A New Stilbene with Tyrosinase Inhibitory Activity from *Chlorophora excelsa*

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A new stilbene, 4-[(2″*E*)-7″-hydroxy-3″,7″-dimethyloct-2″-enyl]-2′,3,4′,5-tetrahydroxy-*trans*-stilbene (1), and the known compound chlorophorin (2) were isolated from the heartwood of *Chlorophora excelsa*. Both 1 and 2 showed tyrosinase inhibitory activity with IC_{50} values of 96 and 1.3 μM, respectively.

Key words *Chlorophora excelsa*; tyrosinase; inhibitor; stilbene; chlorophorin; Moraceae

Tyrosinase (EC 1.14.18.1; PPO) is known to be a key enzyme for melanin biosynthesis in plants, microorganisms and mammalian cells.[1] This enzyme catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity), and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) which, in turn, are polymerized to brown, red or black pigments.[1] Many tyrosinase inhibitors have been tested in cosmetics and pharmaceuticals as a way of preventing overproduction of melanin in epidermal layers.[2] Also, tyrosinase is one of the most important key enzymes in the insect molting process,[3] and investigation of its inhibitors may be important in finding alternative insect control agents.[4] Melanin formation is considered to be deleterious to the color quality of plant-derived food and the prevention of this browning reaction has always been a challenge to food scientists.[5] This broadens the possible use of tyrosinase inhibitors as food additives, in addition to insect control agents and whitening agents. Furthermore, it has been reported that tyrosinase could be central to dopamine neurotoxicity as well as contributing to the neurodegeneration associated with Parkinson's disease.[6] These observations led us to focus on the exploration of tyrosinase inhibitors.

In the course of research on the isolation of biologically active substances from tropical plants, we discovered that the extract of the heartwood of *Chlorophora excelsa* showed potent tyrosinase inhibitory activity. *C. excelsa* (iroko, kambala, African teak), a member of the Moraceae family, is a West African hardwood widely used as a teak substitute in building floors, window frames and boats. The present communication describes the isolation, structural elucidation and tyrosinase inhibitory activity of a new stilbene, chlorophorin hydrate, 4-[(2″*E*)-7″-hydroxy-3″,7″-dimethyloct-2″-enyl]-2′,3,4′,5-tetrahydroxy-*trans*-stilbene (**1**) and the known chlorophorin, 4-geranyl-2′,3,4′,5-tetrahydroxy-*trans*-stilbene (**2**).

The ^{13}C-NMR spectrum of **1** revealed 24 carbon atoms [distortionless enhancement by polarization transfer (DEPT): 9×C, 8×CH, 4×CH_2 and 3×CH_3]. The FAB-MS spectral data ($[M+H]^+ = m/z$ 399) together with the ^{13}C-NMR spectral data suggested the molecular formula $C_{24}H_{30}O_5$. The ^{1}H-NMR spectrum of **1** was very similar to that of **2**,[7] showing a *trans*-stilbene at δ 6.81, 7.25 (2H, AB-system, J=16.6 Hz) and a symmetrically substituted ring A [δ 6.56 (2H, s)], and an ABX pattern for ring B at δ 6.36 (1H, dd, J=2.4, 8.3 Hz), 6.41 (1H, d, J=2.4 Hz), 7.37 (1H, d, J=8.3 Hz). However the aliphatic proton signals were different. From comparison of the ^{13}C- and ^{1}H-NMR data with that of **2**, it was deduced that one of the double bonds in **2** was hydrated to **1**. The ^{13}C-NMR peak at 70.4 ppm indicated that **1** bears an aliphatic hydroxyl group. The hydroxyl group was determined to be at the geranyl group by heteronuclear multiple bond connectivity (HMBC) linkages [C-7″/(H-5″, -6″, -8″, -9″)]. The *E*-configuration of the $\Delta^{2'',3''}$ double bond was deduced from the nuclear Overhauser effect (NOE) correlation between Me-10″ and H-1″. Accordingly, 1 was identified as 4-[(2″*E*)-7″-hydroxy-3″,7″-dimethyloct-2″-enyl]-2′,3,4′,5-tetrahydroxy-*trans*-stilbene. The structure and all assignments were confirmed by HMBC and heteronuclear multiple quantum coherence (HMQC) spectra (Table 1, Fig. 1).

Chlorophorin (**2**) has been reported as the major component about 5% yield in the heartwood of *C. excelsa*[8,9] and as a competitive inhibitor of tyrosinase.[10,11] The effects of **1** and **2** on mushroom tyrosinase were examined, and both compounds showed tyrosinase inhibitory activity with IC_{50} values of 96 and 1.3 μM, respectively. In the current experiment, the IC_{50} value of **2** was below than that of ref. 11 (IC_{50}: 19.2 μM). This difference seems attributable to the difference of 3,4-dihydroxyphenylalanine (DOPA) concentration between 0.83 mM (current experiment) and 1.4 mM (ref. 11) because **2** has been classified as a competitive inhibitor.[11] It should be added for reference that kojic acid, which is known as a potent tyrosinase inhibitor,[2] showed an IC_{50} of 20 μM.

Both **1** and **2** have a 4-substituted resorcinol skeleton (Fig. 1) and showed tyrosinase inhibitory activities. These results supported previous notions about the structure–activity relationship in which 4-substituted resorcinol moiety is important in expressing this activity.[5,11] Some polyphenols with 4-substituted resorcinol skeleton have been reported as a competitive inhibitor.[10—14] Also, 4-substituted alkyl resorcinols (*e.g.*, 4-hexylresorcinol) have been reported to be inhibitors of enzymatic (polyphenol oxidase) browning in food and beverages.[5,15,16]

Interestingly, the hydration of the geranyl side-chain of **1** decreased its inhibitory activity. In the case of the 4-substituted resorcinol-type competitive inhibitor, resorcinol moiety may bind to the binuclear active site and its side chain may be associated with the hydrophobic protein pocket close to the active site.[17,18] In other words, in addition to the stabilizing effect of the resorcinol moiety on the binding site, the side chain of 4-substituted resorcinol seems to be related to its binding affinity by the enzyme, such as hydrophobic-inter-

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Table 1. ^{13}C- and ^{1}H-NMR (400 MHz) Data of **1** [in CD_3COCD_3, Chemical Shifts in δ Values (ppm)]

C/H	δ (ppm) [^{13}C-NMR]	δ (ppm) [^{1}H-NMR]	J (Hz)
C-1	137.8		
CH-2, 6	105.3	6.56 s	
C-3, 5	156.7		
C-4	114.6		
C-1′	117.2		
C-2′	156.4		
CH-3′	103.3	6.41 d	2.4
C-4′	158.6		
CH-5′	108.2	6.36 dd	2.4, 8.3
CH-6′	127.7	7.37 d	8.3
CH-7′	123.1	7.25 d	16.6
CH-8′	125.9	6.81 d	16.6
CH_2-1″	22.9	3.35 d	7.1
CH-2″	123.8	5.30 tq	1.2, 7.3
C-3″	134.5		
CH_2-4″	41.1	1.93 t	7.0
CH_2-5″	23.4	1.42—1.53 m	
CH_2-6″	44.3	1.35—1.40 m	
C-7″	70.4		
CH_3-8″	29.3	1.12 s	
CH_3-9″	29.3	1.12 s	
CH_3-10″	16.2	1.76 br d	0.7

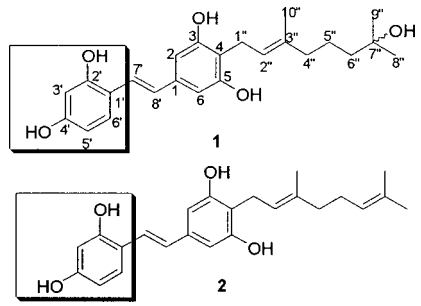

Fig. 1. Structures of **1** and **2**

Boxed portion: 4-substituted resorcinol skeleton.

action. On the basis of the above assumptions, it may be reasonable to conclude that the hydration of the geranyl moieties of **1** may become slightly hard to be embraced by the protein pocket, owing to the change of its side chain properties, such as the decrease of its hydrophobicity. This assumption may provide a clue regarding the interaction of 4-substituted resorcinol inhibitors with the tertiary structure of the enzyme, but this remains unclear since the structure of mushroom tyrosinase used for this study has not yet been established.

Experimental

General All NMR experiments were performed at 400 MHz. Samples were dissolved in acetone-d_6 and chemical shifts were referred to deuterated solvents.

Plant Material The wood of *C. excelsa* (4 kg) was purchased from Kyushu Mokuzai Kogyo Co., Ltd., Japan. The voucher specimen (No. 380) is preserved at the herbarium of the Laboratory of Systematic Forest and Forest Products Science, Kyushu University, in Japan.

Extraction and Identification Air-dried milled heartwood of *C. excelsa* (3.97 kg) was extracted using Et_2O (4.5 l×3), MeOH (4.5 l×2) and then Me_2CO (4.5 l) at ambient temperature and these extracts were combined, and then concentrated to dryness (79 g). The extract (56.6 g) was separated repeatedly by open column chromatography (75 mm i.d.×115 cm) on SiO_2 (2 kg) using EtOAc–hexane gradient and MeOH as an eluent to give three fractions (A—C); Fr. A [6.67 g, SiO_2, EtOAc/hexane, 1/1, *Rf*>0.7, tyrosinase inhibition (IC_{50}>50 μg/ml)], Fr. B [43.6 g, SiO_2, EtOAc/hexane, 1/1, *Rf* 0.6—0.7, tyrosinase inhibition (IC_{50}<50 μg/ml)], Fr. C [6.27 g, SiO_2, EtOAc/hexane, 1/1, *Rf*<0.6, tyrosinase inhibition (IC_{50}<50 μg/ml)]. Fraction B was separated by SiO_2 repeatedly on a smaller scale, to give **2** as yellowish powder, and identified as chlorophorin by comparison of the MS and NMR data with published data.[7] Fraction C was then subjected to silica gel column chromatography and eluted with a solvent mixture of increasing polarity (hexane–EtOAc–MeOH) to give 21 fractions. The Fr. 14 was subsequently subjected to preparative HPLC (Inertsil PREP-ODS: 20 mm i.d.×250 mm) eluting with H_2O/CH_3CN (30/70), 12 ml/min, to give **1** (65 mg) as yellowish powder.

4-[(2″*E*)-7″-Hydroxy-3″,7″-dimethyloct-2″-enyl]-2′,3,4′,5-tetrahydroxy-*trans*-stilbene (**1**): Yellowish powder; FAB-MS *m/z* $[M+H]^+$ 399.

Evaluation of Tyrosinase Inhibitory Activity Although mushroom tyrosinase differs somewhat from other sources, this fungal source was used for the present experiment due to its ready availability. It should be noted that the commercial tyrosinase was reported to contain numerous proteins besides tyrosinase[19] but was used without purification. The temperature was controlled at 25 °C using an Ecoline E100 circulating bath (Lauda Co., Germany) with heater and digital thermometer. The reaction was started by addition of the enzyme. Although tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, the assay was carried out in air-saturated solution. Kojic acid was used as a positive control.[2] The sample was first dissolved in dimethyl sulfoxide (DMSO) and used for the actual experiment diluted to 1/30 normal strength. Controls, without inhibitor, containing DMSO at that concentration were routinely carried out. The assay was performed as previously described.[20] First, 333 μl of 2.5 mM L-DOPA solution was mixed with 600 μl of 0.1 M phosphate buffer ($Na_2HPO_4 \cdot 12H_2O$–$NaH_2PO_4 \cdot 2H_2O$) (pH 6.5), and incubated at 25 °C. Then, 33 μl of the sample solution and 33 μl of the aqueous solution of mushroom tyrosinase (1380 units/ml) was added to the mixture to immediately measure the initial rate of linear increase in optical density at 475 nm based on the formation of dopachrome and using a V530 spectrophotometer (Jasco, Japan). The extent of inhibition by the addition of samples is expressed as the concentration necessary for 50% inhibition (IC_{50}).

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