ),[26,27,47,48] among which the *N*-(*n*-nonyl) derivative (NN-DNJ). Recently, we found that bicyclic sp^2-iminosugar analogues of the parent alkaloid nojirimycin (NJ), with structure of 5-*N*′,6-*X*-(*N*′-alkyliminomethylidene)nojirimycin (X represents O, S or N) behaved as very selective competitive inhibitors of β-glucosidases, including human β-glucocerebrosidase. Preliminary X-ray structural studies of the corresponding enzyme-inhibitor complexes sup-

ported that these compounds bind at the active site of β-glucosidases in a manner that is analogous to that previously encountered for iminosugars such as NN-DNJ.[49] The remarkable selectivity for β-Glu was ascribed to the rigidity of the bicyclic skeleton.[39] Since the synthesis of sp^2 iminosugars is readily amenable to molecular diversity-oriented strategies and biological activity optimization, evaluation of their potential as chemical chaperones for the treatment of Gaucher disease was very appealing.

Prior to this work, a broad screening of bicyclic sp^2-iminosugar structures with different configurational patterns and *N'*-substituents was carried out against a panel of commercial glycosidases.[36] Compounds with hydroxylation profiles analogous to D-glucose (NJ derivatives) or D-galactose (4-*epi*-NJ; galactonojirimycin, GNJ derivatives) and bearing long chain linear *N'*-alkyl substituents were found to be promising candidates in view of their strong and selective inhibition of clan GH-A β-glucosidases to which human acid β-Glu belongs. On those grounds, the *N'*-octyliminomethylidene NJ and GNJ derivatives **1–3** and **4–6**, respectively, were initially considered for additional studies. In a next set of experiments, the bicyclic NJ derivatives **1–3** were found to enhance the activity of several GD β-glucocerebrosidase mutants, whereas compound **5** was the only GNJ analogue that exhibited obvious chaperone activities.

The chaperone effect of compounds **1–3** and **5** was further evaluated and compared with the effect of NN-DNJ in human GD cells by using an in situ cell enzyme assay. The bicyclic sp^2 iminosugars exhibited chaperone activities in five of seven GD cell lines assayed, namely the F213I/F213I, N188S/G193W, G202R/L444P, N370S/N370S and F213I/L444P mutations, whereas they were ineffective in cells with the L444P/L444P and L444P/RecNciI mutations.

The rationale behind the selection of sp^2-iminosugar glycomimetics as chemical chaperone candidates was based on the generally accepted theory that effective competitive inhibitors are more likely to show a potent chaperone effect. This is particularly true for compounds that are more potent β-Glu inhibitors at neutral than at acidic pH values, which emulate the scenarios encountered in the ER and the lysosome, respectively. Our results indicate, however, that the chaperone activity is not directly related to the inhibitory efficiency. Thus, compounds **1–3** and **5**, as well as NN-DNJ, exhibited good inhibitory properties on human normal acid β-Glu and no toxicity in fibroblasts at the range of concentrations tested. They also inhibited the F213I/F213I, N370S/N370S and L444P/L444P mutant β-Glu proteins, with IC_{50} values that showed mutation dependence. Good chaperone activities were achieved on the N370S/N370S mutant in spite of IC_{50} values that were five- to 20-fold higher than those measured on normal and other mutant acid β-Glu. In the case of the L444P/L444P and F213I/F213I mutant acid β-Glu, the IC_{50} values were similar to those measured for the normal enzyme, but the compounds showed no effect on the L444P/L444P whereas they exhibited potent chaperone activity on the F213I/F213I mutant enzyme. Altogether, these results underline that the chaperone activity of a given compound does not solely rely on its binding affinity towards the target enzyme, but that properties such as membrane permeability, metabolism, intracellular localization and the structure of the protein upon binding to the chaperone, among others, might play a decisive role.

No strong variations in the β-glucosidase inhibitory activity were encountered between bicyclic sp^2 iminosugars differing in the endocyclic heteroatom at the five-membered ring in the bicyclic skeleton (O for **1**, S for **2** and **5** or N for **3**). However, the cyclic guanidine derivative **3** was found to be a tenfold weaker inhibitor as compared with the cyclic isourea (**1**) or the cyclic isothiourea derivatives (**2** and **5**) in cases in which the acid β-Glu activities were determined in lysates from human normal and mutant fibroblasts in the presence of increasing concentrations of the compounds. Previous structural and thermodynamic studies suggested that desolvation of the inhibitor probably represents an important contribution to the binding free energy in these family of glycomimetics,[37] a process that is expected to be less favourable for the more basic guanidine functionality. Nevertheless, these differences were not paralleled in the chaperone experiments. Thus, while compound **2** was a 40-fold stronger inhibitor of the F213I/F213I mutant acid β-Glu than **3**, the enzyme activity enhancement achieved with the later compound was slightly higher. It seems that the ratio between the binding affinity to β-Glu in the ER and the lysosome, which can be considered to be proportional to the corresponding ratio of the IC_{50} values at 7.0 and 5.2, is a more critical parameter than the individual inhibition potency at either of those cellular compartments. The fact that NN-DNJ is a less efficient chemical chaperone as compared with the sp^2 iminosugars for the F213I/F213I mutant is also consistent with this observation.

The primary goal of this work was the evaluation of the potential of sp^2 iminosugars as chemical chaperones for a variety of Gaucher mutations in comparison with the classical iminosugar NN-DNJ, already in preclinical studies.[4] It must be stressed, however, that there is an increasing number of reports on new chaperones for GD and that a broader comparative evaluation of the therapeutic potential for different structures would be highly desirable. Synthetic cost and pharmacokinetic properties are also critical questions regarding drug development. The hydrophilic 1-azasugar isofagomine, a specific and potent β-Glu inhibitor (IC_{50} 50 nM), is a molecule of reference at this respect. It can increase the N370S β-Glu activity about two- to threefold by enhancing its cellular folding and trafficking.[45,50,51] Recently, Zheng et al.[52] used quantitative high-throughput screening (qHTS) to find three structural series of potent, selective, nonsugar β-Glu inhibitors. Three compounds from these series, with IC_{50} values 0.031, 0.103 and 0.33 μM, respectively, increased β-Glu activity by 40–90% in N370S mutant cells. By HTS of the 50000-compound Maybridge library, Tropak et al.[23] identified two noncarbohydrate-based inhibitory compounds with IC_{50} values of 5 and 8 μM respectively, which can increase β-Glu activity 50–150% in N370S or F213I GD fibroblasts. This results are, actually, quite similar to ours (40–100% increase for F213I and 40–165% increase for N370S).

Studies on genotype–phenotype relationships in human GD patients have shown that except for the N370S mutations, which are exclusively associated with type 1, the nonneurono-

pathic form of GD, the other three mutations with positive responses to the bicyclic NJ derivatives **1**–**3** and **5** (N188S, G202R, F213I), can be associated with types 2 or 3, neuronopathic forms of the disease,[53–55] which are not responsive to enzyme replacement therapy because the recombinant enzyme cannot cross the blood–brain barrier. We propose the sugar mimics in this study mainly for chemical chaperone therapy of neuronopathic forms of Gaucher disease. Preliminary confocal microscopy studies carried out with a fluorescently labeled derivative of the bicyclic NJ derivative **1** indicated enhanced trafficking of the mutant protein to the lysosome in Gaucher patient cells. Compound **1** also evidenced good properties regarding oral availability and ability to enhance the β-Glu activity in tissues, including brain, as well as the lack of acute toxicity at high doses in normal mice (all procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of Tottori University; data not shown).

As far as we are aware, no glycomimetic-type chaperones, including isofagomine and the sp^2 iminosugars here described, have been reported for the L444P GD mutation. This mutation is not located in the catalytic domain of β-Glu and, consequently, is not sensitive to active site-directed chemical chaperones.[27,50] Mu et al.[56] found that inhibition of L-type Ca^{2+} channels by diltiazem or verapamil can partially restore the L444P mutant β-Glu homeostasis in GD fibroblasts, and they suggested that this effect might be due to a modest up-regulation of a subset of intrinsic molecular chaperones which are essential for the maintenance of cellular protein homeostasis. Recent results by Rigat and Mahuran pointed to a classical glucocerebrosidase chemical chaperone behaviour for diltiazem.[57] However, neither diltiazem nor verapamil were found to enhance mutant enzyme activity in homozygous L444P Gaucher cells. A better understanding of the mechanism of action of intrinsic molecular chaperones would provide us clues to develop nonactive site directed chaperones to broaden the potential of chaperone therapy for GD.

Conclusions

In this study, we have developed a general approach for the synthesis of bicyclic sp^2-iminosugar glycosidase inhibitors that is very well-suited for molecular diversity-oriented strategies. Higher inhibitory selectivities towards β-glucosidase, including the lysosomal human β-glucocerebrosidase, as compared with the structurally related iminosugar NN-DNJ have been achieved. Interestingly, sp^2 iminosugars also were more efficient as chemical chaperones than NN-DNJ for some Gaucher disease mutations. Differences in chaperone activity seem to be related, though not solely, to variations on binding affinity of the glycomimetic to the mutant protein at neutral and acidic pH, a situation that emulates the environment at the ER and the lysosome, respectively.

The body of results presented here stresses the necessity of a thorough mutation-based profiling of chemical chaperones to evaluate their therapeutic potential. The future of chaperone therapy for the treatment of GD and other lysosomal storage disorders depends on our capacity to enlarge the battery of compounds that can promote mutant glycosidase activity enhancements in a highly specific and efficient manner, including active site- and nonactive site-directed chemical chaperones. The development of flexible synthetic strategies suitable for the generation of focused libraries of glycomimetics is therefore mandatory. Physicians could then choose the most suitable chaperone for an individual patient upon his/her mutation. When using active-site chaperones to treat GD, determination of the therapeutically useful doses is critical because high doses would inhibit the acid β-Glu activity heavily and might aggravate the symptoms of GD. While very strong inhibitors can be effective at low doses, moderate inhibitors would allow the use of higher doses. Rather than a very potent inhibitory potency, an ideal active-site chaperone should have a high ratio between the chaperone and inhibition activities.

We have previously demonstrated the effectiveness of chemical chaperone therapy in G_{M1}-gangliosidosis,[58,59] Gaucher disease[20,21] and Fabry's disease.[60] Notably, we recently found that chaperone therapy can prevent neurological deterioration in a G_{M1}-gangliosidosis model mouse expressing R201C mutant human β-galactosidase.[61] In the case of the bicyclic NJ derivatives studied in this work our findings in cultured cells, although indicative of potent chaperone effects for specific GD mutations, do not allow us to predict their pharmacological chaperone efficiency in whole animals and, hence, which structure has the best therapeutic value. We are currently developing a transgenic mouse model that lacks the endogenous wild-type enzyme and expresses instead a mutant human acid β-Glu. The in vivo results will be reported in due curse.

Experimental Section

Materials and methods: The sp^2 iminosugars **1**–**6** were synthesized in our laboratories and their purity was confirmed by spectroscopic techniques and combustion analysis. For the preparation of **2**–**5** the previously reported reaction sequences were followed with slight modification.[37,39] For compound **1**,[32] an advantageous route has been developed, while compound **6** has not been previously reported. NN-DNJ was obtained from United States Biological (Marblehead, MA, USA). Stock solutions of the compounds were prepared in H_2O at 3 mM and stored at −20 °C. Reagents and solvents were purchased from commercial sources and used without further purification. Optical rotations were measured at 22 °C in 1 cm or 1 dm tubes. ^1H (and ^{13}C) NMR spectra were recorded at 300 (75.5) MHz. 2D COSY and HMQC experiments were carried out to assist in signal assignment. TLC was performed with E. Merck precoated TLC plates, silica gel 30F-245, with visualization by UV light and by charring with H_2SO_4 (10%) or cerium(IV) sulfate (0.2% *w/v*)/ammonium molybdate (5% *w/v*) in H_2SO_4 (2 M) or ninhydrin (0.1%) in EtOH. Column chromatography was carried out with Silica Gel 60 (E. Merck, 230–400 mesh). In the FABMS spectra, the primary beam consisted of Xe atoms with a maximum energy of 8 keV. The samples were dissolved in *m*-nitrobenzyl alcohol or thioglycerol as the matrixes and the positive ions were separated and accelerated over a potential of 7 keV. NaI was added as cationizing agent. Dulbecco's Modified Eagle's Medium (DMEM) and foetal

bovine serum (FBS) were obtained from Life Technologies Inc. (Rockville, MD, USA).

5-Deoxy-1,2-*O*-isopropylidene-5-(*N'*-octylureido)-α-D-glucofuranose (8): A solution of 5-azido-5-deoxy-1,2-*O*-isopropylidene-6-*O*-tetrahydropyranyl-α-D-glucofuranose[40] (**7**, 3.0 g, 9.1 mol) in MeOH (50 mL) was hydrogenated at atmospheric pressure for 1 h using Pd (10%)/C (900 mg total) as catalyst. The suspension was filtered through Celite and concentrated. The resulting residue was dissolved in MeOH (100 mL), octyl isocyanate (2.5 mL, 1.0 equiv) was added and the reaction mixture was stirred at RT for 12 h. After conventional work up, the crude material was dissolved in CH_2Cl_2/MeOH (1:1, 120 mL) and *p*-toluenesulfonic acid (2.6 mmol) was added. The reaction mixture was stirred for 2 h at RT, diluted with CH_2Cl_2 (60 mL), washed with saturated, aqueous $NaHCO_3$ (2× 50 mL), dried ($MgSO_4$) and concentrated. The resulting residue was purified by column chromatography (EtOAc/petroleum ether 1:1 → 2:1). Yield: 4.06 g (93%); R_f = 0.45 (EtOAc/petroleum ether 3:1); $[\alpha]_D$ = +41.7 (c = 1.2 in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.85 (d, $J_{1,2}$ = 3.6 Hz, 1H; H-1), 5.80 (br s, 1H; OH), 5.33 (d, $J_{5,NH}$ = 8.1 Hz, 1H; NH), 5.00 (t, $^3J_{NH,CH_2}$ = 5.4 Hz, 1H; N'H), 4.51 (d, 1H; H-2), 4.02 (d, $J_{3,4}$ = 1.8 Hz, 1H; H-3), 3.96 (dd, $J_{4,5}$ = 10.2 Hz, 1H; H-4), 3.92 (dd, $J_{6a,6b}$ = 11.2 Hz, $J_{5,6a}$ = 2.6 Hz, 1H; H-6a), 4.84 (m, 1H; H-5), 3.67 (dd, $J_{5,6b}$ = 2.6 Hz, 1H; H-6b), 3.07 (m, 2H; CH_2N), 1.43, 1.25 (2s, 6H; CMe_2), 1.42 (m, 2H; CH_2CH_2N), 1.21 (m, 10H; CH_2), 0.81 (t, $^3J_{H,H}$ = 6.4 Hz, 3H; CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 159.1 (CO), 111.5 (CMe_2), 105.1 (C-1), 84.6 (C-2), 80.3 (C-4), 74.1 (C-3), 62.3 (C-6), 49.2 (C-5), 40.7 (CH_2N), 31.8, 30.1, 29.3, 29.2, (CH_2), 26.9, 26.7 (CMe_2), 26.0, 22.7 (CH_2), 14.1 (CH_3); FABMS: m/z 397 $[M+Na]^+$, 375 $[M+H]^+$; HRCIMS: calcd for $C_{18}H_{35}N_2O_6$: 375.2495, found 375.2487.

5-Amino-5-deoxy-1,2-*O*-isopropylidene-5-*N*,6-*O*-(*N'*-octylimino-methylidene)-α-D-glucofuranose (9): Methanesulfonic chloride (0.38 mL, 4.85 mmol, 1.1 equiv) was added to a solution of the corresponding ureido derivative **8** (1.65 g, 4.41 mmol) in pyridine (44 mL) at −20 °C, under Ar atmosphere. The reaction mixture was stirred for 12 h and allowed to warm to RT. Then, the solvent was removed under reduced pressure and the resulting residue was co-evaporated several times with toluene. The residue was dissolved in CH_2Cl_2, filtered, concentrated, and purified by column chromatography (EtOAc/EtOH 20:1 and EtOAc/EtOH/H_2O 45:5:3). Yield: 1.26 g (80%); $[\alpha]_D$ = +11.3 (c = 1.5, CH_2Cl_2); R_f = 0.25 (EtOAc/EtOH/H_2O 45:5:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.63 (br s, 1H; OH), 5.86 (d, $J_{1,2}$ = 3.5 Hz, 1H; H-1), 4.62 (m, 1H; H-6a), 4.51 (d, 1H; H-2), 4.49 (dd, $J_{6a,6b}$ = 9.8 Hz, $J_{5,6a}$ = 3.4 Hz, 1H; H-6a), 4.37 (m, 1H; H-5), 4.33 (d, $J_{3,4}$ = 3.1 Hz, 1H; H-3), 4.02 (dd, $J_{4,5}$ = 8.3 Hz, 1H; H-4), 3.18 (t, $^3J_{H,H}$ = 7.3 Hz, 2H; CH_2N), 1.51 (m, 2H; CH_2CH_2N), 1.42, 1.24 (2s, 6H; CMe_2), 1.23 (m, 10H; CH_2), 0.81 (t, $^3J_{H,H}$ = 6.9 Hz, 3H; CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 161.9 (CN), 111.9 (CMe_2), 105.5 (C-1), 85.6 (C-2), 82.5 (C-4), 73.6 (C-3), 70.7 (C-6), 56.1 (C-5), 42.9 (CH_2N), 31.7, 29.4, 29.2, 29.1 (CH_2), 27.0, 26.6 (CMe_2), 22.6 (CH_2CH_3), 14.1 (CH_3); CIMS: m/z 357 $[M+H]^+$; HRCIMS: calcd. for $C_{18}H_{33}N_2O_5$: 357.2389, found 357.2389.

5-*N*,6-*O*-(*N'*-Octyliminomethylidene)nojirimycin (NOI-NJ, 1): The D-glucofuranose precursor **9** (1.09 g, 3.06 mmol) was treated with TFA/H_2O (9:1, 10 mL) for 2 min at 0 °C, concentrated under reduced pressure, coevaporated several times with water, neutralized with Amberlite IRA-68 (OH^-) ion-exchange resin, and subjected to column chromatography (MeCN → MeCN/H_2O 50:1 → MeCN/H_2O 10:1) to give **1** (823 mg, 85%) as an amorphous solid. R_f = 0.39 (MeCN/H_2O/NH_4OH 10:1:1); $[\alpha]_D$ = −5.0 (c = 1.0 in H_2O); ^{1}H NMR (500 MHz, D_2O): δ = 5.74 (d, $J_{1,2}$ = 3.8 Hz, 1H; H-1), 5.22 (t, $J_{6a,6b}$ = $J_{5,6a}$ = 8.9 Hz, 1H; H-6a), 4.90 (t, $J_{5,6b}$ = 8.9 Hz, 1H; H-6b), 4.45 (dt, $J_{4,5}$ = 9.5 Hz, 1H; H-5), 3.95 (t, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, 1H; H-3), 3.83 (dd, 1H; H-2), 3.80 (t, 1H; H-4), 3.57 (td, $^2J_{H,H}$ = 7.1 Hz, $^3J_{H,H}$ = 4.2 Hz, 2H; CH_2N), 1.53 (m, 2H; CH_2CH_2N), 1.24 (m, 10H; 5 CH_2), 0.87 (t, 3H; CH_3); ^{13}C NMR (75.5 MHz, D_2O): δ = 158.7 (CN), 74.9 (C-1), 73.9 (C-2), 73.0 (C-4), 71.9 (C-3), 70.9 (C-6), 56.2 (C-5), 43.2 (CH_2N), 31.2, 28.4, 28.2, 25.8, (5CH_2), 28.3 (CH_2CH_2N), 22.1 (CH_2CH_3), 13.5 (CH_3). FABMS: m/z 317 $[M+H]^+$; elemental analysis calcd (%) for $C_{15}H_{28}N_2O_5$: C 56.94, H 8.92, N 8.85; found: C 56.67, H 8.88, N 8.74.

3-*O*-Benzoyl-1,2-*O*-isopropylidene-6-*O*-trityl-α-D-galactofuranose (11): Trityl chloride (6.5 g, 23 mmol, 1.5 equiv) and DMAP (376 mg, 3.1 mmol, 0.2 equiv) were added to a solution of 3-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-galactofuranose[41] (**10**, 5 g, 15.4 mmol) in CH_2Cl_2 (9.5 mL) and pyridine (25 mL). The solution was stirred at 40 °C under Ar for 18 h, then poured into ice water (10 mL) and the aqueous phase was extracted with CH_2Cl_2 (2×25 mL). The combined organic extracts were washed with iced aqueous AcOH (10%, 2×15 mL), saturated aqueous $NaHCO_3$ (15 mL), dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (EtOAc/petroleum ether 1:4 → 1:3) to give **11** (7.8 g, 90%). R_f = 0.35 (EtOAc/petroleum ether 1:3); $[\alpha]_D$ = 14.6 (c = 1.0 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.00–7.16 (m, 20H; Ph), 5.96 (d, $J_{1,2}$ = 4.0 Hz, 1H; H-1), 5.32 (d, $J_{3,4}$ = 2.5 Hz, 1H; H-3), 4.73 (d, 1H; H-2), 4.41 (dd, $J_{4,5}$ = 6.5 Hz, 1H; H-4), 4.04 (m, 1H; H-5), 3.31 (dd, $J_{6a,6b}$ = 10.0 Hz, $J_{5,6a}$ = 5.0 Hz, 1H; H-6a), 3.27 (dd, $J_{5,6b}$ = 5.5 Hz, 1H; H-6b), 2.69 (d, $J_{OH,5}$ = 5.0 Hz, 1H; OH), 1.57, 1.32 (2s, 6H; CMe_2); ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 165.5 (CO), 143.8–127.0 (Ph), 113.1 (CMe_2), 105.5 (C-1), 86.9 (CPh_3), 86.1 (C-4), 85.3 (C-2), 78.7 (C-3), 69.9 (C-5), 64.9 (C-6), 26.8, 26.1 (CMe_2); IR (KBr): ν_{max} = 3412, 3047, 2991, 1720, 1633, 1490, 1379, 1267, 1069 cm^{-1}; FABMS: m/z 589 $[M+Na]^+$; elemental analysis calcd (%) for $C_{35}H_{34}O_7$: C 74.19, H 6.05; found: C 73.92, H 5.84.

3-*O*-Benzoyl-1,2-*O*-isopropylidene-6-*O*-trityl-β-L-altrofuranose (12): Pyridine (1.38 mL) and trifluoromethanesulfonic anhydride (2.16 mL, 13.1 mmol) were added to a solution of **11** (4.93 g, 8.72 mmol) in CH_2Cl_2 (23 mL) at −25 °C under nitrogen. The reaction mixture was allowed to reach RT and further stirred for 30 min. The mixture was diluted with CH_2Cl_2 (15 mL), washed with iced saturated aqueous $NaHCO_3$ (15 mL), dried ($MgSO_4$), and concentrated. The resulting triflate ester was dissolved in DMF (22 mL), $NaNO_2$ (2.71 g, 39.3 mmol) was added and the reaction mixture was stirred at RT under Ar for 18 h. The solvent was removed under reduced pressure and the resulting residue was dissolved in CH_2Cl_2 (15 mL) and washed with water (8 mL). The organic extract was dried ($MgSO_4$), concentrated and the residue was purified by column chromatography (EtOAc/toluene 1:20 → 1:10) to give **12** (2.24 g, 46%); R_f = 0.64 (EtOAc/toluene 1:10); $[\alpha]_D$ −6.7 (c = 1.0 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.07–7.18 (m, 20H; Ph), 6.00 (d, $J_{1,2}$ = 3.9 Hz, 1H; H-1), 5.68 (d, $J_{3,4}$ = 1.3 Hz, 1H; H-3), 4.61 (d, 1H; H-2), 4.42 (dd, $J_{4,5}$ = 8.7 Hz, 1H; H-4), 4.18 (m, 1H; H-5), 3.46 (dd, $J_{6a,6b}$ = 9.5 Hz, $J_{5,6a}$ = 3.7 Hz, 1H; H-6a), 3.40 (dd, $J_{5,6b}$ = 5.5 Hz, 1H; H-6b), 2.74 (d, $J_{OH,5}$ = 5.4 Hz, 1H; OH), 1.58, 1.36 (2s, 6H; CMe_2); ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 165.6 (CO), 143.8–125.3 (Ph), 112.7 (CMe_2), 106.0 (C-1), 86.9 (CPh_3), 85.9 (C-4), 85.0 (C-2), 78.3 (C-3), 70.4 (C-5), 64.2 (C-6), 26.8, 25.9 (CMe_2); IR (KBr) ν_{max} = 3524, 3059, 2988, 1723, 1601, 1480, 1379, 1267, 1092 cm^{-1}; FABMS: m/z 589 $[M+Na]^+$; elemental analysis calcd (%) for $C_{35}H_{34}O_7$: C 74.19, H 6.05; found: C 74.19, H 5.81.

5-Azido-3-*O*-benzoyl-5-deoxy-1,2-*O*-isopropylidene-α-D-galacto-furanose (13): Pyridine (0.65 mL) and trifluoromethanesulfonic anhydride (0.91 mL, 5.54 mmol) were added to a solution of **12** (1.86 g, 3.69 mmol) in CH_2Cl_2 (11 mL) at −25 °C under nitrogen. The reaction mixture was allowed to reach RT and further stirred for 30 min. The mixture was diluted with CH_2Cl_2 (10 mL), washed with

iced saturated aqueous $NaHCO_3$ (10 mL), dried ($MgSO_4$), and concentrated. The resulting triflate ester was dissolved in DMF (16 mL), NaN_3 (1.2 g, 18.5 mmol) was added and the reaction mixture was stirred at RT under Ar for 18 h. The solvent was removed under diminished pressure and the resulting residue was dissolved in CH_2Cl_2 (15 mL) and washed with water (10 mL). The organic extract was dried ($MgSO_4$) and evaporated. The resulting product was dissolved in CH_2Cl_2 (27 mL) at 0 °C and BF_3/Et_2O (1.08 mL) and MeOH (5.4 mL) were added. The reaction mixture was allowed to reach RT and stirred for 2 h, diluted with CH_2Cl_2 (10 mL), then washed with saturated aqueous $NaHCO_3$ (2×8 mL), dried ($MgSO_4$), and concentrated. The resulting residue was purified by column chromatography (EtOAc/petroleum ether 1:5→1:2) to give **13** (771 mg, 60%); R_f 0.45 (EtOAc/petroleum ether1:2): $[\alpha]_D = -23.9$ ($c = 0.7$ in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.06$–7.45 (m, 5H; Ph), 6.10 (d, $J_{1,2} = 4.0$ Hz, 1H; H-1), 5.39 (d, $J_{3,4} = 1.7$ Hz, 1H; H-3), 4.88 (d, 1H; H-2), 4.33 (dd, $J_{4,5} = 9.2$ Hz, 1H; H-4), 3.95 (m, 2H, H-5; H-6a), 3.86 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6b} = 5.6$ Hz, 1H; H-6b), 2.60 (brs, 1H; OH), 1.65, 1.39 (2s, 6H; CMe_2); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 166.1$ (CO), 133.9–128.6 (Ph), 113.2 (CMe_2), 105.8 (C-1), 86.3 (C-4), 84.4 (C-2), 78.2 (C-3), 63.5 (C-5), 62.7 (C-6), 26.7, 25.8 (CMe_2); IR (KBr) $\nu_{max} =$ 3507, 3031, 2988, 2101, 1723, 1381, 1269, 1113 cm^{-1}; FABMS: *m/z* 372 $[M+Na]^+$; elemental analysis calcd (%) for $C_{16}H_{19}N_3O_6$: C 55.01, H, 5.48, N 12.03; found: C 54.96, H 5.38, N 11.71.

5-Azido-3-*O*-benzoyl-5,6-dideoxy-6-iodo-1,2-*O*-isopropylidene-α-D-galactofuranose (14): I_2 (1.4 g, 5.52 mmol, 2.0 equiv) was slowly added to a solution of PPh_3 (868 mg, 3.31 mmol, 1.2 equiv) and imidazole (470 mg, 6.9 mmol, 2.5 equiv) in dry toluene (29 mL) heated at 50 °C. After stirring for 30 min, a solution of **13** (963 mg, 2.76 mmol) in toluene (9.7 mL) was added dropwise and the reaction mixture was stirred at 70 °C for 2 h. The solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (1:4 EtOAc/petroleum ether) to give **14** (1.06 g, 84%); $R_f = 0.56$ (EtOAc/petroleum ether 1:3); $[\alpha]_D = -12.6$ ($c = 1.0$ in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.07$–7.28 (m, 5H; Ph), 6.05 (d, $J_{1,2} = 4.0$ Hz, 1H; H-1), 5.34 (d, $J_{3,4} = 2.6$ Hz, 1H; H-3), 4.85 (d, 1H; H-2), 4.21 (dd, $J_{4,5} = 7.8$ Hz, 1H; H-4), 3.98 (ddd, $J_{5,6b} =$ 8.1 Hz, $J_{5,6a} = 4.2$ Hz, 1H; H-5), 3.54 (dd, $J_{6a,6b} = 10.8$ Hz, 1H; H-6a), 3.23 (dd, 1H; H-6b), 1.66, 1.39 (2s, 6H; CMe_2); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 165.6$ (CO), 133.8–128.6 (Ph), 113.6 (CMe_2), 105.6 (C-1), 87.2 (C-4), 84.8 (C-2), 78.0 (C-3), 63.5 (C-5), 26.8, 26.0 (CMe_2), 3.4 (C-6); IR (KBr) $\nu_{max} = 3031$, 2936, 2113, 1724, 1602, 1472, 1376, 1263, 1111, 1024 cm^{-1}; FABMS: *m/z* 444 $[M-15]^+$; elemental analysis calcd (%) for $C_{16}H_{18}IN_3O_5$: C 41.85, H 3.95; N 9.15; found: C 41.72, H 3.90, N 9.01.

5,6-Diazido-3-*O*-benzoyl-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-galactofuranose (15): NaN_3 (224 mg, 3.44 mmol) was added to a solution of **14** (316 mg, 0.69 mmol) in DMF (7 mL), and the reaction mixture was stirred at 80 °C under Ar for 12 h. The solvent was removed under reduced pressure and the resulting residue was dissolved in CH_2Cl_2 (20 mL) and washed with water (10 mL). The organic extract was dried ($MgSO_4$), filtered and concentrated. The resulting residue was purified by column chromatography (EtOAc/petroleum ether 1:4) to give **26** (258 mg, 95%); $R_f = 0.52$ (EtOAc/petroleum ether 1:3); $[\alpha]_D = -38.4$ ($c = 1.0$ in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.06$–7.46 (m, 5H; Ph), 6.07 (d, $J_{1,2} = 4.0$ Hz, 1H; H-1), 5.33 (d, $J_{3,4} = 2.3$ Hz, 1H; H-3), 4.86 (d, 1H; H-2), 4.19 (dd, $J_{4,5} = 8.4$ Hz, 1H; H-4), 4.00 (ddd, $J_{5,6b} = 7.4$ Hz, $J_{5,6a} = 4.0$ Hz, 1H; H-5), 3.67 (dd, $J_{6a,6b} = 13.0$ Hz, 1H; H-6a), 3.47 (dd, 1H; H-6b), 1.66, 1.39 (2s, 6H; CMe_2); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 165.6$ (CO), 133.8–128.6 (Ph), 113.5 (CMe_2), 105.7 (C-1), 85.7 (C-4), 84.6 (C-2), 77.8 (C-3), 62.0 (C-5), 51.8 (C-6), 26.8, 25.9 (CMe_2); IR (KBr) $\nu_{max} =$ 3030, 2989, 2102, 1723, 1602, 1450, 1374, 1265, 1112, 1024 cm^{-1}; FABMS: *m/z* 359 $[M-15]^+$; elemental analysis calcd (%) for $C_{16}H_{18}N_6O_5$: C 51.33, H, 4.85, N 22.45, found: C 51.09, H 4.65, N, 21.99.

5,6-Diamino-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-galactofuranose 5,6-(cyclic thiourea) (16): Methanolic NaMeO (1 M, 0.1 equiv per mol of acetate) was added to a solution of **15** (258 mg, 0.69 mmol) in dry MeOH (6 mL). The reaction mixture was stirred at RT for 1 h, then neutralized with Amberlite IRA-120 (H^+) ion-exchange resin, concentrated, and the resulting residue was purified by column chromatography using EtOAc/petroleum ether 1:1 as eluent. A solution of the resulting solid (166 mg, 0.61 mmol) in MeOH (3.3 mL) was hydrogenated at atmospheric pressure for 1 h by using 10% Pd/C (117 mg) as catalyst. The suspension was filtered through Celite and concentrated to give the corresponding diamine that was used in the next step without further purification. CS_2 (0.3 mL, 4.88 mmol, 8 equiv) and DCC (138 mg, 0.67 mmol, 1.1 equiv) were added to a solution of the diamine derivative in CH_2Cl_2 (7 mL). The reaction mixture was stirred for 1 h and then concentrated. The resulting residue was purified by column chromatography (CH_2Cl_2/MeOH 30:1→10:1) to give **16** (124 mg, 69%); $R_f = 0.44$ (CH_2Cl_2/MeOH 10:1); $[\alpha]_D = -123.8$ ($c = 1.0$ in MeOH); ^{1}H NMR (300 MHz, CD_3OD): $\delta = 5.88$ (d, $J_{1,2} = 3.7$ Hz, 1H; H-1), 4.51 (d, 1H; H-2), 4.21 (ddd, $J_{5,6a} = 10.1$ Hz, $J_{4,5} = 9.1$ Hz, $J_{5,6b} = 6.4$ Hz, 1H; H-5), 4.00 (d, $J_{3,4} = 2.2$ Hz, 1H; H-3), 3.86 (dd, 1H; H-4), 3.76 (t, $J_{6a,6b} = 10.1$ Hz, 1H; H-6a), 3.47 (dd, 1H; H-6b), 1.66, 1.39 (2s, 6H; CMe_2); ^{13}C NMR (75.5 MHz, CD_3OD): $\delta = 183.6$ (CS), 113.9 (CMe_2), 107.1 (C-1), 90.3 (C-4), 88.4 (C-2), 76.5 (C-3), 59.9 (C-5), 47.7 (C-6), 27.3, 26.2 (CMe_2); IR (KBr) $\nu_{max} = 3379$, 2989, 1634, 1510, 1381, 1292, 1198, 1068 cm^{-1}; UV (CH_2Cl_2) 242 nm (ε_{mM} 16.8); FABMS: *m/z* 261 $[M+H]^+$; elemental analysis calcd (%) for $C_{10}H_{16}N_2O_4S$: C 46.14, H 6.20, N, 10.76; found: C 45.98; H 6.26, N 10.54.

5,6-Diamino-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-galactofuranose 5,6-(cyclic *S*-methylthiouronium) iodide (17): A solution of **16** (127 mg, 0.49 mmol) and methyl iodide (153 μL, 5 equiv) in MeOH (5 mL) was heated under reflux (70 °C) during 2 h and concentrated. The resulting residue was purified by column chromatography using 70:10:1 CH_2Cl_2/MeOH-H_2O as eluent to give **17** (181 mg, 91%); $R_f = 0.36$ (CH_2Cl_2/MeOH 40:10:1); $[\alpha]_D = -97.8$ ($c =$ 1.0 in MeOH); ^{1}H NMR (300 MHz, CD_3OD): $\delta = 5.91$ (d, $J_{1,2} = 3.7$ Hz, 1H; H-1), 4.55 (m, 1H; H-5), 4.54 (d, 1H; H-2), 4.10 (t, $J_{6a,6b} = J_{5,6a} =$ 11.0 Hz, 1H; H-6a), 4.08 (d, $J_{3,4} = 2.7$ Hz, 1H; H-3), 3.94 (dd, $J_{4,5} =$ 8.7 Hz, 1H; H-4), 3.77 (dd, $J_{5,6b} = 6.8$ Hz, 1H; H-6b), 2.66 (s, 3H; SMe), 1.50, 1.31 (2s, 6H; CMe_2); ^{13}C NMR (75.5 MHz, CD_3OD): $\delta =$ 172.8 (SCN), 114.1 (CMe_2), 107.1 (C-1), 89.5 (C-4), 88.4 (C-2), 76.2 (C-3), 61.3 (C-5), 49.2 (C-6), 27.4, 26.3 (CMe_2), 14.0 (SMe); IR (KBr) $\nu_{max} = 3365$, 2936, 1554, 1378, 1215, 1026 cm^{-1}; FABMS: *m/z* 297 $[M-I+Na]^+$, 275 $[M-I]^+$); elemental analysis calcd (%) for $C_{11}H_{19}IN_2O_4S$: C 32.84, H 4.76, N 6.96; found: C 32.81, H 4.72, N 6.82.

5,6-Diamino-5,6-dideoxy-1,2-*O*-isopropylidene-5,6-di-*N*-(*N'*-octyliminomethylidene)-α-D-galactofuranose hydrochloride (18): A solution of **17** (181 mg, 0.45 mmol) and *n*-octylamine, (1.5 equiv) in DMF (10 mL) was heated at 70 °C under Ar during 18 h and concentrated. The resulting residue was purified by column chromatography (CH_2Cl_2/MeOH/H_2O 90:10:1 → 80:10:1) and freeze dried from an aqueous HCl solution (pH 5) to give **18** (138 mg, 78%), $R_f = 0.38$ (CH_2Cl_2/MeOH/H_2O 70:10:1); $[\alpha]_D = -27.3$ ($c = 1.0$ in MeOH); ^{1}H NMR (300 MHz, CD_3OD): $\delta = 5.93$ (d, $J_{1,2} = 3.7$ Hz, 1H; H-1), 4.57 (d, 1H; H-2), 4.31 (m, 1H; H-5), 4.08 (d, $J_{3,4} = 2.5$ Hz, 1H; H-3), 3.89 (dd, $J_{4,5} = 9.0$ Hz, 1H; H-4), 3.87 (t, $J_{6a,6b} = J_{5,6a} = 10.0$ Hz, 1H; H-6a), 3.23 (t, $J_{5,6b} = 10.0$ Hz, 1H; H-6b), 3.23 (t, $^3J_{H,H} = 7.1$ Hz, 2H;

CH_2N), 1.60 (m, 2H; CH_2CH_2N), 1.53, 1.34 (2s, 6H; CMe_2), 1.35 (m, 10H; CH_2), 0.92 (t, $^3J_{H,H}=7.0$ Hz, 3H; CH_3); ^{13}C NMR (75.5 MHz, CD_3OD): $\delta=160.4$ (CN), 114.0 (CMe_2), 107.1 (C-1), 89.9 (C-4), 88.4 (C-2), 76.3 (C-3), 58.1 (C-5), 46.1 (C-6), 43.9 (CH_2N), 32.9, 30.3, 30.2, 30.1 (CH_2), 27.6 (CH_2), 27.4, 26.2 (CMe_2), 23.6 (CH_2CH_3), 14.4 (CH_3); IR (KBr) $\nu_{max}=3401$, 2924, 1673, 1592, 1462, 1378, 1215, 1015 cm^{-1}; FABMS: *m/z* 356 $[M+H]^+$; elemental analysis calcd (%) for $C_{18}H_{34}ClN_3O_4\cdot H_2O$: C 52.74, H 8.85, N 10.25; found: C 52.61, H 8.62, N 10.24.

6-Amino-6-deoxy-5,6-di-*N*-(*N'*-octyliminomethylidene)galactono-jirimycin hydrochloride (6N-NOI-GNJ, 6): A solution of **18** (0.41 mmol) in 90% TFA–H_2O (1.7 mL) was stirred at 0°C for 1 h, concentrated under reduced pressure, coevaporated several times with water, treated with NaOH 0.1 N until it reached pH 8, subjected to column chromatography ($MeCN/H_2O/NH_4OH$ 4:1:1), then to GPC (Sephadex G-10, 1:1 MeOH/H_2O) and finally freeze dried from an aqueous HCl solution (pH 5) to give **6** (101 mg, 70%) as an amorphous solid. $R_f=0.33$ ($MeCN/H_2O/NH_4OH$ 4:1:1); $[\alpha]_D=-1.9$ ($c=1.0$, H_2O); $[\alpha]_{578}=-3.9$ ($c=1.0$ in H_2O); ^1H NMR (500 MHz, D_2O): $\delta=5.42$ (d, $J_{1,2}=4.0$ Hz, 1H; H-1), 4.28 (t, $J_{5,6a}=J_{5,6b}=9.8$ Hz, 1H; H-5), 4.01 (br s, 1H; H-4), 3.85 (dd, $J_{2,3}=10.3$ Hz, $J_{3,4}=2.8$ Hz, 1H; H-3), 3.77 (dd, 1H; H-2), 3.72 (t, $J_{6a,6b}=9.8$ Hz, 1H; H-6a), 3.59 (t, 1H; H-6b), 3.18 (t, $^3J_{H,H}=7.0$ Hz, 2H; CH_2N), 1.53 (m, 2H; CH_2CH_2N), 1.23 (m, 10H; CH_2), 0.80 (t, $^3J_{H,H}=7.0$ Hz, 3H; CH_3); ^{13}C NMR (125.7 MHz, D_2O): $\delta=155.9$ (CN), 74.1 (C-1), 69.2 (C-3), 68.1 (C-4), 67.8 (C-2), 55.7 (C-5), 42.9 (CH_2N), 41.7 (C-6), 31.0, 28.3, 28.2, 28.0, 25.7 (CH_2), 22.0 (CH_2CH_3), 13.4 (CH_3); FABMS: *m/z* 316 $[M+H-Cl]^+$); elemental analysis calcd (%) for $C_{15}H_{30}ClN_3O_4\cdot H_2O$: C 48.71, H 8.72, N 11.36; found: C 48.84, H 8.72, N 11.25.

General procedure for the inhibition assay against the commercial enzymes: Inhibitory potencies were determined by spectrophotometrically measuring the residual hydrolytic activities of the glycosidases against the respective *o*- (for cytosolic β-glucosidase/β-galactosidase from bovine liver and β-galactosidase from *E. coli*) or *p*-nitrophenyl α- or β-D-glycopyranoside or α,α'-trehalose (for trehalase), in the presence of the corresponding guanidine derivative. Each assay was performed in phosphate buffer at the optimal pH for each enzyme. The K_M values for the different glycosidases used in the tests and the corresponding working pHs are listed herein: α-glucosidase (yeast), $K_M=0.35$ mM (pH 6.8); isomaltase (yeast) $K_M=1.0$ mM (pH 6.8), β-glucosidase (almonds), $K_M=3.5$ mM (pH 7.3); β-glucosidase/β-galactosidase (bovine liver), $K_M=2.0$ mM (pH 7.3); β-galactosidase (*E. coli*), $K_M=0.12$ mM (pH 7.3); α-galactosidase (coffee beans), $K_M=2.0$ mM (pH 6.8); trehalase (pig kidney), $K_M=4.0$ mM (pH 6.2); naringinase (*Penicillium decumbens*), $K_M=2.7$ mM (pH 6.8); α-mannosidase (Jack bean), $K_M=2.0$ mM (pH 5.5); β-mannosidase (*Helix pomatia*), $K_M=0.57$ mM (pH 5.5). The reactions were initiated by addition of enzyme to a solution of the substrate in the absence or presence of various concentrations of inhibitor. After the mixture was incubated for 10–30 min at 37°C or 55°C the reaction was quenched by addition of Na_2CO_3 (1 M) or a solution of Glc-Trinder (Sigma, for trehalase). The absorbance of the resulting mixture was determined at 405 nm or 505 nm. The K_i value and enzyme inhibition mode were determined from the slope of Lineweaver–Burk plots and double reciprocal analysis. Representative examples of the Lineweaver–Burk plots, with typical profiles for competitive inhibition mode, are shown in the Supporting Information (Figure 19S-22S).

Cell cultures: Human skin fibroblasts were cultured in DMEM/FBS (10%) at 37°C in a humidified atmosphere containing CO_2 (5%). For fibroblasts, we used three control cell lines (H8, H22, and H37) and seven lines of GD cells. Six cell lines carried β-Glu mutations of F213I/F213I, G202R/L444P, N188S/G193W, F213 L444P, L444P/RecNcil and L444P/L444P. These cells were from Japanese patients. The other line of GD cells that carried the N370S homozygous mutation was from Caucasian patients (a gift from Prof. C. Kaneski and R. O. Brady). Culture medium was replaced every 2 days with fresh media supplemented with or without compounds at the indicated concentrations. In all cases, informed, signed consent was obtained from either the patient or next of kin.

In vitro enzyme assay: Lysosomal enzyme activities in cell lysates were determined as described.[58,62–64] Briefly, cells were scraped into ice-cold H_2O (10^6 cells mL^{-1}) and lysed by sonication. Insoluble materials were removed by centrifugation at 15000 rpm for 5 min and protein concentrations were determined with a BCA microprotein assay kit (Pierce). The lysates (10 μL) were incubated at 37°C with the substrate solution (20 μL) in citrate buffer (0.1 M, pH 4.5). The substrates were 4-methylumbelliferone-conjugated α-D-glucopyranoside (for α-glucosidase), α-D-galactopyranoside (for α-galactosidase), β-D-galactopyranoside (for β-galactosidase), and *N*-acetyl-β-D-glucosaminide (for β-hexosaminidase). β-Glu activities in cell lysates were determined by using 4-methylumbellifer one-conjugated β-D-glucopyranoside as a substrate. The lysates (10 μL) were incubated at 37°C with the substrate solution (20 μL) in citrate buffer (0.1 M, pH 5.2 or pH 7), supplemented with sodium taurocholate (0.8% *w/v*). The reactions were terminated by adding glycine sodium hydroxide buffer (0.2 mL, 0.2 M, pH 10.7). The liberated 4-methylumbelliferone was measured with a Perkin–Elmer Luminescence Spectrometer (λ_{ex}: 340 nm; λ_{em}: 460 nm). One unit of enzyme activity was defined as nmol of 4-methylumbelliferone released per hour and normalized for the amount of protein contained in the lysates.

In situ cell enzyme assay: β-Glu activities in live cells were estimated by the methods described by Sawkar et al.[26] with modification. Briefly, cells in 96-well assay plates were treated with compounds for 4 days. After washing with PBS, the cells were incubated in PBS (8 μL) and acetate buffer (8 μL, 0.2 M, pH 4.0). The reaction was started by addition 4-methylumbelliferyl-β-D-glucoside (10 μL, 5 mM), followed by incubation at 37°C for 1 h. The reaction was stopped by lysing the cells by the addition of glycine buffer (200 μL, 0.2 M, pH 10.7) and the liberated 4-methylumbelliferone was quantified. Every experiment was performed in parallel with cells that had been preincubated with or without conduritol B epoxide (0.5 mM, CBE, Toronto Research Chemicals, Canada) for 1 h. The CBE-sensitive component was ascribed to lysosomal β-Glu, whereas the CBE-insensitive component was ascribed to nonlysosomal β-Glu.

Cytotoxicity assay: The cytotoxicity assay was performed by using the colorimetric assay reagent TetraColor One (Seikagaku, Tokyo, Japan),[65] according to the manufacturer's instructions. Fibroblasts were seeded on a 96-well assay plate at a density of 3.0×10^4 cells mL^{-1} medium and incubated for 4 days with chaperones. Then, the TetraColor One reagent (10 μL) was added to each well, and cells were further incubated for 2 h. The absorbance at 450 nm was then measured with a reference wavelength at 630 nm in the microplate reader. Measurements were repeated in triplicate and then averaged for each sample.

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[1] E. Beutler, G. A. Grabowski in *The Metabolic and Molecular Bases of Inherited Disease* (Eds.: C. R. Scriver, W. S. Sly, B. Childs, A. L. Beaudet), McGraw–Hill, New York, **2001**, pp. 2641–2670.
[2] M. Jmoudiak, A. H. Futerman, *Br. J. Haematol.* **2005**, *129*, 178–188.
[3] A. H. Futerman, G. van Meer, *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 554–565.
[4] T. D. Butters, *Curr. Opin. Chem. Biol.* **2007**, *11*, 412–418.
[5] E. Beutler, *PLoS Med.* **2004**, *1*, e21.
[6] D. Aviezer, E. Brill-Almon, Y. Shaaltiel, S. Hashmueli, D. Bartfeld, S. Mizrachi, Y. Liberman, A. Freeman, A. Zimran, E. Galun, *PLoS ONE* **2009**, *4*, e4792.
[7] T. D. Butters, R. A. Dwek, F. M. Platt, *Glycobiology* **2005**, *15*, 43R–52R.
[8] N. W. Barton, R. O. Brady, J. M. Dambrosia, A. M. Di Bisceglie, S. H. Doppelt, S. C. Hill, H. J. Mankin, G. J. Murray, R. I. Parker, C. E. Argoff, R. P. Grewal, K. T. Yu, *N. Engl. J. Med.* **1991**, *324*, 1464–1470.
[9] F. M. Platt, G. R. Neises, R. A. Dwek, T. D. Butters, *J. Biol. Chem.* **1994**, *269*, 27108–27114.
[10] T. Cox, R. Lachmann, C. Hollak, J. Aerts, S. van Weely, M. Hrebicek, F. Platt, T. Butters, R. Dwek, C. Moyses, I. Gow, D. Elstein, A. Zimran, *Lancet* **2000**, *355*, 1481–1485.
[11] G. A. Grabowski, N. Leslie, R. Wenstrup, *Blood Rev.* **1998**, *12*, 115–133.
[12] F. M. Platt, M. Jeyakumar, U. Andersson, D. A. Priestman, R. A. Dwek, T. D. Butters, *J. Inherited Metab. Dis.* **2001**, *24*, 275–290.
[13] R. Schiffmann, M. P. Heyes, J. M. Aerts, J. M. Dambrosia, M. C. Patterson, T. DeGraba, C. C. Parker, G. C. Zirzow, K. Oliver, G. Tedeschi, R. O. Brady, N. W. Barton, C. Nagineni, C. R. Kaneski, G. J. Murray, J. J. Higgins, A. Tournay, T. K. Banerjee, C. Kreps, L. J. C. Scott, M. A. McKee, K. Crutchfield, K. Frei, *Ann. Neurol.* **1997**, *42*, 613–621.
[14] C. A. Prows, N. Sánchez, C. Daugherty, G. A. Grabowski, *Am. J. Med. Genet.* **1997**, *71*, 16–21.
[15] M. Aoki, Y. Takahashi, Y. Miwa, S. Iida, K. Sukegawa, T. Horai, T. Orii, N. Kondo, *Eur. J. Pediatr.* **2001**, *160*, 63–64.
[16] J. M. Aerts, C. E. Hollak, R. G. Boot, J. E. Groener, M. Maas, *J. Inherited Metab. Dis.* **2006**, *29*, 449–456.
[17] I. Ron, M. Horowitz, *Hum. Mol. Genet.* **2005**, *14*, 2387–2398.
[18] Z. Q. Yu, A. R. Sawkar, J. W. Kelly, *FEBS J.* **2007**, *274*, 4944–4950.
[19] J. Q. Fan, *Trends Pharmacol. Sci.* **2003**, *24*, 355–360.
[20] H. Lin, Y. Sugimoto, Y. Ohsaki, H. Ninomiya, A. Oka, M. Taniguchi, H. Ida, Y. Eto, S. Ogawa, Y. Matsuzaki, M. Sawa, T. Inoue, K. Higaki, E. Nanba, K. Ohno, Y. Suzuki, *Biochim. Biophys. Acta Mol. Basis Dis.* **2004**, *1689*, 219–228.
[21] K. Lei, H. Ninomiya, M. Suzuki, T. Inoue, M. Sawa, M. Iida, H. Ida, Y. Eto, S. Ogawa, K. Ohno, Y. Suzuki, *Biochim. Biophys. Acta Mol. Basis Dis.* **2007**, *1772*, 587–596.
[22] G. J. Kornhaber, M. B. Tropak, G. H. Maegawa, S. J. Tuske, S. J. Coales, D. J. Mahuran, Y. Hamuro, *ChemBioChem* **2008**, *9*, 2643–2649.
[23] M. B. Tropak, G. J. Kornhaber, B. A. Rigat, G. H. Maegawa, J. D. Buttner, J. E. Blanchard, C. Murphy, S. J. Tuske, S. J. Coales, Y. Hamuro, E. D. Brown, D. J. Mahuran, *ChemBioChem* **2008**, *9*, 2650–2652.
[24] P. Compain, O. R. Martin, C. Boucheron, G. Godin, L. Yu, K. Ikeda, N. Asano, *ChemBioChem* **2006**, *7*, 1356–1359.
[25] L. Yu, K. Ikeda, A. Kato, I. Adachi, G. Godin, P. Compain, O. R. Martin, N. Asano, *Bioorg. Med. Chem.* **2006**, *14*, 7736–7744.
[26] A. R. Sawkar, W. C. Cheng, E. Beutler, C.-H. Wong, W. E. Balch, J. W. Kelly, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15428–15433.
[27] A. R. Sawkar, S. L. Adamski-Werner, W. C. Cheng, C.-H. Wong, E. Beutler, K. P. Zimmer, J. W. Kelly, *Chem. Biol.* **2005**, *12*, 1235–1244.
[28] For a recent monograph, see *Iminosugars: From Synthesis to Therapeutic Applications* (Eds.: P. Compain, O. R. Martin), Wiley-VCH, Weinheim, **2007**.
[29] J. L. Jiménez Blanco, V. M. Díaz Pérez, C. Ortiz Mellet, J. Fuentes, J. M. García Fernández, *Chem. Commun.* **1997**, 1969–1970.
[30] V. M. Díaz Pérez, M. I. García-Moreno, C. Ortiz Mellet, J. Fuentes, J. C. Díaz Arribas, F. J. Cañada, J. M. García Fernández, *J. Org. Chem.* **2000**, *65*, 136–143.
[31] M. I. García-Moreno, P. Díaz-Pérez, C. Ortiz Mellet, J. M. García Fernández, *Chem. Commun.* **2002**, 848–849.
[32] M. I. García-Moreno, P. Díaz-Pérez, C. Ortiz Mellet, J. M. García Fernández, *J. Org. Chem.* **2003**, *68*, 8890–8901.
[33] P. Díaz-Pérez, M. I. García-Moreno, C. Ortiz Mellet, J. M. García Fernández, *Eur. J. Org. Chem.* **2005**, 2903–2913.
[34] M. Aguilar, P. Díaz-Pérez, M. I. García-Moreno, C. Ortiz Mellet, J. M. García Fernández, *J. Org. Chem.* **2008**, *73*, 1995–1998.
[35] E. Sánchez-Fernández, R. Rísquez-Cuadro, M. Aguilar-Moncayo, M. I. García-Moreno, C. Ortiz Mellet, J. M. García Fernández, *Org. Lett.* **2009**, *11*, 3306–3309.
[36] J. M. García Fernández, C. Ortiz Mellet, M. I. García-Moreno, M. Aguilar-Moncayo, Y. Suzuki, K. Ohno, ES Patent P200802988.
[37] M. Aguilar-Moncayo, T. M. Gloster, J. P. Turkenburg, M. I. García-Moreno, C. Ortiz Mellet, G. J. Davies, J. M. García Fernández, *Org. Biomol. Chem.* **2009**, *7*, 2738–2747.
[38] B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, *Nucleic Acids Res.* **2009**, *37*, D233–D238.
[39] B. Brumshtein, M. Aguilar-Moncayo, M. I. García-Moreno, C. Ortiz Mellet, J. M. García Fernández, I. Silman, Y. Shaaltiel, D. Aviezer, J. L. Sussman, A. H. Futerman, *ChemBioChem* **2009**, *10*, 1480–1485.
[40] K. Dax, B. Caigg, V. Grassberger, B. Kölblinger, A. E. Stütz, *J. Carbohydr. Chem.* **1990**, *9*, 479–499.
[41] H. Wang, J. A. Ning, *J. Org. Chem.* **2003**, *68*, 2521–2524.
[42] P. J. Garegg, B. Samuelsson, *J. Chem. Soc. Perkin Trans. 1* **1980**, 2866–2869.
[43] For a review, see P. Molina, M. J. Vilaplana, *Synthesis* **1994**, 1197–1218.
[44] G. Legler, S. Pohl, *Carbohydr. Res.* **1986**, *155*, 119–129.
[45] R. A. Steet, S. Chung, B. Wustman, A. Powe, H. Do, S. A. Kornfeld, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 13813–13818.
[46] S. Ogawa, M. Ashiura, C. Uchida, S. Watanabe, C. Yamazaki, K. Yamagishi, J. Inokuchi, *Bioorg. Med. Chem. Lett.* **1996**, *6*, 929–932.
[47] Z. Q. Yu, A. R. Sawkar, L. J. Whalen, C.-H. Wong, J. W. Kelly, *J. Med. Chem.* **2007**, *50*, 94–100.
[48] G.-N. Wang, G. Reinkensmeier, S.-W. Zhang, J. Zhou, L.-R. Zhang, L.-H. Zhang, T. D. Butters, X.-S. Ye, *J. Med. Chem.* **2009**, *52*, 3146–3149.
[49] H. Dvir, M. Harel, A. A. McCarthy, L. Toker, I. Silman, A. H. Futerman, J. L. Sussman, *EMBO Rep.* **2003**, *4*, 704–709.
[50] H. H. Chang, N. Asano, S. Ishii, Y. Ichikawa, J. Q. Fan, *FEBS J.* **2006**, *273*, 4082–4092.
[51] R. Steet, S. Chung, W. S. Lee, C. W. Pine, H. Do, S. Kornfeld, *Biochem Pharmacol.* **2007**, *73*, 1376–1383.
[52] W. Zheng, J. Padia, D. J. Urban, A. Jadhav, O. Goker-Alpan, A. Simeonov, E. Goldin, D. Auld, M .E. LaMarca, J. Inglese, C. P. Austin, E. Sidransky, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 13192–13197.
[53] Y. Eto, H. Ida, *Neurochem. Res.* **1999**, *24*, 207–211.
[54] K. P. Zimmer, P. le Coutre, H. M. Aerts, K. Harzer, M. Fukuda, J. S. O'Brien, H. Y. Naim, *J. Pathol.* **1999**, *188*, 407–414.
[55] E. Beutler, T. Gelbart, D. Balicki, A. Demina, J. Adusumalli, *Proc. Assoc. Am. Physicians* **1996**, *108*, 179–184.
[56] T. W. Mu, D. M. Fowler, J. W. Kelly, *PLoS Biol.* **2008**, *6*, e26.
[57] B. Rigat, D. Mahuran, *Mol. Genet. Metab.* **2009**, *96*, 225–232.
[58] J. Matsuda, O. Suzuki, A. Oshima, Y. Yamamoto, A. Noguchi, K. Takimoto, M. Itoh, Y. Matsuzaki, Y. Yasuda, S. Ogawa, Y. Sakata, E. Nanba, K. Higaki, Y. Ogawa, L. Tominaga, K. Ohno, H. Iwasaki, H. Watanabe, R. O. Brady, Y. Suzuki, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15912–15917.
[59] H. Iwasaki, H. Watanabe, M. Iida, S. Ogawa, M. Tabe, K. Higaki, E. Nanba, Y. Suzuki, *Brain Dev.* **2006**, *28*, 482–486.
[60] J. Fan, S. Ishii, N. Asano, Y. Suzuki, *Nat. Med.* **1999**, *5*, 112–115.
[61] Y. Suzuki, S. Ichinomiya, M. Kurosawa, M. Ohkubo, H. Watanabe, H. Iwasaki, J. Matsuda, Y. Noguchi, K. Takimoto, M. Itoh, M. Tabe, M. Iida, T. Kubo, S. Ogawa, E. Nanba, K. Higaki, K. Ohno, R. O. Brady, *Ann. Neurol.* **2007**, *62*, 671–675.
[62] A. M. Vaccaro, M. Muscillo, M. Tatti, R. Salvioli, E. Gallozzi, K. Suzuki, *Clin. Biochem.* **1987**, *20*, 429–433.

[63] S. Ichisaka, K. Ohno, I. Yuasa, E. Nanba, H. Sakuraba, Y. Suzuki, *Brain Dev.* **1998**, *20*, 302–306.
[64] Y. Suzuki, A. Tsuji, K. Omura, G. Nakamura, S. Awa, M. Kroos, A. J. Reuser, *Clin. Genet.* **1988**, *33*, 376–385.
[65] K. Kawasaki, A. Nishio, H. Nakamura, K. Uchida, T. Fukui, M. Ohana, H. Yoshizawa, S. Ohashi, H. Tamaki, M. Matsuura, M. Asada, T. Nishi, H. Nakase, S. Toyokuni, W. Liu, J. Yodoi, K. Okazaki, T. Chiba, *Lab Instrum.* **2005**, *85*, 1104–1117.

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REVIEW ARTICLE

Gem-diamine 1-*N*-iminosugars as versatile glycomimetics: synthesis, biological activity and therapeutic potential

Yoshio Nishimura

Iminosugars, which carry a basic nitrogen in the carbohydrate ring, have attracted increasing interest as new glycomimetics. *Gem*-diamine 1-*N*-iminosugars, a new class of iminosugars, have a nitrogen atom in place of the anomeric carbon. Various kinds of 1-*N*-iminosugars have been synthesized from glyconolactones as a chiral source in a totally stereospecific manner and/or by the convergent strategy from siastatin B, a secondary metabolite of *Streptomyces*. The protonated form of 1-*N*-iminosugar mimics the charge at the anomeric position in the transition state of enzymatic glycosidic hydrolysis, resulting in a strong and specific inhibition of glycosidases and glycosyltransferases. They have been recently recognized as a new source of therapeutic drug candidates in a wide range of diseases associated with the carbohydrate metabolism of glycoconjugates, such as tumor metastasis, influenza virus infection, lysosomal storage disorder and so forth.
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Keywords: glycosidase inhibitor; heparanase inhibitor; influenza virus infection; lysosomal storage disease; 1-*N*-iminosugar; siastatin B; tumor metastasis

INTRODUCTION

Iminosugars, which are carbohydrate analogs that most frequently carry the nitrogen atom at the position of the endocyclic oxygen, form the most attractive class of glycomimetics reported so far.[1] Several types of iminosugars have been discovered from natural sources[1,2] since nojirimycin[3] was first isolated as an antibiotic from *Streptomyces* culture in 1966. Many more have also been synthesized on the basis of enzymatic glycoside biosynthesis.[1,4] Of late, they have gained remarkable importance not only as molecular tools to unravel the manner in which glycoconjugates regulate various biological functions, but also as new therapeutic agents in a wide range of diseases associated with the metabolism of carbohydrates.

In 1974, siastatin B (1), an unusual iminosugar, was isolated as an inhibitor against neuraminidases (NAs) from *Streptomyces* culture.[5] Siastatin B (1) also inhibits β-D-glucuronidase and *N*-acetyl-β-D-glucosaminidase. We recognized from biological activity that siastatin B (1) structurally resembles D-glucuronic acid (2) and *N*-acetyl-D-glucosamine (3), as well as *N*-acetylneuraminic acid (4) (Figure 1). It is distinct from the known glycosidase inhibitors, such as nojirimycin, that contain a nitrogen atom in place of the ring oxygen. In the course of our study on siastatin B (1), we proposed a new class of glycosidase inhibitors, *gem*-diamine 1-*N*-iminosugars[6–8] (*gem*-diamine 1-aza-carbasugar in IUPAC nomenclature,[9] cyclic methanediamine monosaccharide, 5) in which the anomeric carbon atom is replaced by nitrogen. We hypothesized that the protonated form of *gem*-diamine 1-*N*-iminosugar 6 may mimic the putative glycopyranosyl cation 7 that was formed during enzymatic glycosidic hydrolysis (Figure 2). This turned out to be the case and led to new findings of highly potent and specific inhibitors of glycosidases and glycosyltransferases, as well as potential therapeutics for tumor metastasis and so forth. On the other hand, the synthetic isofagomine (8), another type of 1-iminosugar, was developed by Bols and colleagues in 1994.[10] The isofagomine type 1 iminosugars showed a potent inhibition of their corresponding β-glycosidase.[11] These findings suggest that 1-iminosugars might provide another alternative to the development of therapeutic agents based on the inhibitors of metabolism of glycoconjugates different from the common iminosugars, such as Zavesca (*N*-*n*-butyl-1-deoxynojirimycin)[12] used for the treatment of Gaucher's disease.

This review describes our current progress in the chemistry, biochemistry and pharmacology of *gem*-diamine 1-*N*-iminosugars.

SYNTHESIS

Various types of iminosugar inhibitors, such as polyfunctional piperidines and pyrrolidines, have been designed on the basis of a flattened, half-chair oxocarbenium ion-like transition state in the reaction catalyzed by glycosidases.[13–15] They are all carbohydrate mimics in which the ring oxygen is replaced by nitrogen. On the other hand, 1-*N*-iminosugars have a unique structure with a nitrogen atom in place of the anomeric carbon atom. *Gem*-diamine 1-*N*-iminosugars

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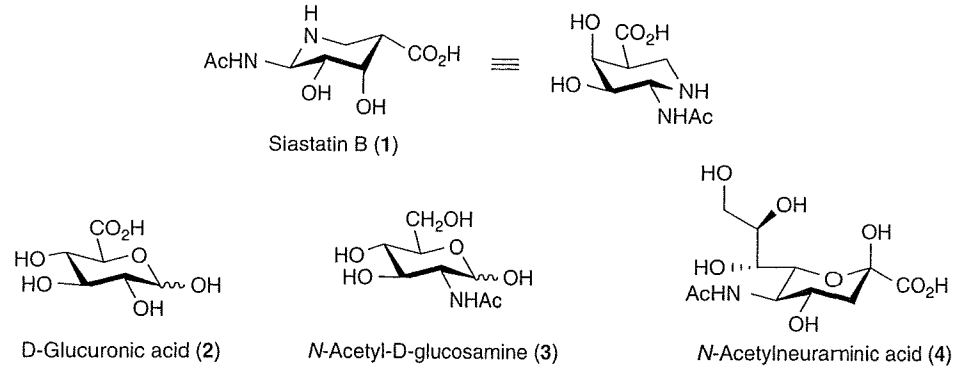


Figure 1 Structural resemblance of siastatin B (**1**) to D-glucuronic acid (**2**), *N*-acetyl-D-glucosamine (**3**) and *N*-acetylneuraminic acid (**4**).

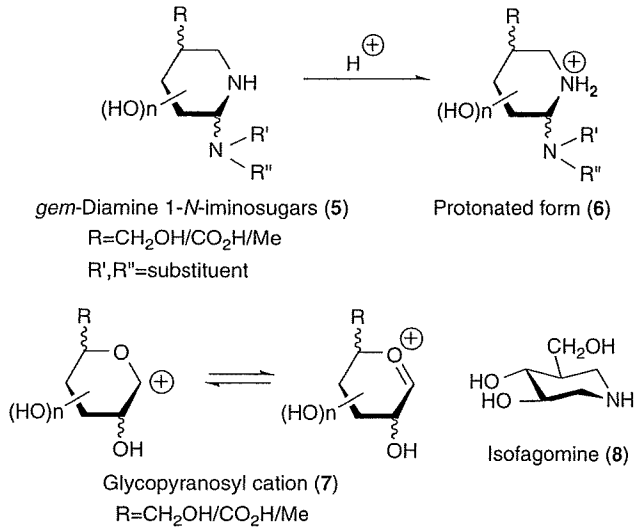


Figure 2 General structures of *gem*-diamine 1-*N*-iminosugars (**5**), its protonated form (**6**), glycopyranosyl cation (**7**), the putative intermediate of enzymatic glycosidic hydrolysis and isofagomine (**8**), another type of 1-iminosugar.

have an especially unusual structure possessing the continuous –CH(OH)–CH(OH)–CH–(NHR)–NH–CH2–CH(CH2OH/COOH)– in a framework. Their multi-functionalized structures with many asymmetric centers in a small molecule and fascinating biological activities have attracted intensive synthetic interest. As the interest in this class of glycomimetics comprised analogs of both D- and L-sugars, we have developed flexible divergent strategies applicable to a wide range of *gem*-diamine 1-*N*-iminosugars using glyconolactones as chiral substrates. Efficient and convenient synthetic methodologies of *gem*-diamine 1-*N*-iminosugars were also developed from natural siastatin B. These convergent strategies using natural siastatin B could be useful and practical for drug development.

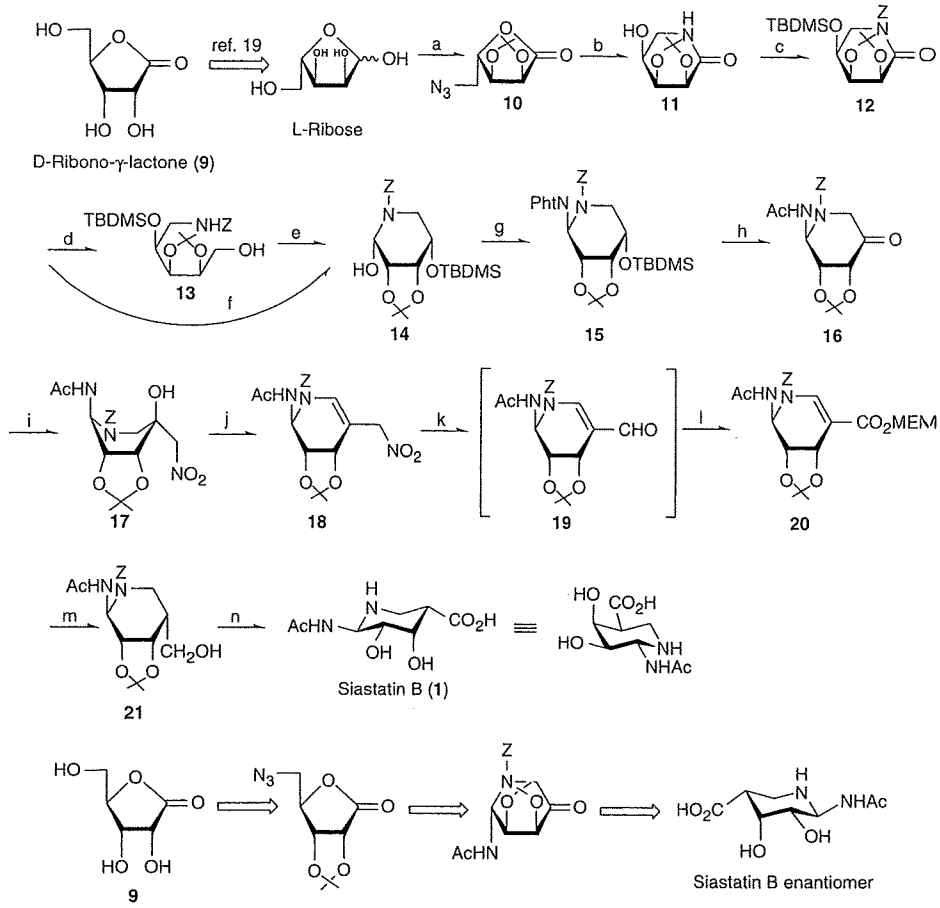
Total synthetic route to *gem*-diamine 1-*N*-iminosugars

The chiron strategy using D-ribono-γ-lactone (**9**) was first adapted for the total synthesis of siastatin B (**1**) and its enantiomer.[16,17] D-galacturonic acid-type 2-acetamido-1-*N*-iminosugar was synthesized in a totally stereospecific and enantiospecific manner as shown in Scheme 1.[18] The strategy involves the formation of the cyclic methanediamine using the Mitsunobu reaction[19] on an aminal (**14**→**15**) and the stereospecific introduction of a carboxylic acid group into a ketone (**16**→**21**) as the key steps. The synthesis of the key intermediate, lactam **11**, commenced with L-ribose, which was transformed into azido-L-ribonolactone **10** by the protection of 2,3-diol, azide formation and oxidation. Hydrogenation of the azide group of **10** resulted in crystalline **11** with ring expansion. Stereospecific introduction of the hydroxyl group at the C-2 position was best achieved by hydride reduction of the protected lactam **12** followed by the Swern oxidation to yield aminal **14**. One-step stereospecific transformation of **12** into **14** was also efficiently achieved by the reduction of L-selectride in tetrahydrofuran. The Mitsunobu reaction with phthalimide in dimethylformamide was proved to quantitatively yield the desired cyclic methanediamine **15**. Replacement of the amino substituent, removal of the *tert*-butyldimethylsilyl (TBDMS) group and oxidation to **16** were carried out straightforwardly. Condensation of **16** with nitromethane was found to proceed smoothly to quantitatively yield **17** as a single stereoisomer. The endocyclic nitro olefin **18** was effectively derived from **17** by acetylation followed by base-catalyzed elimination of the acetoxy group. The crucial intermediate, carboxylate **20**, was obtained through α,β-unsaturated aldehyde **19** generated by simply warming in pyridine. Transformation of **20** into siastatin B (**1**) was best achieved by the following three-step sequences: stereoselective reduction to the α,β-saturated hydroxymethyl compound **21**, oxidation and removal of protecting groups. The antipode of **1** was also synthesized from D-ribono-1,4-lactam using the above-mentioned method. Thus, the total synthesis also elucidated the absolute configuration of siastatin B (**1**) as the (3*S*,4*S*,5*R*,6*R*) isomer.

The strategy of total synthesis of siastatin B (**1**) is applicable to a wide range of D-galacturonic acid-type *gem*-diamine 1-*N*-iminosugars (Schemes 2 and 3).[20,21]

Syntheses of D-galacturonic acid-type 2-acetamido- and trifluoroacetamido-1-*N*-iminosugars (**27a** and **27b**) having a hydroxyl group at the C-5 position and their antipodes **27c** and **27d** are achieved in a straightforward manner. The nitromethane condensation of the ketones **23a** and **23b** stereospecifically proceeded to afford adducts **24a** and **24b**. The *S*-configuration at position C-5 was clarified by an X-ray crystallographic analysis of the antipode of **23a**. Successive sequences of catalytic reduction, ninhydrin oxidation of the resultant aminomethyl group, oxidation of the resultant aldehyde with sodium chlorite and removal of the protecting groups afforded the final products. The antipodes **27c** and **27d** were also synthesized starting from D-ribono-1,4-lactone using similar methods that are mentioned above.

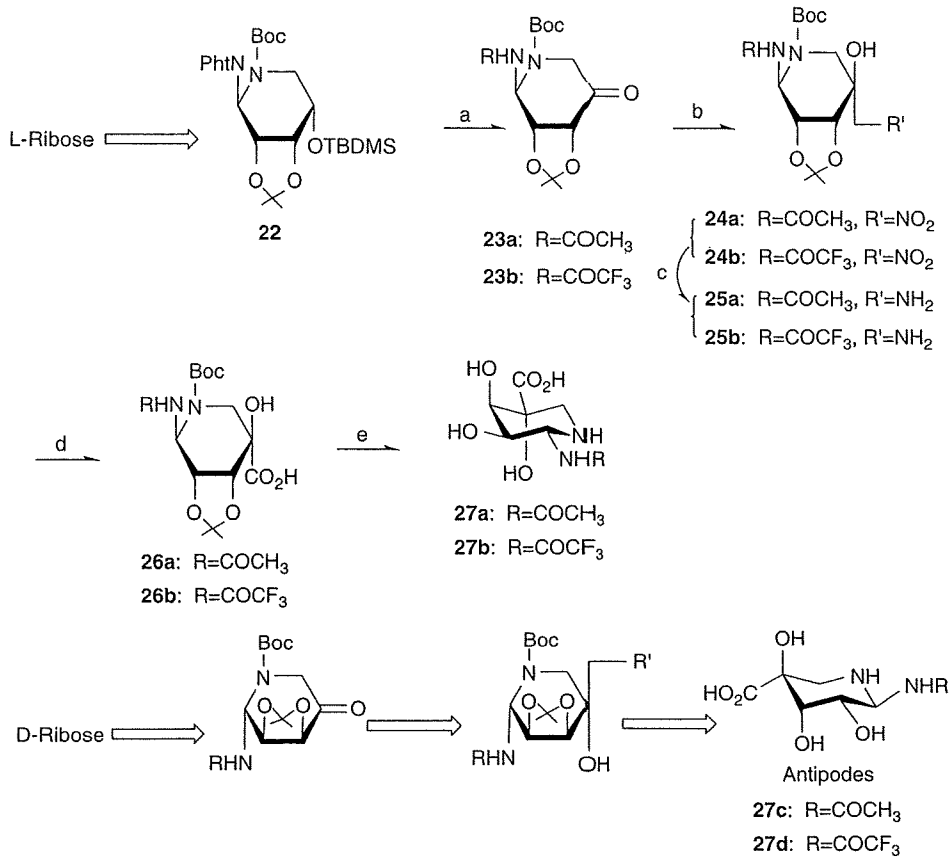
An alternative route from the ketone **23b** to D-galacturonic acid-type 2-trifluoroactamido-1-*N*-iminosugar **32** was also developed



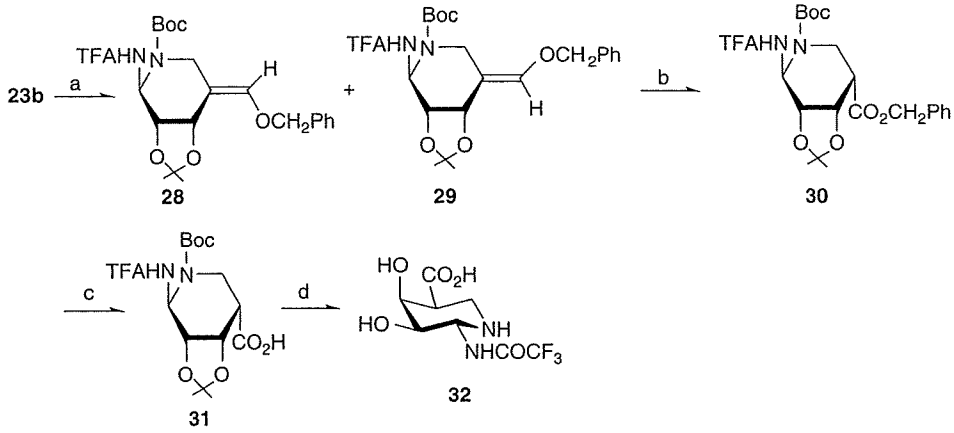
Scheme 1 Reagents and conditions: (a) *p*-TsOH, Me_2CO; MsCl, py; NaN_3, DMSO; CrO_3/py, CH_2Cl_2 (89%); (b) H_2, Raney Ni, MeOH (88%); (c) TBDMSCl, imidazole, DMF; ZCl, NaH, DMF (99%); (d) $NaBH_4$, EtOH (96%); (e) Swern oxidation (88%); (f) L-selectride, THF (88%); (g) phthalimide, Ph_3P, DEAD, DMF (100%); (h) $H_2N{\cdot}NH_2$, MeOH; Ac_2O, py; *n*-Bu_4NF, THF; RuO_4, CH_2Cl_2 (99%); (i) $MeNO_2$, NaH, DME (100%); (j) Ac_2O, *p*-TsOH; K_2CO_3, PhH (100%); (k) py, 38 °C, 1 week (80%); (l) $NaClO_2$–NaH_2PO_4, $CH_3CH{=}CMe_2$, H_2O-*t*-BuOH; MEMCl, *i*-Pr_2NEt, CH_2Cl_2 (55%); (m) $NaBH_4$, THF–CF_3CH_2OH (75%); (n) PDC, DMF; H_2, 10% Pd/C, MeOH; 1 M HCl, then Dowex 50W X_4 (H^+) eluted with 2% NH_4OH (66%).

using the Wacker process oxidation of the enol ethers **28** and **29** as a key step. The Wacker process oxidation stereospecifically proceeded to yield carboxylate **30** as a sole product. The transformation of **30** into **32** was unexceptional.

A flexible synthetic route to four *gem*-diamine 1-*N*-iminosugars of D- and L-uronic acid type (D-glucuronic, D-mannuronic, L-iduronic and L-guluronic acid) from L-galactono-1,4-lactone was also developed in an enantiodivergent manner through a sequence involving as the key steps (1) the formation of *gem*-diamine 1-*N*-iminopyranose ring by the Mitsunobu reaction of an aminal (**44**→**45, 46**) and (2) the flexible introduction of a carboxylic acid group by the Wittig reaction on a ketone, followed by hydroboration and oxidation, as well as the Sharpless oxidation (**45** and **46**→**47, 48** and **55, 56**) (Schemes 4 and 5).[22,23] The diastereoselective construction of amino and carboxylic acid substituents at positions C-2 and C-5, respectively, on the versatile aminal **44** led to the formation of four enantiomerically pure stereoisomers (**51, 54, 61** and **66**). The Wittig reaction on the ketone **37** derived from L-galactono-1,4-lactone resulted in the methylene derivative **38**, which was converted into the diol **39**. The monoalcohol **40** was successfully obtained by the Luche reduction of the labile aldehyde generated by the periodate oxidation of **39**. Conversion of the hydroxyl group of **40** to the azide group was best achieved from the corresponding sulfonate by one-pot reaction *in situ*. Hydrogenation of the azide group of **41** with sodium hydrogentelluride (NaTeH) was found to proceed preferentially without any effect on the reduction of the methylene group. The pivotal intermediate, aminal **44** was obtained as an epimeric mixture by the removal of a TBDMS group and the Swern oxidation. The Mitsunobu reaction with phthalimide afforded both desired epimers of iminophthalimides **45** and **46** in a 3:1 ratio. The absolute stereochemistry and a boat conformer of **45** were clarified by an X-ray crystallographic analysis. Another epimer **46** was assigned its stereochemistry and boat conformation by the hydrogen-1 nuclear magnetic resonance (^{1}H-NMR) spectrum. Hydroboration of **45** followed by oxidation efficiently yielded the D-*gluco* isomer **47** and the L-*idulo* isomer **48** in a 2:9 ratio. Hydrazinolysis of **47** and conventional trifluoroacetylation furnished the trifluoroacetamide **49**. The ruthenium tetraoxide-catalyzed Sharpless oxidation effectively yielded the carboxylic acid **50**. Removal of the protecting groups of **50** resulted in D-glucuronic acid-type 2-trifluoroacetamido-1-*N*-iminosugar **51**. The same sequences of reactions also successfully resulted in L-iduronic acid-type 2-trifluoroacetamido-1-*N*-iminosugar **54** from **48**. The ^{1}H-NMR spectrum of **51** showed the $^4C_1$-conformation, whereas the ^{1}H-NMR spectrum of **54** indicated the boat conformation. On the other hand, D-mannuronic



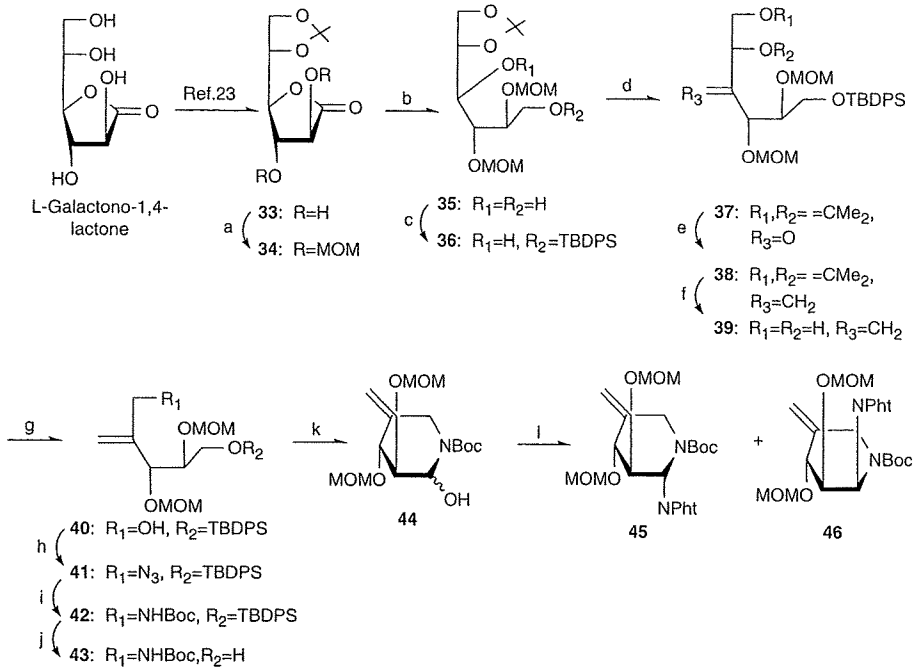
Scheme 2 Reagents and conditions: (a) $H_2N{\cdot}NH_2$, MeOH; Ac_2O, py (or CF_3CO_2Et, *i*-Pr_2NEt, DMF): *n*-Bu_4NF, THF; RuO_4, CH_2Cl_2 (81 and 91%); (b) CH_3NO_2, NaH, DME (69 and 74%); (c) H_2, Raney Ni, MeOH (100 and 98%); (d) ninhydrin, $NaHCO_3$, MeOH/H_2O; $NaClO_2$, NaH_2PO_4, $MeCH{=}CMe_2$, *t*-BuOH/H_2O (38 and 43%); (e) 4 M HCl/dioxane (92 and 96%).



Scheme 3 Reagents and conditions: (a) $PhCH_2OCH_2PPh_3Cl$, PhLi, THF (48%); (b) $PdCl_2$, CuCl, O_2, DMF/H_2O (46%); (c) H_2, Pd/C, EtOAc (92%); (d) 4 M HCl/dioxane (96%).

acid-type and L-guluronic acid-type 1-*N*-iminosugars **61** and **66** were prepared in a straightforward manner by a similar sequence of structure transformation, except for the protection of the hydroxyl groups of **55** and **56** before hydrazinolysis of the phthalimide group for improvement in yield. The ^{1}H-NMR spectra of **61** and **66** showed the boat and $^1C_4$-conformations, respectively.

An enantioselective synthesis of L-fucose-type *gem*-diamine 1-*N*-iminosugars from D-ribono-γ-lactone was developed that used the Mitsunobu reaction on an aminal in the *gem*-diamine 1-*N*-iminopyranose ring formation (**74**→**75**) and a stereospecific reduction of an *exo*-methylene group to form the correct configuration of L-fucose (**75**→**76**) (Scheme 6).[24,25] The synthesis of the pivotal intermediate,



Scheme 4 Reagents and conditions: (a) $MeOCH_2Cl$, *n*-Bu_4NI, *i*-Pr_2NEt, 70 °C, 81%; (b) $LiAlH_4$, THF, 100%; (c) *t*-$Bu(Ph_2)SiCl$, *i*-Pr_2NEt, DMAP, CH_2Cl_2, 99.7%; (d) Dess–Martin periodinane, CH_2Cl_2, 93%; (e) Ph_3PMeBr, *n*-BuLi, THF, −78 °C, 96%; (f) 80% AcOH, rt, 99%; (g) $NaIO_4$, $MeCN/H_2O$; $NaBH_4$, $CeCl_3$, MeOH, 88%; (h) MsCl, py; NaN_3, DMF, 88.7%; (i) Te, $NaBH_4$, EtOH; $(t\text{-BuCO})_2O$, *i*-Pr_2NEt, DMF, 88%; (j) *n*-Bu_4NF, THF, 100%; (k) $(COCl)_2$, DMSO, CH_2Cl_2, 93%; (l) PPh_3, DEAD, phthalimide, DMF, **45**: 61.4%; **46**: 20%.

aminal **74** began with the known lactam **67**. Transformation of **67** into the diol **70** was unexceptional. The Dess–Martin oxidation of **70** followed by the Wittig reaction with methylenetriphenylphosphorane yielded the *exo*-methylene **72**. Removal of a protecting group of **72** and the Swern oxidation resulted in the desired aminal **74** as a sole product. The Mitsunobu reaction of **74** with phthalimide efficiently afforded the iminophthalimide **75**. The catalytic hydrogenation of **75** yielded the desired product **76**, its epimer **77** and the rearranged derivative **78** in a ratio of 15:1:3. Compound **78** was also successfully converted into the desired **76** on the same hydrogenation. The expected stereochemistry and a boat conformation of **76** were clarified by an X-ray crystallographic analysis. Hydrazinolysis of **76** yielded the amine **79**, which was transformed into the acetamide **80**, the trifluoroacetamide **81** and the trichloroacetamide **82**. Removal of the protecting groups resulted in L-fucose-type 2-acetamido, 2-trifluoroacetamido and 2-trichloroacetamido-1-*N*-iminosugars **83**, **84** and **85**. Other L-fucose-type 1-*N*-iminosugars **86**, **87** and **88** were also obtained from the intermediates **76**, **77** and **75**, respectively, by conventional transformation. The ^{1}H-NMR spectra of **83**, **84**, **85** and **86** showed $^1C_4$-conformations.

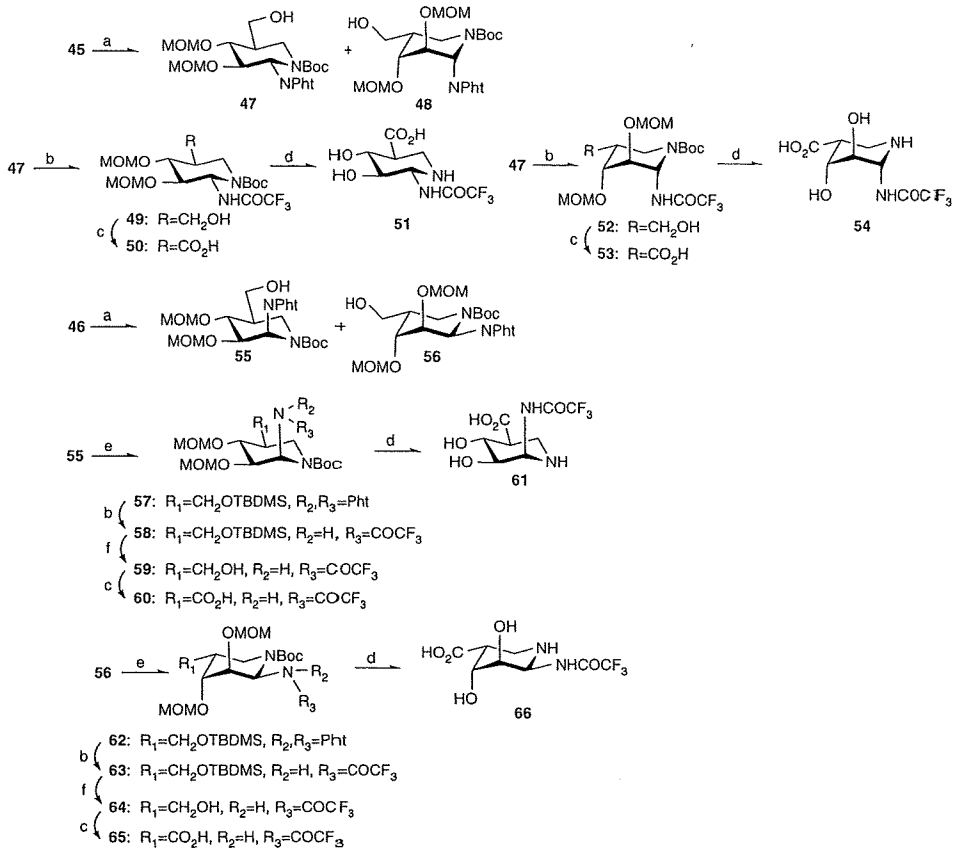
Intermediates prepared during the total synthetic route to uronic acid-type *gem*-diamine 1-*N*-iminosugars are also available for the synthesis of various kinds of glycose and glycosamine-type *gem*-diamine 1-*N*-iminoisugars (Scheme 7).[22]

Semi-synthetic route to *gem*-diamine 1-*N*-iminosugars

Natural siastatin B (**1**) can also serve as a starting material in a simple and easy route to D-galacturonic acid-type *gem*-diamine 1-*N*-iminosugars (Scheme 8).[26,27] Transketalization using chlorotrimethylsilane successfully proceeded to yield the ketal **94**. A sequence of esterification, hydride reduction and hydrazinolysis efficiently afforded the amino alcohol **97**, which was converted into the trifluoroacetamide **98**. The ruthenium-catalyzed Sharpless oxidation followed by the removal of the protecting group resulted in the desired product **32**. 2-Trichloroacetamido, guanidino and phthaloyl analogs **106**, **107** and **108** were also prepared using similar methods.

Configurational inversion of the carboxyl group of siastatin B (**1**) leads to *gem*-diamine 1-*N*-iminosugars corresponding to L-sugars.[28,29] The intramolecular Michael addition of *O*-imidate to the α,β-unsaturated ester through *cis* oxiamination[30] (Overman rearrangement, **110**→**111**) as a key step effectively yielded L-uronic acid-type *gem*-diamine 1-*N*-iminosugars (Schemes 9 and 10). The α,β-unsaturated ester **110** readily available from siastatin B (**1**) smoothly underwent *cis* oxiamination through the conjugate addition of the intermediate imidate anion to result in the desired oxazoline **111** in a good yield and a trace amount of its epimer. Hydrolysis of **111** afforded the trichloroacetamides **112** and **113**, which were converted into the amines **114** and **115**, respectively, on reductive cleavage of the trichloroacetamide group. Removal of the protecting groups of **114** and **115** resulted in L-alturonic acid-type and L-mannuronic acid-type 2-acetamido-1-*N*-iminosugars **116** and **117**, respectively. Another type of L-alturonic acid-type 2-acetamido-1-*N*-iminosugar **119** with a guanidine group was also obtained by the conventional method. The ^{1}H-NMR spectra of **116**, **117** and **119** showed $^1C_4$-conformations. 2-Trifluoroactamide analogs **130** and **133** were also prepared by a similar sequence of reactions using the α,β-unsaturated ester **123** readily available from **97**.

Siastatin B (**1**) has the correct configuration corresponding to D-galactose- and D-galactosamine-type gem-diamine 1-*N*-iminosugars. Therefore, the various kinds of D-glycose and D-glycosamine-type *gem*-diamine 1-*N*-iminosugars could be obtainable by a semi-synthetic method starting from **1** (Schemes 11 and 12)[30,31]



Scheme 5 Reagents and conditions: (a) $BH_3{\cdot}Me_2S$, THF; H_2O_2, 2 M NaOH/H_2O, **47**: 16.6%; **48**: 77.1%; **55**: 50%; **56**: 38%; (b) $H_2NNH_2{\cdot}xH_2O$, MeOH; $(CF_3CO)_2O$, py, CH_2Cl_2, **49**: 90%; **52**: 87%; **58**: 88%; **63**: 79%; (c) RuO_2, $NaIO_4$, CCl_4/MeCN/H_2O, **50**: 91%; **53**: 90%, **60**: 92%; **65**: 87%; (d) 4 M HCl/dioxane, **51**: 99.7%; **54**: 99%; **61**: 99.7%; **66**: 91%; (e) *t*-Bu(Me$_2$)SiCl, imidazole, DMF, **57**: 91%; **62**: 100%; (f) *n*-Bu_4NF, THF, **59**: 93%; **64**: 100%.

Configurational inversions at the C-4 position of **144** and **145** by two-step reactions led to the facile synthesis of D-glucosamine and glucose-type *gem*-diamine 1-*N*-iminosugars **150** and **151**, respectively (Scheme 13).[32]

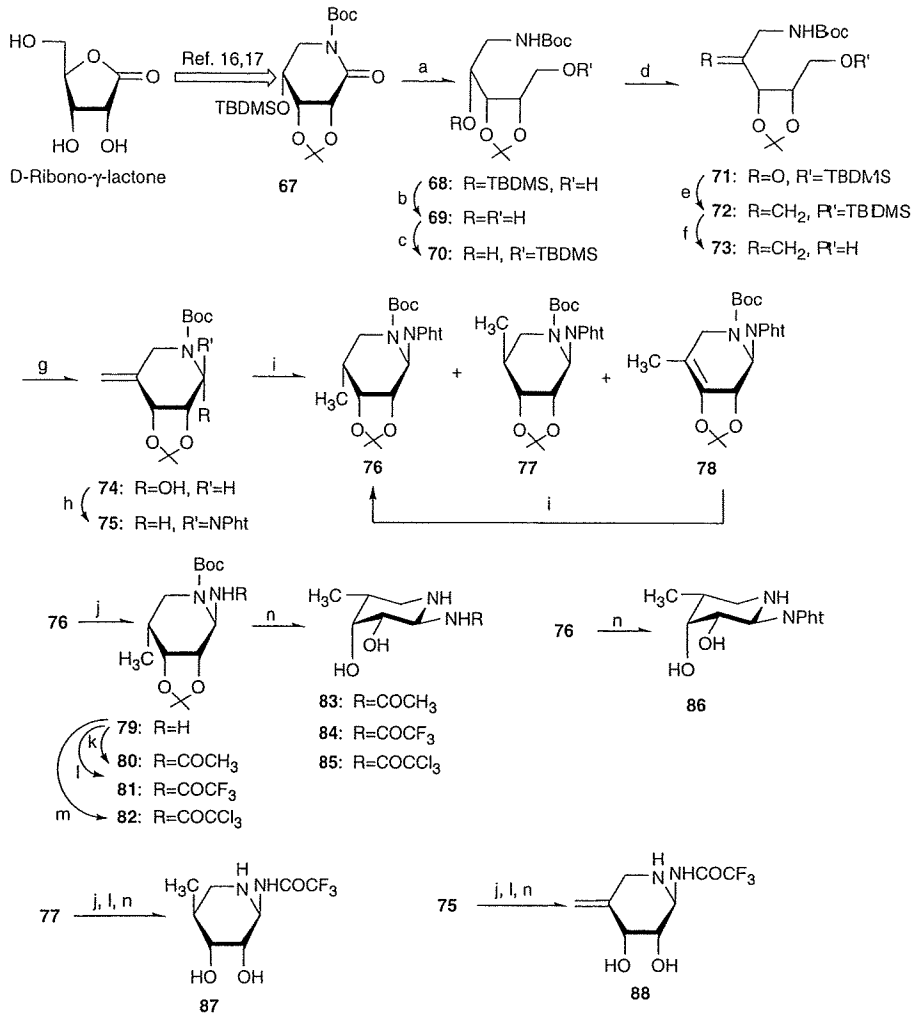
BIOLOGICAL ACTIVITY

Glycoconjugates such as glycoprotein, glycolipid and proteoglycan are ubiquitous in nearly all forms of life and are involved in cell-to-cell communication, cell-to-cell recognition, cell adhesion, cell growth regulation, differentiation and transport. Specific inhibitors of glycosidase and glycosyltransferases are useful for unraveling the manner in which glycoconjugates regulate biological function, and also for developing new drugs for a wide range of diseases associated with both the biosynthesis and degradation of glycoconjugates, namely cancer, tumor metastasis, diabetes, lysosomal storage disorders, viral and bacterial infections and so forth. Iminosugars generally show potent and specific inhibition against glycosidases and glycosyltransferases from various organisms.[1,33–35] Of these, *gem*-diamine 1-*N*-iminosugars have been proven to be highly potent and specific inhibitors of glycosidases, glycosyltransferases and sulfotransferases, and also potential therapeutics for tumor metastasis, lysosomal storage disorders and other diseases.[1,6,36–38]

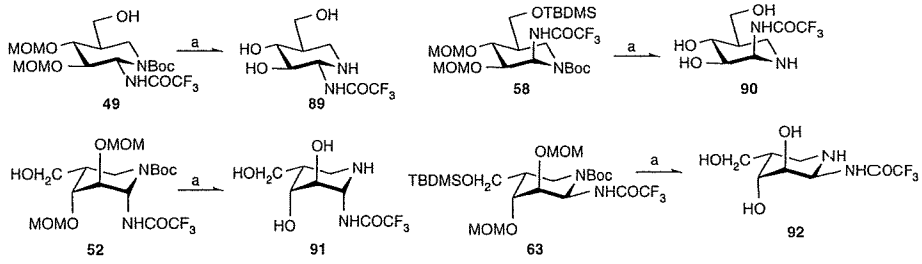
Glycosidase, glycosyltransferase and sulfotransferase inhibitory activity

The inhibitory activities of L-fucose-type *gem*-diamine 1-*N*-iminosugars against glycosidases are summarized in Table 1.[24,25]

The L-fucose-type 2-trifluoroacetamide **84** showed a very strong and specific inhibition against α-L-fucosidase from bovine kidney. Compound **84** was proved to be a competitive inhibitor by the Lineweaver–Burk plot, and the Ki value of **84** was determined as 5×10^{-9} M by the Dixon plot.[25] The 2-trichloroacetamide **85** and 2-phthalimide **86** also strongly affected α-L-fucosidase equivalent to the trifluoroacetamide **84**. Compounds **84**, **85** and **86** have been proven to smoothly undergo the Amadori rearrangement to yield the common intermediates, the hemiaminal **152** and the hydrated ketone **153** at pH 6.3 (Figure 3).[39] The time-course evaluation of inhibitory activities of **84**, **85** and **86** in the medium indicates that the hemiaminal and the hydrated ketone generated in the medium strongly inhibit α-L-fucosidase as the real active form. The ^{1}H-NMR spectra of the hemiaminal **152** and the hydrated ketone **153** also clearly show their $^1C_4$-conformation. Interestingly, the hemiaminal **152** has the same structure as that of synthetic L-fuconoeuromycin.[11,40] L-fuco-noeuromycin shows a potent inhibition against α-L-fucosidase equivalent to compounds **84**, **85** and **86**. On the other hand, the stable acetamide **83** in the medium shows a moderate inhibition against α-L-fucosidase. These results support the hypothesis that the protonated *gem*-diamine 1-*N*-iminosugars may mimic the presumed glycosyl cation **7** in the transition state of the enzymatic reaction as shown in Figure 2. Compounds **87** and **88** show a weak inhibition against α-L-fucosidase. These results also indicate that the 5-methyl group, its stereochemistry and the $^1C_4$-conformation have important roles as major factors for potency and specificity.



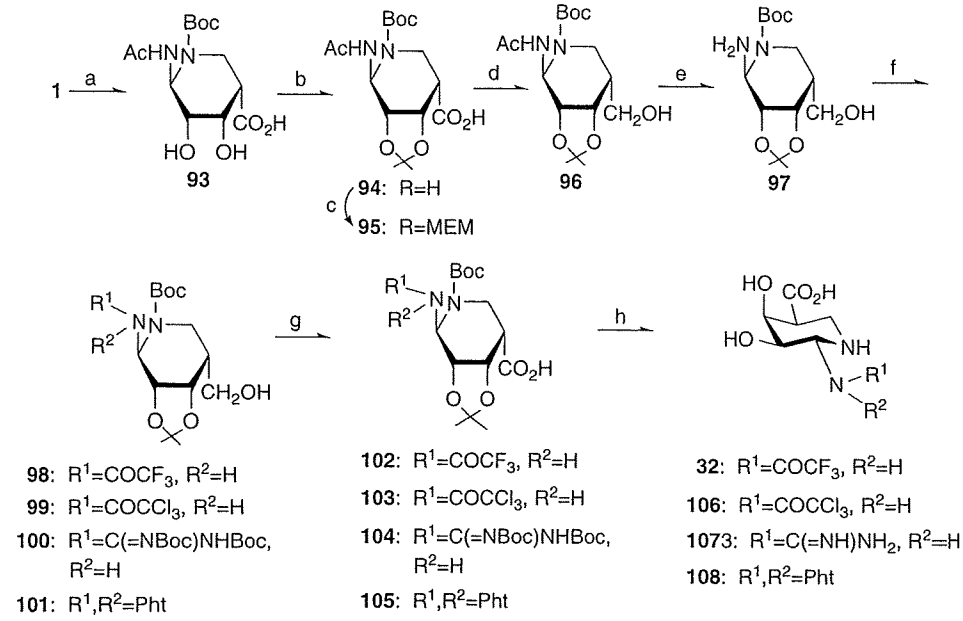
Scheme 6 Reagents and conditions: (a) $NaBH_4$, EtOH, 0 °C to rt; (b) *n*-Bu_4NF, THF, rt; (c) *t*-$BuMe_2SiCl$, imidazole, DMF, rt; (d) Dess–Martin periodinane, CH_2Cl_2; (e) Ph_3PMeBr, $(Me_3Si)_2NLi$, THF, 0 °C to rt; (f) *n*-Bu_4NF, THF, rt; (g) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −78 °C to rt; (h) phthalimide, Ph_3P, DEAD, DMF, rt; (i) H_2/Pd-C, MeOH, rt; (j) $H_2NNH_2{\cdot}xH_2O$, MeOH, rt; (k) Ac_2O, DMAP, py, rt; (l) $(CF_3CO)_2O$, py, CH_2Cl_2, rt; (m) CCl_3COCl, py, CH_2Cl_2, 0 °C; (n) 4 M HCl/dioxane, 0 °C to rt.



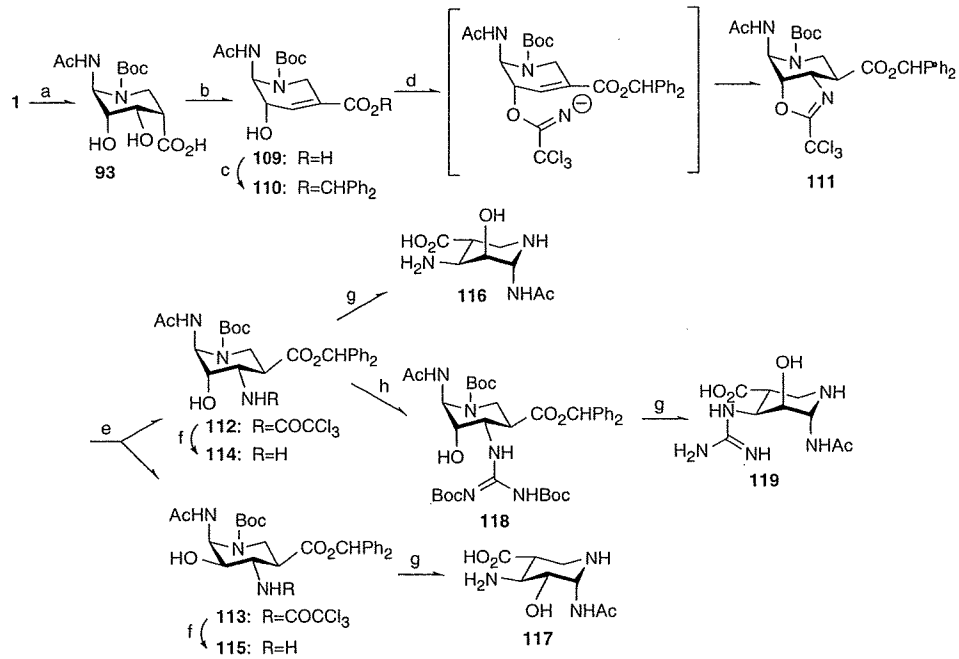
Scheme 7 Reagents and conditions: (a) 4 M HCl/dioxane.

The inhibitory activities of uronic acid-type *gem*-diamine 1-*N*-iminosugars against glycosidases are summarized in Table 2.[7,20–22,26,27]

D-glucuronic acid-type 2-trifluoroacetamido-1-*N*-iminosugar **51** shows a very strong inhibition against β-glucuronidase. D-uronic acid-type *gem*-diamine 1-*N*-iminosugars, similar to the L-fucose-type *gem*-diamine mentioned above, have been proven to smoothly undergo the Amadori rearrangement to yield the hydrated ketone **155** or its derivative **156** through a hemiaminal **154** at pH > 5.0 (Figure 4).[39] The time-course evaluation of the inhibitory activity in the medium also indicates that the hemiaminal and hydrated ketone generated in the medium strongly inhibits glycuronidases. It is reasonable to expect that the hemiaminal and hydrated ketone and

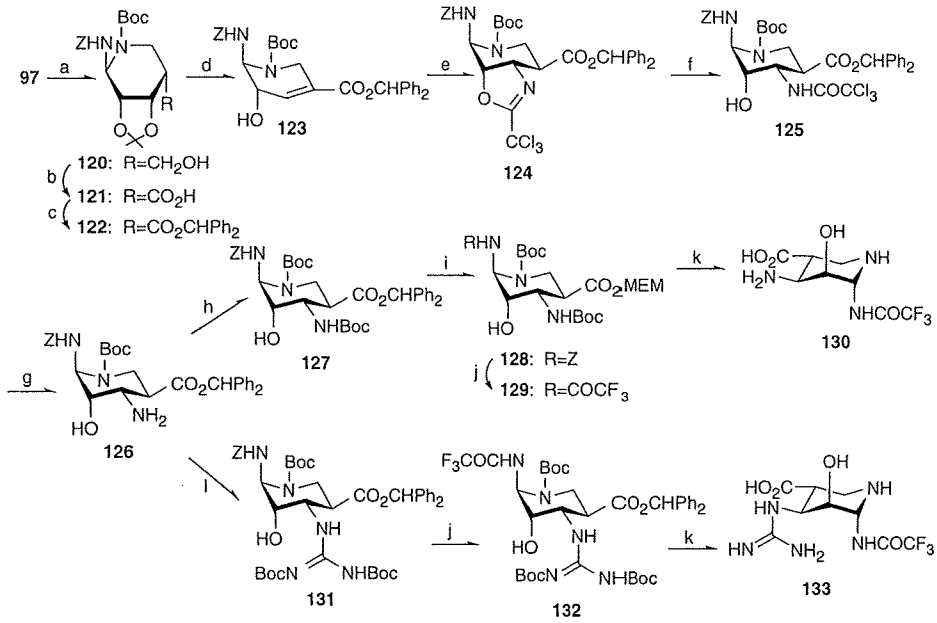


Scheme 8 Reagents and conditions: (a) $(t\text{-BuOCO})_2O$, $i\text{-Pr}_2NEt$, DMF (91%); (b) MeCH(OMe)CH_2OMe, TMSCl, DMF (98%); (c) MEMCl, $i\text{-Pr}_2NEt$, DMF (83%); (d) NaBH4, CF_3CH_2OH/THF (99%); (e) $H_2NNH_2{\cdot}xH_2O$ (54%; recovery, 80%); (f) CF_3CO_2Et, $i\text{-Pr}_2NEt$, DMF; or CCl_3COCl, py, CH_2Cl_2; or $(BocNH)_2CS$, $HgCl_2$, Et_3N, DMF; or Phthalic anhydride, Et3N, DMF, 120 °C (81, 88, 94, 60%); (g) RuO_2, $NaIO_4$, CCl_4/MeCN/H_2O (77, 76, 69, 75%); (h) 4 M HCl/dioxane (97, 100, 94, 95%).

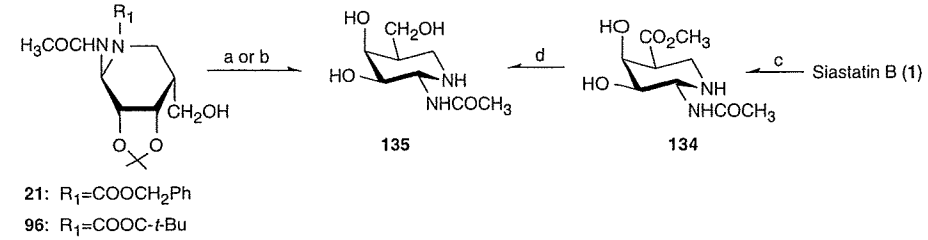


Scheme 9 Reagents and conditions: (a) $(t\text{-BuOCO})_2O$, $i\text{-Pr}_2NEt$, MeOH (93%); (b) Ac_2O, $i\text{-Pr}_2NEt$, DMF; MeONa, MeOH (90%); (c) $PhCN_2$, CH_2Cl_2/MeOH (92%); (d) CCl_3CN, DBU, CH_2Cl_2 (76%); (e) *p*-TsOH, py/H_2O (**112**, 77%; **113**, 9%); (f) $NaBH_4$, EtOH (~62%); (g) 4 M HCl/dioxane (~96%); (h) $(BocNH)_2CS$, $HgCl_2$, Et_3N, DMF (88%).

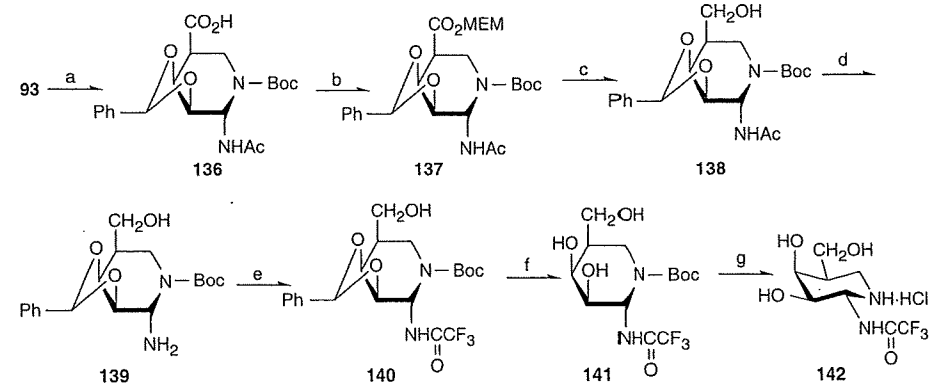
its equivalent generated from **51** in the medium should closely mimic a glycopyranosyl cation that could adapt either a chair-like or a flattened conformational **157** and **158**, respectively, in the putative transition state of β-glucuronidase hydrolysis (Figure 5). D-galacturonic acid-type 2-trifluoroacetamido-, 2-trichloroacetamido-, 2-guanidino- and 2-phthalimido-1-*N*-iminosugars **27b**, **32**, **106**, **107** and **108** also strongly inhibit glucuronidase. These results indicate that β-glucuronidase roughly recognizes the configuration of the 4-OH group of glycopyranose and/or that the binding group of β-glucuronidase to the 4-OH has no important role in the specificity. Interestingly, D-mannuronic acid-type 2-trifluoroacetamido-1-*N*-iminosugar **61** also shows a strong inhibition equivalent to those of



Scheme 10 Reagents and conditions: (a) $Ph_2CH_2CO_2Cl$, *i*-Pr_2NEt, MeOH (86%); (b) RuO_2, $NaIO_4$, $MeCN/CCl_4/H_2O$ (80%); (c) Ph_2CN_2, CH_2Cl_2/MeOH (94%); (d) *t*-BuOK, THF (70%); (e) CCl_3CN, DBU, C6H6 (70%); (f) *p*-TsOH, py/H_2O (95%); (g) $NaBH_4$, EtOH (68%); (h) $(t\text{-BuOCO})_2O$, *i*-Pr_2NEt, MeOH (91%); (i) 1 M NaOH/MeOH; MEMCl, *i*-Pr_2NEt, CH_2Cl_2 (83%); (j) H_2, 10% Pd/C, MeCN; $(CF_3CO)_2O$, py (~68%); (k) 4 M HCl/dioxane (~100%); (l) $(BocNH)_2CS$, $HgCl_2$, Et3N, DMF (98%).

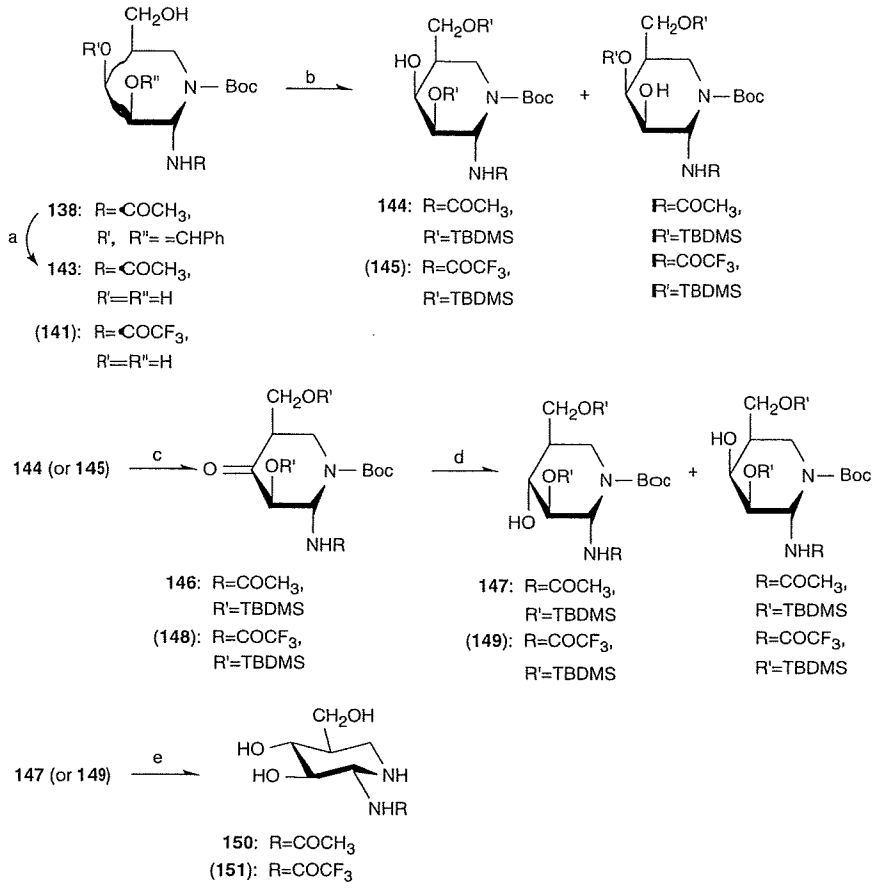


Scheme 11 Reagents and conditions: (a) H_2, 5% Pd/C, MeOH; 4 M HCl/dioxane (78%); (b) 4 M HCl/dioxane(63%); (c) Amberlist 15 (H^+), MeOH (84%); (d) $NaBH_4$, EtOH (90%).



Scheme 12 Reagents and conditions: (a) $(MeO)_2CHPh$, TMSCl, DMF (92%); (b) $MeOCH_2CH_2OCH_2Cl$, *i*-Pr_2NEt, DMF, rt, 98%; (c) $NaBH_4$, CF_3CH_2OH/THF, rt, 94%; (d) $H_2NNH_2{\cdot}xH_2O$, 70 °C, 83%; (e) CF_3CO_2Et, *i*-Pr_2NEt, DMF, 60 °C, 73%; (f) H_2/10% Pd-C, MeOH, rt, 92%; (g) 4 M HCl/dioxane, rt, 80%.

D-glucuronic- and D-galacturonic acid-type 1-*N*-iminosugars **51**, **32**, **106**, **107** and **108**. The ^{1}H-NMR spectrum of **61** shows the adoption of a boat conformation that is different from the chair conformations of D-glucuronic acid- and D-galacturonic acid-type 1-*N*-iminosugars in solution, suggesting that the hemiaminal generated from **61** may mimic the flattened conformation of cation **158** in the transition state



Scheme 13 Reagents and conditions: (a) H_2, 10% Pd/C, MeOH, 94%; (b) TBDMSCl, imidazole, DMF, rt, (**141**⇝**145**, 58%; **143**⇝**144**, 50%); (c) Dess–Martin periodinane, CH_2Cl_2, rt, (**145**⇝**148**, 97%; **144**⇝**146**, 98%); (d) $LiBH_4$, MeCN, −50 °C, (**148**⇝**149**, 88%; **146**⇝**147**, 74%); (e) 4 M HCl/dioxane, rt, (**147**⇝**150**, 91%; **149**⇝**151**, 80%).

Table 1 Inhibitory activities (IC_{50} (M) and Ki (M)) of L-fucose-type *gem*-diamine 1-*N*-iminosugars against glycosidases

Enzyme	*83*	*84*	*85*	*86*	*87*	*88*
α-L-fucosidase[a]	4.8×10^{-7}	1.1×10^{-8} (5×10^{-9})[j]	9.0×10^{-9}	1.3×10^{-8}	1.8×10^{-6}	7.0×10^{-7}
α-D-glucosidase[b]	1.8×10^{-4}	4.7×10^{-5}	NT	NT	NT	NT
β-D-glucosidase[c]	1.0×10^{-5}	1.2×10^{-4}	NT	NT	NT	NT
α-D-mannosidase[d]	$>2.2\times10^{-4}$	$>1.8\times10^{-4}$	NT	NT	NT	NT
β-D-mannosidase[e]	$>2.2\times10^{-4}$	$>1.8\times10^{-4}$	NT	NT	NT	NT
α-D-galactosidase[f]	$>2.2\times10^{-4}$	$>1.8\times10^{-4}$	NT	NT	NT	NT
β-D-galactosidase[f]	$>2.2\times10^{-4}$	$>1.8\times10^{-4}$	NT	NT	NT	NT
β-D-glucuronidase[g]	$>2.2\times10^{-4}$	$>1.8\times10^{-4}$	NT	NT	NT	NT
α-D-NAc-galactosaminidase[h]	$>2.2\times10^{-4}$	$>1.8\times10^{-4}$	NT	NT	NT	NT
β-D-NAc-glucosaminidase[i]	$>2.2\times10^{-4}$	$>1.8\times10^{-4}$	NT	NT	NT	NT

Abbreviations: IC_{50}, half maximal inhibitory concentration; NT, not tested.
[a]Bovine kidney.
[b]Baker's yeast.
[c]Almonds.
[d]Jack beans.
[e]Snail.
[f]*Escherichia coli.*
[g]Bovine liver.
[h]Chicken liver.
[i]Bovine epididymis.
[j]Ki (M).

(Figure 5). On the other hand, the stable analogs **1** and **27a** in the media expectedly show only a weak inhibitory activity against β-glucuronidase.

The typical analogs (**32**, **51**, **61**) of D-uronic acid-type *gem*-diamine 1-*N*-iminosugars also inhibit recombinant human heparanase from human melanoma A375M cells transfected with

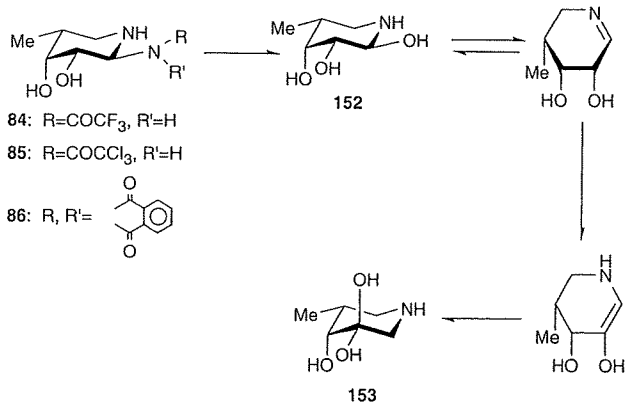


Figure 3 Structural changes of L-fucose-type *gem*-diamine 1-*N*-iminosugars in medium at pH 6.3.

pBK-CMV expression vectors containing the heparanase cDNA (Table 3).[22,41]

Heparanase is an *endo*-β-glucuronidase that specifically cleaves the β-1,4 linkage between D-glucuronic acid and *N*-acetyl-D-glucosamine of heparan sulfate (HS) side chains of HS proteoglycans (HSPGs). The relationships between activity against heparanase and the inhibitor structures are also similar to those discussed regarding the inhibition against *exo*-β-glucuronidase from bovine liver. The weaker activity against heparanase than that against *exo*-β-glucuronidase suggests that heparanase should simultaneously recognize D-glucuronic acid and the adjacent glycoses on both sides of D-glucuronic acid. As expected, all of the L-uronic acid-type *gem*-diamine 1-*N*-iminosugars (**54, 66, 116, 117, 119, 130** and **133**) show no remarkable inhibition against these D-sugar hydrolases. These results indicate that glycohydrolases recognize precisely the absolute configurations of *gem*-diamine 1-*N*-iminosugars corresponding to the D- and L-sugars for specificity and potency.

Table 2 Inhibitory activities (IC_{50} (M)) of D-uronic acid-type *gem*-diamine 1-*N*-iminosugars against D-glycosidase

Enzyme	*1*	*27a*	*27b*	*32*	*106*	*107*	*108*	*51*	*61*
β-D-glucuronidase[a]	7.1×10^{-5}	1.2×10^{-4}	6.2×10^{-8}	6.5×10^{-8}	9.2×10^{-8}	1.3×10^{-7}	6.8×10^{-8}	6.5×10^{-8}	6.5×10^{-8}
α-D-glucosidase[b]	$>3.3\times10^{-3}$ (Ni)	Ni	2.4×10^{-7}	Ni	NT	NT	NT	Ni	Ni
β-D-glucosidase[c]	Ni	Ni	Ni	1.3×10^{-5}	NT	NT	NT	9.8×10^{-5}	3.6×10^{-5}
α-D-mannosidase[d]	Ni	Ni	Ni	Ni	NT	NT	NT	Ni	Ni
β-D-mannosidase[e]	Ni	Ni	Ni	Ni	NT	NT	NT	Ni	Ni
α-D-galactosidase[f]	Ni	Ni	Ni	1.3×10^{-6}	NT	NT	NT	Ni	Ni
β-D-galactosidase[f]	Ni	Ni	Ni	Ni	NT	NT	NT	Ni	Ni
α-D-NAc-galactosaminidase[g]	Ni	Ni	Ni	Ni	NT	NT	NT	Ni	Ni
β-D-NAc-glucosaminidase[h]	Ni	Ni	Ni	Ni	NT	NT	NT	Ni	Ni

Abbreviations: IC_{50}, half maximal inhibitory concentration; Ni, no inhibition at 3.3×10^{-3} M; NT: not tested.
[a]Bovine liver.
[b]Baker's yeast.
[c]Almonds.
[d]Jack beans.
[e]Snail.
[f]*Aspergillus niger.*
[g]Chicken liver.
[h]Bovine epididymis.

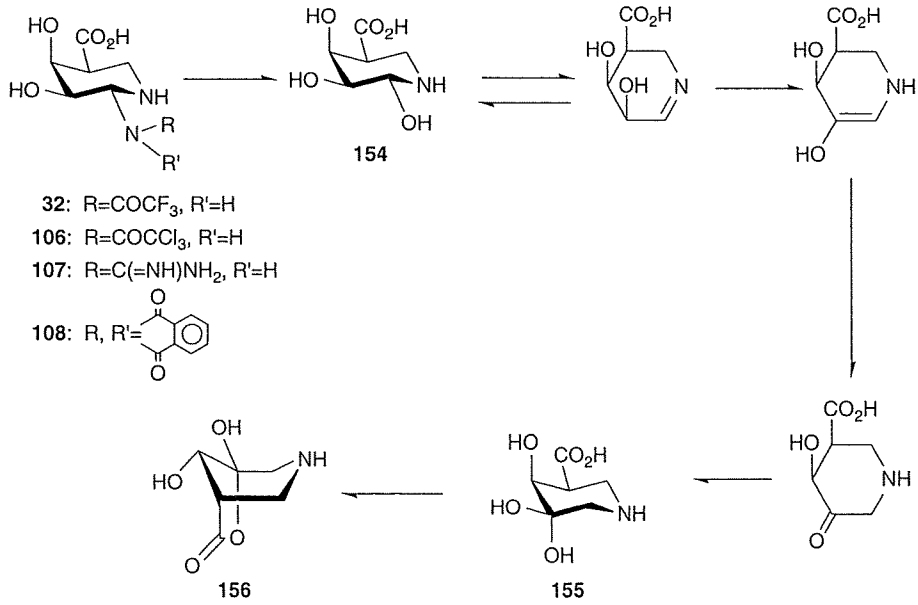
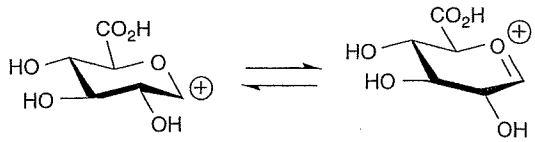


Figure 4 Structural changes of D-galacturonic acid-type *gem*-diamine 1-*N*-iminosugars in medium at pH 5.0.



157 158

Figure 5 Possible conformations **157** and **158** of the glycosyl cation formed as an intermediate in β-glucuronidase hydrolysis.

Table 3 Inhibitory activities (IC_{50}) of uronic acid-type *gem*-diamine 1-*N*-iminosugars against human heparanase (endo β-D-glucuronidase)

Compounds	*IC_{50} (μM)*
32	1.02± 0.29
51	10.5± 1.07
54	Ni
61	28.99± 11.41
66	Ni

Abbreviations: IC_{50}, half maximal inhibitory concentration; FITC, fluorescein isothiocyanate. Ni, no inhibition at 3.3 mM; buffer: 50 mM AcNA, pH 4.2, 0.02% CHAPS. Enzyme: heparanase 0.26 μg protein per tube; substrate: FITC-heparan sulfate 0.5 μl (5 μg HS). Incubation time: 37 °C, 2 h.

Table 4 Inhibitory activities (IC_{50} (M)) of D-glycose- and D-glycosamine-type *gem*-diamine 1-*N*-iminosugars against D-glycosidases

Enzyme	*135*	*142*	*150*	*151*
α-D-glucosidase[a]	$>3.9\times10^{-4}$	$>3.2\times10^{-4}$	2.9×10^{-6}	1.9×10^{-7}
β-D-glucosidase[b]	7.9×10^{-5}	4.8×10^{-7}	5.4×10^{-6}	4.2×10^{-7}
α-D-galactosidase[c]	2.5×10^{-5}	3.4×10^{-7}	$>3.9\times10^{-4}$	$>3.2\times10^{-4}$
β-D-galactosidase[c]	1.7×10^{-5}	1.7×10^{-7}	$>3.9\times10^{-4}$	1.9×10^{-4}
α-D-mannosidase[d]	$>3.9\times10^{-4}$	$>3.2\times10^{-4}$	2.5×10^{-4}	2.2×10^{-5}
β-D-mannosidase[e]	$>3.9\times10^{-4}$	1.3×10^{-4}	3.8×10^{-5}	3.2×10^{-6}
α-D-NAc-galactosaminidase[f]	3.3×10^{-7}	2.2×10^{-6}	$>3.9\times10^{-4}$	$>3.2\times10^{-4}$
β-D-NAc-glucosaminidase[g]	2.7×10^{-6}	$>3.2\times10^{-4}$	1.2×10^{-6}	$>3.2\times10^{-4}$
β-D-glucuronidase[h]	$>3.9\times10^{-4}$	$>3.2\times10^{-4}$	$>3.9\times10^{-4}$	$>3.2\times10^{-4}$

Abbreviations: IC_{50}, half maximal inhibitory concentration.
[a]Baker's yeast.
[b]Almonds.
[c]*Aspergillus niger.*
[d]Jack beans.
[e]Snail.
[f]Chicken liver.
[g]Bovine epididymis.
[h]Bovine liver.

The inhibitory activities of D-glycose- and D-glycosamine-type *gem*-diamine 1-*N*-iminosugars against glycosidases are summarized in Table 4.[30–32]

As expected, D-glucose-type 2-trifluoroacetamide **151** inhibits α- and β-D-glucosidases very strongly and specifically, and D-glucosamine-type 2-acetamide **150** shows a strong and specific inhibition against β-D-*N*-acetylglucosaminidase. On the other hand, D-galactose-type 2-trifluroacetamide **142** strongly inhibits not only α– and β-D-galactosidases but also β-D-glucosidase. D-galactosamine-type 2-acetamide **135** also strongly inhibits both α-D-*N*-acetylgalactosaminidase and β-D-*N*-acetylglucosaminidase. These results seem to indicate that the hemiaminals of the glycose-type inhibitors generated in the media mimic a transient intermediate **7** (Figure 2) in the hydrolysis of glycosidases, and that the glycosamine-type inhibitors themselves mimic a glycopyranoside in its grand state during the hydrolysis of glycosaminidases. These results also suggest that the axial 4-OH group is the main determinant for specificity and potency of the inhibitors against D-galacto-type hydrolases. However, D-gluco-type hydrolases may roughly recognize the stereochemistry of the 4-OH group and accept both axial and equatorial configurations. *N*-acetyl-glycosaminidases also recognize the 2-*N*-acetyl group precisely.

Table 5 Effects of 27b and CDP on [^{14}C]NeuAc incorporation into lactosylceramide (LacCer) as an exogenous acceptor

Compound	*Treatment*	*[^{14}C]NeuAc incorporated into GM3 c.p.m. mg^{-1} lipid added*	*%*
—	0	850	100
27b	1.3 mM	961	110
	4.3 mM	659	78
	13 mM	13	1.5
CDP	13 mM	7	0.82

Abbreviations: CDP, cytidine 5′-diphosphate.
The sialyltransferase activity was determined according to the method of Hakomori *et al.*[34] using mouse mammary carcinoma mutant cell line (FUA 1.69), which shows high activity of CMP-sialic acid:LacCer 2,3-sialosyltransferase.

D-galacturonic acid-type 2-trifluoroacetamido-1-*N*-iminosugar having a hydroxyl group at the C-5 position **27b** inhibits sialyltransferase nearly as well as cytidine 5′-diphosphate, a standard inhibitor, in the mouse mammary carcinoma mutant cell line (FUA169),[42] which has a high transfer activity of sialic acid to lactosylceramide [Galβ1—4Glcβ1—1Cer] to form ganglioside GM3 [NeuAcα2—3Galβ1—4Glcβ1—1Cer] (Table 5). This result suggests that **27b** may resemble a *gem*-diamine 5-*N*-iminosugar and mimic sialic acid (**4**) in the sialyltransferase reaction (Figure 6). This is similar to the method in which siastatin B (**1**) mimics **4** in NA (*N*-acetylsialidase) hydrolysis (Figure 1).

L-altruronic acid-type 2-acetamido-1-*N*-iminosugar **119** and its 1-*N*-2-ethlybutyrylamide (**159**) also inhibit HS 2-*O*-sulfotransferase (HS 2-*O*-ST) over 80% at 25 μM.[43] HS 2-*O*-ST transfers the sulfate group from the sulfate donor, adenosine 3′-phosphate-5′-phosphosulfate, to the 2-OH group of L-iduronic acid of HS, which is composed of a repeating disaccharide unit comprising glucosamine (GlcN) and hexuronic acid (D-glucuronic acid or its C-5 epimer, L-iduronic acid).[44] This result indicates that HS 2-*O*-ST recognizes **119** and **159** as L-iduronic acid. Molecular modeling using PM3 in MOPAC shows the structural similarity between α-L-iduronic acid and **119** (Figure 7).[29] As shown in Figure 7, **119** superimposes well on α-L-iduronic acid and the acetamido and guanidino moieties of **119** are also topographically equivalent to the hydroxyl moieties of α-L-iduronic acid.

Inhibition of esophageal keratinocyte differentiation

Recently, it has been clarified that heparanase is localized in the cell nucleus of the normal esophageal epithelium and esophageal cancer,[45] and that its expression is correlated with cell differentiation.[46] On esophageal cell differentiation, heparanase is translocated from the cytoplasm to the nucleus. On such translocation, heparanase degrades the glycan chain of HS in the nucleus, and changes in the expression of keratinocyte differentiation markers such as p27 and involucrin are observed. D-galacturonic acid-type *gem*-diamine 1-*N*-iminosugar **32** inhibits efficiently this degradation and induction in the nucleus.[47] It has been shown that heparanase regulates the