α-Glucosidase Inhibitor from Kothala-himbutu (*Salacia reticulata* WIGHT)

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A polyhydroxylated cyclic 13-membered sulfoxide (**1**) was isolated from an aqueous extract of Kothala-himbutu (*Salacia reticulata* WIGHT). The structure of compound **1** was elucidated by 1D and 2D NMR and APCI-MS methods. The α-glucosidase inhibitory activity of compound **1** (IC_{50}: maltase, 0.227 μM; sucrase, 0.186 μM; isomaltase, 0.099 μM) was much greater than the inhibitory activity of salacinol and kotalanol, which were previously isolated from Kothala-himbutu.

Kothala-himbutu (*Salacia reticulata* WIGHT, family Hippocrateaceae), a popular medicinal plant in Sri Lanka, the southern region of India, and Malaysia, is used to treat diabetes mellitus.[1–3] Since the hypoglycemic effect of this plant in rats was first reported,[2] many subsequent investigations have shown that the hypoglycemic effect of this plant can be largely attributed to inhibition of carbohydrate absorption from the small intestine.[3] Recently, salacinol and kotalanol (Figure 1), which occur naturally in *Salacia* spp. such as *S. reticulata*, *S. oblonga*, and *S. chinensis*, were identified as potent inhibitors of glucosidase, showing better activity than that of acarbose.[3,4] Chemical modification of salacinol has been vigorously pursued by Pinto et al.[5] Numerous studies investigating the compounds mentioned above have been reported.[3–7] Nevertheless, the remaining problem is that salacinol and kotalanol do not precisely account for the total α-glucosidase inhibition associated with Kothala-himbutu. This is because less than 10% of the total inhibitory activity of the starting extract was ascribed to salacinol and kotalanol, which was estimated from the data reported earlier.[4] This observation suggests that other compounds in Kothala-himbutu also possess inhibitory activity.

This study revealed the presence of an unknown inhibitor, which was determined to be a polyhydroxylated cyclic 13-membered sulfoxide. The α-glucosidase inhibitory activity of this new compound was confirmed to be more potent than those of previously identified compounds.

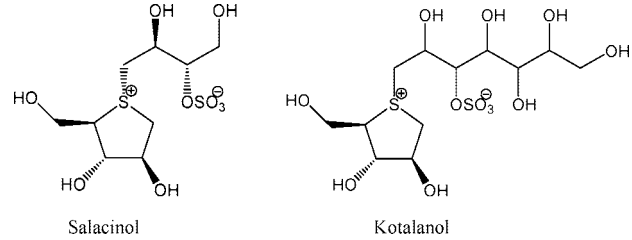

Figure 1. Structures of salacinol and kotalanol.

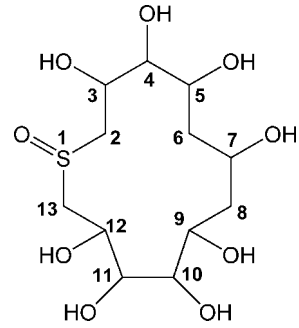

Figure 2. Structure of compound **1**.

Results and Discussion

The structure of the novel inhibitor isolated from an aqueous extract of Kothala-himbutu is shown in Figure 2. The inhibitor was isolated using the procedures shown in Figure 3. α-Glucosidase (maltase and sucrase) inhibitory activity was evaluated for each fraction obtained during the isolation procedure. First, the dried stems (1.5 kg) were extracted with H_2O at 90 °C for 2 h. The resulting aqueous extract (100 g, IC_{50}: maltase, 10.2 μg/mL; sucrase, 6.9 μg/mL) was separated by column chromatography using a Diaion HP-20 and a Chromatrex NH-DM1020SG to give the principle inhibitory fraction, Fr 1-2 (20.0 g, IC_{50}: maltase, 3.61 μg/mL; sucrase, 2.50 μg/mL). In addition, this fraction was subjected to HPLC using a YMC-Pack Polyamine-II column to give five fractions; inhibitory activity was found in several of these fractions. Fr1-2-1 (0.9 g, IC_{50}: maltase, 0.20 μg/mL; sucrase, 0.14 μg/mL) contained the majority of the inhibitory activity. Fr1-2-1 also contained inactive substances. Additional separation by HPLC using a Daisopak SP-120−5-ODS-BP column was necessary to give Fr1-2-1-2-3, i.e., compound **1** (241.8 mg), which possessed potent inhibitory activity.

Compound **1** showed marked α-glucosidase inhibitory activity (IC_{50}: maltase, 0.227 μM; sucrase, 0.186 μM; isomaltase, 0.099 μM), but did not inhibit α-amylase from porcine pancreas (data not shown). The α-glucosidase inhibitory activity of compound **1** was greater than those of salacinol (IC_{50}: maltase, 9.58 μM; sucrase, 2.51 μM; isomaltase, 1.77 μM) and kotalanol (IC_{50}: maltase, 6.60 μM; sucrase, 1.37 μM; isomaltase, 4.48 μM).[3] Relative to salacinol, the IC_{50} values of **1** for maltase, sucrase, and isomaltase were increased 42-, 14-, and 18-fold, respectively. Moreover, to characterize the inhibition of maltase, sucrase, and isomaltase, **1** was incubated with increasing concentrations of each substrate. Lineweaver−Burk plots clearly indicated competitive inhibition for each α-glucosidase, and the K_i values for maltase, sucrase, and isomaltase inhibited by **1** were determined to be 1.1×10^{-7}, 5.2×10^{-8}, and 4.2×10^{-7} M, respectively (Figure 4 and Table 1). A total activity of 26.6% was recovered in Fr1-2-1-2-3 (compound **1**) from the starting material, but only 2.1% of the total activity was recovered in Fr1-2-3, containing salacinol. These results adequately account for the α-glucosidase inhibitory effect of aqueous extracts of Kothala-himbutu.

Compound **1** was obtained as a colorless, amorphous solid with a positive specific rotation $[\alpha]_D^{20}$ +12.1 (*c* 0.31 in H_2O). The

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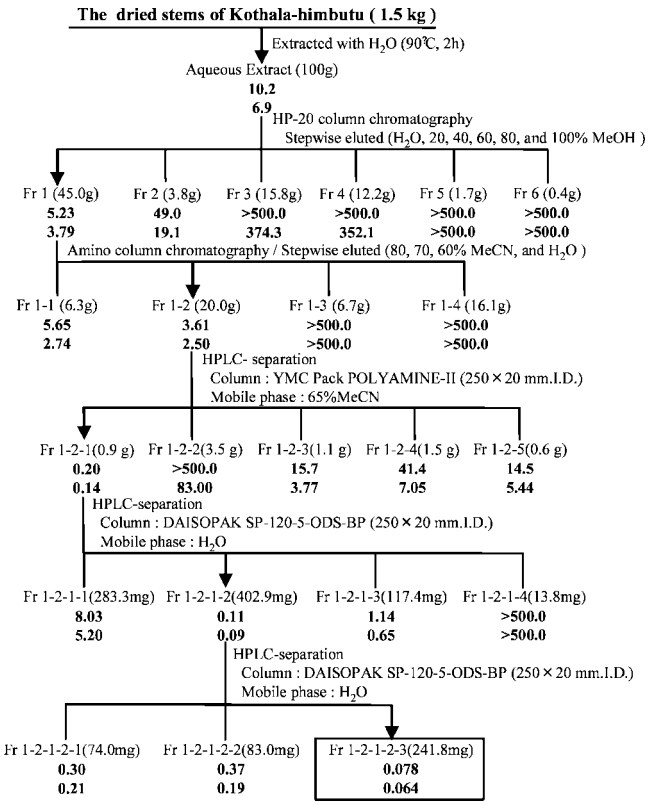

Figure 3. Isolation scheme of compound **1** from an aqueous extract of Kothala-himbutu and α-glucosidase inhibitory activity for each fractionation.

positive-ion FABMS of compound **1** demonstrated a quasi-molecular ion peak at *m/z* 345 $[M + H]^+$, while a quasi-molecular ion peak at *m/z* 343 $[M - H]^-$ was detected in the negative-ion FABMS. These data strongly suggest that the molecular weight of compound **1** is 344. The IR spectrum of compound **1** showed absorption bands corresponding to hydroxy groups at 3397−3349 cm^{-1}, methylene groups at 1406 cm^{-1}, and a sulfoxide group at 1071 cm^{-1}. The fact that absorption bands corresponding to sulfate were not detected in this spectrum suggests that the structure of compound **1** is different from those of salacinol and kotalanol.

The ^1H and ^{13}C NMR resonances (Table 2) of compound **1** were assigned to two methylenes bonded to the heteroatom [δ_H 3.79–3.75 2H, m, H-2); δ_H 3.82 (1H, dd, 13.4, 3.2 Hz, H-13a), 3.63 (1H, dd, 13.4, 8.8 Hz, H-13b)], two unbounded methylenes [δ_H 3.96 (1H, dd, 13.2, 4.6 Hz, H-6a); δ_H 3.78 (1H, dt, 13.2, 2.2 Hz, H-6b); δ_H 3.51 (2H, d, 6.6 Hz, H-8)], and eight methines in the other parts of the macrocycle [δ_H 4.59 (1H, q, 3.4 Hz, H-3), 4.28 (1H, br t, 3.4 Hz, H-4), 4.07 (1H, dt, 8.8, 2.4 Hz, H-12), 3.94 (1H, m, H-5), 3.79 (1H, dt, 6.6, 2.2 Hz, H-7), 3.73 (1H, t, 9.5 Hz, H-10), 3.72 (1H, br d, 9.5 Hz, H-11), 3.49 (1H, t, 9.5 Hz, H-9)]. These signals were confirmed by $^1H-^1H$ COSY and HMQC experiments (data not shown). On the basis of the observed chemical shifts and IR spectrum, it was concluded that the compound contained eight methines adjacent to hydroxy groups. Furthermore, the cross-peaks in the $^1H-^1H$ COSY spectrum indicated the presence of four partial structures, arbitrarily labeled **a** to **d** in Figure 5. In the HMBC experiment (Figure 6), correlations were detected between **d**C-13Ha, -13Hb (3.82, 3.63 ppm) and **a**C-2 (46.9 ppm), **d**C-12H (4.07 ppm) and **c**C-10 (66.9 ppm), **a**C-6H(3.96 ppm) and **b**C-7 (68.9 ppm), and **b**C-8H (3.51 ppm) and **c**C-9 (68.0 ppm).

At this stage in the structure elucidation procedure, the molecular weight of the structure shown in Figure 5, not including the X group, was determined to be 296. Subsequently, HR-APCI-MS analysis established the molecular formula of the structure to be $C_{12}H_{24}O_9S$. Thus, the calculated and predicted figures were in agreement. In addition, the IR spectrum confirmed that the X group was a sulfoxide group. On the basis of the chemical shifts of **a**C-2 and **d**C-13, the HMBC correlations of **d**H-13a and **d**H-13b to **a**C-2,

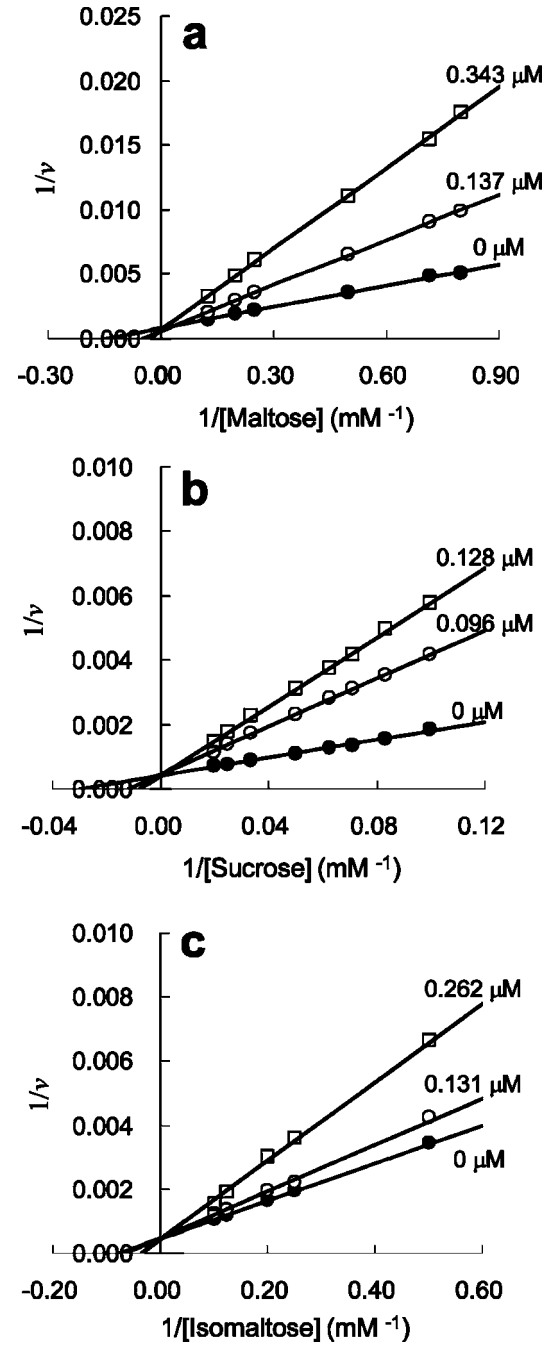

Figure 4. Lineweaver−Burk plots of inhibition by compound **1** for rat intestinal maltase (a), sucrase (b), and isomaltase (c). The initial velocity (v) is defined as number of glucosidic bonds (μmol) cleaved by 1 mg of protein per hour. The concentrations of compound **1** are indicated in the figures. The other experimental conditions are shown in the Experimental Section.

Table 1. K_i values of Compound **1**, Salacinol, and Kotalanol for Rat Intestinal Glucosidase

	K_i (M)		
substrate	compound **1**	salacinol	kotalanol
maltose	1.1×10^{-7}	9.7×10^{-7}	5.4×10^{-7a}
sucrose	5.2×10^{-8}	2.0×10^{-7}	4.2×10^{-7a}
isomaltose	4.2×10^{-7}	1.1×10^{-6}	4.2×10^{-6a}

[a] K_i value of kotalanol was taken from ref 3.

Table 2. ^{1}H and ^{13}C NMR Data for Compound **1** (δ in ppm and J in Hz, D_2O, at 25 °C)

position	δ_H	δ_C
2	3.79−3.75 (2H, m)	46.9
3	4.59 (1H, q, 3.4 Hz)	75.8
4	4.28 (1H, br t, 3.4 Hz)	76.4
5	3.94 (1H, m)	68.8
6	3.96 (1H, dd, 13.2, 4.6 Hz)	58.0
	3.78 (1H, dt, 13.2, 2.2 Hz)	
7	3.79 (1H, dt, 6.6, 2.2 Hz)	68.9
8	3.51 (2H, d, 6.6 Hz)	62.0
9	3.49 (1H, t, 9.5 Hz)	68.0
10	3.73 (1H, t, 9.5 Hz)	66.9
11	3.72 (1H, br d, 9.5 Hz)	70.4
12	4.07 (1H, dt, 8.8, 2.4 Hz)	66.3
13	3.82 (1H, dd, 13.4, 3.2 Hz)	49.3
	3.63 (1H, dd, 13.4, 8.8 Hz)	

and the disappearance of the correlation between the **a**C-2 and **d**C-13 methylene protons, it was concluded that the X group was positioned between partial structures **a** and **d** as shown in Figure 5.

Salacinol, which is a five-membered thiocyclitol with a sulfate group, has also been investigated with respect to derivatization and

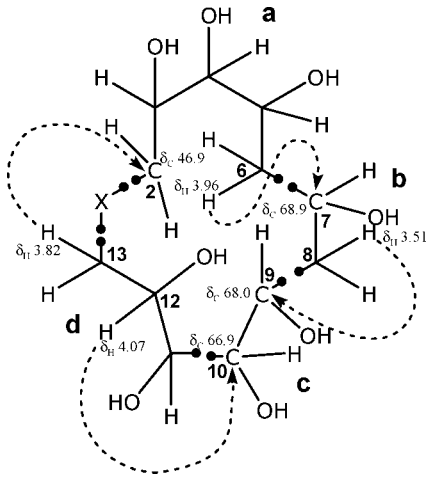

Figure 5. Partial structures and HMBC correlations of compound **1**.

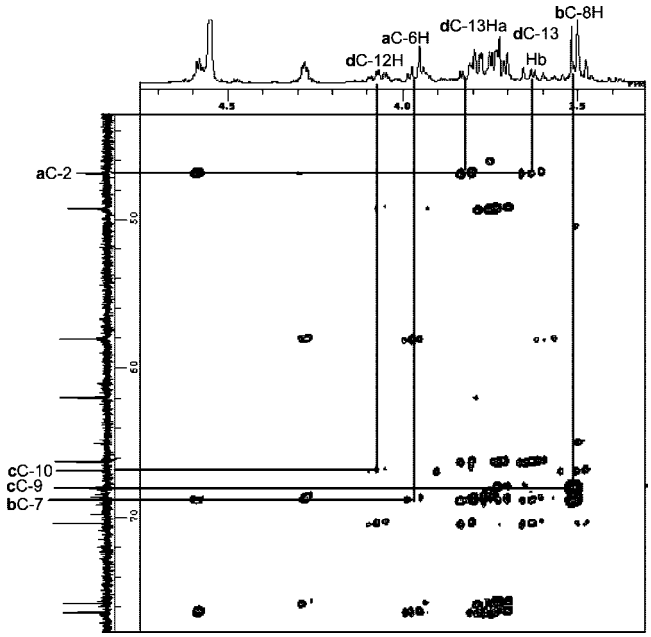

Figure 6. HMBC spectrum of compound **1** in D_2O at 25 °C. This spectrum was recorded on a JEOL AL-400 MHz spectrometer.

structure−activity correlations. Johnston et al. recently reported that the stereochemistry of the polyhydroxylated side chain was very important for inhibitory activity.[7] We speculate that not only the orientation of the OH group but also the ring structure and the sulfoxide groups might be important for inhibitory activity in compound **1**. Further characterization of compound **1**—such as the elucidation of its stereochemistry and other physiological effects—is required. These studies are currently in progress.

Experimental Section

General Experimental Procedures. Optical rotations were determined using a JASCO DIP-360 polarimeter (H_2O, c in g/100 mL). IR spectra were recorded on a JASCO FT/IR-460 plus infrared spectrometer. ^{1}H and ^{13}C NMR spectra were measured with a JEOL AL-400 instrument at 400 and 100 MHz in D_2O at 25 °C. Chemical shifts were determined using acetone as the internal reference (δ_H: 2.04 ppm, δ_C: 29.8, 206.0 ppm). ^{1}H NMR assignments were determined by ^{1}H−^{1}H COSY experiments. ^{13}C NMR assignments were made using data collected in the HMQC and HMBC experiments. FABMS was recorded on a JEOL 700QQ instrument, and HR-APCI-MS was recorded on a JEOL JMS-T100LC instrument. Chromatorex NH-DM1020SG (100−200 mesh; Fuji Silysia Chemical) and a synthetic absorbent (Diaion HP-20; Mitsubishi Chemical Co.) were used for open column chromatography. HPLC was performed using a Shimadzu Class-VP HPLC instrument equipped with a YMC-Pack polyamine-II column (250 × 20 mm i.d.; YMC Co., Ltd.), a Daisopak SP-120−5-ODS-BP column (250 × 20 mm i.d.; Daiso Co., Ltd.), and a Shimadzu RID-10A detector.

Plant Materials. The stems of Kothala-himbutu (*Salacia reticulata* WIGHT), collected in Anamaduwa, Kurunegala, Sri Lanka, by Eco Tech Create 21, were purchased from Kothalahimbutu Sales, Inc., Japan. The botanical identity of the plant was confirmed by comparing TLC fingerprints with those of authentic samples from the herbarium at Industrial Technology Institute in Sri Lanka.

Extraction and Isolation. The starting material for the isolation procedure used in this experiment was an aqueous extract of stems of *S. reticulata* from Sri Lanka. The stems were extracted with H_2O at 90 °C for 2 h, and after removal of the solvent, lyophilization gave the extract (100 g, 6.7% yield). A 20% solution of the extract (in H_2O, 500 mL) was precipitated, followed by centrifugation at 4300*g* for 30 min. The supernatant was evaporated under reduced pressure and then was lyophilized to yield 82.2 g of soluble fraction. The soluble fraction was loaded onto a synthetic absorbent column (500 × 80 mm i.d., Diaion HP-20) and eluted with H_2O (4 L) and 20%, 40%, 60%, 80%, and 100% MeOH (2 L), respectively, to give six fractions [Fr1 (45.0 g), Fr2 (3.8 g), Fr3 (15.8 g), Fr4 (12.2 g), Fr5 (1.7 g), Fr6 (0.4 g)]. Fr1 was subjected to amino column chromatography (500 × 40 mm i.d.; Chromatorex NH-DM1020SG) and eluted stepwise with 80% (6 L), 70% (9 L), and 60% MeCN (6 L) and H_2O (12 L), respectively, to give four fractions [Fr1-1 (6.3 g), Fr1-2 (20.0 g), Fr1-3 (6.7 g), and Fr1-4 (16.1 g)]. Fr1-2 (70% MeCN effluent) was separated using HPLC with an YMC-Pack polyamine-II column (250 × 20 mm i.d., YMC Co., Ltd.) under the conditions described above. The column was eluted with 65% MeCN at a flow rate of 4.5 mL/min. The effluent was monitored by refractive index detection, and the resulting spectrum indicated five fractions [Fr1-2-1 (0.9 g), Fr1-2-2 (3.5 g), Fr1-2-3 (1.1 g), Fr1-2-4 (1.5 g), and Fr1-2-5 (0.6 g)]. In addition, Fr1-2-1 was loaded onto a Daisopak SP-120-5-ODS-BP HPLC column (250 × 20 mm i.d., Daiso Co., Ltd.) and was eluted with distilled H_2O at a flow rate of 5 mL/min. Inactive substances were removed by repeating the method described above. The separated effluent was evaluated by determination of α-glucosidase inhibition. It was clear that the new inhibitory compound **1** (241.8 mg, 0.016% yield from raw material) was included in the Kothala-himbutu extract. Fr1-2-3 contained salacinol (252.2 mg). The structure and purity were ascertained by NMR spectroscopy and HPLC and used as a standard for the measurements of α-glucosidase inhibitory activity.

Assessment of α-Glucosidase Inhibitory Activity. α-Glucosidase inhibitory activity was measured as follows. An enzyme complex solution prepared with 0.1 M K_3PO_4 buffer (5 mM EDTA, pH 7) from rat intestinal acetone powder (Sigma Aldrich Japan Co.) showed maltase (140 mU/mg protein), sucrase (150 mU/mg protein), and isomaltase (90 mU/mg protein) activities, which were respectively measured using maltose (25 mM), sucrose (100 mM), and isomaltose (25 mM) as substrates. The reaction solution for determination of inhibitory activity

consisted of a crude enzyme solution (20 μL), test sample (in DMSO, 10 μL), and substrate (in 0.1 M K_3PO_4 buffer, 25 μL). Tris-HCl buffer (2 M, 50 μL) was added to stop the reaction after 30 min of incubation. The glucose concentrations of each test material were then measured using a glucose oxidase method (glucose CII-test, Wako Pure Industries). The IC_{50} values were determined graphically by plotting percent inhibition versus the log of the test compound concentrations. For the kinetic analysis of enzyme inhibition (maltase, sucrase, and isomaltase) by compound **1**, the following concentrations of compound **1** and substrate were used: maltase, 0.137−0.343 μM and 0.8−4.0 mM maltose; sucrase, 0.096−0.128 μM and 10−50 mM sucrose; and isomaltase, 0.131−0.262 μM and 1.3−10 mM isomaltose.

Compound 1: colorless, amorphous solid, $[\alpha]_D^{20}$ +12.1 (*c* 0.31, H_2O); IR (KBr) ν_{max} 3397−3349, 2933, 1417, 1406, 1071, 1046 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HR-APCI-MS *m/z* 345.1217 [M + H]$^+$ (calcd for $C_{12}H_{25}O_9S$ 345.1219).

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