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MEDICINAL FOODSTUFFS. XXXII.[1] NOVEL SESQUITERPENE GLYCOSIDE SULFATE, FUKINOSIDE A, WITH ANTIALLERGIC ACTIVITY FROM JAPANESE BUTTERBUR (*PETASITES JAPONICUS*)

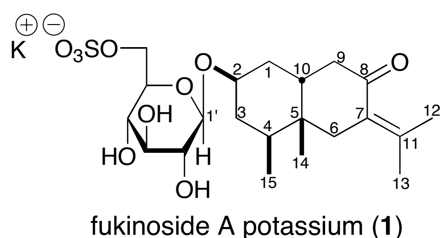
Masayuki Yoshikawa,*[,a)] Toshio Morikawa,[a,b)] Junji Tanaka,[c)]
and Hiroshi Shimoda[c)]

a) Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607-8412, Japan
b) Pharmaceutical Research and Technology Institute, Kinki University, 3-4-1 Kowakae, Higashi-osaka, Osaka 577-8502, Japan
c) Research and Development Division, Oryza Oil & Fat Chemical Company, Ltd., 1 Numata Kitagata-cho, Ichinomiya, Aichi 493-8001, Japan

Abstract — Novel sesquiterpene glycoside sulfate, fukinoside A, was isolated as the potassium salt from the aerial parts of *Petasites japonicus*. The absolute sterostructure of fukinoside A was elucidated on the basis of chemical and physicochemical evidence. In addition, fukinoside A was found to inhibit release of *β*-hexosaminidase, as a marker of antigen-induced degranulation, in RBL-2H3 cells.

The Compositae plant *Petasites* (*P.*) *japonicus* MAXIM. (Japanese butterbur in English, Fuki in Japanese) has been cultivated as a vegetable in Japan. The fresh stems of *P. japonicus* have been used as a food garnish in Japanese-style dishes. On the other hand, the rhizomes of this plant have been used for the treatment of tonsillitis, contusions, and poisonous-snake bite in China.[2] In previous studies, several sesquiterpenes, triterpenes, anthraquinones, and phenolic compounds were isolated from the rhizomes of *P. japonicus*.[3–14] In the course of our characterization studies on the bioactive constituents in medicinal foodstuffs,[1,15–19] we have reported that the 70% aqueous ethanol extract from the dried aerial parts of *P. japonicus* was found to show an anti-allergic effect.[20] As a continuing study on this herbal medicine, we have isolated a novel eremophilane-type sesquiterpene glycoside sulfate named fukinoside A as the potassium salt (**1**). In addition, **1** was found to show inhibitory effect on the release of *β*-hexosaminidase in RBL-2H3 cells. This paper deals with the absolute stereostructure elucidation of **1** as well as the inhibitory effect of **1** on the release of *β*-hexosaminidase in RBL-2H3 cells.

The dried aerial parts of *P. japonicus* (cultivated in Aichi prefecture, Japan) were extracted with 70% aqueous ethanol (EtOH) at 70 ºC for 2 h to give an aqueous EtOH extract (12.4% from this herbal medicine). The aqueous ethanolic extract was partitioned in an ethyl acetate (EtOAc)/water mixture to



give an EtOAc-soluble fraction (1.0%) and an aqueous layer. The aqueous layer was extracted with *n*-butanol (*n*-BuOH) to give *n*-BuOH and H_2O-soluble fractions (0.7, 9.4%, respectively). In our previous report, 2*β*-hydroxyfukinone (**2**), (+)-fukinone, fukinolic acid, chlorogenic acid, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid methyl ester, caffeic acid, dotorioside II, and mussaendoside R were isolated from this extract.[20] Continuing the isolation study for this herbal medicine, the *n*-BuOH-soluble fraction was subjected to silica gel and ODS column chromatography and finally HPLC to furnish fukinoside A potassium (**1**, 0.0005%).

Absolute Stereostructure of 1

Fukinoside A was isolated as the potassium salt with positive optical rotation ($[\alpha]_D^{26}$ +12.4°, in MeOH). In the positive-ion fast atom bombardment (FAB)-MS of **1**, quasimolecular ion peaks were observed at *m/z* 539 $(M+Na)^+$ and *m/z* 555 $(M+K)^+$. On the other hand, quasimolecular ion peaks were observed at *m/z* 477 $(M–K)^-$ and *m/z* 515 $(M–H)^-$ in the negative-ion FAB-MS. High-resolution MS analysis of quasimolecular ion peaks in the positive-ion FAB-MS revealed the molecular formula of **1** to be $C_{21}H_{33}KO_{10}S$, so that the presence of a potassium sulfate function in **1** was confirmed.[21] The IR spectrum of **1** showed absorption bands at 3454, 1684, 1256, 1065, and 1042 cm^{-1} ascribable to hydroxyl, *α*,*β*-unsaturated carbonyl, sulfate, and ether functions, while its UV spectrum indicated the presence of enone chromophore with absorption maximum at 251 (log *ε* 3.79) nm. Solvolysis[22,23] of **1** with pyridine–1,4-dioxane (4:1, v/v) gave **1a** as shown in Figure 2. Acid hydrolysis of **1a** with 1 M hydrochloric acid (HCl) liberated 2*β*-hydroxyfukinone (**2**)[24] as an aglycone, whose absolute configuration was left uncharacterized, together with D-glucose, which was identified by HPLC analysis using an optical rotation detector.[18,19] The ^1H-NMR (CD_3OD) and ^{13}C-NMR (Table 1) spectra of **1**, which were assigned by various NMR experiments,[25] showed signals assignable to four methyls [*δ* 0.91 (3H, d, *J* = 6.7 Hz, 15-H_3), 1.00 (3H, s, 14-H_3), 1.83 (3H, s, 13-H_3),1.92 (3H, d, *J* = 1.8 Hz, 12-H_3)], four methylenes [*δ* 1.35, 1.90 (1H each, both m, 3*β*-H and 3*α*-H), 1.78 (2H, m, 1-H_2), 2.07, 2.73 (1H each, both d, *J* = 15.0 Hz, 6*β*-H and 6*α*-H), 2.31 (1H, dd, *J* = 5.5, 16.5 Hz, 9*α*-H), 2.59 (1H, dd, *J* = 12.8, 16.5 Hz, 9*β*-H)], two methines and a methine bearing an oxygen function [*δ* 1.78 (1H, m, 4-H), 2.02 (1H, m, 10-H), 3.97 (1H, m, 2-H)], and four quaternary carbons (5, 7, 8, and 11-C) together with a hexose moiety [*δ* 4.15 (1H, dd, *J* = 5.5, 11.0 Hz), 4.29 (1H, dd, *J* = 1.8, 11.0 Hz), 6'-H_2], 4.40 (1H, d, *J* = 7.6 Hz, 1'-H)].

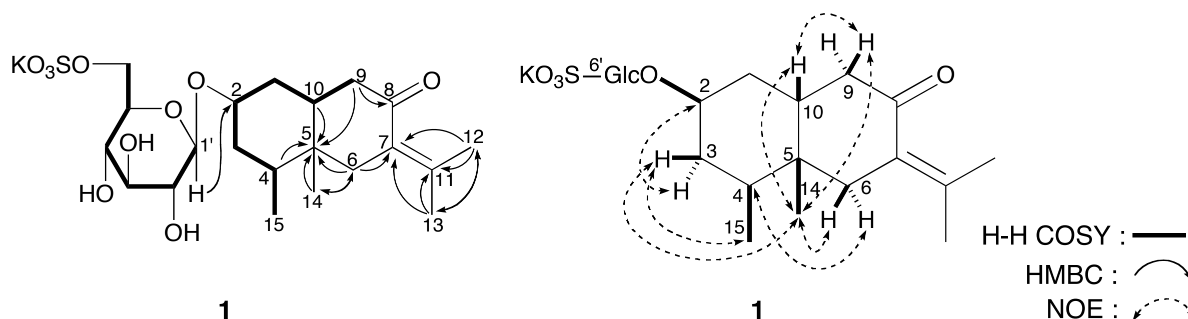


Figure 1. H–H COSY, HMBC, and NOE correlations of **1**

The proton and carbon signals due to the 6'-position in the ^{1}H-NMR and ^{13}C-NMR spectra of **1** were observed at lower fields compared to those of **1a** {δ [3.63 (1H, dd, J = 5.8, 11.9 Hz), 3.85 (1H, dd, J = 1.8, 11.9 Hz), 6'-H_2]; δ_C 62.9 (6'-C)}, so that the position of the potassium sulfate function in **1** was clarified to be the 6'-position. The eremophil-7(11)-en-8-one type sesquiterpene structure of **1** was constructed on the basis of ^{1}H—^{1}H correlation spectroscopy (^{1}H—^{1}H COSY) and heteronuclear multiple bond correlation (HMBC) experiments (Figure 1). Thus, the ^{1}H—^{1}H COSY experiment on **1** indicated the presence of partial structures written in bold lines, and in the HMBC experiment, long-range correlations were observed between the following protons and carbons (1'-H and 2-C; 4-H, 6-H_2, 9-H_2, 10-H, 14-H_3 and 5-C; 14-H_3 and 6-C; 6-H_2, 12-H_3, 13-H_3 and 7-C; 9-H_2 and 8-C, 12-H_3, 13-H_3 and 11-C; 6-H_2 and 14-C). Furthermore, the stereostructure of **1** was characterized on the basis of the nuclear Overhauser enhancement spectroscopy (NOESY) experiment, in which NOE correlations were observed between the following proton pairs of **1** (2-H and 3α-H; 3β-H and 14-H_3, 15-H_3; 4-H and 6α-H; 6β-H and 14-H_3; 9β-H and 10-H, 14-H_3; 10-H and 14-H_3).

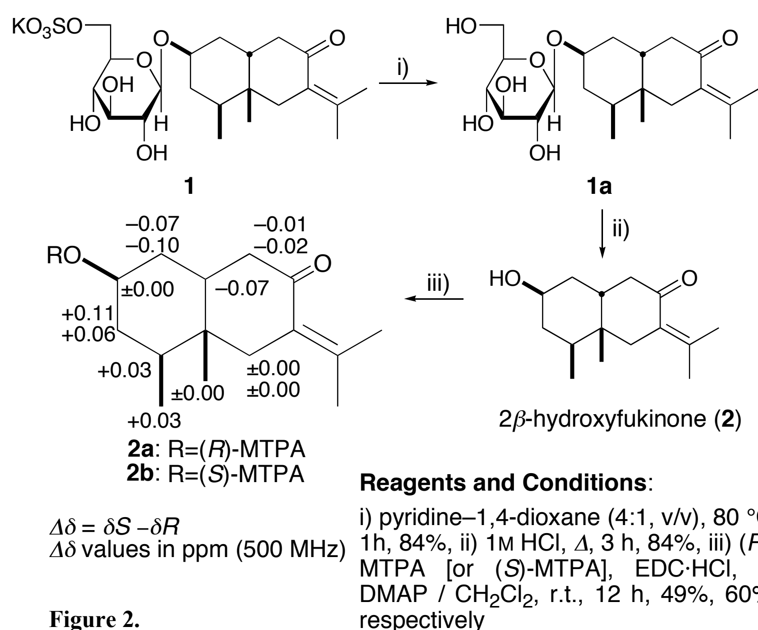


Figure 2.

Table 1. ^{13}C-NMR Data for **1**, **1a**, and **2**

	1[a]	**1a**[a]	**2**[b]	**2**[c]
C-1	35.0	35.1	36.0	37.0
C-2	75.0	74.2	66.4	65.4
C-3	37.1	37.1	39.8	41.0
C-4	31.9	31.8	31.0	31.2
C-5	37.6	37.7	36.4	36.7
C-6	41.2	41.3	40.3	40.6
C-7	131.8	131.9	130.5	131.4
C-8	207.7	207.8	205.0	204.2
C-9	45.3	45.4	44.4	45.0
C-10	43.9	44.0	42.5	43.0
C-11	142.0	142.1	140.9	139.2
C-12	22.7	22.7	22.6	22.5
C-13	21.9	21.8	21.7	21.4
C-14	21.1	21.2	20.9	21.0
C-15	16.7	16.8	16.2	16.5
C-1'	102.5	102.2		
C-2'	74.9	75.2		
C-3'	77.7	77.9		
C-4'	71.4	71.8		
C-5'	75.7	78.1		
C-6'	68.2	62.9		

Measured in [a]CD_3OD, [b]$CDCl_3$, and [c]pryidine-d_5 (125 MHz).

Finally, the absolute configurations of **1** and **2** were characterized by the application of the modified Mosher's method.[26] Namely, treatment of **2** with (R)- or (S)-2-methoxy-2-trifluoromethylphenylacetic acid [(R)- or (S)-MTPA] in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and 4-dimethylaminopyridine (4-DMAP) yielded the MTPA esters (**2a**, **2b**), respectively, whose ^{1}H-NMR data showed an acylation shift at the 2-position in **2**. As shown in Figure 2, the signals due to protons attached to the 3, 4, and 15-positions in the 2-(S)-MTPA ester (**2b**) were observed at lower fields compared with those of the 2-(R)-MTPA ester (**2a**) [$\Delta\delta$: positive], while the signals due to protons on the 1, 9, and 10-positions in **2b** were observed at higher fields compared with those of **2a** [$\Delta\delta$: negative].

Consequently, the absolute configuration at the 2-position of **2** was determined as *R* configuration and the absolute stereostructures of **1** and **2** were elucidated to be as shown.

Inhibitory Effect of 1 on the Release of *β*-Hexosaminidase in RBL-2H3 Cells

Histamine, which is released from mast cells stimulated by an antigen or a degranulation inducer, is usually determined as a degranulation marker *in vitro* experiments on immediate allergic reactions. *β*-Hexosaminidase is also stored in the secretory granules of mast cells and is released concomitantly with histamine when mast cells are immunologically activated.[27,28] Therefore it is generally accepted that *β*-hexosaminidase is a degranulation marker of mast cells. As a part of our characterization studies on the bioactive components of natural medicines, we previously reported several inhibitors of the release of *β*-hexosaminidase such as diarylheptanoids,[19,29,30] sesquiterpenes,[31] diterpenes,[32] flavonoids,[33] anthraquinones,[34] stilbenes,[35] phenanthrenes,[35] phenylpropanoids,[36] and alkaloids.[37,38] In our previous report, several constituents from *P. japonicus* showed inhibitory activities on the release of *β*-hexosaminidase in RBL-2H3 cells.[20] Compound (**1**) (IC_{50} = 16.6 μM) was also inhibited on the release of *β*-hexosaminidase and this activity was stronger than that of antiallergic compounds, tranilast[36] (IC_{50} = 282 μM) and ketotifen fumarate[36] (IC_{50} = 158 μM).

EXPERIMENTAL

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (*l* = 5 cm); UV spectra, Shimazdu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; ^{1}H-NMR spectra, JNM-LA500 (500 MHz) spectrometer; ^{13}C-NMR spectra, JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; HPLC detector, Shimadzu RID-6A refractive index and SPD-10A UV-VIS detectors; HPLC column, GL Science Inertsil ODS-3 (250 × 4.6 mm i.d.) and (250 × 10 mm i.d.) columns were used for analytical and preparative purposes, respectively.

The following experimental conditions were used for chromatography: ordinary-phase column chromatography; Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh), reversed-phase column chromatography; Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh): TLC, pre-coated TLC plates with Silica gel $60F_{254}$ (Merck, 0.25 mm) (normal-phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed-phase); HPTLC, pre-coated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm) (reversed-phase). Detection was done by spraying with 1% $Ce(SO_4)_2$-10% aqueous H_2SO_4, followed by heating.

Extraction and Isolation

The dried aerial parts of *P. japonicus* (cultivated in Aichi prefecture, Japan) were extracted with 70% aqueous EtOH at 70 ºC for 2 h to give an aqueous EtOH extract (12.4% from this herbal medicine). The aqueous EtOH extract was partitioned in an EtOAc/water mixture to give an EtOAc-soluble fraction (1.0%) and aqueous layer. The aqueous layer was extracted with *n*-BuOH to give *n*-BuOH and H_2O-soluble fractions (0.7, 9.4%, respectively), which were described previously.[20] The *n*-BuOH-soluble

fraction (17.0 g) was to reversed-phase ODS column chromatography [300 g, MeOH–H_2O (15:85 → 70:30) → MeOH] to afford 16 fractions [fr. 1 (3.47 g), fr. 2 (1.85 g), fr. 3 (0.90 g),fr. 4 (0.95 g), fr. 5 (0.41 g), fr. 6 (0.25 g), fr. 7 (0.39 g), fr. 8 (0.48 g), fr. 9 (0.33 g), fr. 10 (0.40 g), fr. 11 (0.27 g), fr. 12 (0.26 g), fr. 13 (0.30 g), fr. 14 (0.29 g), fr. 15 (0.06 g), and fr. 16 (0.26 g)]. Fraction 4 (0.95 g) was further purified by HLPC [Inertsil ODS-3 (GL Science), MeOH–H_2O (30:70, v/v)] to give **1** (12 mg, 0.0005%).

Fukinoside A potassium (**1**): A white powder, $[\alpha]_D^{26}$ +12.4° (*c*=1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{21}H_{33}KO_{10}SNa$ $(M+Na)^+$: 539.1330. Found: 539.1326; Calcd for $C_{21}H_{33}KO_{10}SK$ $(M+K)^+$: 555.1069. Found: 555.1063. UV [nm (log *ε*), MeOH]: 251 (3.79). IR (KBr): 3454, 1684, 1256, 1065, 1042 cm^{-1}. ^1H-NMR (CD_3OD, 500 MHz) *δ*: 0.91 (3H, d, *J* = 6.7 Hz, 15-H_3), 1.00 (3H, s, 14-H_3), 1.35 (1H, m, 3*β*-H), 1.78 (3H, m, 1-H_2 and 4-H), 1.83 (3H, s, 13-H_3), 1.90 (1H, m, 3*α*-H), 1.92 (3H, d, *J* = 1.8 Hz, 12-H_3), 2.02 (1H, m, 10-H), 2.07, 2.73 (1H each, both d, *J* = 15.0 Hz, 6*β*-H and 6*α*-H), 2.31 (1H, dd, *J* = 5.5, 16.5 Hz, 9*α*-H), 2.59 (1H, dd, *J* = 12.8, 16.5 Hz, 9*β*-H), 3.16 (1H, dd, *J* = 7.6, 8.8 Hz, 2'-H), 3.35 (1H, dd, *J* = 7.9, 8.6 Hz, 4'-H), 3.37 (1H, dd, *J* = 8.6, 8.8 Hz, 3'-H), 3.48 (1H, m, 5'-H), 3.97 (1H, m, 2-H), [4.15 (1H, dd, *J* = 5.5, 11.0 Hz), 4.29 (1H, dd, *J* = 1.8, 11.0 Hz), 6'-H_2], 4.40 (1H, d, *J* = 7.6 Hz, 1'-H). ^{13}C-NMR (CD_3OD, 125 MHz) *δ*c: given in Table 1. Positive-ion FAB-MS: *m/z* 539 $(M+Na)^+$, 555 $(M+K)^+$. Negative-ion FAB-MS: *m/z* 477 $(M-K)^-$, 515 $(M-H)^-$.

Solvolysis of 1

A solution of **1** (10.0 mg) in pyridine–1,4-dioxane (4:1, v/v, 3.0 mL) was heated at 80 °C for 1 h. After removal of the solvent under reduced pressure, the residue was subjected to HPLC [MeOH–H_2O (50:50, v/v)] to furnish **1a** (6.5 mg, 84%).

1a: A white powder, $[\alpha]_D^{17}$ –4.0° (*c*=0.23, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{21}H_{34}O_7Na$ $(M+Na)^+$: 421.2202. Found: 421.2208. IR (KBr): 3415, 1684, 1076 cm^{-1}. ^1H-NMR (CD_3OD, 500 MHz) *δ*: 0.91 (3H, d, *J* = 6.7 Hz, 15-H_3), 1.01 (3H, s, 14-H_3), 1.33 (1H, m, 3*β*-H), 1.72, 1.80 (1H each, both m, 1-H_2), 1.80 (1H, m, 4-H), 1.83 (3H, s, 13-H_3), 1.90 (3H, s, 12-H_3), 1.93 (1H, m, 3*α*-H), 2.02 (1H, m, 10-H), 2.06, 2.76 (1H each, both d, *J* = 14.9 Hz, 6*β*-H and 6*α*-H), 2.27 (1H, dd, *J* = 5.5, 16.5 Hz, 9*α*-H), 2.61 (1H, dd, *J* = 12.5, 16.5 Hz, 9*β*-H), 3.12 (1H, dd, *J* = 8.0, 9.1 Hz, 2'-H), 3.24 (1H, m, 5'-H), 3.32 (1H, m, 4'-H), 3.35 (1H, m, 3'-H), [3.63 (1H, dd, *J* = 5.8, 11.9 Hz), 3.85 (1H, dd, *J* = 1.8, 11.9 Hz), 6'-H_2], 4.02 (1H, m, 2-H), 4.38 (1H, d, *J* = 8.0 Hz, 1'-H). ^{13}C-NMR (CD_3OD, 125 MHz) *δ*c: given in Table 1. Positive-ion FAB-MS: *m/z* 421 $(M+Na)^+$.

Acid Hydrolysis of 1a

A solution of **1a** (6.0 mg) in 1 M HCl (0.5 mL) was heated under reflux for 3 h. After cooling, the reaction mixture was poured into ice-water and neutralized with Amberlite IRA-400 (OH^- form), and the resin was removed by filtration. Then, the filtrate was extracted with EtOAc. The aqueous layer was subjected to HPLC analysis under the following conditions: HPLC column, Kaseisorb LC NH_2-60-5, 4.6

mm i.d. × 250 mm (Tokyo Kasei Co., Ltd., Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan)]; mobile phase, $CH_3CN–H_2O$ (75:25, v/v); flow rate 0.8 mL/min; column temperature, room temperature. Identification of D-glucose present in the aqueous layer was carried out by comparison of its retention time and optical rotation with those of an authentic sample. t_R: 12.3 min (positive optical rotation). The EtOAc layer was evaporated *in vacuo* gave the residue, which was purified by HPLC [$MeOH–H_2O$ (85:15, v/v)] to give 2β-hydroxyfukinone (**2**, 3.0 mg, 84%).

2β-Hydroxyfukinone (**2**): ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.89 (3H, d, J = 6.4 Hz, 15-H_3), 0.99 (3H, s, 14-H_3), 1.31 (1H, m, 3β-H), 1.68 (2H, m, 1-H_2), 1.78 (1H, m, 3α-H), 1.81 (1H, m, 4-H), 1.81 (3H, s, 13-H_3), 1.94 (3H, d, J = 1.8 Hz, 12-H_3), 2.03 (1H, m, 10-H), 2.07, 2.67 (1H each, both d, J = 15.9 Hz, 6β-H and 6α-H), 2.31 (1H, dd, J = 5.5, 16.5 Hz, 9α-H), 2.49 (1H, dd, J = 12.8, 16.5 Hz, 9β-H), 3.89 (1H, m, 2-H). ^{1}H-NMR (pyridine-d_5, 500 MHz) δ: 0.81 (3H, d, J = 6.7 Hz, 15-H_3), 0.91 (3H, s, 14-H_3), 1.56 (1H, br dd, J = *ca.* 11, 13 Hz, 3β-H), 1.71 (3H, d, J = 0.9 Hz, 13-H_3), 1.75 (1H, m, 4-H), 1.82, 1.93 (1H each, both m, 1-H_2), 1.91 (1H, m, 3α-H), 1.91 (1H, m, 10-H), 1.97 (1H, d-like, 6β-H), 2.06 (3H, d, J = 1.9 Hz, 12-H_3), 2.61 (1H, d, J = 15.6 Hz, 6α-H), 2.38 (1H, dd, J = 5.2, 16.2 Hz, 9α-H), 2.65 (1H, dd, J = 12.5, 16.2 Hz, 9β-H), 4.09 (1H, m, 2-H). ^{13}C-NMR ($CDCl_3$ and pyridine-d_5, 125 MHz) δc: given in Table 1.

Preparation of the (*R*)-MTPA Ester (2a) and (*S*)-MTPA Ester (2b) from 2

A solution of **2** (2.0 mg) in CH_2Cl_2 (0.5 mL) was treated with (*R*)-α-methoxy-α-trifluoromethylphenylacetic acid [(*R*)-MTPA, 9.9 mg] in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 8.1 mg) and 4-dimethylaminopyridine (4-DMAP, 3.1 mg), and the mixture was stirred at room temperature for 12 h. The reaction mixture was poured into ice-water and the whole was extracted with EtOAc. The EtOAc extract was successively washed with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ powder and filtered. Removal of the solvent from the filtrate under reduced pressure furnished a residue, which was separated by ordinary-phase silica-gel column chromatography [*n*-hexane–EtOAc (10:1, v/v)] to give **2a** (1.9 mg, 49%). Using a similar procedure, (*S*)-MTPA esters [**2b** (2.3 mg, 60%)] was obtained from **2** (2.0 mg), using (*S*)-MTPA (9.9 mg), EDC·HCl (8.1 mg), and 4-DMAP (3.1 mg).

2a: ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.87 (3H, d, J = 6.8 Hz, 15-H_3), 0.98 (3H, s, 14-H_3), 1.40, 1.88 (1H each, both m, 3-H_2), 1.74, 1.88 (1H each, both m, 1-H_2), 1.81 (3H, s, 13-H_3), 1.88 (1H, m, 4-H), 1.95 (3H, d, J = 1.6 Hz, 12-H_3), [2.07 (1H, d-like), 2.69 (1H, d, J = 15.5 Hz), 6-H_2], 2.10 (1H, m, 10-H), [2.38 (1H, dd, J = 4.8, 16.1 Hz), 2.56 (1H, dd, J = 12.2, 16.1 Hz), 9-H_2], 3.55 (3H, s, –OCH_3), 5.23 (1H, m, 2-H), [7.41 (3H, m), 7.51 (2H, m), Ph-H].

2b: ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.90 (3H, d, J = 6.7 Hz, 15-H_3), 0.98 (3H, s, 14-H_3), 1.51, 1.94 (1H each, both m, 3-H_2), 1.67, 1.78 (1H each, both m, 1-H_2), 1.81 (3H, s, 13-H_3), 1.91 (1H, m, 4-H), 1.95 (3H, d, J = 1.8 Hz, 12-H_3), [2.07 (1H, d-like), 2.69 (1H, d, J = 15.3 Hz), 6-H_2], 2.03 (1H, m, 10-H), [2.37 (1H, dd, J = 5.8, 16.5 Hz), 2.54 (1H, dd, J = 12.5, 16.5 Hz), 9-H_2], 3.55 (3H, s, –OCH_3), 5.23 (1H, m, 2-H), [7.41 (3H, m), 7.51 (2H, m), Ph-H].

Bioassay

Inhibitory effect on the release of β-hexosaminidase in RBL-2H3 cells was assayed by the method described in a previous paper.[20]

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