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Preparation and biological activity of manno- and galacto-validamines, new 5a-carba-glycosylamines as α-glycosidase inhibitors

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Abstract

Manno- and galacto-validamines, which are epimers of validamine, were semi-synthesized by the configurational inversion of validamine, a pseudo-sugar analogue of α-D-glucopyranose that has inhibitory activity for α-glucosidases. The inhibitory activities of these analogues were determined against several mannosidases and galactosidases. Manno-validamine shows potent inhibition for the α-mannosidases (competitive, $K_i = 4.6 \times 10^{-5}$ M for jack beans, and competitive, $K_i = 2.8 \times 10^{-5}$ M for almonds), and galacto-validamine shows weak inhibition for the α-galactosidases (coffee bean and *E. coli*). The inhibitory effect of the epimers on the N-linked oligosaccharide-processing mannosidases involved in glycoprotein biosynthesis and lysosomal mannosidase from rat liver were also examined. Manno-validamine shows potent inhibition on the endoplasmic reticulumal α-mannosidase (competitive, $K_i = 1.2 \times 10^{-6}$ M), Golgi mannosidases IA, II (competitive, $K_i = 2.8 \times 10^{-5}$ M), and lysosomal α-mannosidase (competitive, $K_i = 1.7 \times 10^{-5}$ M). © 1997 Elsevier Science Ltd.

Keywords: Pseudo-sugar; Manno-validamine; Galacto-validamine; α-Glycosidase inhibitor

1. Introduction

Validamine (**1a**), hydroxyvalidamine (**1b**), valienamine (**2**) and valiolamine (**1c**), which are pseudo sugar analogues of α-D-glucose in which the ring oxygen is replaced with a carbon atom, were first isolated by the chemical or microbial degradation of validamycins [1,2]. They are stronger α-specific glucosidase inhibitors of a new class [3] and show competitive inhibition of small intestinal carbohydrolases such as sucrase, maltase, glucoamylase, isomaltase and trehalase [4], as well as N-linked oligosaccharide-processing glucosidases I and II that are involved in glycoprotein biosynthesis in the rat [5].

Manno- (**1d**) and galacto-validamine (**1e**), which are epimers of validamine, are pseudo-sugar analogues of α-D-mannose and α-D-galactose, respectively. Some related compounds have been chemically synthesized by other groups [6–14]. As to manno-analogue, S. Ogawa et al. reported a synthesis

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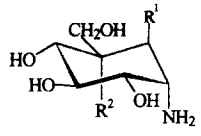
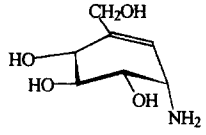
of the racemic *N*-acetyl-tetra-*O*-acetate from *exo*-2-*endo*-3-diacetoxy-*endo*-5-acetoxymethyl-7-oxabicyclo [2.2.1]heptane; however, the inhibitory effects on glycosidases for these compounds are not well-known, with only a few exceptions [13,14]. We semi-synthesized the two epimers **1d** and **1e** by the configurational inversion of validamine, which is easily prepared by the microbial degradation of validamycins [15].



Validamine (**1a**): $R_1 = H, R_2 = H$
Hydroxyvalidamine (**1b**): $R_1 = OH, R_2 = H$
Valiolamine (**1c**): $R_1 = H, R_2 = OH$



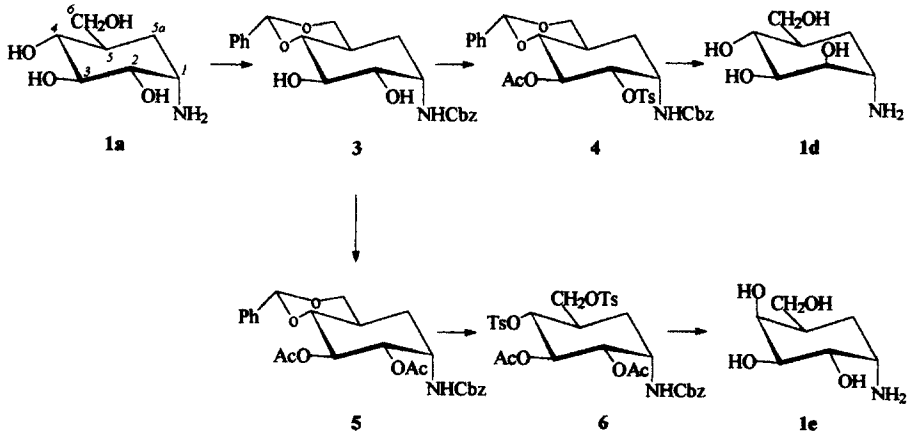
Valienamine (**2**)

We determined their inhibitory activities against some commercially available mannosidases and galactosidases. The inhibitory effect of the validamine epimers on the *N*-linked oligosaccharide-processing mannosidases IA and II in glycoprotein biosynthesis and lysosomal α-mannosidase from rat liver was also elucidated by a comparison with swainsonine and deoxymannojirimycin.

2. Results and discussion

The synthesis of **1d** and **1e** began with 4,6-*O*-benzylidene-*N*-benzyloxycarbonyl-5a-carba-D-glucopyranosylamine (**3**) [16]. See Scheme 1. Selective 2-*O*-toluenesulfonylation of 3 with a slight excess of *p*-toluenesulfonyl chloride in pyridine at room temperature, followed by acetylation, gave the 3-*O*-acetyl-2-*O*-tosyl derivative **4** in 64% overall yield. Configurational inversion at C-2 of **4** with excess sodium benzoate afforded the 2-epimeric derivative, which was sequentially deprotected without separation [debenzylidenation with N HCl in MeOH, deacetylation and *N*-debenzyloxycarbonation with 10% $Ba(OH)_2$]. The product was separated from the byproducts and validamine by CM-Sephadex C-25 (NH_4^+ form) chromatography with 0.02 N NH_4OH. The faster eluting fraction was lyophilized to give manno-validamine (**1d**) in 13% yield. The structure of **1d** was proved by 400 MHz ^1H NMR spectroscopy and a $^{13}C-^1H$ COSY experiment. The observed chemical signals and coupling constants for H-1 (δ 3.16, ddd, $J_{1,2}$ 3.5, $J_{1,6eq}$ 3.5, $J_{1,6ax}$ 3.5 Hz), H-2 (δ 3.87, dd, $J_{2,3}$ 3.0 Hz), H-3 (δ 3.73, dd, $J_{3,4}$ 9.2 Hz) and H-4 (δ 3.61, dd, $J_{4,5}$ 9.5 Hz) indicate the α-manno configuration. Other ^1H and ^{13}C NMR data are given in the Experimental section and are consistent with the structure assigned.

Galacto-validamine (**1e**) was prepared as follows. The 4,7-*O*-benzylidene-*N*-benzyloxycarbonyl derivative **3** was acetylated to give the 2,3-diacetate **5**. Debenzylidation with 0.2 N HCl in MeOH gave the 2,3-diacetate. *p*-Toluenesulfonylation of the diacetate with TsCl in pyridine gave the 4,7-di-*O*-tosyl derivative **6**. Configurational inversion at C-4 of **6** with excess sodium benzoate in Me_2NCHO afforded the 4-epimeric derivative, which was deprotected with 10% $Ba(OH)_2$. The product was separated from the byproducts and from validamine by CM-Sephadex C-25 (NH_4^+ form) chromatography with 0.02 N NH_4OH. Validamine was eluted first, and the next fraction that eluted was lyophilized to give galacto-validamine (**1e**) in 23% yield. The ^1H NMR spectrum showed H-2 (δ 3.76, dd, $J_{2,3}$ 10.0 Hz), H-3 (δ 3.67,



Scheme 1.

dd, $J_{3,4}$ 3.0 Hz) and H-4 (δ 4.40, dd, $J_{4,5}$ 3.5 Hz), which indicated the galacto-configuration for **3**. The observed signal (δ 4.40) for H-4 in lower field is characteristic of the galactose configuration.

The inhibitory effects of manno- and galacto-validamines on commercially available mannosidases and galactosidases from various organisms were examined, and the results are presented in Table 1. The inhibitory activities were assayed at 37 °C and at the pH optimum of each enzyme with the respective *p*-nitrophenyl glycosides as substrates. The dissociation constants for competitive inhibition were calculated from the Lineweaver–Burk plots. The data demonstrate that manno-validamine shows potent inhibition for the α-mannosidases (competitive, $K_i = 4.6 \times 10^{-5}$ M for jack beans, and competitive, $K_i = 2.8 \times 10^{-5}$ M for almonds), and galacto-validamine showed weak inhibition for the α-galactosidases (coffee bean, *E. coli*). Galacto-validamine also showed moderate inhibition for the β-galactosidase of *Aspergillus niger*.

The inhibitory effect of the epimers on rat liver α-mannosidases was also elucidated by comparison with swainsonine and deoxymannojirimycin. The results are presented in Table 2. The activities of the endoplasmic reticulumal (ER), Golgi II and lysosomal α-mannosidases were assayed by the common methods in the previous literature, and the Golgi mannosidase IA was assayed by the HPLC method with fluorescence using a pyridylamino derivative of $Man_6GlcNAc_2$ as a substrate. Detailed descriptions are given in the experimental section.

Manno-validamine showed potent inhibition of the endoplasmic reticulum α-mannosidase (competitive, $K_i = 1.2 \times 10^{-6}$ M), Golgi mannosidases IA, II (competitive, $K_i = 2.8 \times 10^{-5}$ M) and lysosomal (competitive, $K_i = 1.7 \times 10^{-5}$ M), though swainsonine showed specifically potent inhibition on Golgi

Table 1
Inhibitory effects of manno- and galacto-validamines on various mannosidases and galactosidase

		IC_{50} (M) [a]		
		Manno-validamine	Galacta-validamines	validamine
α-mannosidases				
Jack beans	(pH 4.5)	5.6×10^{-5}	NI [b]	NI
Almonds	(pH 4.5)	3.6×10^{-5}	NI	NI
Turbo cornutus	(pH 4.0)	4.0×10^{-5}NI	NI	
β-mannosidase				
Snail	(pH 4.0	NI	NI	NI
α-galactosidases				
Coffee beans	(pH 6.5)	NI	5.0×10^{-4}	NI
E. coli	(pH 6.5)	NI	8.9×10^{-4}	NI
Asp. niger	(pH 4.0)	NI	NI	NI
β-galactosidases				
Jack beans	(pH 3.5)	NI	NI	NI
E. coli	(pH 6.8)	NI	NI	NI
Asp. niger	(pH 4.0)	NI	2.1×10^{-4}	NI

[a] Concentration giving 50% inhibition.
[b] NI = no inhibition (less than 50% inhibition at 2.0×10^{-3} M).

Table 2
Inhibitory effect of manno-validamine on rat liver α-mannosidases

	IC_{50} (M) [a]		
	Manno-validamine	Deoxymannojirimycin	Swainsonine
ER-α-mannosidase, pH 6.5	8.0×10^{-6}	NI [b]	3.0×10^{-5}
Golgi-α-mannosidase IA, pH 5.5	3.4×10^{-5}	2.8×10^{-5}	NI
Golgi-α-mannosidase II, pH 5.5	3.0×10^{-5}	7.4×10^{-5}	2.0×10^{-8}
Lysosomal-α-mannosidase, pH 4.5	2.4×10^{-5}	6.4×10^{-4}	3.6×10^{-8}

[a] Concentration giving 50% inhibition.
[b] NI = no inhibition (less than 50% inhibition at 2.0×10^{-3} M).

mannosidase II and lysosomal α-mannosidase, and deoxymannojirimycin showed little or no inhibition on ER α-mannosidase. There are significant differences in the inhibitory specificity between manno-validamine and the other analogues. Manno-validamine may be useful as a tool to establish the function of α-mannosidases that are involved in the trimming process involved in glycoprotein biosynthesis. More studies are indicated to determine the more potent derivatives of manno-validamine, such as N-substituted derivatives, which were done in the case of validamine, valienamine and valiolamine by the chemical modifications [16,17].

3. Experimental

General methods.—Melting points were determined with a Yanaco MP-S3 micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a Jasco DIP-370 digital polarimeter in H_2O at 25 °C. ^1H and ^{13}C NMR spectra were recorded on a Jeol JNM-GX 400 spectrometer for solution in D_2O using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the internal standard. Silica gel TLC was performed on E. Merck Silica Gel $60F_{254}$ plates using the solvent system 4:1:1 PrOH–AcOH–H_2O.

3-O-Acetyl 4,6-O-benzylidene-N-benzyloxycarbonyl 2-O-(p-tolylsufonyl)-5a-carba-D-glucopyranosylamine (**4**).—A solution of *p*-toluenesulfonyl chloride (3.5 g, 18.4 mmol) in CH_2Cl_2 (20 mL) was added dropwise to a cooled solution of **3** (5.0 g, 12.5 mmol) in dry pyridine (30 mL) at 0 °C over 30 min, and the mixture was stirred for 14 h at room temperature. Acetic anhydride (10 mL) was then added to the mixture at 0 °C. The mixture was stirred for 15 h at room temperature and then poured into CH_2Cl_2(300 mL). The organic layer was successively washed with aqueous $NaHCO_3$ and water and evaporated. The residue was chromatographed on silica gel (200 g) using 10:1 $CHCl_3$–EtOAc to give **4** (4.8 g, 64.3%): mp 124–125 °C; $[\alpha]_D$ +41.7° (*c* 1, $CHCl_3$); ^1H NMR (90 MHz, $CDCl_3$): δ 1.90 (s, 3 H, CH_3CO–), 1.13–2.09 (m, 3 H), 2.40 (s, 3 H, CH_3–Ph), 3.51 (m, 2 H, H-2 and H-4), 4.12 (d, 1 H), 4.27 (d, 1 H), 4.70 (m, 1 H), 5.31 (d, 1 H, H-3), 7.24–7.75 (m, 14 H, Ph). Anal. Calcd for $C_{27}H_{35}NO_7Si$: C, 63.10; H, 5.55; N, 2.73. Found: C, 62.80; H, 5.15; N, 2.31.

5a-Carba-D-mannopyranosylamine (manno-validamine, **1d**).—A mixture of **4** (3.5 g, 6.8 mmol) and sodium benzoate (3.5 g, 24.3 mmol) in Me_2NCHO was heated with stirring for 120 h at 140 °C and evaporated. The residue was dissolved in MeOH (20 mL) and N HCl (6 mL), refluxed for 30 min, and then evaporated. The residue was treated with 10% $Ba(OH)_2$ (30 mL) at 80 °C for 4 h, filtered and concentrated. To separate from the byproducts and validamine, the residue was chromatographed on CM-Sephadex C-25 (NH_4^+ form, 180 mL) with 0.02 N NH_4OH. The faster eluted fraction (TLC, R_f = 0.39, 4:1:1 PrOH–AcOH–H_2O) was lyophilized to give **1d** as a white solid in 13% yield: $[\alpha]_D$ +8.2° (*c* 1, water); ^1H NMR (400 MHz, D_2O): δ 1.63 (dt, *J* 13.0, 3.5 Hz, H-6_{eq}), 1.74 (ddd, *J* 13.0, 12.5, 3.5 Hz, H-6_{ax}), 1.81 (m, H-5), 3.16 (ddd, *J* 3.5, 3.5, 3.5 Hz, H-1), 3.61 (dd, *J* 9.5, 9.2 Hz, H-4), 3.64 (dd, *J* 11.0, 3.5 Hz, H-7a), 3.73 (dd, *J* 3.0, 9.2 Hz, H-3), 3.76 (dd, *J* 11.0, 3.5 Hz, H-7b), 3.87 (dd, *J* 3.5, 3.0 Hz, H-2); ^{13}C NMR (100 MHz, D_2O): δ (from DSS) 30.30 (t, C-6), 41.68 (d, C-5), 52.59 (d, C-1), 65.36 (t, C-7), 73.28 (d, C-4), 74.77 (d, C-3), 76.39 (d, C-2). Anal. Calcd for $C_7H_{15}NO_4 \cdot H_2O$: C, 43.07; H, 8.78; N, 7.17. Found: C, 42.63; H, 9.25; N, 7.01.

2,3-Di-O-acetyl-4,6-O-benzylidene-N-benzyloxy carbonyl-5a-carba-D-glucopyranosylamine (**5**).—To a solution of **3** (5.0 g, 12.5 mmol) in dry pyridine (40 mL) was added acetic anhydride (5 mL, 49.0 mmol). The mixture was stirred for 8 h at room temperature, quenched with water (10 mL), and concentrated to give an oil. The oil was dissolved in CH_2Cl_2 (50 mL), and the solution was washed with water, dried over $MgSO_4$, and evaporated to give **5** (5.4 g, 89.6%) as an amorphous solid: $[\alpha]_D$ +45.5° (*c* 1, $CHCl_3$). Anal. Calcd for $C_{26}H_{29}NO_8$: C, 64.59; H, 6.05; N, 2.90. Found: C, 64.66; H, 6.21; N, 2.89.

2,3-Di-O-acetyl-N-benzyloxycarbonyl-4,6-di-O-(p-tolylsulfonyl)-5a-carba-D-glucosylamine (**6**).—A solution of 4 (5.0 g, 9.7 mmol) in MeOH and 0.5 N HCl was refluxed for 30 min, then diluted with aqueous $NaHCO_3$, and extracted with EtOAc (300 mL). The organic layer was washed with water, dried over $MgSO_4$, and evaporated. A solution of *p*-toluenesulfonyl chloride (5.0 g, 26.2 mmol) in CH_2Cl_2 (20 mL) was added dropwise to a cooled solution of the residue in dry pyridine (20 mL) at 0 °C over 30 min, and the mixture was stirred for 14 h at room temperature and then poured into CH_2Cl_2 (300 mL). The organic layer was successively washed with aqueous $NaHCO_3$, water, and evaporated. The residue was purified by silica gel chromatography using 5:1 benzene–EtOAc to give **6** (4.5 g, 85.6%), as an amorphous solid: $[\alpha]_D$ +41.8° (*c* 1, $CHCl_3$);

^{1}H NMR (90 MHz, $CDCl_3$): δ 1.91 (3 H, s), 1.96 (3 H, s), 2.42 (3 H, s, CH_3–Ph), 2.44 (3 H, s, CH_3–Ph), 5.03 (2 H, s, –CH_2–Ph), 7.30–7.34 (9 H, m), 7.68 (2 H, d), 7.76 (2 H, d). Anal. Calcd for $C_{25}H_{41}NO_8Si_2$: C, 55.63; H, 7.66; N, 2.59. Found: C, 55.35; H, 7.52; N, 2.48.

*5a-Carba-*D*-mannopyranosylamine (galacto-validamine,* **1e**).—A mixture of **5** (3.5 g, 7.2 mmol) and sodium benzoate (3.0 g, 20.8 mmol) in Me_2NCHO (100 mL) was heated with stirring for 120 h at 140 °C and evaporated. The residue treated with 10% $Ba(OH)_2$ (30 mL) at 80 °C for 4 h, filtered and concentrated. The residue was chromatographed on CM-Sephadex C-25 (NH_4^+ form, 180 mL) with 0.02 N NH_4OH. Validamine was first eluted and the subsequently eluted fraction (TLC, $R_f = 0.37$, 4:1:1 PrOH–AcOH–H_2O) was lyophilized to give **1d** (0.22 g, 23%) as a white solid: $[\alpha]_D$ +73.8° (*c* 1, H_2O); ^{1}H NMR (400 MHz, D_2O): δ 1.58 (overlapped, H-6_{eq}), 1.59 (ddd, *J* 14.0, 12.5, 3.6 Hz, H-6_{ax}), 1.97 (m, H-5), 3.27 (ddd, *J* 3.5, 3.5, 4.0 Hz, H-1), 3.48 (dd, *J* 11.0, 6.0 Hz, H-7a), 3.62 (dd, *J* 11.0, 8.0 Hz, 7b-H), 3.67 (dd, *J* 10.0, 3.0 Hz, 3 H), 3.76 (dd, *J* 4.0, 10.0 Hz, 2 H), 4.40 (dd, *J* 3.0, 3.5 Hz, H-4); ^{13}C NMR (100 MHz, D_2O): δ (from DSS) 29.42 (t, C-6), 38.84 (d, C-5), 65.29 (t, C-7), 72.76 (d, C-4), 73.46 (d, C-3), 73.50 (d, C-2). Anal Calcd for $C_7H_{15}NO_4 \cdot H_2O$: C, 43.07; H, 8.78; N, 7.17. Found: C, 42.84; H, 8.99; N, 7.04.

Enzymes and activity measurements.—Hydrolytic activities of α-mannosidases, from jack beans (Sigma, M7257), almond (Sigma, M1266) Turbo cornutus (Sigma, M0893) and the endoplasmic reticulum α-mannosidase, Golgi α-mannosidase II, the lysosomal α-mannosidase from rat liver, and the activities of β-mannosidase from snail (Sigma, M9400), and α-galactosidases from coffee beans (Sigma, G8507), *Escherichia coli* (Sigma, G6762), *Aspergillus niger* (Sigma, G3397) and β-galactosidases from jack beans (Sigma, G0884), *Escherichia coli* (Sigma, G6008), *Aspergillus niger* (Sigma, G3522) were assayed at 37 °C by recording of A_{400} with the respective 2 mM *p*-nitrophenyl (PNP) glycosides as substrates. When the steady-state hydrolysis rate in the presence of inhibitor was approached slowly, the rate was calculated from the slope of asymptotes drawn to the product curves. The activity of mannosidase IA from rat liver was determined by HPLC analysis. To a substrate solution of pyridylamino–$Man_6GlcNAc_2$ (0.5 nmol, Takara Shuzo Co. Ltd., Japan) in acetate buffer (pH 5.5) was added the enzyme solution of Golgi α-mannosidase IA, and the mixture was incubated at 37 °C for 4–8 h. A 2 μL sample was injected onto the HPLC column, and the decrease of substrate was determined by comparing the peak area with that of the initial sample. HPLC analysis was performed on a μ-Bondasphere 5 μ C_{18} column (Waters) with 100 mM acetic acid–triethylamine (pH 4.0) as a solvent at a flow rate of 0.5 mL/min using a fluorescence spectrometer (Ex 320 nm, Em 400 nm, Hitachi F-1050).

Preparation of endoplasmic reticulum α-mannosidase.—The rough endoplasmic reticulum fraction was prepared from livers of fed male Wistar rats (250–350 g) by the procedure of Kreibach et al. [18]. The enzyme was partially purified according to the method of Bischoff et al. [19]. In this procedure, The α-mannosidase-active fraction that did not bind to a ConA-Sepharose column was referred to as the endoplasmic reticulum α-mannosidase. The specific activity towards 2 mM PNP-α-mannoside as a substrate was 2.4 nmol/min/mg protein.

Preparation of Golgi mannosidases IA and II.—The preparation of rat Golgi membranes was based on method of Leelavathi et al. [20], and the enzymes were partially purified according to the procedure described by Tulsiani et al. [21] with some modification. The highly purified Golgi membranes were suspended in 50 mM Tris-HCl buffer (pH 7.2) containing 5 mM $MgCl_2$ and 0.3% Triton X-100 with stirring at 4 °C for 1 h. The suspension was centrifuged at 108 000 × *g* for 1 h. The supernatant solution was dialyzed against 10 mM phosphate buffer (pH 5.8) containing 5 mM $MgCl_2$ and 0.1% Triton X-100 and applied to a cellulose phosphate column (15 × 1 cm) equilibrated with the same buffer. After washing the column with the buffer (120 mL), it was eluted with 300 mL of 10 mM phosphate buffer (pH 7.2) containing 5 mM $MgCl_2$ and 0.1% Triton X-100, followed 500 mL of the same buffer containing 500 mM NaCl. Fractions (8.4 mL) were collected at a flow rate of 20 mL/h. The fractions (Nos. 22–24) of the first buffer (pH 7.2) did not show PNP-α-mannosidase activity, but showed the $Man_6GlcNAc_2$-α-mannosidase activity using pyridylamino–$Man_6GlcNAc_2$ by the HPLC method. The fractions were concentrated to 1 mL and referred to as mannosidase IA. The salt-eluted fractions (Nos. 51–52, Mannosidases 1B and II) that showed PNP-α-mannosidase activity were dialyzed for 5 h against 50 mM Tris-HCl buffer (pH 7.6) containing 5 mM $MgCl_2$ and 0.1% Triton X-100. The dialyzed enzyme was applied to a second cellulose phosphate column (10 × 1 cm) equilibrated with the same buffer. After

washing (80 mL), the column was eluted with 250 mL of a NaCl gradient (0–0.5 M) in the buffer. Fractions (5 mL) were collected and analyzed for the $Man_6GlcNAc_2$-α-mannosidase and PNP-α-mannosidase activity. The wash fractions (mannosidase 1B) showed little of either activity. The salt-eluted fractions (Nos. 25–36) were concentrated to 2 mL and referred to as mannosidase II. The specific activity towards PNP-α-mannoside as substrate was 2 nmol/min/mg protein.

Preparation of lysosomal α-mannosidase.—The enzyme was partially purified according to the method of Opheim et al. [22]. In this procedure, the last two steps (Dowex 50W and affinity chromatography) were omitted. In brief, The extract of the mitochondrial-lysosomal fraction was adjusted to 40% ammonium sulfate saturation. The suspension was stirred at 4 °C for 15 min and then centrifuged at $22\,000 \times g$ for 15 min. The supernatant solution was brought to 65% saturation with ammonium sulfate, stirred for 15 min, and centrifuged. The pellet was dissolved in 10 mM acetate buffer (pH 6.0) containing 1 mM $MgSO_4$ and 1 mM 2-mercaptoethanol. Cold acetone (−20 °C) was added with stirring until the acetone contraction was 40%. After 10 min stirring, the suspension was centrifuged at $22\,000 \times g$ for 10 min. The pellet was dissolved in the buffer containing 50 mM NaCl and applied to a cellulose phosphate column (1.5 × 7 cm) equilibrated with the same buffer. The column was developed with a linear salt gradient of NaCl (50–300 mM), and 13 mL fractions were collected. Fractions (Nos. 26–38) were applied to a hydroxylapatite column (2 × 3 cm) equilibrated with 10 mM phosphate buffer (pH 6.0) containing 1 mM $MgSO_4$, 1 mM 2-mercaptoethanol and 300 mM NaCl. A gradient (50–400 mM sodium phosphate) was applied and 10-mL fractions were collected. Fractions (Nos. 11–17) were concentrated to 5 mL and referred to as the lysosomal α-mannosidase. The specific activity was 775 nmol/min/mg protein with PNP-α-mannoside as a substrate.

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