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α-Glucosidase Inhibitors from *Dendrobium tortile*

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Abstract: From the whole plant of *Dendrobium tortile*, a new compound, namely 4-(2-hydroxypropyl)-2(5*H*)-furanone, was isolated, together with six known compounds, which included *trans*-tetracosylferulate (**2**), *cis*-docosylferulate (**3**), *p*-hydroxybenzaldehyde (**4**), 3,4-dihydroxy-3,4′-dimethoxybibenzyl (**5**), (2*S*)-eriodictyol (**6**) and dendrofalconerol A (**7**). The structures of these compounds were determined through analysis of 1-D and 2-D NMR and HR-ESI-MS data. All of the isolates were evaluated for their α-glucosidase inhibitory activity. Compound **7** showed strong α-glucosidase inhibitory activity when compared with the positive control acarbose, whereas compounds **5** and **6** exhibited appreciable effects. An enzyme kinetic study revealed that compound **7** is a non-competitive inhibitor of α-glucosidase. This is the first report of the chemical constituents with biological activity from *D. tortile*.

Keywords: *Dendrobium tortile*; Orchidaceae; α-glucosidase; 2(5*H*)-furanone; bisbibenzyl.

1. Introduction

Dendrobium is one of the largest genera in Orchidaceous family. The genus is comprised of about 1,100 species, and 150 species have been identified in Thailand [1]. The stems of several *Dendrobium* species, locally known as "Shi-hu", have been used in Traditional Chinese Medicine for reducing fever, nourishing the stomach and promoting saliva production [2]. In Thailand, the dried stems of *D. draconis* have been used for blood tonic effect in the form of tea [3]. Recent pharmacological investigations have shown that *Dendrobium* plants possess a wide variety of biological activities, including cytotoxic, antioxidative, antimalarial, antifibrotic and hypoglycemic activities [4].

Diabetes mellitus (DM) is a metabolic disorder characterized by high blood glucose level, which is usually accompanied with weight loss and frequent urination. People with DM are at risk for long-term complications related to nephropathy, retinopathy and neuropathy [5]. The treatment of DM is based on hypoglycemic agents and insulin injections. There are several classes of hypoglycemic agents, for example insulin sensitizers, secretagogues and α-glucosidase inhibitors [6].

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α-Glucosidase, a membrane bound enzyme in the small intestine, is responsible for digesting starch and disaccharides into glucose. Inhibition of this enzyme can significantly reduce the postprandial increase of blood glucose levels after meal [7]. In addition, some α-glucosidase inhibitors such as acarbose and miglitol have been used as anti-obesity medication [8]. Due to the above-mentioned potential applications, the number of reports on natural compounds with α-glucosidase inhibitory activity have continuously increased [9], and in this regard several α-glucosidase inhibitors have been identified from *Dendrobium*. [10-11].

Dendrobium totile Lindl. is an orchid that is widely distributed in Thailand [12]. Prior to this investigation, there were no reports on the chemical and biological studies of this plant. As part of our ongoing research on bioactive constituents from *Dendrobium* plants [13-14], a MeOH extract prepared from the whole plant of *D. totile* was evaluated for α-glucosidase inhibitory activity and found to show 70% inhibition at a concentration of 2 mg/mL. Herein, we present results on the isolation and characterization of the compounds responsible for this activity.

The methanol extract of *D. tortile* was suspended in water and then partitioned with EtOAc and butanol to give an EtOAc extract, a butanol extract and an aqueous extract after removal of the solvent. These extracts were then evaluated for α-glucosidase inhibitory activity. It was found that the EtOAc extract showed the strongest α-glucosidase inhibitory effect with 98% inhibition at a concentration of 2 mg/mL, whereas the butanol extract and the aqueous extract exhibited only 26% and 46% inhibition, respectively. Therefore, the EtOAc extract was selected for further chemical investigation.

2. Materials and Methods

2.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were obtained on a Milton Roy Spectronic 300 Array spectrophotometer, and IR spectra on a Perkin-Elmer FT-IR 1760X spectrophotometer. Mass spectra were recorded on a Bruker micro TOF mass spectrometer (ESI-MS). NMR spectra were recorded on a Bruker Avance DPX-300 FT-NMR spectrometer. Microtiter plate reading was performed on a Perkin-Elmer Victor™ 1420 multilabel counter. Vacuum-liquid column chromatography (VLC) and column chromatography (CC) were performed on silica gel 60 (Merck, Kieselgel 60, 70-320 mesh), silica gel 60 (Merck, Kieselgel 60, 230-400 mesh) and Sephadex LH-20 (25-100 μm, Pharmacia Fine Chemical Co. Ltd.). Yeast α-glucosidase enzyme and *p*-nitrophenol-α-D-glucopyranoside were purchased from Sigma Chemical, Inc. (St. Louis, MO , USA), and acarbose from Fluka Chemical (Buchs, Switzerland).

2.2. Plant material

The whole plant of *D. totile* was purchased from Jatujak market, Bangkok, in October 2012. Authentication was performed by comparison with herbarium specimens at the Department of National Park, Wildlife and Plant Conservation, Ministry of National Resources and Environment. A voucher specimen (BS-DT-102555) has been deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.3. Extraction and isolation

The dried and powdered whole plant of *D. tortile* (1.5 kg) was macerated with MeOH (3 x 10 L) to obtain a MeOH extract (153 g) after removal of the solvent. This material was suspended in water and then partitioned with EtOAc and butanol to give an EtOAc extract (33 g), a butanol extract (54 g) and an aqueous extract (61 g), respectively, after evaporation of the solvent. The EtOAc extract was then separated by vacuum liquid chromatography (silica gel, acetone-hexane, gradient) to give 8

fractions (A-H). Fraction C (4.2 g) was fractionated on a silica gel column (acetone-hexane, gradient) to give 76 fractions. Fractions 19-22 were combined and then subjected to column chromatography (CC) over silica gel, eluted with an EtOAc-hexane gradient to give *trans*-tetracosylferulate (**2**) (18 mg) and *cis*-docosylferulate (**3**) (2 mg), respectively. Fraction D (2.1 g) was separated by CC (silica gel, acetone-hexane, gradient) and then further purified on Sephadex LH20 to yield *p*-hydroxybenzaldehyde (**4**) (4 mg). Fraction F (10.4 g) was subjected to CC (silica gel, acetone-hexane, gradient) to give 5 fractions (F1-F5). 3,4-Dihydroxy-3,4′-dimethoxybibenzyl (**5**) (31 mg) was obtained from fraction F2 after purification on Sephadex LH-20 (acetone). Fraction F3 (50 mg) was fractionated by CC (silica gel, acetone-hexane, gradient) and then purified on Sephadex LH-20 (acetone) to yield (2*S*)-eriodictyol (**6**) (5 mg). Separation of fraction F4 (75 mg) by CC (silica gel, acetone-hexane, gradient) gave dendrofalconerol A (**7**) (14 mg). Fraction G (3g) was separated on a silica gel column (acetone-hexane, gradient) and then further purified on Sephadex LH-20 (acetone) to furnish **1** (32 mg).

Compound (**1**): 4-(2-Hydroxypropyl)-2(5*H*)-furanone**,** Yellow oil; UV (MeOH) λ_{max} nm (log ε): 222 (3.8)**;** HR-ESIMS: *m*/*z* 165.0525 $[M+Na]^+$ calcd. for $C_7H_{10}O_3Na$, 165.0527**;** IR (film) ν_{max} cm^{-1}: 3395, 2980, 2934, 1701, 1697, 1391, 1274, 1252, 1133, 1042, 998, 841**;** $[\alpha]_D^{20}$: –162.0 (*c* = 0.05, MeOH)**;** ^{1}H NMR (300 MHz, acetone-d_6): δ 1.37 (3H, d, *J* = 6.3 Hz, H_3-8), 2.23 (1H, dd, *J* = 17.7, 6.6 Hz, H-6), 2.36 (1H, dd, *J* = 17.7, 4.2 Hz, H-6), 4.24 (2H, br s, H_2-5), 4.53 (1H, m, H-7), 5.93 (1H, br s, H-3); ^{13}C NMR (75 MHz, acetone-d_6): δ 20.0 (C-8), 31.3 (C-6), 62.8 (C-5), 73.6 (C-7), 112.4 (C-3), 161.2 (C-4), 164.5 (C-2).

2.4. Assay for α-glucosidase inhibitory activity

The α-glucosidase inhibition assay was performed as described previously with slight modification [10]. The activity of α-glucosidase was assayed by observing the liberation of *p*-nitrophenol from the substrate *p*-nitrophenol-α-D-glucopyranoside, using 0.04 U/mL of the enzyme and 1 mM of *p*-nitrophenol-α-D-glucopyranoside in 0.1 M phosphate buffer (pH 6.8). The test samples were each dissolved in 5% DMSO. The test was initiated by adding 10 μL of the sample solution and 40 μL of α-glucosidase in phosphate buffer (pH 6.8) to a 96-well plate, and the mixture was pre-incubated at 37 °C for 10 minutes. Then 50 μL of *p*-nitrophenol-α-D-glucopyranoside was added, and the reaction was allowed to proceed at 37 °C for 20 minutes. Finally Na_2CO_3 (100 μL, 0.1 mM) solution was added. The mixture was measured with a micro-plate reader at 405 nm. Acarbose was used as the positive control. The kinetic study of enzyme inhibition was performed by analyzing the double reciprocal Lineweaver-Burk plot data. The experiment was performed by varying the concentration of substrate *p*-nitrophenol-*α*-D-glucopyranoside (0.25, 0.5, 1.0,-2.0 mM) in the absence or presence of dendrofalconerol A at two different concentrations (15 and 30 μM).

Data were expressed as means ± SD. Statistical analysis was performed by Student's t test.

3. Results and Discussion

3.1. Structure elucidation

Phytochemical investigation of the EtOAc extract resulted in the isolation of a new compound, 4-(2-hydroxypropyl)-2(5*H*)-furanone (**1**), along with 6 known compounds, which include *trans*-tetracosylferulate (**2**) [15], *cis*-docosylferulate (**3**) [16], *p*-hydroxybenzaldehyde (**4**) [17], 3,4-dihydroxy-3,4′-dimethoxybibenzyl (**5**) [18], (2*S*)-eriodictyol (**6**) [19] and dendrofalconerol A (**7**) [20]

(Figure 1). The structures of these compounds were determined through extensive spectroscopic studies, including 1D-and 2D-NMR, as well as HR-ESI-MS experiments.

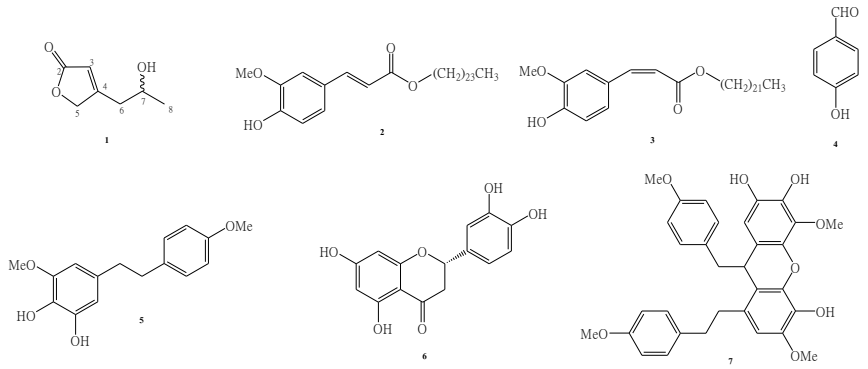

Figure 1. Structures of compounds (**1**-**7**) from *Dendrobium tortile.*

Compound **1** was obtained as a yellow oil. The positive HR-ESI-MS showed an $[M+Na]^+$ ion at *m/z* 165.0525 (calcd. for $C_7H_{10}O_3Na$, 165.0527), suggesting the molecular formula $C_7H_{10}O_3$. The UV spectrum displayed a maximal absorption at 222 nm. The IR spectrum exhibited strong absorption bands for carbonyl (1701 cm^{-1}) and hydroxyl groups (3395 cm^{-1}). The ^1H NMR signals at δ 4.24 (2H, br s, H_2-5) and 5.93 (1H, br s, H-3), and the ^{13}C NMR resonances at δ 164.5 (C-2), 161.2 (C-4), 112.4 (C-3) and 62.8 (C-5) suggested the presence of a 2(5*H*)-furanone structure [21]. This was confirmed by the HMBC correlations from C-2 to H-3, and from C-3 to H-5. The ^1H NMR and ^1H-^1H COSY spectra also exhibited signals for a 2-hydroxy-propyl group at δ 1.37 (3H, d, *J* = 6.3 Hz, H_3-8), 2.23 (1H, dd, *J* = 17.7, 6.6 Hz, H-6), 2.36 (1H, dd, *J* = 17.7, 4.2 Hz, H-6) and 4.53 (1H, m, H-7) which correlated with the ^{13}C NMR signals at δ 20.0 (C-8), 31.3 (C-6), and 73.6 (C-7), respectively, in the HSQC spectrum. The 2-hydroxy-propyl group should be attached to C-4 of the 2(5*H*)-furanone nucleus, as indicated from the HMBC correlations from H_2-6 to C-3, C-5 and C-8. Based on the above spectral evidence, the structure of **1** was established as 4-(2-hydroxypropyl)-2(5*H*)-furanone. Furanones have been rarely identified in *Dendrobium*. Most of them have been found as volatile components and detected by GC-MS analysis [22].

3.2. α-Glucosidase inhibitory activity

All the isolated compounds (**1**-**7**) were evaluated for their α-glucosidase inhibitory activity. In this study, each compound was initially tested at 200 μg/mL. An IC_{50} was determined if the compound showed more than 50% inhibition of the enzyme. The results are summarized in Table 1

Dendrofalconerol A (**7**) showed strong α-glucosidase inhibitory activity with an IC_{50} value of 18.0 μM, as compared with the positive control acarbose (IC_{50} 392.0 μM). 3,4-Dihydroxy-3,4′-dimethoxybibenzyl (**5**) and (2*S*)-eriodictyol (**6**) exhibited appreciable effects (IC_{50} 324.6 and 276.2 μM, respectively). It is interesting to note that the bisbibenzyl **7,** which is a dimer of **5**, was 18-fold more inhibitory than the monomer (**5**). There are several reports that indicated that stilbene dimers are stronger α-glucosidase inhibitors than their corresponding monomers due to the additional hydroxyl groups in the structures [23-25]. A molecular docking investigation of bibenzyls on α-glucosidase enzyme has shown that the formation of H-bonding between hydroxyl groups and the amide backbone of the enzyme played a key role in the binding modes of bibenzyls [26]. It could be proposed that dendrofalconerol A (**7**) could similarly bind to the enzyme through hydrogen bonding interaction. In a previous report, some stilbene dimers isolated from *Dendrobium loddigesii* showed strong α-glucosidase inhibition [11]. Our results confirmed the potential of dimeric stilbenes as α-glucosidase inhibitors.

Table 1. α-Glucosidase inhibitory activity of compounds **1**-**7**.

Compounds	IC_{50} (μM)
4-(2-Hydroxypropyl)-2(5*H*)-furanone (**1**)	NA
trans-Tetracosylferulate (**2**)	NA
cis-Docosylferulate (**3**)	NA
p-Hydroxybenzaldehyde (**4**)	NA
3,4-Dihydroxy-3,4′-dimethoxybibenzyl (**5**)	324.6 ± 34.8
(2*S*)-Eriodictyol (**6**)	276.2 ± 25.5
Dendrofalconerol A (**7**)	18.0 ± 0.8
Acarbose	392.0 ± 15.4

NA = no inhibitory activity

In order to investigate the mechanism of enzyme inhibition, a kinetic study of dendrofalconerol A (**7**) was conducted using yeast α-glucosidase. The experiment was performed by varying the concentration of the substrate *p*-nitrophenol-α-D-glucopyranoside (0.25-2.0 mM) in the absence or presence of compound **7** at two different concentrations (15 and 30 μM). Figure 2A displays a Lineweaver-Burk plot of the α-glucosidase inhibitory activity of compound **7**. As summarized in Table 2, the different concentrations of **7** decreased the V_{max} but did not affect the K_m value of the enzyme. These results indicated that dendrofalconerol A (**7**) is a non-competitive inhibitor with K_i value 2.0 μM. The inhibition mechanism of compound **7** on α-glucosidase enzyme was evaluated by analysis of plots of the initial velocity with different enzyme concentrations (0.01, 0.02, 0.04 and 0.08 U/mL) in the absence or presence of two concentrations of the inhibitor (15 and 30 μM) as shown in Figure 2B. It can be seen that the plots provided a family of straight lines and all of which passed through the origin. Moreover, increasing of concentrations of compound **7** resulted in lowering of the line slope. Therefore it was concluded that this compound was reversible an α-glucosidase inhibitor [26].

The combined effects of acarbose and dendrofalconerol A (**7**) were investigated. The assay was performed by adding different combinations of acarbose (100 μM) and compound **7** (6 or 9 μM) in the assay system [27]. The results (Figure 3) show that the combination of acarbose (100 μM) and compound **7** (6 μM) significantly increased the percentage of α-glucosidase inhibition when compared with acarbose or compound **7** alone whereas compound **7** at 9 μM did not cause significant change in the percentage enzyme inhibition. These data suggest that compound **7** at low concentration show additive effect on α-glucosidase inhibition when combined with a low concentration of acarbose.

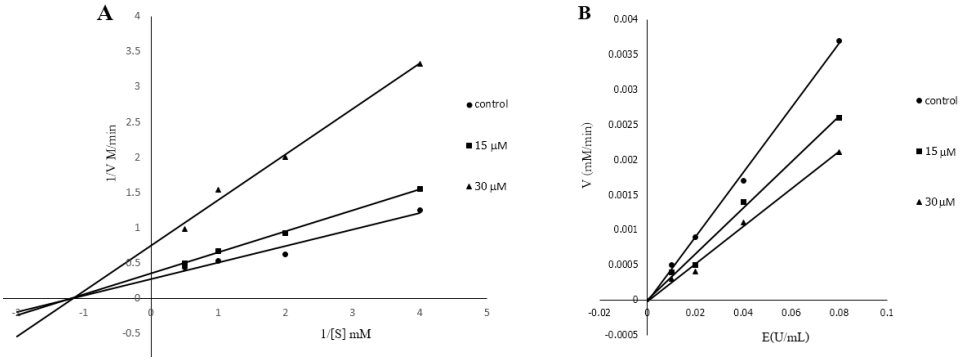

Figure 2. (A) Lineweaver-Burk plot analysis of the inhibition kinetic of dendrofalconerol A (**7**)
(B) α-Glucosidase inhibition at different concentrations of **7.**

Table 2. Kinetic parameters of α-glucosidase in the presence of dendrofalconerol A (**7**).

Inhibitor	Dose (μM)	V_{max} (M/min)	K_m (mM)	K_i (μM)
None	-	3.7	0.8	-
Dendrofalconerol A (**7**)	15	2.8	0.8	2.0
	30	1.3	0.8	

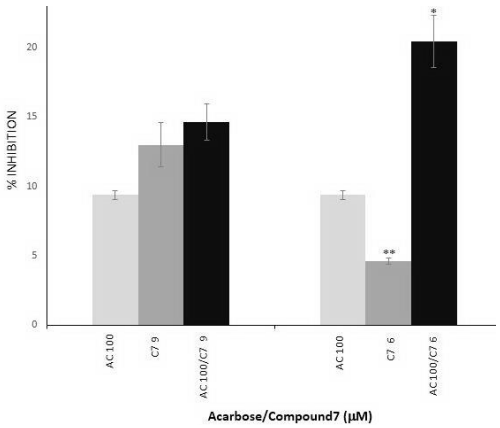

Figure 3. The combined effects of dendrofalconerol A (**7**) and acarbose on α-glucosidase inhibition. * $P < 0.05$ compared with acarbose at 100 μM. ** $P < 0.05$ compared with compound **7** at 6 μM + acarbose at 100 μM.

Currently, examples of clinically useful α-glucosidase inhibitors include acarbose, miglitol and voglibose. Among these drugs acarbose remains the most thoroughly investigated [28]. Several animal model studies on the effects of acarbose on the long-term complications of diabetes have been reported. It was demonstrated that acarbose can improve glycemic control in both induced and spontaneous rat and mouse models. Acarbose treatment also improved blood glucose levels, β-cell function and protein glycation as well as reduced insulin resistance [29]. Prolonged administration of acarbose has been associated with symptoms of carbohydrate malabsorption such as diarrhea and flatulence [30].

Nowadays, there are only a few α-glucosidase inhibitors approved for type 2 diabetes treatment although plenty have been discovered. This might be due to the lack of evidence for the beneficial effects of these drugs on the morbidity, mortality and quality of life in type 2 diabetes patients [31]. Moreover, the gastro-intestinal side effects of α-glucosidase inhibitors (flatulence, diarrhea, stomachache) are clinically relevant and may limit long-term compliance to therapy. Other possible disadvantages are the relatively higher costs compared to sulphonylurea and the need for a 3-times daily dosage schedule as opposed to thiazolidinediones or sulphonylurea that may be taken once or twice daily [31]. Although the potential of α-glucosidase inhibitors as drugs is rather low, the investigation for new α-glucosidase inhibitors from natural sources still remains attractive since the discovery of active compounds would be beneficial for the development of functional foods.

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