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2,5-Dideoxy-2,5-imino-D-altritol as a new class of pharmacological chaperone for Fabry disease

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ABSTRACT

Chromatographic separation of the extract from roots of *Adenophora triphylla* resulted in the isolation of two pyrrolidines, six piperidines, and two piperidine glycosides. The structures of new iminosugars were elucidated by spectroscopic methods as 2,5-dideoxy-2,5-imino-D-altritol (DIA) (**2**), β-1-*C*-butenyl-1-deoxygalactonojirimycin (**8**), 2,3-dideoxy-β-1-*C*-ethyl-1-deoxygalactonojirimycin (**9**), and 6-*O*-β-D-glucopyranosyl-2,3-dideoxy-β-1-*C*-ethyl-1-deoxygalactonojirimycin (**10**). β-1-*C*-Butyl-1-deoxygalactonojirimycin (**7**) and compound **8** were found to be better inhibitors of α-galactosidase than *N*-butyl-1-deoxygalactonojirimycin. The present work elucidated that DIA was a powerful competitive inhibitor of human lysosome α-galactosidase A (α-Gal A) with a K_i value of 0.5 μM. Furthermore, DIA improved the thermostability of α-Gal A in vitro and increased intracellular α-Gal A activity by 9.6-fold in Fabry R301Q lymphoblasts after incubation for 3 days. These experimental results suggested that DIA would act as a specific pharmacological chaperone to promote the smooth escape from the endoplasmic reticulum (ER) quality control system and to accelerate transport and maturation of the mutant enzyme.

1. Introduction

Fabry disease is a lysosomal storage disorder caused by an X-linked inherited deficiency of α-galactosidase A (α-Gal A). Deficiency of the enzyme activity results in the progressive accumulation of globotriaosylceramide (Gb3) in the lysosomes of vascular endothelial cells.[1] More than 400 mutations have been identified in the α-Gal A gene, and 57% of mutations are missense. Furthermore, structural studies revealed that the majority of the mutations do not directly contribute to the catalytic function of the enzyme, but rather to the maintenance of the tertiary structure.[2,3] Although enzyme replacement therapy has been approved for the treatment of Fabry disease, it has no therapeutic benefit for the treatment of stroke and fails to remove accumulated Gb3 from kidney podocytes and blood vessel walls. In recent years, a remarkable progress has been made in developing a molecular therapy for Fabry disease. Residual α-Gal A activity in lymphoblasts derived from Fabry patients and in tissues of R301Q α-Gal A transgenic mice was enhanced by treatment with 1-deoxygalactonojirimycin (DGJ), a competitive inhibitor of the enzyme.[4] The inhibitor appears to act as a template that stabilizes the native folding state in the endoplasmic reticulum (ER) by occupying the active site of the mutant enzyme, thus allowing its maturation and trafficking to the lysosome.[5] To establish the concept of using competitive inhibitors as specific pharmacological chaperones, a number of naturally occurring and chemically synthesized compounds were tested for intracellular enhancement of the α-Gal A activity in Fabry lymphoblasts.[6] However, the initial studies using glycosidase inhibitors as pharmacological chaperones have focused on six-membered sugar mimetics.[7–9] With respect to the five-membered sugar mimetics, there is little evaluation as pharmacological chaperones. In this paper, we describe the isolation, structural determination, and glycosidase inhibitory activity of three new six-membered iminosugars with a 1-C-substitution and one five-membered iminosugar, 2,5-dideoxy-2,5-imino-D-altritol (DIA), which is not a new compound but the first report as a natural product, from *Adenophora triphylla* var. *japonica* (Campanulaceae). Furthermore, we report for the first time that DIA is an excellent pharmacological chaperone which effectively enhances intracellular α-Gal A activity in Fabry lymphoblasts.

2. Results and discussion

2.1. Isolation and structural determination

The roots (10 kg) of *A. triphylla* were extracted with 50% aqueous MeOH, and the extract was subjected a variety of chromatographic

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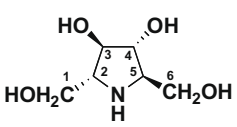
steps with ion-exchange resins to give alkaloids **1** (1140 mg), **2** (191 mg), **3** (130 mg), **4** (149 mg), **5** (32 mg), **6** (6 mg), **7** (103 mg), **8** (49 mg), **9** (14 mg), and **10** (125 mg). The ^{1}H and ^{13}C NMR spectra of alkaloids **1** and **3**–**7** were in accord with those of 2,5-dideoxy-2,5-imino-D-mannitol (DMDP), 1-deoxynojirimycin, 1-deoxymannojirimycin, α-1-*C*-ethyl-fagomine, 1-*O*-β-D-glucopyranosyladenophorine, and β-1-*C*-butyl-1-deoxygalactonojirimycin, respectively, which have been isolated previously from *A. triphylla*.[10,11] The structural determination of the new alkaloids **2**, **8**, **9**, and **10** is described below.



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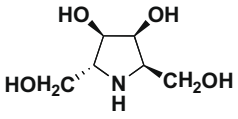

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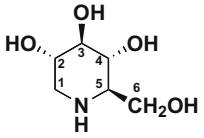

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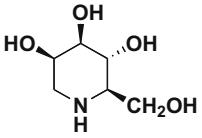

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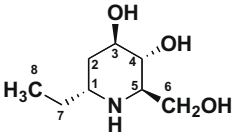

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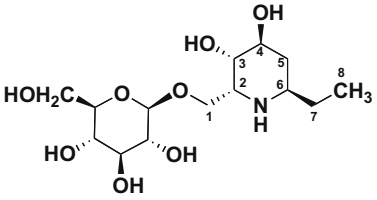

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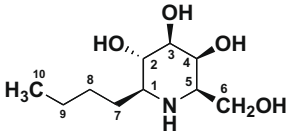

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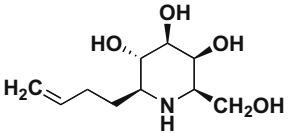

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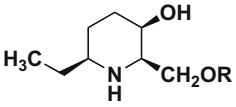

9 R = H
10 R = β-D-glucopyranoside

The ^{1}H NMR and ^{13}C NMR spectral data of **2** were superimposable with those of 2,5-dideoxy-2,5-imino-D-altritol, which has been chemically synthesized.[12] Furthermore, the optical rotation {$[\alpha]_D$ +34.2 (*c* 0.83, H_2O)} of **2** was identical to that of the synthetic compound {$[\alpha]_D$ +34.8 (*c* 0.70, H_2O)}. Hence, compound **2** was determined to be 2,5-dideoxy-2,5-imino-D-altritol (DIA).

The HRFABMS (*m/z* 218.1393 $[M+H]^+$) and 10 resonances in the ^{13}C NMR spectrum of **8** established that the molecular formula was $C_{10}H_{19}NO_4$. The complete connectivity of the carbon and hydrogen atoms was defined from ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, and HMBC spectroscopic data. The COSY and HMBC spectra elucidated that the carbon signals of δ 32.2, 33.0, 117.7, and 141.9 are derived from the butenyl side chain at C-1. The remaining carbon signals (δ 61.1, 61.5, 64.3, 71.7, 74.9, 77.8) were completely in accord with the corresponding signals (δ 61.1, 61.8, 64.2, 71.8, 74.9, 77.9 of β-1-*C*-butyl-1-deoxygalactonojirimycin (**7**). The coupling patterns of H-4 (dd, $J_{3,4}$ = 3.2 Hz, $J_{4,5}$ = 1.4 Hz) and H-3 (dd, $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 3.2 Hz) indicated the equatorial orientation of H-4 and the axial orientations of H-2 and H-3. Furthermore, the strong NOE between H-1 and H-5 indicated that these protons are 1,3-diaxial. Hence, the structure of **8** was determined to be β-1-*C*-butenyl-1-deoxygalactonojirimycin.

The HRFABMS (*m/z* 160.1338 $[M+H]^+$) and eight resonances in the ^{13}C NMR spectrum of **9** established that the molecular formula was $C_8H_{17}NO_4$. The complete connectivity of the carbon and hydrogen atoms was defined from ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, and HMBC spectroscopic data. The COSY and HMBC spectra elucidated that the carbon signals of δ 12.6 and 29.7 are derived from the ethyl side chain at C-1. The relatively high-field methine carbons at δ 52.6 (C-1) and 59.3 (C-5) must be bonded to the nitrogen of the piperidine ring. The strong NOE between H-1 and H-5 indicated that these protons are 1,3-diaxial. The strong NOE between H-1 and H-5 and the coupling patterns of H-4 (ddd, $J_{3a,4}$ = 3.3 Hz, $J_{3b,4}$ = 4.1 Hz, $J_{4,5}$ = 1.5 Hz) indicated the equatorial orientation of H-4. Hence, the structure of **9** was determined to be 2,3-dideoxy-β-1-*C*-ethyl-1-deoxygalactonojirimycin.

The HRFABMS (*m/z* 322.1874 $[M+H]^+$) and 14 resonances in the ^{13}C NMR spectrum of **10** established that the molecular formula was $C_{14}H_{27}NO_7$. The characteristic anomeric proton (d, $J_{1',2'}$ = 8.2 Hz) and carbon (δ 106.0) signals suggested that **10** was a glycoside of an alkaloid. After acid hydrolysis of this glycoside using Dowex 50 W-X2 (H^+) resin, D-glucose was detected in the filtrate by the D-glucose oxidase peroxidase method. On the other hand, the alkaloid part was displaced from the resin with 0.5 M NH_4OH, concentrated to dryness, and confirmed as 2,3-dideoxy-β-1-*C*-ethyldeoxygalactonojirimycin (**9**) by direct comparison of its NMR spectra with those of **9**. The chemical shift (δ 4.43) and $^{3}J_{H,H}$ coupling (8.2 Hz) of the anomeric proton indicated that its glucosidic linkage is β. Introduction of a β-D-glucopyranosyl group into **9** produced a 9.3 ppm downfield shift for C-6 and a 1.6 ppm upfield shift for C-5. From these results, the linkage site of the β-D-glucopyranosyl group is C-6 and the structure of **10** was determined to be 6-*O*-β-D-glucopyranosyl-2,3-dideoxy-β-1-*C*-ethyl-1-deoxygalactonojirimycin.

2.2. In vitro glycosidase inhibitory activities

The IC_{50} values of the alkaloids isolated from *A. triphylla* toward various glycosidases are shown in Table 1. β-1-*C*-Butyl-DGJ (**7**) and β-1-*C*-butenyl-DGJ (**8**) showed potent inhibitory activity toward coffee beans α-galactosidase, with IC_{50} values of 1.2 and 2.0 μM, respectively. We previously reported that β-1-*C*-butyl-DNJ and β-1-*C*-ethyl-1-deoxymannojirimycin abolished their inhibition

Table 1
Concentration of iminosugars giving 50% inhibition of various glycosidases

Enzyme	IC_{50} (μM)					
	2	7	8	9	10	DGJ
α-Glucosidase						
Yeast	66	NI[a]	NI	NI	NI	NI
Rice	171	10	45	NI	670	NI
Rat intestinal maltase	74	112	239	NI	NI	NI
β-Glucosidase						
Almond	40	NI	NI	NI	321	NI
C. saccharolyticum	19	152	1000	NI	NI	367
Bovine liver	444	NI	329	950	NI	NI
α-Galactosidase						
Coffee beans	0.78	1.2	2.0	NI	318	0.003
Human lysosome	0.69	15	39	NI	224	0.07
β-Galactosidase						
Bovine liver	190	370	476	332	NI	NI
Rat intestinal lactase	7.2	NI	NI	NI	NI	72
α-Mannosidase						
Jack beans	64	NI	NI	NI	NI	NI
β-Mannosidase						
Rat epididymis	NI	NI	NI	NI	NI	NI
α-L-Fucosidase						
Bovine epididymis	503	NI	NI	NI	NI	NI
Trehalase						
Porcine kidney	631	NI	NI	NI	NI	NI
Amyloglucosidase						
Aspergillus niger	NI	NI	NI	NI	NI	NI

[a] NI: no inhibition (less than 50% inhibition at 1000 μM).

toward α-glucosidases and α-mannosidases, respectively,[13,14] while the present study revealed that β-1-*C*-butyl-DGJ (**7**) and β-1-*C*-butenyl-DGJ (**8**) retained their inhibitory potential toward α-galactosidase. Furthermore, both compounds were more potent inhibitors of the enzyme than *N*-butyl-DGJ with an IC_{50} value of 14 μM. New alkaloids 2,3-dideoxy-β-1-*C*-ethyl-DGJ (**9**) and 6-*O*-β-D-glucopyranosyl-2,3-dideoxy-β-1-*C*-ethyl-DGJ (**10**) markedly lowered their inhibition toward this enzyme. DMDP (**1**) is known to be a potent inhibitor of yeast α-glucosidase, mammalian β-glucosidase, and β-galactosidase.[15] In contrast, DIA (**2**) showed a significant inhibition against coffee bean and human lysosome α-galactosidases, with IC_{50} values of 0.78 and 0.69 μM, respectively. DIA is the most potent inhibitor of α-galactosidase among five-membered iminosugars reported to date. Fabry disease is a lysosomal storage disorder caused by deficiency of lysosomal α-galactosidase A (α-Gal A), an enzyme responsible for hydrolysis of the terminal α-galactosyl residue in glycosphingolipids. Studies on residual α-Gal A activity of mutant enzymes in many Fabry patients revealed that some had kinetic properties similar to those of normal α-Gal A, but were significantly less stable.[16] We have previously reported that a potent competitive inhibitor appears to act as a template that stabilizes the native folding state by occupying the active site of the unstable enzyme.[4] We therefore focused on a strong competitive inhibitor, DIA (**2**), and studied whether this compound could act as pharmacological chaperone. We first determined the inhibition constant (K_i) and the mode of inhibition of DIA by the Lineweaver–Burk plots, and found to inhibit α-Gal A in a competitive manner, with K_i value of 0.5 μM (Fig. 1).

2.3. In vitro stabilization of α-Gal A by DIA and effect of DIA on α-Gal A activity in Fabry lymphoblasts

The effect of DIA on the stabilization of α-Gal A at 48 °C was studied in vitro (Fig. 2). The enzyme α-Gal A was incubated in 100 mM sodium citrate buffer (pH 4.6) containing 1 mg/mL of BSA for 0, 20, 40, and 60 min and the remaining enzyme activity

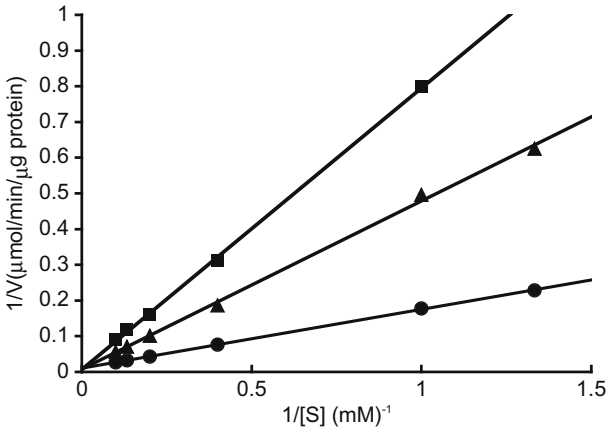

Figure 1. Lineweaver–Burk plots of 2,5-dideoxy-2,5-imino-D-altritol (**2**) of human lysosome α-galactosidase A. Increasing concentration of PNP-α-D-galactopyranoside were used to determine the K_m and K_i values and the data were plotted as $1/V$ versus $1/[S]$. Concentrations of **2** were 0 μM (*J*): closed circle, 1.0 μM (*H*): closed triangle, 2.5 μM (*H*): closed diamond. The calculated K_m and K_i values were 16 mM and 0.5 μM, respectively.

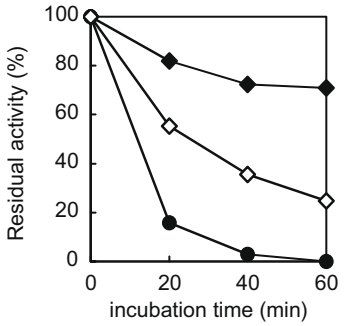

Figure 2. Effect of DIA (**2**) on the in vitro thermostability. Human lysosome α-galactosidase A (α-Gal A) was incubated at 48 °C in 100 mM sodium citrate buffer (pH 4.6) containing 1 mg/mL of BSA at various incubation time with or without 2,5-dideoxy-2,5-imino-D-altritol (**2**). Concentrations of **2** were 0 μM (*J*): closed circle, 10 μM (◇), 100 μM (♦).

was determined with *p*-nitrophenyl-α-D-galactopyranoside as substrate. The enzyme activity was lost within 60 min under incubation without DIA, while the incubation with DIA effectively stabilized α-Gal A in a dose-dependent manner. The enzyme activity remained over 70% even under the incubation for 60 min in the presence of 100 μM DIA. Thus, DIA occupied the catalytic center of α-Gal A and enhanced the thermostability. DIA that showed strong competitive inhibition and improvement of thermostability was further tested for enhancement of intracellular α-Gal A activity in Fabry R301Q lymphoblasts. Treatment with DIA for 3 days dose-dependently increased intracellular α-Gal A activity, with maximal increase of 9.6-fold at 500 μM (Fig. 3). In conclusion, this study indicates that the five-membered iminosugar DIA could as a specific pharmacological chaperone to effectively enhance intracellular enzyme activity in Fabry R301Q lymphoblasts.

3. Experimental section

3.1. General experimental procedures

Optical rotations were measured with a JASCO DIP-370 digital polarimeter (Tokyo, Japan). ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker DRX500 spectrometer. Chemical shifts are expressed in ppm downfield from sodium 3-(trimethylsilyl)-propionate (TSP) in D_2O as internal standard. The assignment of proton and carbon NMR signals was determined

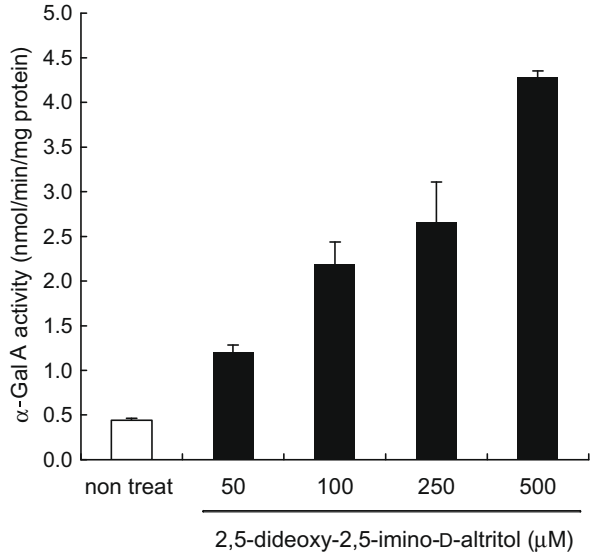

Figure 3. Influence of DIA (**2**) on α-Gal A activity in Fabry R301Q lymphoblasts. DIA was added to the culture medium of R301Q cells at a concentration of 50–500 μM. Cells were subsequently incubated for 3 days and cell growth was not affected by the inclusion of DIA.

from extensive homonuclear decoupling experiments, and the DEPT, ^{1}H–^{13}C COSY, HMQC, and HMBC spectroscopic data. FABMS were measured using glycerol as a matrix on a JEOL JMS-700 spectrometer. The purity of samples was checked by HPTLC on Silica Gel $60F_{254}$ (E. Merck) using the solvent system PrOH/AcOH/H_2O (4:1:1), and a chlorine-*o*-tolidine reagent or iodine vapor was used for detection.

3.2. Plant material

Roots of *A. triphylla* var. *japonica* were purchased at a herbal medicine shop (Uchida Wakanyaku Co., Tokyo, Japan) in May 2008. A voucher specimen (no. RJN2008012) is deposited at the herbarium of the Institute of Biological, Environmental and Rural Sciences, Aberystwyth, UK.

3.3. Extraction and isolation

The bulbs (10 kg) of *A. triphylla* were extracted with 50% aqueous MeOH. The filtrate was applied to a column of Amberlite IR-120B (2000 mL, H^+ form). The 0.5 M NH_4OH eluate was concentrated to give a brown oil (89.3 g). The oil was further applied to Amberlite IRA-400J (OH^- form) to remove amino acids and pigments, and eluted with H_2O. This eluate was concentrated and chromatographed over an Amberlite CG-50 column (2.0 × 55 cm, (NH_4^+ form) with H_2O as eluant (fraction size 8 mL). Fractions were divided into three pools: I (fractions 18–50, 28.5 g), II (fractions 51–150, 2.7 g), and III (fractions 32–41, 1.4 g). Each pool was further chromatographed with Dowex 1-X2 (OH^- form) with H_2O as eluant and/or Amberlite CG-50 column (NH_4^+ form) with H_2O and 0.5 M NH_4OH as eluents to give alkaloids **8** (49 mg), β-1-*C*-butyl-D-1-deoxygalactonojirimycin (103 mg), DNJ (130 mg), and 1-*O*-β-D-glucopyranosyladenophorine (6 mg) from pool I, 1-deoxymannojirimycin (149 mg), DMDP (1140 mg), α-1-*C*-ethyl-fagomine (32 mg), and **10** (125 mg) from pool II, **9** (14 mg), and **2** (191 mg) from pool III.

3.3.1. 2,5-Dideoxy-2,5-imino-D-altritol (DIA) (2)

Colorless powder; $[\alpha]_D$ +34.2 (*c* 0.83, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 3.19 (1H, ddd, *J* = 3.9, 4.2, 5.9 Hz, H-5), 3.36 (1H, ddd, *J* = 4.1, 6.6, 6.6 Hz, H-2), 3.67 (1H, dd, *J* = 5.9, 11.7 Hz, H-6a), 3.67 (1H, dd, *J* = 6.6, 11.2 Hz, H-1a), 3.78 (1H, dd, *J* = 3.9, 11.7 Hz, H-6b), 3.82 (1H, dd, *J* = 6.6, 11.2 Hz, H-1b), 4.03 (1H, dd, *J* = 4.2, 8.5 Hz, H-4), 4.21 (1H, dd, *J* = 4.1, 4.2 Hz, H-3); ^{13}C NMR (125 MHz, D_2O) δ 62.8 (C-2), 63.2 (C-1), 64.3 (C-5), 64.6 (C-6), 74.7 (C-3), 76.3 (C-4); HRFABMS *m/z* 164.0925 $[M+H]^+$ (calcd for $C_6H_{14}NO_4$ 164.0923).

3.3.2. β-1-*C*-Butenyl-1-deoxy-1-galactonojirimycin (8)

Colorless powder; $[\alpha]_D$ −10.8 (*c* 0.10, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 1.51 (1H, m, H-7a), 1.93 (1H, m, H-7b), 2.15 (1H, m, H-8a), 2.24 (1H, m, H-8b), 2.51 (1H, ddd, *J* = 3.2, 8.3, 9.6 Hz, H-1), 2.80 (1H, ddd, *J* = 1.4, 6.4, 6.9 Hz, H-5), 3.42 (1H, t, *J* = 9.6 Hz, H-2), 3.51 (1H, dd, *J* = 3.2, 9.6 Hz, H-3), 3.64 (1H, dd, *J* = 6.9, 11.0 Hz, H-6a), 3.68 (1H, dd, *J* = 6.4, 11.0 Hz, H-6b), 3.99 (1H, dd, *J* = 1.4, 3.2 Hz, H-4), 5.04 (1H, dd, *J* = 1.8, 10.5 Hz, H-10a), 5.14 (1H, dd, *J* = 1.8, 17.4 Hz, H-10b), 5.94 (1H, dd t, *J* = 6.4, 10.5, 17.4 Hz, H-9); ^{13}C NMR (125 MHz, D_2O) δ 32.2 (C-7), 33.0 (C-8), 61.1 (C-5), 61.5 (C-1), 64.3 (C-6), 71.7 (C-4), 74.9 (C-2), 77.8 (C-3), 117.7 (C-10), 141.9 (C-9); HRFABMS *m/z* 218.1393 $[M+H]^+$ (calcd for $C_{10}H_{20}NO_4$ 218.1392).

3.3.3. 2,3-Dideoxy-β-1-*C*-ethyl-1-deoxygalactonojirimycin (9)

Colorless powder; $[\alpha]_D$ +11.8 (*c* 1.89, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 93Ht *J* = 73 Hz CH_3), 1.27 (1H, m, H-2a), 1.42–1.48 (2H, m, H-7a, H-7b), 1.52 (1H, m, H-2b), 1.80–1.92 (2H, m, H-3a, H-3b), 2.81 (1H, m, H-5), 3.19 (1H, m, H-1), 3.74 (1H, dd, *J* = 4.6, 11.5 Hz, H-6a), 3.87 (1H, dd, *J* = 9.6, 11.5 Hz, H-6b), 3.98 (1H, DDD, *J* = 1.5, 3.3, 4.1 Hz, H-4); ^{13}C NMR (125 MHz, D_2O) δ 12.6 (C-8), 29.7 (C-2, 3, 7), 52.6 (C-1), 59.3 (C-5), 60.2 (C-6), 70.5 (C-4); HRFABMS *m/z* 160.1338 $[M+H]^+$ (calcd for $C_8H_{18}NO_2$ 160.1335).

3.3.4. 6-*O*-β-D-Glucopyranosyl-2,3-dideoxy-β-1-*C*-ethyl-1-deoxygalactonojirimycin (10)

Colorless powder; $[\alpha]_D$ −1.0 (*c* 3.15, H_2O); HRFABMS *m/z* 322.1874 $[M+H]^+$ ($C_{14}H_{28}NO_7$ requires 322.1866); ^{1}H NMR (500 MHz, D_2O) δ 0.86 (3H, t, *J* = 7.3 Hz, CH_3), 1.28 (1H, m, H-2a), 1.45 (2H, H-7a, H-7b), 1.53 (1H, m, H-2b), 1.80 (1H, m, H-3a), 1.86 (1H, m, H-3b), 2.87 (1H, m, H-1), 3.25 (1H, dd, *J* = 8.2, 9.2 Hz, H-2′), 3.34 (1H, t, *J* = 9.2 Hz), 3.37–3.44 (2H, m, H-5, H-5′), 3.45 (1H, t, *J* = 9.2 Hz, H-3′), 3.63 (1H, dd, *J* = 5.5, 12.4 Hz, H-6′a), 3.87 (1H, dd, *J* = 2.3, 12.4 Hz, H-6′b), 3.91 (1H, dd, *J* = 10.1, 11.0 Hz, H-6a), 3.95 (1H, m, H-4), 4.03 (1H, dd, *J* = 3.7, 11.0 Hz, H-6b), 4.43 (1H, d, *J* = 8.2 Hz, H-1′); ^{13}C NMR (125 MHz, D_2O) δ 12.7 (C-8), 28.4 (C-7), 29.2 (C-2, C-3), 53.7 (C-1), 57.7 (C-5), 63.5 (C-6′), 69.4 (C-4), 69.5 (C-6), 72.4 (C-4′), 76.0 (C-2′), 78.4 (C-3′), 78.8 (C-5′), 106.0 (C-1′); HRFABMS *m/z* 322.1874 $[M+H]^+$ (calcd for $C_{14}H_{28}NO_7$ 322.1866).

3.4. Preparation of *N*-butyl-1-deoxygalactonojirimycin

1-Deoxygalactonojirimycin was synthesized in a highly stereocontrolled mode from *cis*-4,5-oriented dioxanylpiperidene as a chiral building block according to the literature.[17] The N-butylation of DGJ was prepared by treated with the butyl bromide and K_2CO_3 in dimethylformamide. The reaction mixture was evaporated in vacuo, and the residual syrup was resolved in MeOH and applied to an Amberlist 15 column (H^+ form), eluted with 0.5 M NH_4OH and concentrated. The eluate was finally purified by Dowex 1-X2 (OH^- form) and Amberlite CG-50 column (NH_4^+ form) chromatography with water as eluent.

3.4.1. *N*-Butyl-1-deoxygalactonojirimycin (*N*B-DGJ)

Colorless powder; HRFABMS *m/z* 220.1547 $[M+H]^+$ ($C_{10}H_{22}NO_4$ requires 220.1549); ^{13}C NMR (125 MHz, D_2O) δ 16.1, 23.0, 27.9, 55.0 (*N*-butyl), 58.6 (C-1), 63.3 (C-6), 65.5 (C-5), 69.9 (C-2), 73.0 (C-4), 77.9 (C-3).

3.5. Biological assays

The enzymes α-glucosidase (from rice, assayed at pH 5.0; from yeast, assayed at pH 6.8), β-glucosidase (from almond, pH 5.0; from *C. saccharolyticum*, pH 5.0; bovine liver, pH 6.8), α-galactosidase (from coffee bean, pH 6.5), β-galactosidase (from bovine liver, pH 6.8), α-mannosidase (from jack beans, pH 4.5), β-mannosidase (from rat epididymis, pH 4.5), α-L-fucosidase (from bovine epididymis, pH 5.5), trehalase (from porcine kidney, pH 6.5), amyloglucosidase (from *Aspergillus niger*, pH 4.5), *p*-nitrophenyl glycosides, and various disaccharides were purchased from Sigma–Aldrich Co. Brush border membranes were prepared from the rat small intestine according to the method of Kessler et al.,[18] and were assayed at pH 5.8 for rat intestinal maltase using maltose. For rice α-glucosidase and rat intestinal maltase activities, the reaction mixture (0.2 mL) contained 25 mM maltose and the appropriate amount of enzyme, and the incubations were performed for 10–30 min at 37 °C. The reaction was stopped by heating at 100 °C for 3 min. After centrifugation (600 g; 10 min), 0.05 mL of the resulting reaction mixture were added to 3 mL of the Glucose CII-test Wako (Wako Pure Chemical Ind., Osaka, Japan). The absorbance at 505 nm was measured to determine the amount of the released D-glucose. Other glycosidase activities were determined using an appropriate *p*-nitrophenyl glycoside as substrate at the optimum pH of each enzyme. The reaction mixture (1 mL) contained 2 mM of the substrate and the appropriate amount of enzyme. The reaction was stopped by adding 2 mL of 400 mM Na_2CO_3. The released *p*-nitrophenol was measured spectrometrically at 400 nm.

The preparation and in vitro enzyme assay of α-Gal A were performed according to the methods described in our previous paper (Asano, N., 2000). The Fabry R301Q lymphoblasts were cultured in DMDM (Dulbecco's modified Eagle's medium; Sigma–Aldrich Co.) supplemented with 10% FCS. Cells were cultured in a water-jacket incubator at 37 °C under 5% CO_2 in the presence or absence of DIA for 3 days. The intracellular α-Gal A assay was performed as described previously.[6]

3.6. Thermostability of α-Gal A

The enzyme α-Gal A was incubated at 48 °C in 100 mM sodium citrate buffer (pH 4.6) containing 1 mg/mL of BSA at various incubation time. After incubation, an aliquot was diluted with fourfold volume of 100 mM sodium citrate buffer (pH 4.6), and then the remaining α-Gal A activity was assayed immediately using *p*-nitrophenyl-α-D-galactoside as substrate.

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