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Potent α-glucosidase inhibitors purified from the red alga *Grateloupia elliptica*

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ABSTRACT

Diabetes mellitus is a most serious and chronic disease whose incidence rates are increasing with incidences of obesity and aging of the general population over the world. One therapeutic approach for decreasing postprandial hyperglycemia is to retard absorption of glucose by inhibition of α-glucosidase. Two bromophenols, 2,4,6-tribromophenol and 2,4-dibromophenol, were purified from the red alga *Grateloupia elliptica*. IC_{50} values of 2,4,6-tribromophenol and 2,4-dibromophenol were 60.3 and 110.4 μM against *Saccharomyces cerevisiae* α-glucosidase, and 130.3 and 230.3 μM against *Bacillus stearothermophilus* α-glucosidase, respectively. In addition, both mildly inhibited rat-intestinal sucrase (IC_{50} of 4.2 and 3.6 mM) and rat-intestinal maltase (IC_{50} of 5.0 and 4.8 mM). Therefore, bromophenols of *G. elliptica* have potential as natural nutraceuticals to prevent diabetes mellitus because of their high α-glucosidase inhibitory activity.

1. Introduction

Diabetes mellitus is a most serious and chronic disease whose incidence rates are increasing with increasing levels of obesity and also with aging of the general population over the world. Currently, an estimated 150 million people worldwide have diabetes and that this will increase to 220 million by 2010 and 300 million by 2025 (Li et al., 2005). Globally, type II diabetes (noninsulin-dependent diabetes mellitus) accounts for greater than 90% of the cases (Zimmet et al., 2001; Tewari et al., 2003). Postprandial hyperglycemia plays an important role in development of type II diabetes and complications associated with the diseases, such as micro- and macrovascular diseases (Baron, 1998). The best way to control the postprandial plasma glucose level is to medicate in combination with dietary restriction and an exercise program (Yki, 1990). Although several drugs for type II diabetes exist today, they have drawbacks such as liver toxicity and adverse gastrointestinal symptoms, thereby raising the symptoms and risk factors of heart disease (Tewari et al., 2003).

One therapeutic approach to decrease postprandial hyperglycemia is to retard absorption of glucose via inhibition of carbohydrate-hydrolysing enzymes, such as α-glucosidase, in the digestive organs (Holman et al., 1999). Glycosidases are well known targets in the design and development of antidiabetic, antiviral, antibacterial, and anticancer agents. In type II diabetes, delaying glucose absorption after meals by inhibition of α-glucosidase is known to be beneficial in therapy (Tewari et al., 2003).

α-Glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) is an exo-type carbohydrase that catalyzes the liberation of α-glucose from the non-reducing end of the substrate. This enzyme is widely distributed in microorganisms, plants, and animal tissues, although the substrate specificity of α-glucosidase differs greatly depending on the source (Kimura et al., 2004). Three types of α-glucosidase inhibitors exist: polyhydroxylated *N*-substituted heterocyclic compounds; polyhydroxylated cycloalkenes; and oligomers of pseudosugars. Most inhibit α-glucosidases by mimicking the pyranosyl moiety of the α-glucosidase. There are reports of α-glucosidase inhibitors such as acarbose (Truscheit et al., 1981; Wehmeier and Piepersberg, 2004) and voglibose (Luo et al., 2001) from microorganisms and nojirimycin (Inouye et al., 1968; Reese and Parrish, 1971; Asano et al., 1994) and 1-deoxynojirimycin (Asano et al., 1994) from plants, as well as effects of α-glucosidase inhibitor in wheat kernels on blood glucose levels after food uptake (Maeda et al., 1985).

Seaweeds are known to contain α-glucosidase inhibitors (Kurihara et al., 1995, 1999a,b; Xu et al., 2003; Kurata et al., 1997). Red algae of the family *Rhodomelaceae* contain bromophenols with α-glucosidase inhibitory activity; one of the family *Rhodomelaceae* bears a 3,4-dihydroxybenzyl skeleton (Fenical, 1975; Kurihara et al., 1999a,b). Bromophenols of the family *Rhodomelaceae* are divided into three groups according to the number of Br atoms in a benzene ring: three, two, and one Br atoms. The objectives of this study were thus to; screen α-glucosidase inhibitors from 10 different seaweeds collected in the eastern coastal area of the Korean

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peninsula; to analyze the structure and biological activity of α-glucosidase inhibitors of the red alga *Grateloupia elliptica* (*Halymeniaceae*); and to compare the *G. elliptica* results with known α-glucosidase inhibitors.

2. Results and discussion

2.1. Extraction and isolation of α-glucosidase inhibitors

Compounds with α-glucosidase inhibitory activity were preliminarily screened and purified from ten different seaweeds (two green, four brown, and four red). The α-glucosidase inhibitory activity of the MeOH · H_2O (4:1, v/v) extract of *Polyopes lancifolia* at 5 mg/ml was highest at 52.2%, followed by *G. elliptica* (42.0%), *Sargassum thunbergii* (24.3%), and *Grateloupia. lanceolata* (22.0%) in decreasing order (Table 1). With the exception of *S. thunbergii*, these are all red algae. The MeOH · H_2O (4:1, v/v) extract of *G. elliptica* was further purified using a series of solvent partitions and Sephadex LH-20 chromatographic separations. Although *P. lancifolia* had the highest α-glucosidase inhibitory activity, it was difficult to harvest because of its very low amount of bioactive product. Therefore, *G. elliptica* was selected as raw material for purification of the α-glucosidase inhibitor used in this study. The remaining eight seaweed lines were not examined further.

In the solvent fractionation of α-glucosidase inhibitors in *G. elliptica*, the presumed inhibitor was concentrated into the EtOAc-soluble fraction followed by BuOH, even though the hexane fraction resulted in the highest solute yield (52.0%), followed by the $CHCl_3$ (26.2%), EtOAc (16.5%), BuOH (5.2%), and water (1.2%) fractions (Table 2). Lipids, chlorophyll, refined oil, and sterols were dissolved and fractionated in *n*-hexane; resin is usually extracted using $CHCl_3$. In addition, polyphenols such as flavonoids and tannins are typically extracted in EtOAc and water-soluble components are shifted over to the BuOH fraction (Suffness et al., 1989). A large quantity of the solute in the MeOH · H_2O (4:1, v/v) extract was shifted over to *n*-hexane (52%) and $CHCl_3$ (26.2%), but neither had any α-glucosidase inhibitory activity at 0.01 and 0.1 mg/ml (Table 2). Since the α-glucosidase inhibitory activity of the EtOAc fraction was the highest (Table 2), the main α-glucosidase inhibitor of *G. elliptica* was provisionally considered to be a phenolic compound.

2.2. Purification and identification of α-glucosidase inhibitors

The EtOAc fraction in *G. elliptica* was further purified to isolate the compounds with α-glucosidase inhibitory activity using silica gel and Sephadex LH-20 chromatographic separations, respectively. Each chromatographic fraction was loaded on the plate of a silica gel TLC and then developed with toluene:EtOAc:HOAc (5:7:1, v/v). The TLC plate was then sprayed with $FeCl_3$ solution to analyze the spot pattern, with any positive reaction of blue color indicating the presence of a phenol (Kurihara et al., 1999a). Two kinds of positive spots among 30 fractions of Sephadex LH-20 chromatographic separations were confirmed and classified as compound **1** (30.1 mg) and compound **2** (22.1 mg), respectively. Both showed characteristic patterns for Br-containing compounds in EI-MS. Compound **1** showed cluster peaks at *m*/*z* 312, 314, 316, and 318, which suggested it was tribrominated, whereas compound **2** had cluster peaks at *m*/*z* 234, 236, and 238, which indicates that it was dibrominated. Compound **2** lacked only one Br atom relative to compound **1**, because the difference between both was 78 mass units. The ^1H and ^{13}C NMR spectra of compound **1** showed a simple, one aromatic proton and four aromatic carbon signals, respectively, suggesting a symmetrical structure. Hence, compound **1** was 2,4,6-tribromophenol (Fig. 1). The ^1H and ^{13}C NMR resonances for compound **2** showed three aromatic protons and six aromatic carbon signals, respectively. These results suggested that compound **2** had an asymmetrical structure; therefore, compound **2** was identified as 2,4-dibromophenol (Fig. 1).

The structures of both α-glucosidase inhibitors – 2,4,6-tribromophenol (**1**) and 2,4-dibromophenol (**2**) – purified from *G. elliptica* (*Halymeniaceae*) were different from those reported by Kurihara et al. (1999a, b). Those researchers had purified bromophenols with 3 Br (2,3,6-tribromo-4,5-dihydroxybenzyl alcohol) and 2 Br (2,3-dibromo-4,5-dihydroxybenzyl alcohol) atoms from *Symphyocladia latiuscula* and *Odonthalia corymbifera* (*Rhodomelaceae*) collected in southern Hokkaido in Japan.

Table 1
α-Glucosidase inhibitory activity of 80% methanol extract from different seaweeds

Seaweeds	α-Glucosidase inhibitory activity[a] (%)
Ulva pertusa	8.3 ± 0.9[b]
Ostria costata	4.0 ± 0.4
Sargassum horneri	12.0 ± 0.9
Sargassum thunbergii	24.3 ± 1.3
Sargassum nigrifolium	12.3 ± 1.0
Myelophycus simplex	13.5 ± 1.1
Grateloupia elliptica	42.0 ± 2.5
Plocamium elfairiae	18.0 ± 1.2
Grateloupia lanceolata	22.0 ± 1.4
Polyopes lancifolia	52.2 ± 2.7

[a] Experiments were conducted in triplicate.
[b] Data shown are mean ± standard deviation.

Table 2
α-Glucosidase inhibitory activities of the solvent-partitioned fractions of *Grateloupia elliptica* at different concentrations

Fractions	Yield (%)	α-Glucosidase inhibitory activity[a] (%)		
		0.01 mg/ml[b]	0.1 mg/ml	0.8 mg/ml
Hexane	52.0 ± 2.4	NI[c]	NI	2
Chloroform	26.2 ± 1.3	NI	NI	4
Ethyl acetate	16.5 ± 0.6	27.3 ± 1.3[a]	54.3 ± 4.2[a]	64.8 ± 5.3[a]
Butanol	5.2 ± 0.5	12.4 ± 0.9[b]	27.9 ± 2.3[b]	24.2 ± 1.9[b]
Water	1.2 ± 0.3	6.2 ± 0.5[c]	8.1 ± 0.8[c]	16.4 ± 1.3[c]

[a] Values are expressed as mean ± SD. Means in the same column with different superscripts are significantly different ($p < 0.05$).
[b] The final concentration in the reaction mixture.
[c] No inhibition.

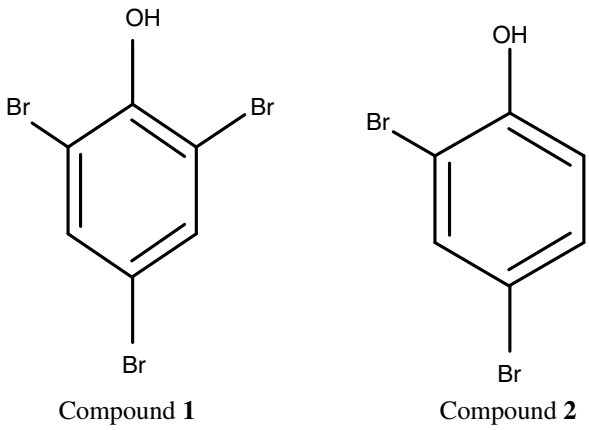

Fig. 1. Bromophenols purified from *Grateloupia elliptica*.

2.3. Assay for α-glucosidase inhibitory activity

The α-glucosidases of *S. cerevisiae and Bacillu. stearothermophilus* were used to investigate the inhibitory activity of the isolated compounds. α-Glucosidase inhibitory activity of *G. elliptica* compounds against α-glucosidases was determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as a substrate and this was compared with acarbose and voglibose (Table 3). The IC_{50} values of compounds **1** and **2** against *S. cerevisiae* α-glucosidase were 60.3 and 110.4 μM, respectively, which were lower than the 130.3 and 230.3 μM that was presented against the *B. stearothermophilus* α-glucosidase. The IC_{50} value (60.3 μM) of compound **1** of *G. elliptica* against the *S. cerevisiae* α-glucosidase was higher than that of the 2,3,6-tribromo-4,5-dihydroxybenzyl alcohol from *S. latiuscula* (11 μM) and lower than that of 2,3-dibromo-4,5-dihydroxybenzyl alcohol from *O. corymbifera* (89 μM) (Kurihara et al., 1999a), whereas compound **2** (110.4 μM) was higher than those from both *S. latiuscula* and *O. corymbifera*. The α-glucosidase inhibitory activities of compound **1** against *S. cerevisiae* and *B. stearothermophilus* α-glucosidases were also higher than that for compound **2**. This finding was similar to the result of Kurihara et al. (1999a), in which inhibitory potencies of the bromophenol increased with increasing degree of bromo-substitution per benzene ring and with decreasing degree of methyl-substitution. By contrast, the commercial inhibitors, acarbose and voglibose, exhibited no α-glucosidase inhibitory activities against *S. cerevisiae* and *B. stearothermophilus* (Table 3). Haslam (1974), Stern et al. (1996), and Cogoli and Semenza (1975) reported the same phenomenon: Voglibose and acarbose had high inhibitory effects on mammalian α-glucosidase, but no inhibitory activity against *S. cerevisiae* α-glucosidase. In contrast, Blanco and Iturbe (1981) showed that (+)-catechin, an inhibitor of *S. cerevisiae* α-glucosidase, had no inhibitory effect on enzymes from mammalian species.

In this study, compounds from the red alga *G. elliptica* were shown to be effective inhibitors against α-glucosidase. The compounds exhibited very powerful inhibitory activity against both *S. cerevisiae* and *B. stearothermophilus* α-glucosidases. Polyphenolic compounds, such as tannins from terrestrial plants and phlorotannins from marine algae, are known to be associated with a variety of proteins to form complexes (Stern et al., 1996). The hydroxy groups of bromophenols may, therefore, have an important role in promoting inhibitory activity (Pierpoint, 1969). For example, *o*-quinones derived from catechols are covalently bound to protein amino acids and thiol groups. Therefore, bromophenols should bind to active or binding sites of the enzymes, resulting in inhibition of the enzyme activity. The K_I (inhibition constant) values of compounds **1** and **2** were 15.2 and 92.1 μM, respectively (Table 4), which shows that compound **1** was a more effective inhibitor than compound **2**. Kurihara et al. (1999a) reported K_I values of 7.0 μM for a bromophenol with three Br atoms from *S. latiuscula* and 72 μM for a bromophenol with two Br atoms from *O. corymbifera*. Compounds **1** and **2** showed a mixed type of inhibition against *S. cerevisiae* α-glucosidase (Fig. 2, and Table 4) that was characterized by a combination of competitive and noncompetitive inhibition (Li et al., 2006). This is the same result as reported by Kurihara et al. (1999a) for *S. latiuscula* and *O. corymbifera* bromophenols.

Table 4
Inhibition constant (K_I value) and mode of isolated compounds purified from *Grateloupia elliptica* against *Saccharomyces cerevisiae* α-glucosidase

Compounds	K_I [1,2] (μM)	Inhibition mode[3]
1	15.2 ± 2.2[b]	Mixed inhibition
2	92.1 ± 3.4[a]	Mixed inhibition

[1] Values are expressed as mean ± SD. Means in the same column with different superscripts are significantly different ($p < 0.05$).
[2] K_I value was determined by Dixon plot analysis.
[3] Inhibition mode was determined by Lineweaver–Burk plot.

2.4. Inhibitory activity against rat-intestinal sucrase and maltase

The inhibitory activities of *G. elliptica* compounds against rat-intestinal sucrase and maltase were also compared with those of acarbose and voglibose (Table 3). The IC_{50} values of compounds **1** and **2** were 4.2 and 3.6 mM against sucrase and 5.0 and 4.8 mM against maltase, respectively; thus, compound **1** was a less effective inhibitor than compound **2**. The IC_{50} values of acarbose and voglibose were 0.02 and 0.06 mM against sucrase and 0.08 and 0.04 mM against maltase, respectively. Hence, acarbose and voglibose were better inhibitors against mammalian α-glucosidase than the algal compounds. In previous studies, most mammalian α-glucosidase inhibitors did not effectively inhibit microbial α-glucosidases, whereas catechin, an inhibitor of *S. cerevisiae* α-glucosidase, did not inhibit mammalian α-glucosidases (Blanco and Iturbe, 1981). Therefore, the higher mammalian α-glucosidase inhibitory activity of acarbose and voglibose compared to algal compounds is likely due to the substrate specificities that depend on the source of α-glucosidases. Kurihara et al. (1999a) showed that the bromophenols of Rhodomelaceae algae moderately inhibited rat-intestinal α-glucosidase (sucrase and maltase), as was also shown in this study. This suggests that binding of algal compounds is less specific to the enzyme because they can bind to various proteins included in the crude enzyme solution. Bacterial, yeast, and insect enzymes, named α-glucosidase I, show higher activity toward heterogeneous substrates such as sucrose and *p*NPG, and either no or less activity toward homogeneous substrates such as maltooligosaccharides; this implies that α-glucosidase I recognizes the "glucosyl structure" in the substrate (Kimura, 2000). The mold, plant, and mammalian enzymes, named α-glucosidase II, hydrolyze homogeneous substrates more rapidly than heterogeneous substrates, indicating that this class of α-glucosidases recognizes the "maltostructure" (Kimura et al., 2004). The hydrolysis of *p*-nitrophenol 2-deoxy-α-D-arabino-hexopyranoside was catalyzed by α-glucosidase II (Nishio et al., 2002), but no such reaction was observed with

Table 3
IC_{50} values of isolated compounds purified from *Grateloupia elliptica* against α-glucosidases

Inhibitors	IC_{50}[A,B]			
	S. cerevisiae α-glucosidase (μM)	*B. stearothermophilus* α-glucosidase (μM)	Rat-intestinal sucrase (mM)	Rat-intestinal maltase (mM)
Compound **1**	60.3 ± 3.5[a]	130.3 ± 5.4[b]	4.20 ± 0.25[a]	5.00 ± 0.30[a]
Compound **2**	110.4 ± 5.0[b]	230.3 ± 6.5[a]	3.60 ± 0.22[b]	4.80 ± 0.29[b]
Acarbose	NI[C]	NI	0.02 ± 0.01[d]	0.08 ± 0.01[c]
Voglibose	NI	NI	0.06 ± 0.01[c]	0.04 ± 0.01[d]

[A] Values are expressed as mean ± SD. Means in the same column with different superscripts are significantly different ($p < 0.05$).
[B] The IC_{50} value is defined as the inhibitor concentration to inhibit 50% of its activity under assayed conditions.
[C] NI: no inhibition.

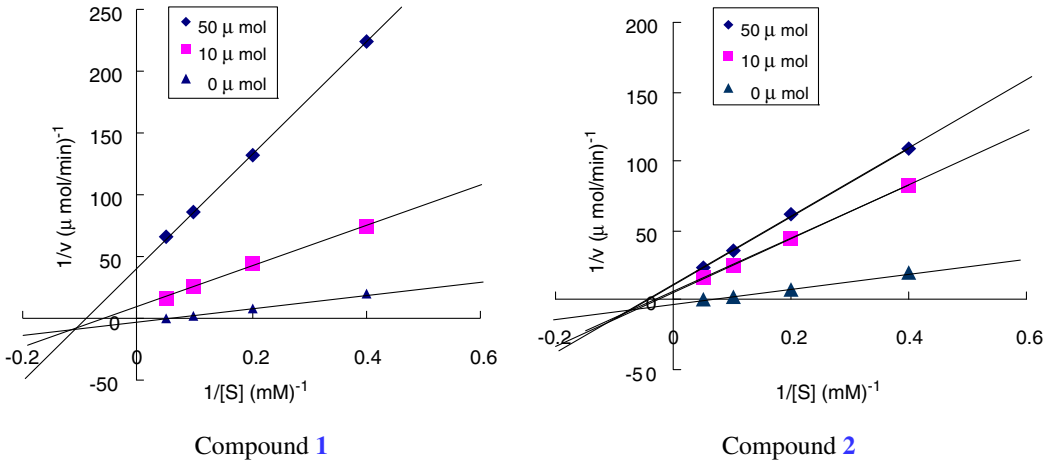

Fig. 2. Lineweaver–Burk plot of *Saccharomyces cerevisiae* α-glucosidase inhibition of bromophenols **1** and **2** purified from *Grateloupia elliptica* at different concentrations of pNPG.

α-glucosidase I, suggesting that the 2-OH groups in the glucose moiety are essential for α-glucosidase I (Kimura et al., 2004). The α-glucosidase II catalyzed the hydration of D-glucal to produce 2-deoxy-α-D-arabino-hexose, but α-glucosidase I yielded no detectable hydration product (Chiba et al., 1988). The strong enzymatic inhibitory activity against microbial α-glucosidases shown by algal compounds, which is much better than that of commercial inhibitors such as acarbose and voglibose at low concentration, will decrease the blood glucose level, adverse gastrointestinal effects, and abdominal discomfort caused by acarbose and voglibose (Tewari et al., 2003). Therefore, algal compounds can potentially be developed as novel natural nutraceuticals because of their high inhibitory activity against α-glucosidase. However, since the results reported herein were obtained in vitro, further studies need to be conducted in vivo. This is because if the structure of algal compounds is degraded in the human body by stomach acid or digestive enzymes, their inhibitory activity could be altered.

3. Conclusions

One of the therapeutic approaches for preventing diabetes mellitus is to retard absorption of glucose via inhibition of α-glucosidase. Hence, the search for α-glucosidase inhibitors in marine organisms is important because they are expected to suppress the postprandial hyperglycemia of diabetic patients. In this study, α-glucosidase inhibitors were screened and isolated from 10 different seaweeds harvested from the eastern coastal area of the Korean peninsula. Two compounds with α-glucosidase inhibitory activity – 2,4,6-tribromophenol (**1**) and 2,4-dibromophenol (**2**) – were purified and identified from the red alga *G. elliptica*. These compounds exhibited a mixed type of inhibition against *S. cerevisiae* and *B. stearothermophilus* α-glucosidase. 2,4,6-Tribromophenol (**1**) resulted in as higher inhibitory activity than 2,4-dibromophenol (**2**) against *S. cerevisiae* and *B. stearothermophilus* α-glucosidases, whereas the reverse was true against rat-intestinal sucrase and maltase. Therefore, bromophenols of *G. elliptica* can potentially be developed as a novel natural nutraceutical to prevent diabetes mellitus because of their high α-glucosidase inhibitory activity.

The search for α-glucosidase inhibitors in marine organisms is important because they are expected to suppress the postprandial hyperglycemia of diabetic patients. Although it is still not clear whether algal α-glucosidase inhibitors can suppress hyperglycemia, algal bromophenols greatly inhibited yeast α-glucosidase in this study.

4. Experimental

4.1. Reagents

p-Nitrophenyl α-D-glucopyranoside (*p*NPG), *S. cerevisiae* α-glucosidase, *B. stearothermophilus* α-glucosidase, rat intestinal acetone powder, and a glucose assay kit (GAGO-20) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sephadex LH-20 and Sephacryl HR-100 columns were purchased from Pharmacia Biotech Ltd. (Uppsala, Sweden). All other chemicals used in this study were of analytical grade.

4.2. Algal samples

Two green algae (*Ulva pertusa* and *Ostria costata*), four brown algae (*Sargassum horneri*, *S. thunbergii*, *S. nigrifolium*, and *Myelophycus simplex*), and four red algae (*G. elliptica*, *Plocamium elfairiae*, *G. lanceolata*, and *P. lancifolia*) were harvested in the eastern coastal area of the Korean peninsula in July and August, 2005. Fresh algae were individually washed with tap H_2O and air-dried in the shade at room temperature. Dried samples were individually cut into small pieces (2 × 3 cm), homogenized, sifted with a 500 μm sieve, and stored at −40 °C until needed.

4.3. Extraction and purification of α-glucosidase inhibitors

Each algal sample (1 kg) was soaked in MeOH · H_2O (8:2, v/v, 1 l) and individually extracted under conditions where the solvent was heated until reflux began, this being maintained for 3 h. Each resulting residue was dissolved in MeOH · H_2O (8:2, v/v, 500 ml) and extracted as above, with the extracts individually filtered using Whatman No. paper (Maidstone, England). Each combined supernatant was then evaporated to ~200 ml under reduced pressure at a temperature of 40 °C. Specifically, the concentrated extract (200 ml) of *G. elliptica* was further purified using a series of solvent partitions. At first, the *G. elliptica* extract (200 ml) was added to the same amount of distilled H_2O. The whole (400 ml) was then partitioned using *n*-hexane (400 ml) by standing for 30 min at room temperature after vigorous shaking (hexane fraction), which was repeated five times until any color in the hexane layer was absent. The aqueous layer of the hexane fraction was then partitioned using $CHCl_3$, EtOAc, and BuOH in the same order as mentioned above. The EtOAc extract in MeOH (30 ml) was loaded onto a silica gel column fraction (2.0 × 15.0 cm) and then eluted with $CHCl_3$-MeOH (7:3, v/v, 50 ml). Each $CHCl_3$-MeOH was concentrated using

a vacuum evaporator and loaded onto a plate of silica gel TLC which was then developed with toluene:EtOAc:HOAc = 5:7:1 solvent. The plate was sprayed with $FeCl_3$ solution to analyze the spot pattern. The fractions showing inhibitory activity were further purified by Sephadex LH-20 (3.0 × 30.0 cm) chromatography eluted with MeOH to obtain the α-glucosidase inhibitor.

4.4. Assay for α-glucosidase inhibitory activity

The inhibitory activity of α-glucosidase was determined according to the modified method of Kurihara et al. (1995). 3 mM *p*-nitrophenyl α-D-glucopyranoside (0.01 ml) and 20 U/ml α-glucosidase (0.01 ml) in 0.01 M phosphate buffer (pH 7) were added to the sample solution (2.2 ml) to start the reaction. Each reaction was carried out at 37 °C for 30 min and stopped by adding 0.1 M Na_2CO_3 (2 ml). Enzymatic activity was quantified by measuring absorbance at 405 nm. One unit of α-glucosidase activity was defined as amount of enzyme liberating *p*-nitrophenol (1.0 μM) per min. The IC_{50} value was defined as the concentration of α-glucosidase inhibitor that inhibited 50% of α-glucosidase activity.

4.5. Purification of rat intestinal α-glucosidase

Rat intestinal α-glucosidase was purified according to the modified method of Kurihara et al. (1999a). Commercial rat intestine acetone powder (10 g) was dissolved in buffer A (100 ml) (0.1 M potassium phosphate buffer containing 5 mM EDTA, pH 7.0), sonicated at 4 °C for 15 s, and then centrifuged at 27,000*g* at 4 °C for 60 min to obtain supernatant A. The precipitate was dissolved in buffer A (100 ml), sonicated, and then centrifuged (32,000*g*, 60 min, 4 °C) to obtain supernatant B. The combined supernatant was dialyzed against buffer B (0.05 M potassium phosphate buffer containing 0.4 mM EDTA, pH 7.0) for 48 h. This was concentrated to 20 ml by ultrafiltration (cut off membrane 10 kDa) and then loaded onto a Sephacryl HR-100 column (2.6 × 60.0 cm) equilibrated with buffer B in advance. Rat intestinal α-glucosidase was eluted with buffer B at a flow rate of 0.2 ml/min.

4.6. Inhibitory assay for rat intestinal α-glucosidase (sucrase and maltase) activity

Rat intestinal α-glucosidase inhibitory activity was determined according to the modified method of Kurihara et al. (1999a). Sucrase activity was determined in a mixture of 500 mM sucrose (0.1 ml), the isolated compound in MeOH (0.05 ml), and 0.1 M maleate buffer (pH 6.0, 0.75 ml). The mixture was preincubated at 37 °C for 5 min, and reaction was initiated by adding rat intestinal α-glucosidase (0.1 ml) to the reaction mixture. The mixture was incubated at 37 °C for 60 min. The reaction was terminated by adding 2.0 M maleate–Tris–NaOH buffer (pH 7.4, 1.0 ml). To measure maltase activity, maltose (500 mM) was used instead of sucrose.

The reaction mixture was extracted with Et_2O to remove bromophenols because it hindered the determination of the quantity of glucose released. Et_2O in the reaction mixture was removed under a N_2 gas stream. The glucose release in the solution was determined using a glucose assay kit based on the glucose oxidase/peroxidase method. One unit of α-glucosidase activity was defined as the amount of enzyme that liberated 1.0 μM of substrate per min. One unit of α-glucosidase inhibitory activity was defined as a 1 unit decrease in α-glucosidase activity.

4.7. Kinetics of α-glucosidase inhibitors

The enzyme-inhibitor reaction was performed according to the mentioned above at 10 and 50 μM of inhibitor. The type of inhibition was determined using the Lineweaver–Burk plot (Lineweaver and Burk, 1934), double reciprocal plot of the substrate concentration and velocity.

4.8. Identification of α-glucosidase inhibitors

The structures of isolated compounds were identified by spectroscopic methods, including ^1H NMR, ^{13}C NMR, and EI-MS. ^1H and ^{13}C NMR spectra were recorded in methanol-d_1 (MeOD) on a Bruker DRX-600 spectrometer (Bruker, Karlsruhe, Germany). Mass spectra were recorded on JEOL JMS-AX500 and JEOL JMS-SX102A spectrometers (Jeol, Tokyo, Japan).

4.9. Statistical analysis

Data were analyzed with Duncan's multiple comparison test ($p \leqslant 0.05$) using the SPSS software package version 10.0 (SPSS Inc., Chicago, IL, USA).

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