Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 2396–2402
Bioorganic & Medicinal Chemistry

Synthesis and evaluation of 2′,4′,6′-trihydroxychalcones as a new class of tyrosinase inhibitors

Nishida Jun, Gao Hong and Kawabata Jun*

Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan

Received 26 October 2006; revised 11 January 2007; accepted 12 January 2007
Available online 17 January 2007

Abstract—In this study, we synthesized a series of hydroxychalcones and examined their tyrosinase inhibitory activity. The results showed that 2′,4′,6′-trihydroxychalcone (**1**), 2,2′,3,4′,6′-pentahydroxychalcone (**4**), 2′,3,4,4′,5,6′-hexahydroxychalcone (**5**), 2′,4′,6′-trihydroxy- 3,4-dimethoxychalcone (**9**) and 2,2′,4,4′,6′-pentahydroxychalcone (**15**) exhibited high inhibitory effects on tyrosinase with respect to L-tyrosine as a substrate. By the structure–activity relationship study, it was suggested that the 2′,4′,6′-trihydroxyl substructure in the chalcone skeleton were efficacious for the inhibition of tyrosinase activity. And also, the catechol structure on B-ring of chalcones was not advantageous for the inhibitory potency. Furthermore, **15** (IC_{50} = 1 μM) was found to show the highest activity out of a set of 15 hydroxychalcones, even better than both 2,2′,4,4′-tetrahydroxychalcone (**13**, IC_{50} = 5 μM) and kojic acid (**16**, IC_{50} = 12 μM), which were known as potent tyrosinase inhibitors. Kinetic study revealed that **15** acts as a competitive inhibitor of tyrosinase with K_i value of 3.1 μM.

1. Introduction

Tyrosinase (EC 1. 14. 18. 1) is a copper-containing enzyme, which catalyzes two distinct reactions of melanin synthesis, the hydroxylation of L-tyrosine by monophenolase action and the oxidation of L-DOPA to the corresponding *o*-dopaquinone by diphenolase action.[1] These processes are involved in local hyperpigmentations such as melasma, ephelide, and lentigo.[2,3] Therefore, tyrosinase inhibitors have become increasingly important for medicinal and cosmetic products that may be used to prevent or treat pigmentation disorders.[4] Also, melanin formation is considered to be deleterious to the color quality of fruits and vegetables, hence an inhibition of this enzyme should be useful for browning control of plant-derived food in the food industry.[5] Furthermore, tyrosinase is one of the most important key enzymes in insect molting process, and investigating its inhibitors may be important in finding alternative insect control agents.[6,7]

Many efforts have been addressed to the search for effective and safe tyrosinase inhibitors from natural materials, and chalcones are one of the most numerous and best-studied groups of plant polyphenols. Recently, it has been reported that some chalcones possessing a 2,4-substituted resorcinol moiety act as potential inhibitors of tyrosinase and the position of the hydroxyl group attached to the chalcone rings is of major importance in that activity.[8–10]

We have designed hydroxychalcones in order to improve and evaluate their antioxidant activity. A series of 2′,4′,6′-trihydroxychalcones were synthesized and the mechanism of their DPPH radical scavenging action was evaluated in alcoholic and non-alcoholic solvents.[11] As a part of our research on the new biological and physiological properties of hydroxychalcones, we have been interested in the inhibitory effect on melanin formation by 2′,4′,6′-trihydroxychalcones. In this study, we evaluated the tyrosinase inhibitory activity of 2′,4′,6′-trihydroxychalcones, and the structure–activity relationship on the enzyme inhibitory activity was investigated.

2. Results and discussion

2.1. Chemistry

Chalcones **1**–**14** were prepared through the Claisen–Schmidt condensation of the corresponding acetophenones

Keywords: Tyrosinase inhibitor; Monophenolase; Hydroxychalcones; Hyperpigmentation; Structure–activity relationship.
* Corresponding author. Tel./fax: +81 11 706 2496; e-mail addresses: gaohong@chem.agr.hokudai.ac.jp; junk@ chem.agr.hokudai.ac.jp

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.01.017

and benzaldehydes as described previously.[11] The efficient total synthesis of 2,2′,4,4′,6′-pentahydroxychalcone (**15**) was carried out as follows (Scheme 1). In this case, an acid treatment of **15a** could not afford directly the expected **15**. The main product was the 2′,4′,5,7-tetrahydroxyflavanone (**15b**), which was then treated with KOH in ethanol to give **15** in a high yield. All the synthesized chalcones were characterized by spectroscopic data.

2.2. Biology

The synthesized chalcones **1**–**15** and kojic acid (**16**), a well-known tyrosinase inhibitor, were tested for their enzymatic inhibitory activities against tyrosinase by the usual procedure.[12] As shown in Figure 1, 2′,4′,6′-trihydroxychalcone (**1**) inhibited 72% of tyrosinase activity at a concentration of 400 μM, where 50% inhibitory concentration (IC_{50}) was calculated to be 120 μM. On the other hand, compound **2** lacking a hydroxyl group at position 4′ and compound **3** lacking a hydroxyl group at position 6′ exhibited a loss of activity (less than 20% inhibition at a concentration of 400 μM). Hence, these results indicate that all of the hydroxyl groups in **1** are necessary for the activity. Further evidence supporting this rationale is that A-ring-hydroxylated or methoxylated chalcones **10**–**12**, which do not possess a 2′,4′,6′-trihydroxylated A-ring structure, showed no activity.

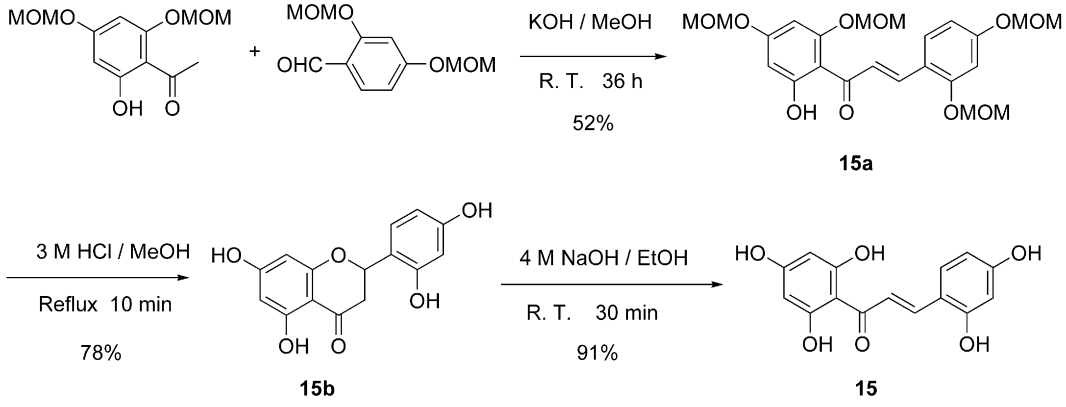
Having identified the importance of the 2′,4′,6′-trihydroxyl group of **1**, we decided to explore incorporation of additional hydroxyl groups onto positions 3 and 4 at B-ring of **1** to afford **6**. Unfortunately, this led to a reduction in potency, as compared with **1**, whereas 3,4-dimethoxylated **9** (IC_{50} = 150 μM) showed a comparable activity to **1**.

Monophenolase activity of tyrosinase is characterized by a lag time, derived from oxidative hydroxylation of monophenolic substrates to *o*-diphenols. Like compound **6**, chalcones **7**, **8**, and **12** showed no inhibitory effect and the lag time was not observed for these compounds (Fig. 2). It was reported that *o*-diphenols (catechols) acted as an alternative co-factor to initiate monophenolase activity.[12] Judging from the structure of **6**–**8** and **12** which possess chatechol structure on B-ring, we concluded that **6**–**8** and **12** worked as co-factors for the tyrosinase reaction, and thus, the catechol structure on B-ring of chalcones was not advantageous for the inhibitory potency.

To further investigate the effect of hydroxyl substituents on B-ring of **1**, the B-ring-hydroxylated 2′,4′,6′-trihydroxychalcones **4**, **5**, and **15** were examined for the tyrosinase inhibitory activity. As shown in Figure 1, the inhibitory effects of **4** (IC_{50} = 193 μM) and **5** (IC_{50} = 200 μM) were slightly lower than that of **1**, while **15** (IC_{50} = 1 μM) showed the most potent activity among the tested chalcones. The activity of **15** was 10-fold stronger than that of kojic acid (**16**, IC_{50} = 12 μM). A 2,4-hydroxylated resorcinol structure on B-ring of chalcones has been reported to be effective for tyrosinase inhibition, in which 2,2′,4,4′-tetrahydroxychalcone (**13**) was found to be a potent tyrosinase inhibitor with respect to L-tyrosine as a substrate.[9] Comparing the tyrosinase inhibitory activity of **15** with those of **13** and **14**, each of them possessing the resorcinol on B-ring, revealed that **15** had the highest activity (IC_{50} = 1 μM) compared to **13** (IC_{50} = 5 μM) and **14** (IC_{50} = 3.1 μM), emphasizing the enhanced potential of 2′,4′,6′-trihydroxy structure as eminent tyrosinase inhibitors.

Among the tested hydroxychalcones **1**–**15**, 2,2′,4,4′,6′-pentahydroxychalcone **15** showed the highest monophenolase inhibitory activity, and hence, we carried out the steady-state kinetic analysis of **15** for tyrosinase inhibition with respect to L-tyrosine as a substrate. Lineweaver–Burk plots for the inhibition of tyrosinase (monophenolase) by **15** were obtained with variable concentrations of **15** and the substrate (Fig. 3). The intersection of these lines on the vertical axis indicated that **15** was a competitive inhibitor of tyrosinase with respect to L-tyrosine as a substrate, with a K_i value of 3.1 μM. These data strongly suggested that **15** effectively inhibited the enzyme by binding to its active site.

In conclusion, the results show that 2′,4′,6′-trihydroxychalcones represent a new class of tyrosinase inhibitors,



Scheme 1. Synthesis of 2,2′,4,4′,6′-pentahydroxychalcone **15**.

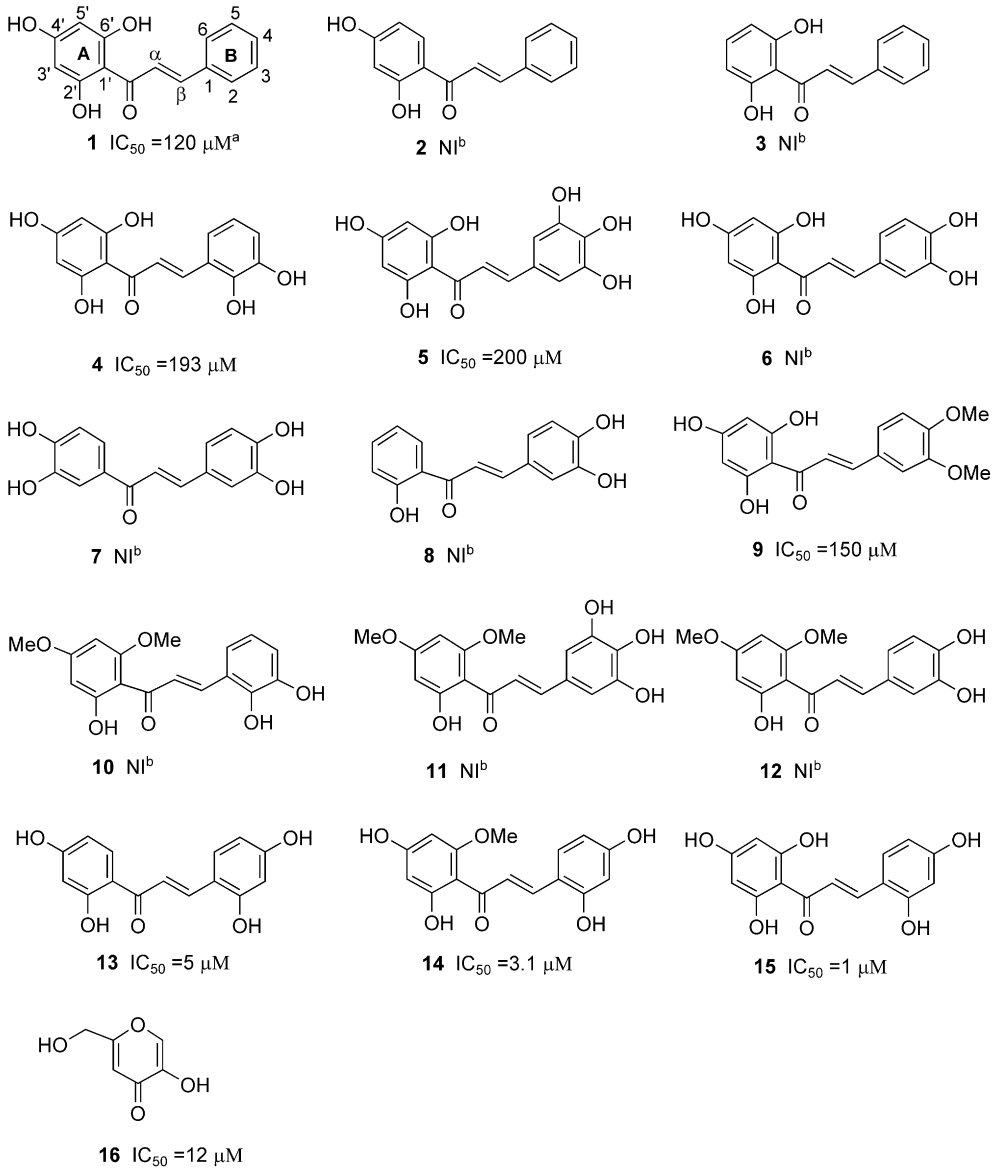


Figure 1. Structures and inhibitory activity against mushroom tyrosinase (monophenolase) of hydroxychalcones **1**–**15**, and kojic acid (**16**). [a]Each IC_{50} was calculated from time/absorption plot for five different concentrations of test compounds 20 min after addition of a solution of tyrosinase (250 U/mL) in a 50-mM solution of phosphate buffer (pH 6.8) at 25 °C. [b]Less than 20% inhibition at a concentration of 400 μM.

and the number and position of hydroxyl substituent in 2′,4′,6′-trihydroxychalcones are important for the inhibition of tyrosinase activity.

3. General experimental

NMR spectra were recorded with a Bruker AMX500 (^{1}H, 500 MHz) instrument. Chemical shifts were calculated from the residual solvent signals of δ_H 3.30 ppm in methanol-d_4, δ_H 7.24 ppm in chloroform-*d*. Field desorption (FD), FD-high resolution (HR), electron ionization (EI), and EI-HR mass spectra (MS) were obtained on a Jeol JMS-SX102A instrument. Melting points were measured on a hot stage and were uncorrected. The following experimental conditions were used for chromatography: ordinary phase column chromatography; Silica gel Wakogel C-300 (Wako Pure Chem. Co., Osaka, Japan, 40–64 mesh). TLC, precoated TLC plates with Silica gel 60 F_{254} (Merck, 0.25 mm or 0.5 mm thickness, normal phase). Detection was done by UV lamp (254 nm). All reagents were of reagent grade and were purchased from Wako Pure Chem. Co., Osaka, Japan, unless otherwise stated. Acetone and tetrahydrofuran were dried by storage over 3A molecular sieves. All solvents were distilled before use. All nonaqueous reactions were performed in dry glassware.

3.1. General procedure for the preparation of compounds 1a–15a

To a stirred solution of KOH (2.0 g, 45.6 mmol) in water (2 mL) cooled to 0 °C in an ice bath was added

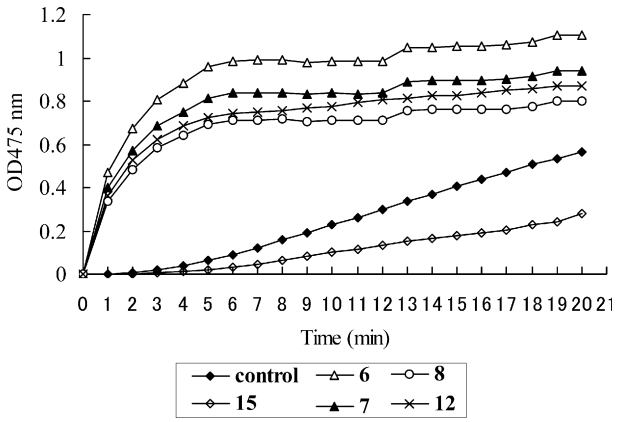


Figure 2. Lag-period of L-dopaquinone formation. L-Tyrosine (0.8 mM) was incubated with tyrosinase in the absence (control, ♦) or presence of 2′,3,4,4′,6′-pentahydroxychalcone **6** (△, 400 μM), 3,3′,4,4′-tetrahydroxychalcone **7** (▲, 400 μM), 2′,3,4-trihydroxychalcone **8** (○, 400 μM), 2′,3,4-trihydroxy-4′,6′-dimethoxychalcone **12** (×, 400 μM), and 2,2′,4,4′,6′-pentahydroxychalcone **15** (◇, 1 μM).

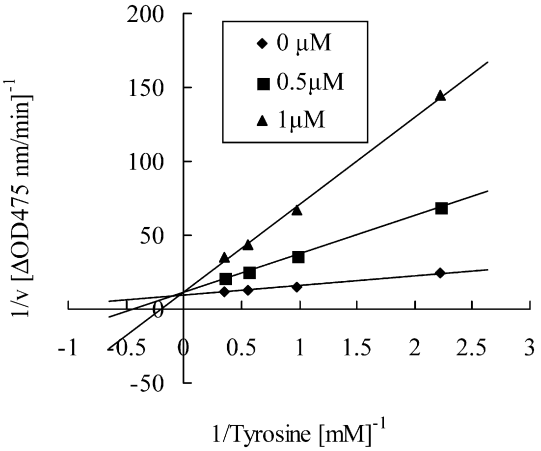


Figure 3. Lineweaver–Burk plots for the monophenolase inhibitory activity of 2,2′,4,4′,6′-pentahydroxychalcone (**15**) with respect to L-tyrosine as a substrate.

dropwise a solution of actetophenone (1.5 mmol) and aldehyde (1.0 mmol) in methanol (10 mL) under argon. The reaction mixture was kept at 0 °C for 3 h and then at room temperature for 72 h. The mixture was poured into ice-water (10 mL), adjusted to pH 3–4 with 1 M HCl, and then extracted with ethyl acetate. The organic layer was successively washed with water and saturated brine, dried over anhydrous Na_2SO_4. After concentration under reduced pressure, the resultant was recrystallized from hexane-ethyl acetate.

3.1.1. 2′-Hydroxy-4′,6′-bis(methoxymethoxy)chalcone (1a). Yield: 55%; yellow powders; mp 49–50 °C; EI-HR-MS *m/z*: 344.1270 (calcd for $C_{19}H_{20}O_6$, 344.1260); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.49 and 3.54 (each 3H, s, $2\times OCH_3$), 5.19 and 5.30 (each 2H, s, $2\times OCH_2O$), 6.26 (1H, d, J = 2.5 Hz, H-3′ or 5′), 6.33 (1H, d, J = 2.5 Hz, H-3′ or 5′), 7.40–7.62 (5H, m, H-2 ~ 6), 7.80 (1H, d, J = 15.7 Hz, H-β), 7.93 (1H, d, J = 15.7 Hz, H-α).

3.1.2. 2′-Hydroxy-4′-methoxymethoxychalcone (2a). Yield: 51%; yellow oil; FD-MS *m/z* (%): 284 (100, $[M]^+$); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.47 (3H, s, OCH_3), 5.21 (2H, s, OCH_2O), 6.58 (1H, dd, J = 8.8, 2.3 Hz, H-5′), 6.63 (1H, d, J = 2.3 Hz, H-3′), 7.41–7.42 (3H, m, H-3, 4 and 5), 7.57 (1H, d, J = 15.5 Hz, H-β), 7.63–7.65 (2H, m, H-2 and 6), 7.84 (1H, d, J = 8.8, H-6′), 7.88 (1H, d, J = 15.5 Hz, H-α).

3.1.3. 2′-Hydroxy-6′-methoxymethoxychalcone (3a). Yield: 75%; yellow oil; FD-MS *m/z* (%): 284 (100, $[M]^+$); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.52 (3H, s, OCH_3), 5.30 (2H, s, OCH_2O), 6.60 (1H, d, J = 8.4 Hz, H-3′), 6.66 (1H, d, J = 8.4 Hz, H-5′), 7.33 (1H, t, J = 8.4, H-4′), 7.40–7.41 (3H, m, H-3, 4 and 5), 7.60–7.62 (2H, m, H-2 and 6), 7.81 (1H, d, J = 15.5 Hz, H-β), 7.89 (1H, d, J = 15.5 Hz, H-α).

3.1.4. 2′-Hydroxy-2,3,4′,6′-tetrakis(methoxymethoxy)-chalcone (4a). Yield: 88%; yellow powders; mp 67–68 °C; EI-HR-MS *m/z*: 464.1682 (calcd for $C_{23}H_{28}O_{10}$, 464.1682); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.48, 3.51, 3.52 and 3.64 (each 3H, s, $4\times OCH_3$), 5.19, 5.19, 5.22 and 5.29 (each 2H, s, $4\times OCH_2O$), 6.26 (1H, d, J = 2.5 Hz, H-5′), 6.32 (1H, d, J = 2.5 Hz, H-3′), 7.08 (1H, dd, J = 8.1, 7.9 Hz, H-5), 7.20 (1H, dd, J = 8.1, 1.2 Hz, H-6), 7.32 (1H, dd, J = 7.9, 1.2 Hz, H-4), 7.92 (1H, d, J = 15.8 Hz, H-β), 8.22 (1H, d, J = 15.8 Hz, H-α).

3.1.5. 2′-Hydroxy-3,4,4′,5,6′-pentakis(methoxymethoxy)-chalcone (5a). Yield: 65%; yellow powders; mp 89–90 °C; EI-HR-MS *m/z*: 524.1908 (calcd for $C_{25}H_{32}O_{12}$, 524.1894); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.48 (3H, s, OCH_3), 3.51 (6H, s, $2\times OCH_3$), 3.54 and 3.62 (each 3H, s, $2\times OCH_3$), 5.19 and 5.19 (each 2H, s, $2\times OCH_2O$), 5.23 (4H, s, $2\times OCH_2O$), 5.30 (2H, s, OCH_2O), 6.28 (1H, d, J = 2.5 Hz, H-5′), 6.31 (1H, d, J = 2.5 Hz, H-3′), 7.16 (2H, s, H-2 and 6), 7.69 (2H, d, J = 15.5 Hz, H-β), 7.86 (1H, d, J = 15.5 Hz, H-α).

3.1.6. 2′-Hydroxy-3,4,4′,6′-tetrakis(methoxymethoxy)-chalcone (6a). Yield: 74%; yellow powders; mp 87–88 °C; EI-HR-MS *m/z*: 464.1691 (calcd for $C_{23}H_{28}O_{10}$, 464.1682); ^{1}H NMR (acetone-d_6) δ (ppm): 3.45, 3.47, 3.50 and 3.56 (each 3H, s, $4\times OCH_3$), 5.26, 5.27, 5.28 and 5.42 (each 2H, s, $4\times OCH_2O$), 6.24 (1H, d, J = 2.2 Hz, H-5′), 6.34 (1H, d, J = 2.2 Hz, H-3′), 7.20 (1H, d, J = 8.6 Hz, H-5), 7.34 (1H, dd, J = 8.6, 2.2 Hz, H-6), 7.56 (1H, d, J = 2.2 Hz, H-2), 7.74 (1H, d, J = 15.8 Hz, H-β), 7.96 (1H, d, J = 15.8 Hz, H-α).

3.1.7. 3,3′,4,4′-Tetrakis(methoxymethoxy)chalcone (7a). Yield: 55%; yellow powders; mp 51–52 °C; EI-HR-MS *m/z*: 448.1685 (calcd for $C_{23}H_{28}O_9$, 448.1733); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.53, 3.53, 3.55 and 3.55 (each 3H, s, OCH_3), 5.29 (4H, s, $2\times OCH_2$), 5.31, 5.33 (each 2H, s, OCH_2O), 7.19 (1H, d, J = 8.3 Hz, H-5 or 5′), 7.24 (1H, d, J = 8.4 Hz, H-5 or 5′), 7.26 (1H, dd, H = 8.3, 2.2 Hz, H-6 or 6′), 7.37 (1H, d, J = 15.5 Hz, H-β), 7.46 (1H, d, J = 2.2 Hz, H-2 or 2′), 7.68 (1H, dd, J = 8.4, 2.2 Hz, H-6 or 6′), 7.72 (1H, d, J = 15.5 Hz, H-α), 7.84 (1H, d, J = 2.2 Hz, H-2 or 2′).

3.1.8. 2′-Hydroy-3,4-bis(methoxymethoxy)chalcone (8a). Yield: 55%; yellow powders; mp 81–82 °C; EI-HR-MS *m/z*: 344.1263 (calcd for $C_{19}H_{20}O_6$, 344.1260); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.53 and 3.56 (each 3H, s, $2\times OCH_3$), 5.30 and 5.31 (each 2H, s, $2\times OCH_2O$), 6.95 (1H, t, J = 8.1 Hz, H-4′), 7.02 (1H, d, J = 8.3 Hz, H-5), 7.21 (1H, d, J = 8.1 Hz, H-3′), 7.30 (1H, dd, J = 8.3, 2.0 Hz, H-6), 7.48–7.49 (2H, m, H-2 and 5′), 7.53 (1 H, d, J = 15.5 Hz, H-β), 7.87 (1 H, d, J = 15.5 Hz, H-α), 7.92 (1H, dd, J = 8.1, 2.2 Hz, H-6′).

3.1.9. 2′-Hydroxy-3,4-dimethoxy-4′,6′-bis(methoxymethoxy)chalcone (9a). Yield: 83%; yellow powders; mp 85–86 °C; EI-HR-MS *m/z*: 404.1444 (calcd for $C_{21}H_{24}O_8$, 404.1471); ^{1}H NMR δ (chloroform-*d*) δ (ppm): 3.48 and 3.54 (each 3H, s, $2\times OCH_3$), 3.93 (6H, s, $2\times OCH_3$), 5.19 and 5.28 (each 2H, s, $2\times OCH_2O$), 6.21 (1H, d, J = 2.4 Hz, H-3′), 6.31 (1H, d, J = 2.4 Hz, H-5′), 6.90 (1H, d, J = 8.4 Hz, H-5), 7.14 (1H, d, J = 1.7 Hz, H-2), 7.21 (1H, dd, J = 8.4, 1.7 Hz, H-6), 7.77 (1H, d, J = 15.5 Hz, H-β), 7.85 (1H, d, J = 15.5 Hz, H-α).

3.1.10. 2′-Hydroxy-4′,6′-dimethoxy-2,3-bis(methoxymethoxy)-chalcone (10a). Yield: 88%; yellow powders; mp 94–95 °C; EI-HR-MS *m/z*: 404.1440 (calcd for $C_{21}H_{24}O_8$, 404.1471); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.51, 3.64, 3.83 and 3.90 (each 3H, s, $4\times OCH_3$), 5.19 and 5.22 (each 2H, s, $2\times OCH_2O$), 5.96 (1H, d, J = 2.3 Hz, H-5′), 6.11 (1H, d, J = 2.3 Hz, H-3′), 7.08 (1H, dd, J = 8.1, 7.9 Hz, H-5), 7.19 (1H, dd, J = 8.1, 1.5 Hz, H-4), 7.33 (1H, dd, J = 7.9, 1.5 Hz, H-6), 7.90 (1H, d, J = 15.8 Hz, H-β), 8.20 (1H, d, J = 15.8 Hz, H-α).

3.1.11. 2′-Hydroxy-4′,6′-dimethoxy-3,4,5-tris(methoxymethoxy)-chalcone (11a). Yield: 64%; yellow powders; mp 110–111 °C; EI-HR-MS *m/z*: 464.1635 (calcd for $C_{23}H_{28}O_{10}$, 464.1682); ^{1}H NMR (acetone-d_6) δ (ppm): 3.51 (6H, s, $2\times OCH_3$), 3.57, 3.87 and 4.00 (each 3H, s, $3\times OCH_3$), 5.15 (2H, s, OCH_2O), 5.30 (4H, s, $2\times OCH_2O$), 6.09 (1H, d, J = 2.2 Hz, H-5′), 6.12 (1H, d, J = 2.2 Hz, H-3′), 7.24 (2H, s, H-2 and 6), 7.66 (1H, d, J = 15.5 Hz, H-β), 7.94 (1H, d, J = 15.5 Hz, H-α).

3.1.12. 2′-Hydroxy-4′,6′-dimethoxy-3,4-bis(methoxymethoxy)chalcone (12a). Yield: 97%; yellow powders; mp 112–113 °C; EI-HR-MS *m/z*: 404.1450 (calcd for $C_{21}H_{24}O_8$, 404.1471); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.52, 3.55, 3.84 and 3.92 (each 3H, s, $4\times OCH_3$), 5.28 (4H, s, $2\times OCH_2O$), 5.97 (1H, d, J = 2.2 Hz, H-5′), 6.11 (1H, d, J = 2.2 Hz, H-3′), 7.18 (1H, d, J = 8.6 Hz, H-5), 7.22 (1H, dd, J = 8.6, 1.9 Hz, H-6), 7.51 (1H, d, J = 1.9 Hz, H-2), 7.73 (1H, d, J = 15.5 Hz, H-β), 7.84 (1H, d, J = 15.5 Hz, H-α).

3.1.13. 2,2′,4,4′-Tetrakis(methoxymethoxy)chalcone (13a). Yield: 56%; yellow oil; EI-HR-MS *m/z*: 404.1486 (calcd for $C_{21}H_{24}O_8$, 404.1471); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.49, 3.50 and 3.53 (each 3H, s, $3\times OCH_3$), 5.21, 5.22 and 5.29 (each 2H, s, $3\times OCH_2O$), 6.58 (1H, d, J = 8.8, 2.4 Hz, H-5 or 5′), 6.64 (1H, d, J = 2.4 Hz, H-3 or 3′), 6.75 (1H, dd, J = 8.8, 2.4 Hz, H-5 or 5′), 6.87 (1H, d, J = 2.4 Hz, H-3 or 3′), 7.58 (1H, d, J = 15.5 Hz, H-β), 7.61 (1H, d, J = 8.8 Hz, H-6 or 6′), 7.83 (1H, d, J = 8.8 Hz, H-6 or 6′), 8.20 (1H, d, J = 15.5 Hz, H-α).

3.1.14. 2′-Methoxy-2,4,4′,6′-tetrakis(methoxymethoxy)-chalcone (14a). Yield: 85%; yellow oil; EI-HR-MS *m/z*: 478.1885 (calcd for $C_{24}H_{30}O_{10}$, 474.1839); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.39, 3.44, 3.47, 3.50 and 3.75 (each 3H, s, $5\times OCH_3$), 5.10, 5.17, 5.18 and 5.19 (each 2H, s, $4\times OCH_2O$), 6.36 (1H, d, J = 2.0 Hz, H-5′), 6.51 (1H, d, J = 2.0 Hz, H-3′), 6.70 (1H, dd, J = 8.6, 2.0 Hz, H-5), 6.80 (1H, d, J = 2.0 Hz, H-3), 6.97 (1H, d, J = 16.0, H-β), 7.48 (1H, d, J = 8.6 Hz, H-6), 7.67 (1H, d, J = 16.0 Hz, H-α).

3.1.15. 2′-Hydroxy-2,4,4′,6′-tetrakis(methoxymethoxy)chalcone (15a). Yield: 52%; yellow powders; mp 84–85 °C; EI-HR-MS *m/z*: 464.1639 (calcd for $C_{23}H_{28}O_{10}$, 464.1682); ^{1}H NMR (chloroform-*d*) δ (ppm): 3.48, 3.49, 3.51 and 3.53 (each 3H, s, $4\times OCH_3$), 5.19, 5.20, 5.26 and 5.28 (each 2H, s, $4\times OCH_2O$), 6.25 (1H, d, J = 2.2 Hz, H-3′), 6.32 (1H, d, J = 2.2 Hz, H-5′), 6.74 (1H, dd, J = 8.7, 2.3 Hz, H-5), 6.86 (1H, d, J = 2.3 Hz, H-3), 7.57 (1H, d, J = 8.7 Hz, H-6), 7.87 (1H, d, J = 15.8 Hz, H-β), 8.17 (1H, d, J = 15.8 Hz, H-β).

3.2. General procedure for the preparation of compounds 1–14

To a stirred solution of chalcones **1a**–**14a** (0.25 mmol) in methanol (5 mL) was added dropwise 3 M HCl (2 mL). The mixture was refluxed for 10 min, diluted with water, and extracted with ethyl acetate. The organic layer was successively washed with water and brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (chloroform/methanol as the eluent) to give the corresponding chalcones **1**–**14**.

3.2.1. 2′,4′,6′-Trihydroxychalcone (1). Yield: 91%; yellow powders; mp 175–176 °C; EI-HR-MS *m/z*: 256.0689 (calcd for $C_{15}H_{12}O_4$, 256.0736); ^{1}H NMR (methanol-d_4) δ (ppm): 5.85 (2H, s, H-3′ and 5′), 7.38–7.62 (5H, m, H-2 ~ 6), 7.72 (1H, d, J = 15.8 Hz, H-β), 8.21 (1H, d, J = 15.8 Hz, H-α).

3.2.2. 2′,4′-Dihydroxychalcone (2). Yield: 91%; yellow powders; mp 152–153°C; EI-HR-MS *m/z*: 240.0776 (calcd for $C_{15}H_{12}O_3$, 240.0787); ^{1}H NMR (acetone-d_6) δ (ppm): 6.38 (1H, d, J = 2.3 Hz, H-3′), 6.48 (1H, dd, J = 8.8, 2.3 Hz, H-5′), 7.45–7.85 (5H, m, H-2 ~ 6), 7.87 (1H, d, J = 15.5, H-β), 7.96 (1H, d, J = 15.5, H-α), 8.16 (1H, d, J = 8.8 Hz, H-6′).

3.2.3. 2′,6′-Dihydroxychalcone (3). Yield: 85%; yellow powders; mp 169–170 °C; EI-HR-MS *m/z*: 240.0769 (calcd for $C_{15}H_{12}O_3$, 240.0787); ^{1}H NMR (acetone-d_6) δ (ppm): 6.47 (2H, d, J = 8.4 Hz, H-3′and 5′), 7.29 (1H, t, J = 8.4, H-4′), 7.30–7.73 (5H, m, H-2 ~ 6), 7.83 (1H, d, J = 15.5 Hz, H-β), 8.24 (1H, d, J = 15.5 Hz, H-α).

3.2.4. 2′,3,4,4′,6′-Pentahydroxychalcone (4). Yield: 41%; yellow powders; mp 157–158 °C; EI-HR-MS *m/z*: 288.0602 (calcd for $C_{15}H_{12}O_6$, 288.0634); ^{1}H NMR (acetone-d_6) δ (ppm): 5.95 (2H, s, H-3′ and 5′), 6.88 (1H, d, J = 8.1 Hz, H-5), 7.07 (1H, dd, J = 8.1, 1.8 Hz, H-6), 7.19 (1H, d, J = 1.8 Hz, H-2), 7.69 (1H, d, J = 15.5 Hz, H-β), 8.05 (1H, d, J = 15.5 Hz, H-α).

3.2.5. 2′,3,4,4′,5,6′-Hexahydroxychalcone (5). Yield: 25%; yellow powders; mp 179–180 °C; EI-HR-MS *m/z*: 304.0566 (calcd for $C_{15}H_{12}O_7$, 304.0583); ^{1}H NMR (acetone-d_6) δ (ppm): 5.95 (2H, s, H-3′ and 5′), 6.77 (2H, s, H-2 and 6), 7.61 (1H, d, J = 15.6 Hz, H-β), 8.03 (1H, d, J = 15.6 Hz, H-α).

3.2.6. 2′,3,4,4′,6′-Pentahydroxychalcone (6). Yield: 41%; yellow powders; mp 157–158 °C; EI-HR-MS *m/z*: 288.0602 (calcd for $C_{15}H_{12}O_6$, 288.0634); ^{1}H NMR (acetone-d_6) δ (ppm): 5.95 (2H, s, H-3′ and 5′), 6.88 (1H, d, J = 8.1 Hz, H-5), 7.07 (1H, dd, J = 8.1, 1.8 Hz, H-6), 7.19 (1H, d, J = 1.8 Hz, H-2), 7.69 (1H, d, J = 15.5 Hz, H-β), 8.05 (1H, d, J = 15.5 Hz, H-α).

3.2.7. 3,3′,4,4′-Tetrahydroxychalcone (7). Yield: 63%; yellow powders; mp 170–171 °C; EI-HR-MS *m/z*: 272.0650 (calcd for $C_{15}H_{12}O_5$, 272.0685); ^{1}H NMR (methanol-d_4) δ (ppm): 6.76 (1H, d, J = 8.1 Hz, H-5 or 5′), 6.82 (1H, d, J = 8.2 Hz, H-5 or 5′), 7.03 (1H, dd, J = 8.1, 2.2 Hz, H-6 or 6′), 7.12 (1H, d, J = 2.2 Hz, H-2 or 2′), 7.41 (1H, d, J = 15.5 Hz, H-β), 7.45 (1H, d, J = 2.2 Hz, H-2 or 2′), 7.49 (1H, dd, J = 8.2, 2.2 Hz, H-6 or 6′), 7.57 (1H, d, J = 15.5 Hz, H-α).

3.2.8. 2′,3,4-Trihydroxychalcone (8). Yield: 74%; yellow powders; mp 169–170 °C; EI-HR-MS *m/z*: 256.0714 (calcd for $C_{15}H_{12}O_4$, 256.0736); ^{1}H NMR (methanol-d_4) δ (ppm): 6.83 (1H, d, J = 8.3 Hz, H-5), 6.94–6.99 (2H, m, H-3′ and 5′), 7.15 (1H, dd, J = 8.3, 2.3 Hz, H-6), 7.22 (1H, d, J = 2.3 Hz, H-2), 7.49 (1H, dt, J = 7.4 (t), 1.7 Hz, H-4′), 7.65 (1H, d, J = 15.3 Hz, H-β), 7.81 (1H, d, J = 15.3 Hz, H-α), 8.08 (1H, dd, J = 7.9, 1.7 Hz, H-6′).

3.2.9. 2′,4′,6′-Trihydroxy-3,4-dimethoxychalcone (9). Yield: 53%; yellow powders; mp 91–92 °C; EI-HR-MS *m/z*: 316.0943 (calcd for $C_{17}H_{16}O_6$, 316.0947); ^{1}H NMR (methanol-d_4) δ (ppm): 3.87 and 3.90 (each 3H, s, $2\times OCH_3$), 5.84 (2H, s, H-3′ and 5′), 6.99 (1H, d, J = 8.3 Hz, H-5), 7.22 (1H, dd, J = 8.3, 2.0 Hz, H-6), 7.22 (1H, d, J = 2.0 Hz, H-2), 7.69 (1H, d, J = 15.5 Hz, H-β), 8.10 (1H, d, J = 15.5 Hz, H-α).

3.2.10. 2,2′,3-Trihydroxy-4′,6′-dimethoxychalcone (10). Yield: 60%; yellow powders; mp 175–176 °C; EI-HR-MS *m/z*: 316.0918 (calcd for $C_{17}H_{16}O_6$, 316.0947); ^{1}H NMR (acetone-d_6) δ (ppm): 3.83, 3.94 (each 3H, s, $2\times OCH_3$), 6.04 (1H, d, J = 2.4 Hz, H-5′), 6.07 (1H, d, J = 2.4 Hz, H-3′), 6.68 (1H, dd, J = 8.1, 7.2 Hz, H-5), 6.87 (1H, d, J = 7.2 Hz, H-4), 7.11 (1H, d, J = 8.1 Hz, H-6), 8.12 (2H, m, H-α and β).

3.2.11. 2′,3,4,5,-Tetrahydroxy-4′,6′-dimethoxychalcone (11). Yield: 25%; yellow powders; mp 91–92 °C; EI-HR-MS *m/z*: 316.0943 (calcd for $C_{17}H_{16}O_6$, 316.0947); ^{1}H NMR (methanol-d_4) δ (ppm): 3.87 and 3.90 (each 3H, s, $2\times OCH_3$), 5.84 (2H, s, H-3′ and 5′), 6.99 (1H, d, J = 8.3 Hz, H-5), 7.22 (1H, dd, J = 8.3, 2.0 Hz, H-6), 7.22 (1H, d, J = 2.0 Hz, H-2), 7.69 (1H, d, J = 15.5 Hz, H-β), 8.10 (1H, d, J = 15.5 Hz, H-α).

3.2.12. 2′,3,4-Trihydroxy-4′,6′-dimethoxychalcone (12). Yield: 67%; yellow powders; mp 174–175 °C; EI-HR-MS *m/z*: 316.0926 (calcd for $C_{17}H_{16}O_6$, 316.0947); ^{1}H NMR (acetone-d_6) δ (ppm): 3.86 and 4.00 (each 3H, s, $2\times OCH_3$), 6.08 (1H, d, J = 2.5 Hz, H-5′), 6.11 (1H, d, J = 2.5 Hz, H-3′), 6.89 (1H, d, J = 8.1 Hz, H-5), 7.11 (1H , dd, J = 8.1, 2.0 Hz, H-6), 7.24 (1H, d, J = 2.0 Hz, H-2), 7.69 (1H, d, J = 15.5 Hz, H-β), 7.83 (1H, d, J = 15.5 Hz, H-α).

3.2.13. 2,2′,4,4′-Tetrahydroxychalcone (13). Yield: 53%; yellow powders; mp 178–179 °C; EI-HR-MS *m/z*: 272.0670 (calcd for $C_{15}H_{12}O_5$, 272.0685); ^{1}H NMR (methanol-d_4) δ (ppm): 6.27 (1H, d, J = 2.4 Hz, H-3 or 3′), 6.34 (1H, d, J = 2.4 Hz, H-3 or 3′), 6.36 (1H, dd, J = 8.6, 2.4 Hz, H-5 or 5′), 6.39 (1H, dd, J = 8.8, 2.5 Hz, H-5 or 5′), 7.50 (1H, d, J = 8.6 Hz, H-6 or 6′), 7.69 (1H, d, J = 15.2 Hz, H-β), 7.88 (1H, d, J = 8.8 Hz, H-6 or 6′), 8.08 (1H, d, J = 15.2 Hz, H-α).

3.2.14. 2,2′,4,4′-Tetrahydroxy-6′-methoxychalcone (14). Yield: 12%; yellow powders; FD-HR-MS *m/z*: 302.0806 (calcd for $C_{16}H_{14}O_6$, 302.0790); ^{1}H NMR (acetone-d_6) δ (ppm): 3.93 (3H, s, OCH_3), 5.97 (1H, d, J = 2.2 Hz, H-5′), 6.04 (1H, d, J = 2.2 Hz, H-3′), 6.44 (1H, dd, J = 8.1, 1.9 Hz, H-5), 6.49 (1H, d, J = 1.9 Hz, H-3), 6.53 (1H, d, J = 8.1 Hz, H-6), 7.98 (1H, d, J = 15.7 Hz, H-β), 8.14 (1H, d, J = 15.7 Hz, H-α).

3.3. The preparation of 2,2′,4,4′,6′-pentahydroxychalcone 15

To a stirred solution of**15a** (232 mg, 0.5 mmol) in methanol (10 mL) was added dropwise 3 M HCl (4 mL). The mixture was refluxed for 10 min, diluted with water, and extracted with ethyl acetate. The organic layer was successively washed with water and brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (chloroform/methanol = 1:50) to give 112 mg of 2′,4′,6,8-hydroxyflavanone **15b** in 78% yield: FD-MS *m/z* (%): 288 (100, $[M]^+$); ^{1}H NMR (acetone-d_6) δ (ppm): 2.66 (1H, dd, J = 16, 2.9 Hz, H-3_{eq}), 3.16 (1H, dd, J = 13.6, 16 Hz, H-3_{ax}), 5.69 (1H, dd, J = 13.6, 2.9 Hz, H-2), 5.95 (2H, s, H-6 and 8), 6.42 (1 H, dd, J = 8.4, 1.8 Hz, H-5′), 6.48 (1H, d, J = 1.8 Hz, H-3′), 7.48 (1H, d, J = 8.4 Hz, H-6′).

To a stirred solution of 4 M KOH (10 mL) cooled to 0 °C in an ice bath was added dropwise a solution of **15a** (72 mg, 0.25 mmol) in ethanol (10 mL) under argon, and then the reaction mixture was kept at room temperature for 30 min. The mixture was poured into ice-water (10 mL), adjusted to pH 3–4 with 1 M HCl, and then extracted with ethyl acetate. The organic layer was successively washed with water and saturated brine, dried over anhydrous Na_2SO_4. After evaporation, the residue

was purified by silica gel column chromatography (chloroform/methanol/formic acid = 4:1:0.1, $R_f = 0.49$) to give 25 mg of **15**. Yield: 35%; yellow powders; mp 140–141 °C; EI-HR-MS *m/z*: 288.0650 (calcd for $C_{15}H_{12}O_6$, 288.0634); ^{1}H NMR (methanol-d_4) δ (ppm): 5.82 (2 H, s, H-3′ and 5′), 6.31 (1H, d, $J = 2.2$ Hz, H-3), 6.49 (1H, dd, $J = 9.1$, 2.2 Hz, H-5), 7.40 (1H, d, $J = 9.1$ Hz, H-6), 8.09 (2H, m, H-α and β).

3.4. Tyrosinase inhibition assay

Tyrosinase inhibition assays were performed according to a modified method described by Kubo.[12] Mushroom tyrosinase (EC 1. 14. 18. 1, Sigma Product T3824 with an activity of 3320 U/mg) was used for the bioassay in this study. The tested compounds were first dissolved in DMSO and diluted 25 times with water in the individual experiment before use. The activity was expressed as the sample concentration that gave a 50% inhibition in the enzyme activity (IC_{50}).

For the measurement of diphenolase inhibitory activity, 0.1 mL of a sample solution and 1.5 mL of 1.5 M L-DOPA solution (0.1 M phosphate buffer, pH 6.8) were mixed with 0.4 mL H_2O, and preincubated at 25 °C for 5 min. Then, 0.5 mL of tyrosinase solution (125 U) was added and the reaction was monitored using a Hitachi UV-3210 spectrophotometer, at 475 nm from 0.5 to 4 min, on the base of formation of dopachrome.

For the measurement of monophenolase inhibitory activity, 0.1 mL of a sample solution and 1 mL of 2 M L-tyrosine solution were mixed with 0.9 mL of 0.1 M phosphate buffer (pH 6.8), and pre-incubated at 25 °C for 5 min. Then, 0.5 mL of tyrosinase solution (250 U) was added and the reaction was monitored at 475 nm for 20 min for detection of dopachrome formed.

Acknowledgments

We are grateful to Mr. Kenji Watanabe and Dr. Eri Fukushi, GC-MS & NMR Laboratory, Faculty of Agriculture, Hokkaido University, for their skillful mass spectral measurements.

References and notes

1. Sánchez-Ferrer, Á.; Rodríguez-López, J. N.; García-Cánovas, F.; García-Garmona, F. *Biochim. Biophys. Acta* **1995**, *1247*, 1.
2. Shin, N. H.; Ryu, S. Y.; Choi, E. J.; Kang, S. H.; Chang, I. M.; Min, K. R.; Kim, Y. *Biochem. Biophys. Res. Commun.* **1998**, *243*, 801.
3. Iozumi, K.; Hoganson, G. E.; Pennella, R.; Everett, M. A.; Fuller, B. B. *J. Invest. Dermatol.* **1993**, *100*, 806.
4. Briganti, S.; Camera, E.; Picardo, M. *Pigment Cell Res.* **2003**, *16*, 101.
5. (a) Friedman, M. *J. Agric. Food Chem.* **1996**, *44*, 631; (b) Sugumaran, M. *FEBS Lett.* **1991**, *293*, 4–10.
6. Miyazawa, M.; Oshima, T.; Koshio, K.; Itsuzaki, Y.; Anzai, J. *J. Agric. Food Chem.* **2003**, *51*, 6953–6956.
7. Kubo, I.; Kinst-Hori, I. *J. Agric. Food Chem.* **1999**, *47*, 4121–4125.
8. Nerya, O.; Vaya, J.; Musa, R.; Izrael, S.; Ben-Arie, R.; Tamir, S. *J. Agric. Food Chem.* **2003**, *51*, 1201–1207.
9. Nerya, O.; Musa, R.; Khatid, S.; Tamir, S.; Vaya, J. *Phytochemistry* **2004**, *65*, 1389–1395.
10. Khatid, S.; Nerya, O.; Musa, R.; Shmuel, M.; Tamir, S.; Vaya, J. *Bioorg. Med. Chem.* **2005**, *13*, 433–441.
11. Nishida, J.; Kawabata, J. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 193–202.
12. Kubo, I.; Kinst-Hori, I.; Chaudhuri, S. K.; Kubo, Y.; Sánchez, Y.; Ogura, T. *Bioorg. Med. Chem.* **2000**, *8*, 1749.