Antidiabetic Principles of Natural Medicines. IV.[1] Aldose Reductase and α-Glucosidase Inhibitors from the Roots of *Salacia oblonga* WALL. (Celastraceae): Structure of a New Friedelane-Type Triterpene, Kotalagenin 16-Acetate

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The aqueous methanolic extract of an Indian natural medicine, the roots of *Salacia oblonga* WALL. (Celastraceae), was found to show inhibitory activity on the increase in serum glucose level in sucrose- and maltose-loaded rats. The water-soluble and ethyl acetate-soluble portions from the aqueous methanolic extract showed inhibitory activities on α-glucosidase and aldose reductase, respectively. From the water-soluble portion, potent α-glucosidase inhibitors, salacinol and kotalanol, were isolated, together with nine sugar related components, while a new friedelane-type triterpene, kotalagenin 16-acetate, was isolated from the ethyl acetate-soluble portion along with known diterpenes and triterpenes. The structure of kotalagenin 16-acetate was elucidated on the basis of physicochemical evidence. Principal components from this natural medicine were examined in terms of inhibitory activity on aldose reductase, and the diterpene and triterpene constituents, including the new kotalagenin 16-acetate, were found to be responsible components for the inhibitory activity on aldose reductase.

Key words *Salacia oblonga*; kotalagenin 16-acetate; friedelane-type triterpene; aldose reductase inhibitor; antidiabetic principle; salacinol

Salacia (*S.*) *oblonga* WALL. (Celastraceae), called Chundan in Tamil and Ponkoranti in Malayalam, is distributed in the south region of India. The roots of *S. oblonga* have been extensively used as a remedy for gonorrhea, rheumatism, itch, asthma, and diabetes in the Ayurvedic system of traditional Indian medicine. Recently, it was reported that the rootbark powder of this natural medicine showed anti-inflammatory effects,[2] but its chemical constituents including the active component, have been left uncharacterized. During the course of characterization studies on the bioactive compounds of medicinal foodstuffs[3] and natural medicines,[4] we have isolated two potent α-glucosidase inhibitors, salacinol (**8**) and kotalanol **9**, from the stems of *Salacia reticulata* WIGHT collected in Sri Lanka.[5] As a continuation of our screening for antidiabetic agents in natural medicine, we have found that the methanolic extract from the roots of *S. oblonga* exhibited an inhibitory effect on the increase of serum glucose levels in sucrose- and maltose-loaded rats. The methanolic extract and its water-soluble portion showed a potent inhibitory effect on α-glucosidase, and **8** and **9** were isolated from the water-soluble portion. On the other hand, the methanolic extract and ethyl acetate-soluble portions were also found to show inhibitory activity on aldose reductase, which is related to chronic diabetic complications such as peripheral neuropathy, retinopathy, and cataracts. From the ethyl acetate-soluble portion, a new friedelane-type triterpene called kotalagenin 16-acetate (**1**) was isolated together with three triterpenes, two diterpenes, and catechin. This paper deals with the isolation of α-glucosidase inhibitors and the structural elucidation of a new triterpene (**1**). We also describe the inhibitory activity of principal components against aldose reductase.

The roots of *S. oblonga*, collected in Tamil Nade Prefecture, India, were extracted with hot aqueous methanol. The aqueous methanol extract was found to exhibit inhibitory activity on increases in serum glucose levels in sucrose (1 g/kg)- and maltose (0.5 g/kg, 1 g/kg)-loaded rats after a single oral administration of a 250 or 500 mg/kg dose, as shown in Tables 1 and 2. The extract also inhibited rat small intestinal α-glucosidase and rat lens aldose reductase (Tables 3 and 4). The methanolic extract was partitioned into an ethyl acetate–water mixture. The water-soluble portion showed potent inhibitory activity against α-glucosidase, as shown in Table 3, while the ethyl acetate-soluble portion in particular showed inhibitory activity on the aldose reductase (Table 4). The ethyl acetate-soluble portion was subjected to normal-phase and reversed-phase silica gel column chromatography, and finally HPLC, to give kotalagenin 16-acetate (**1**, 0.0009%), 26-hydroxy-1,3-friedelanedione[6] (**2**, 0.0013%), maytenfolic acid[7] (**3**, 0.0011%), 3β,22α-dihydroxyolean-12-en-29-oic acid[8] (**4**, 0.0008%), 19-hydroxyferruginol[9] (**5**, 0.0004%), lambertic acid[10] (**6**, 0.0023%), and (−)-4′-*O*-methylepigallocatechin[11] (**7**, 0.0016%). The water-soluble portion was also separated by normal-phase silica gel (SiO_2 and NH Chromatorex) column chromatography and HPLC to give salacinol[5] (**8**, 0.017%), kotalanol[5] (**9**, 0.0002%), glycerol (0.010%), dulcitol (**10**, 0.035%), D-fructose (0.089%), D-glucose (0.087%), sucrose (0.020%), galactinol[12] (**11**, 0.0040%), 3-*O*-α-D-galactopyranosyl(1→6)-*O*-β-D-galactopyranosyl-*sn*-glycerol[13] (**12**, 0.0037%), raffinose[14] (**13**, 0.024%), and stachyose[14] (**14**, 0.052%).

Structure of 1 Kotalagenin 16-acetate (**1**) was isolated as a white powder with a positive optical rotation ($[\alpha]_D^{26}$ +29.1°). The positive-ion FAB-MS of **1** showed quasimolecular ion peaks at *m/z* 515 $(M+H)^+$, 537 $(M+Na)^+$, and 559 $(M+2Na-H)^+$, and high-resolution MS analysis of the quasimolecular ion peak $(M+H)^+$ revealed the molecular formula of **1** to be $C_{32}H_{50}O_5$. The IR spectrum of **1** showed absorption bands at 3569, 1718, 1709, 1619, 1273, and 1246 cm^{-1} assignable to hydroxyl, ketone, carbonyl, and

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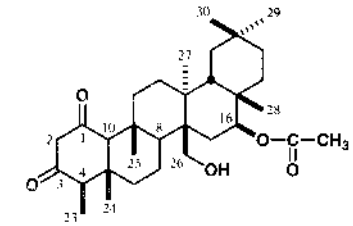
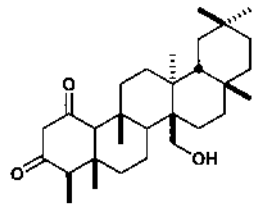
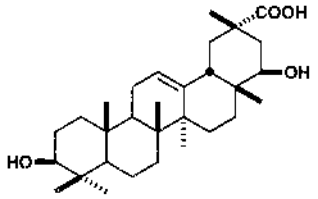


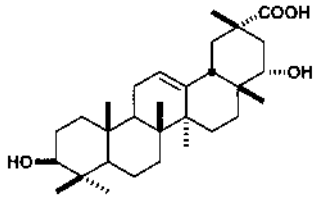
kotalagenin 16-acetate (**1**)



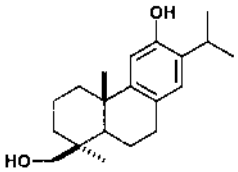
26-hydroxy-1,3-friedelanedione (**2**)



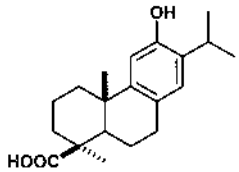
maytenfolic acid (**3**)



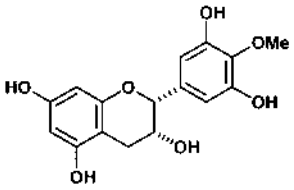
3β,22α-dihydroxyolean-12-en-29-oic acid (**4**)



19-hydroxyferruginol (**5**)

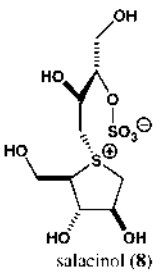


lambertic acid (**6**)

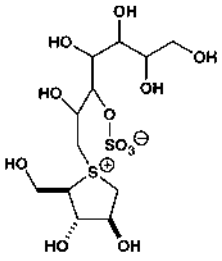


(−)-4'-*O*-methylepigallocatechin (**7**)

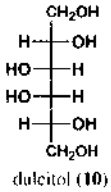
Chart 1



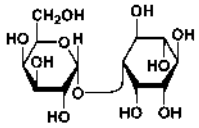
salacinol (**8**)



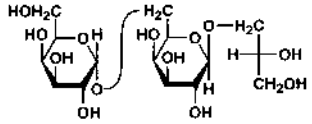
kotalanol (**9**)



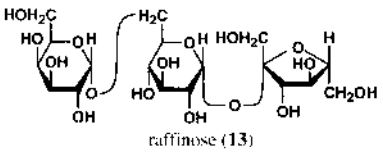
duleitol (**10**)



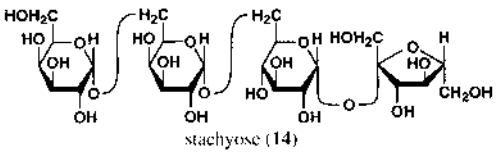
galactinol (**11**)



3-*O*-α-D-galactopyranosyl (1→6)-
O-β-D-galactopyranosyl-*sn*-glycerol (**12**)



raffinose (**13**)



stachyose (**14**)

Chart 2

olefin functions. The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 5) spectra of **1**, which were assigned with the aid of various NMR analytical methods,[15] showed signals assignable to a methine bearing an acetoxyl group [δ 2.02 (s, Ac), 5.22 (dd-like, 16-H)], two isolated methylenes [δ 3.26, 3.49 (both d, J=15.9 Hz, 1-H_2), 4.05, 4.20 (both d, J=11.5 Hz, 26-H_2)], six tertiary methyls [δ 0.70, 0.94, 0.98, 1.09, 1.22, 1.38 (all s, 24, 30, 29, 27, 28, 25-H_3)], a secondary methyl [δ 1.04 (d, J=6.7 Hz, 23-H_3)], and two ketocarbonyl carbons. The planar structure of **1**, including the positions of two carbonyl groups, and an acetoxyl group was clarified by a heteronuclear multiple bond correlation (HMBC) experiment on **1**, which showed long-range correlations between the following protons and carbons: 2-H_2, 10-H and 1-C; 2-H_2, 4-H, 23-H_3 and 3-C; 28-H_3 and 16-C. The stereostructure of **1** was clarified by a nuclear Overhauser enhancement spectroscopy

Table 1. Inhibitory Effect of MeOH Extract from *S. oblonga* on Serum Glucose Levels in Sucrose-Loaded Rats

	Dose (mg/kg, *p.o.*)	*n*	Serum glucose concentration (mg/dl)		
			0.5 h	1.0 h	2.0 h
Control (normal)	—	6	72.6±4.0[a)]	84.5±3.8	78.3±4.0[a)]
Control (sucrose-loaded, 1 g/kg)	—	6	176.3±6.8	146.8±3.4	118.3±2.2
MeOH extract	100	6	111.0±6.4[a)]	110.2±2.7[a)]	99.2±3.8[b)]
	200	6	82.3±3.8[a)]	91.8±5.5[a)]	88.2±6.1[a)]

Significant differences from the controls at *a*) $p<0.01$, *b*) $p<0.05$.

Table 2. Inhibitory Effect of MeOH Extract from *S. oblonga* on Serum Glucose Levels in Maltose-Loaded Rats

	Dose (mg/kg, *p.o.*)	*n*	Serum glucose concentration (mg/dl)		
			0.5 h	1.0 h	2.0 h
Control (normal)	—	5	62.5±8.1[a)]	68.7±4.2[a)]	65.2±7.0
Control (maltose-loaded, 0.5 g/kg)	—	5	142.8±5.2	119.8±2.8	87.2±10.9
MeOH extract	100	5	123.9±10.5	118.3±8.5	93.1±7.2
	200	5	86.7±4.1[a)]	95.3±2.8[b)]	86.0±5.8
Control (maltose-loaded, 1.0 g/kg)	—	5	198.4±6.7	153.4±8.7	99.5±6.6
MeOH extract	100	5	158.9±11.8[b)]	145.1±5.9	102.0±7.6
	200	5	118.4±8.9[a)]	146.1±6.2	118.9±4.4

Significant differences from the controls at *a*) $p<0.01$, *b*) $p<0.05$.

Table 3. Inhibitory Activity of the Methanolic Extract and the Ethyl Acetate- and Water-Soluble Portions from *S. oblonga* for α-Glucosidase

	IC_{50} (μg/ml)	
	Sucrose	Maltose
80% aqueous MeOH extract	24	66
AcOEt-soluble portion	400	>400
H_2O-soluble portion	25	49

Table 4. Inhibitory Activity of the Methanolic Extract and the Ethyl Acetate- and Water-Soluble Portions from *S. oblonga* for Rat Lens Aldose Reductase

	IC_{50} (μg/ml)
MeOH extract	3.4
AcOEt-soluble portion	3.1
H_2O-soluble portion	6.0

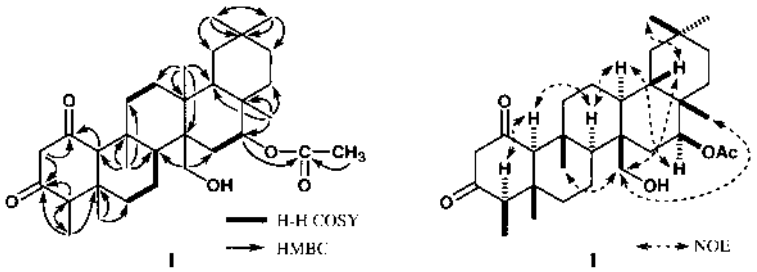


Fig. 1

(NOESY) experiment, which showed nuclear Overhauser effect (NOE) correlations between the following protons (10-H and 4, 8-H; 18-H and 26-H_2, 30-H_3; 26-H_2 and 25, 28-H_3; 27-H_3 and 8, 16-H). This evidence and comparison of the ^{1}H- and ^{13}C-NMR data for **1** with those for known friedelane-type triterpenes such as **2** led us to formulate the structure of kotalagenin 16-acetate as 16-acetoxy-26-hydroxy-1,3-friedelanedione (**1**).

Inhibitory Effect of Constituents from the Roots of *S. oblonga* on Rat Lens Aldose Reductase Aldose reductase as a key enzyme in the polyol pathway is reported to catalyze the reduction of glucose to sorbitol. Sorbitol does not readily diffuse across cell membranes, and the intracellular accumulation of sorbitol has been implicated in the chronic complications of diabetes. Since the roots of *S. oblonga* have been proven useful for the treatment of diabetes in Ayurvedic traditional medicine, we examined the inhibitory activity of its extracts (*vide ante*) and the components isolated from the active fractions, the ethyl acetate- and water-soluble portions, on rat lens aldose reductase. As shown in Table 6, triterpenoids (**1**, **3**, **4**) and diterpenoids (**5**, **6**) isolated from the ethyl acetate-soluble portion showed inhibitory activity on rat lens aldose reductase, while the constituents (**8**—**14**) from the water-soluble portion exhibited little activity.

Experimental

The following instruments were used to obtain physical data: melting points, Yanagimoto micro-melting point apparatus MP-500D (values are uncorrected); specific rotations, Horiba SEPA-300 digital polarimeter (l=5

Table 5. ^{13}C-NMR Data for **1** and **2**

	1	2		1	2
C-1	202.8	202.8	C-17	35.0	30.4
C-2	60.6	60.6	C-18	45.5	43.5
C-3	204.0	204.1	C-19	35.7	35.4
C-4	58.9	58.9	C-20	28.1	28.3
C-5	38.1	38.1	C-21	31.8	32.9
C-6	41.5	41.7	C-22	34.9	39.1
C-7	20.4	20.4	C-23	7.3	7.3
C-8	52.4	52.0	C-24	15.8	15.7
C-9	37.1	37.1	C-25	18.2	17.8
C-10	72.3	72.4	C-26	63.2	64.0
C-11	34.9	35.0	C-27	20.9	19.7
C-12	29.9	29.9	C-28	25.9	31.7
C-13	39.7	39.7	C-29	37.8	34.5
C-14	42.9	42.0	C-30	30.4	32.0
C-15	31.3	20.1	C-1′	171.3	
C-16	78.1	35.3	C-2′	21.3	

Table 6. Inhibitory Activity of Chemical Constituents from *S. oblonga* on Rat Lens Aldose Reductase

	Inhibition (%)	
	Concentration (μM)	
	30	100
Kotalagenin 16-acetate (**1**)	21.8	48.2
26-Hydroxy-1,3-friedelanedione (**2**)	15.8	15.6
Maytenfolic acid (**3**)	38.5	54.6
3β,22α-Dihydroxyolean-12-en-29-oic acid (**4**)	55.9	75.9
19-Hydroxyferruginol (**5**)	21.4	38.5
Lambertic acid (**6**)	17.7	35.2
(−)-4′-*O*-Methylepigallocatechin (**7**)	11.0	6.9
Salacinol (**8**)	−1.2	−3.6
Kotalanol (**9**)	−2.4	0.0
Galactinol (**11**)	−1.3	11.3
3-*O*-α-D-Galactopyranosyl(1→6)-*O*-β-D-galactopyranosyl-*sn*-glycerol (**12**)	4.0	12.3
Raffinose (**13**)	20.0	11.3
Stachyose (**14**)	−2.0	14.7

cm); IR spectra, Shimadzu FTIR-8100 spectrometer; FAB-MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; ^{1}H-NMR spectra, JEOL EX-270 (270 MHz) and JNM LA-500 (500 MHz) spectrometers; ^{13}C-NMR spectra, JEOL EX-270 (68 MHz) and JNM LA-500 (125 MHz) spectrometers with tetramethylsilane as an internal standard.

The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography, silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh); reversed-phase silica gel 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) (ordinary phase) and silica gel RP-18 60F$_{254}$ (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, pre-coated TLC plates with silica gel RP-18 60WF$_{254S}$ (Merck, 0.25 mm); detection was achieved by spraying with 1% Ce(SO$_4$)$_2$–10% aqueous H$_2$SO$_4$ and heating.

Isolation of 1 and Known Constituents (2—18) from *S. oblonga* The roots of *S. oblonga* (7.0 kg, collected in Tamil Nude Prefecture, India) were cut and extracted three times with MeOH–H$_2$O (80 : 20, v/v) under reflux. Evaporation of the solvent under reduced pressure provided an aqueous MeOH extract (800 g, 11.4%). The aqueous MeOH extract (420 g) was partitioned into an AcOEt–H$_2$O (1 : 1, v/v) solution. Removal of the solvent under reduced pressure from the AcOEt-soluble portion and aqueous phase yielded 60 g (1.63%) and 289 g (7.83%) of residue, respectively.

The AcOEt extract was subjected to normal-phase silica gel column chromatography [1.1 kg, *n*-hexane–AcOEt (5 : 1→2 : 1, v/v)→CHCl$_3$–MeOH (10 : 1→5 : 1, v/v)→CHCl$_3$–MeOH–H$_2$O (6 : 4 : 1→5 : 5 : 1, v/v)→MeOH→MeOH–H$_2$O (80 : 20→70 : 30, v/v)] to give nine fractions [fr. 1 (1.26 g), fr. 2 (7.6 g), fr. 3 (6.6 g), fr. 4 (8.0 g), fr. 5 (6.0 g), fr. 6 (4.1 g), fr. 7 (6.3 g), fr. 8 (8.0 g), fr. 9 (1.8 g)]. Fraction 2 (7.0 g) was separated by reversed-phase silica gel column chromatography [150 g, MeOH–H$_2$O (95 : 5, v/v)→MeOH] to give fr. 2-1 (189 mg), fr. 2-2 (1.0 g), fr. 2-3 (2.7 g), fr. 2-4 (350 mg), and fr. 2-5 (2.4 g). Fraction 2-2 (150 mg) was purified by HPLC [MeOH–H$_2$O (90 : 10, v/v)] to give 26-hydroxy-1,3-friedelanedione (**2**, 90 mg, 0.0013%). Fraction 3 (5.2 g) was separated by reversed-phase silica gel column chromatography [150 g, MeOH–H$_2$O (95 : 5, v/v)→MeOH] to give fr. 3-1 (449 mg), fr. 3-2 (547 mg), fr. 3-3 (1.5 g), fr. 3-4 (1.3 g), fr. 3-5 (1.1 g), fr. 3-6 (875 mg). Fraction 3-1 (410 mg) was purified by HPLC [MeOH–H$_2$O (80 : 20, v/v)] to give 19-hydroxyferruginol (**5**, 30 mg, 0.0004%) and lambertic acid (**6**, 164 mg, 0.0023%). Fraction 3-2 (500 mg) was purified by HPLC [MeOH–H$_2$O (85 : 15, v/v)] to give kotalagenin 16-acetate (**1**, 63 mg, 0.0009%). Fraction 5 (5.1 g) was separated by reversed-phase silica gel column chromatography [150 g, MeOH–H$_2$O (30 : 70, v/v)→MeOH] and HPLC [MeOH–H$_2$O (30 : 70, v/v)] to give (−)-4′-*O*-methylepigallocatechin (**7**, 115 mg, 0.0016%). Fraction 6 (3.9 g) was separated by reversed-phase silica gel column chromatography [100 g, MeOH–H$_2$O (50 : 50→90 : 10, v/v)→MeOH] and HPLC [MeOH–H$_2$O (85 : 15, v/v)] to give maytenfolic acid (**3**, 76 mg, 0.0011%) and 3β,22α-dihydroxyolean-12-en-29-oic acid (**4**, 57 mg, 0.0008%).

The H$_2$O extract (50 g) was treated with MeOH, and the suspension was filtered to remove the MeOH-unsoluble portion (8.6 g, 1.1%). After removal of the solvent under reduced pressure, the MeOH-soluble portion (41 g, 5.5%) was obtained. The MeOH-soluble portion (31 g) was subjected to normal-phase silica gel column chromatography [1.5 kg, CHCl$_3$–MeOH–H$_2$O (6 : 4 : 1→5 : 5 : 1→3 : 7 : 1, v/v)→MeOH→acetone–H$_2$O (1 : 1, v/v)] to give eight fractions [fr. 1 (1.78 g, 0.30%), fr. 2 (1.22 g, 0.20%), fr. 3 (2.46 g, 0.41%), fr. 4 (3.69 g, 0.61%), fr. 5 (3.96 g, 0.66%), fr. 6 (13.5 g, 2.25%), fr. 7 (0.87 g, 0.14%), fr. 8 (0.74 g, 0.12%)]. Fraction 3 (700 mg) was purified by normal-phase silica gel column chromatography [2 g, NH Chromatorex, CH$_3$CN–H$_2$O (70 : 30, v/v)→MeOH→H$_2$O] and HPLC [YMC-Pack Polyamine II (250×20 mm i.d.), CH$_3$CN–H$_2$O (70 : 30, v/v)] to give salacinol (**8**, 30 mg, 0.017%), glycerol (15 mg, 0.010%), D-fructose (152 mg, 0.089%), D-glucose (148 mg, 0.087%), and sucrose (34 mg, 0.020%). Fraction 4 (2.6g) was purified by reversed-phase silica gel column chromatography (52 g, H$_2$O→MeOH) and HPLC [CH$_3$CN–H$_2$O (75 : 25, v/v)] to give dulcitol (**10**, 68 mg, 0.035%). Fraction 5 (4.0 g) was purified by reversed-phase silica gel column chromatography [80 g, H$_2$O→MeOH] and HPLC [CH$_3$CN–H$_2$O (60 : 40, v/v)] to give kotalanol (**9**, 12 mg, 0.0002%), galactinol (**11**, 16 mg, 0.0040%), 3-*O*-α-D-galactopyranosyl(1→6)-*O*-β-D-galactopyranosyl-*sn*-glycerol (**12**, 15 mg, 0.0037%), raffinose (**13**, 100 mg, 0.024%), and stachyose (**14**, 210 mg, 0.052%). The known compounds (**2**—**7**, **10**—**14**, glycerol, and saccharides) were identified by comparison of their physical data ([α]$_D$, IR, ^{1}H-NMR, ^{13}C-NMR) with reported values[7—14] and authentic samples.

1: A white powder, $[\alpha]_D^{26}$ +29.1° (c=0.1, MeOH). IR (KBr) : 3569, 1718, 1709, 1619, 1273, 1246 cm^{-1}. High-resolution positive-ion FAB-MS: Calcd for $C_{32}H_{51}O_5$ (M+H)$^+$: 515.3737. Found: 515.3744. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.70, 0.94, 0.98, 1.09, 1.22, 1.38 (3H each, all s, 24, 29, 30, 27, 28, 25-H$_3$), 1.04 (3H, d, J=6.7 Hz, 23-H$_3$), 1.61 (1H, dd-like, 18-H), 2.02 (3H, s, Ac), 2.41 (1H, s, 10-H), 2.58 (1H, d, J=6.7 Hz, 4-H), 3.26, 3.49 (1H each, both d, J=15.9 Hz, 1-H$_2$), 4.05, 4.20 (1H each both d, J=11.5 Hz, 26-H$_2$), 5,22 (1H, dd-like, 16-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 5. Positive-ion FAB-MS *m/z*: 515 (M+H)$^+$, 537 (M+Na)$^+$, 559 (M+2Na−H)$^+$.

Bioassay Methods Inhibitory Activity on the Increase of Serum Glucose Level in Oral Sucrose- and Maltose-Loaded Rats: Male Wistar rats (Kiwa Laboratory Animals, Ltd., Wakayama, Japan) weighing 130—170 g were starved for 20—24 h prior to the study but were allowed access to water *ad libitum*. The test samples were suspended in 5% gum arabic solution (5 ml/kg), and then orally administered to the rats at each dose. Thirty min thereafter, a water solution (5 ml/kg) of sucrose (1 g/kg) or maltose (0.5 g/kg, 1 g/kg) was orally administered. Blood (0.4 ml) was collected from the retro-orbital sinus at 0.5, 1.0, and 2.0 h after the administration of sucrose or maltose, and the serum glucose concentration was assayed by the enzymatic glucose oxidase method.

Statistics: Statistical significance of differences was estimated by analysis of variance (ANOVA) followed by Dunnett's test.[16] Results are expressed as the mean±S.E. (Tables 1 and 2).

α-Glucosidase Inhibitory Activity: Rat small intestinal brush border membrane vesicles[17] were used in the preparation of small intestinal α-glucosidase of maltase, and of sucrase. The reaction was performed by slight

modification of the procedure of Dahlqvist.[18] The substrate (maltose, 37 mM; sucrose, 37 mM), test compound and the enzyme in 0.1 M maleate buffer (pH 6.0) were incubated together at 37 °C. After 30 min of incubation, 0.8 ml of water was added to the test tube, and the tube was immediately immersed in boiling water for 2 min, then cooled with water. Glucose concentration was determined by the glucose oxidase method.

Aldose Reductase Inhibitory Activity: Aldose reductase activity was assayed according to the method described by Dufrane *et al.*[19] with slight modifications. Lenses of Wistar rats were homogenized in 135 mM Na, K-phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol, then centrifuged at 100000×***g*** for 30 min. The supernatant fluid was used as the enzyme fraction (1.1 mg protein/ml). The incubation mixture contained 135 mM Na, K-phosphate buffer (pH 7.0), 100 mM Li_2SO_4, 0.03 mM NADPH, 1 mM DL-glyceraldehyde as a substrate, and 100 μl of the enzyme fraction, with or without 25 μl of sample solution in a total volume of 0.5 ml. Test samples were dissolved in dimethyl sulfoxide. The reaction was initiated by the addition of NADPH at 30 °C. After 30 min of incubation, the reaction was stopped by the addition of 150 μl of 0.5 N HCl. Then, 0.5 ml of 6 N NaOH containing 10 mM imidazole was added, and the solution was heated at 60 °C for 10 min to convert NADP to a fluorescent product. The fluorescence was measured at room temperature using a spectrofluorometer (Type 650-10, Hitachi, Japan) with an excitation wavelength of 360 nm, and an emission wavelength of 460 nm.

References and Notes

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