CHAPTER III

EXPERIMENTAL

1. General Experimental Procedures

1.1 Thin-Layer Chromatography (TLC)

Technique: One dimension, ascending.

Adsorbent: Silica gel GF$_{254}$ (E. Merck) precoated plate.

Layer thicknes: 250 μm

Distance: 8 cm

Detection: 1. Ultraviolet light (254 and 365 nm).

                  2. 20% sulfuric acid in methanol and heating.

                  3. Iodine vapor.

1.2 Column Chromatography

1.2.1 Vacuum Liquid Column Chromatography

Adsorbent: Silica gel (No. 1.07734), particle size 0.063-0.200 nm (70-230 mesh ASTM) (E. Merck).

Packing method: Dry packing with sintered glass filter column.

Sample loading: The sample was dissolved in a small volume of organic solvent, mixed and triturated with a small quantity of adsorbent, dried and then applied gently on top of the column.

Detection: Fractions were monitored by TLC technique.

1.2.2 Flash Column Chromatography

Adsorbent: Silica gel (No. 1.09385), particle size 0.040-0.063 mm (230-400 mesh ASTM) (E. Merck)

Packing method: Wet packing.

Sample loading: The sample was dissolved in a small volume of eluent and applied gently on top of the column.

Detection: Fractions were monitored by TLC technique.

1.2.3 Gel Filtration Chromatography

Gel filter: Sephadex LH-20 (Pharmacia Biotech AB)

Packing method: Sephadex gel was suspended in the eluant and kept overnight to swell prior to use. The slurry of adsorbent was poured in to the column and allowed to set tightly.

Sample loading: The sample was dissolved in a small volume of eluent and applied gently on top of the column.

Detection: Fractions were monitored by TLC technique.

2. Spectroscopy and Physical Constants

2.1 Infrared (IR) Absorption Spectroscopy

IR spectra (KBr disc or film) were recorded on a Perkin Elmer FT-IR 1760X Spectrometer (Scientific and Technological research Equipment Center, Chulalongkorn University).

2.2 Mass Spectroscopy

High Resolution mass spectra were obtained in the Time-of-Flight technique (TOF) manner with a Micromass mass spectrometer and Bruker micrOTOF mass spectrometer (BIOTEC).

2.3 Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H- and ^{13}C-NMR) Spectroscopy

^{1}H NMR (300 MHz), ^{13}C NMR (75 MHz), DEPT 90 and 135, ^{1}H,^{1}H-COSY, HMQC and HMBC spectra were obtained on a Bruker Avance DPX-300 FT-NMR

spectrometer, (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

Deuterated solvents for NMR spectra were $CDCl_3$ and DMSO-d_6. Chemical shifts were reported in ppm scale using the solvent signal as reference signal and TMS as the internal standard. Proton-detected heteronuclear correlations were measured using HMQC (optimized for $^1J_{HC} = 145$ Hz) and HMBC (optimized for $^nJ_{HC} = 4$ Hz) pulse sequences.

2.4 Melting Points

Melting points were obtained on a Yamaco Micro melting point apparatus (Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health).

2.5 Elemental Analysis

Elemental Analysis was performed on Perkin Elmer Series II CHNS/O Analyser 2400 (Scientific and Technological research Equipment Center, Chulalongkorn University).

3. Chemical and Biological Reagents

Acetic anhydride	Fluka
Benzoyl chloride	TCI
m-, and p-Bromo benzoyl chloride	TCI
o- and m-Methoxy benzoyl chloride	TCI
m-, and p-Nitro benzoyl chloride	TCI
Decanoic anhydride	Aldrich
3,4-Dihydroxy-L-phenylalanine (L-DOPA)	Sigma
Disodium hydrogen phosphate	Fisher Scientific
Hexanoic anhydride	Aldrich
Iodine	Fluka
Kojic acid	Sigma
10% Palladium on activated charcoal	Merck

Mushroom tyrosinase (EC 1.14.18.1)	Sigma
Palmitic anhydride	Fluka
Porcine liver esterase (EC 3.1.1.1)	Sigma
PT-40 (licorice extract)	Maruzen
Quercetin	Sigma
Sodium chloride	Riedel-de Haen
Sodium dihydrogen phosphate	BDH
Sodium sulfate, anhydrous	Merck
Stearic anhydride	Fluka
Sulfuric acid concentrated	Baker

4. Solvents

All solvents used were either analytical or laboratory grade and were redistilled prior to use. For chemical reactions, pyridine was dried with molecular sieve type 4A.

5. Isolation of Glabridin (4) from Licorice Extract

Licorice extract, PT-40 (13.014 g) was fractionated with vacuum liquid column chromatography using a sinter glass filter column of silica gel (No. 1.07734, 270 g). Elution was performed in a polarity gradient manner with mixtures of CH_2Cl_2/EtOAc (10:0 to 0:10) and methanol. The eluates were collected (200 ml each) and examined by TLC technique (silica gel, $CHCl_3$/EtOAc = 8:2). Twenty four fractions were collected and those of similar chromatographic pattern were combined. Based on bioassay guided fractionation, three active fractions with tyrosinase inhibitory activities (F02 = 6.412 g, F03 = 1.272 g and F04 = 1.720 g) were further isolated.

Each fraction of F02 and F03 was dissolved in $CHCl_3$ to give a white precipitate and solution. The white precipitate was collected and crystallized in hexane to yield white crystals of compound 4. The chloroform soluble of F02 was separated by a silica gel flash column using gradient elution of hexane/EtOAc (9:1 to 6:4). The fractions were combined according to their TLC patterns and afforded the pale yellow precipitates, which was purified by crystallization in hexane to give white crystals of compound 4. In the same manner, the chloroform soluble of F03 and

fraction F04 was separately isolated using flash column chromatography, eluted with the mixture of hexane/EtOAc (6:4) and purified by crystallization in hexane to give white crystals of compound **4** as shown in scheme 1. The compound **4** obtained was later identified as glabridin (3.7624 g, 28.91 % of the dry weight of licorice extract).

Glabridin (4) (3.7624 g, 28.91%)

^{1}H-NMR (300 MHz, DMSO-d_6, Figure 21) δ: 1.33 (3H x 2, s, H-5″ and H-6″), 2.69 (dd, J = 15.6, 10.2 Hz, H-4ax), 2.88 (dd, J = 15.6, 4.2 Hz, H-4eq), 3.29 (m, H-3), 3.92 (dd, J = 10.2, 10.2 Hz, H-2ax), 4.23 (br d, J = 10.2 Hz, H-2eq), 5.63 (d, J = 9.9 Hz, H-3″), 6.18 (dd, J = 8.2, 1.3 Hz, H-5′), 6.27(d, J = 8.2 Hz, H-6), 6.32 (d, J = 1.3 Hz, H-3′), 6.53 (d, J = 9.9 Hz, H-4″), 6.81 (d, J = 8.2 Hz, H-6′), 6.84 (d, J = 8.2 Hz, H-5), 9.08 (br s, OH), 9.35 (br s, OH).

^{13}C-NMR (75 MHz, DMSO-d_6, Figure 22) δ: 27.33 (C5″/C6″), 27.45 (C5″/C6″), 30.09 (C-4), 31.01 (C-3), 69.85 (C-2), 75.33 (C-2″), 102.64 (C-3′), 106.42 (C-5′), 108.21 (C-6), 109.15 (C-8), 114.84 (C-10), 116.53 (C-4″), 117.59 (C-1′), 127.68 (C-6′), 129.31 (C-3″), 129.44 (C-5), 149.36 (C-9), 151.34 (C-7), 155.96 (C-2′), 156.98 (C-4′).

IR ν (cm^{-1}): 3528, 3351, 2965, 1607, 1480, 1171, 1114 (Figure 27)
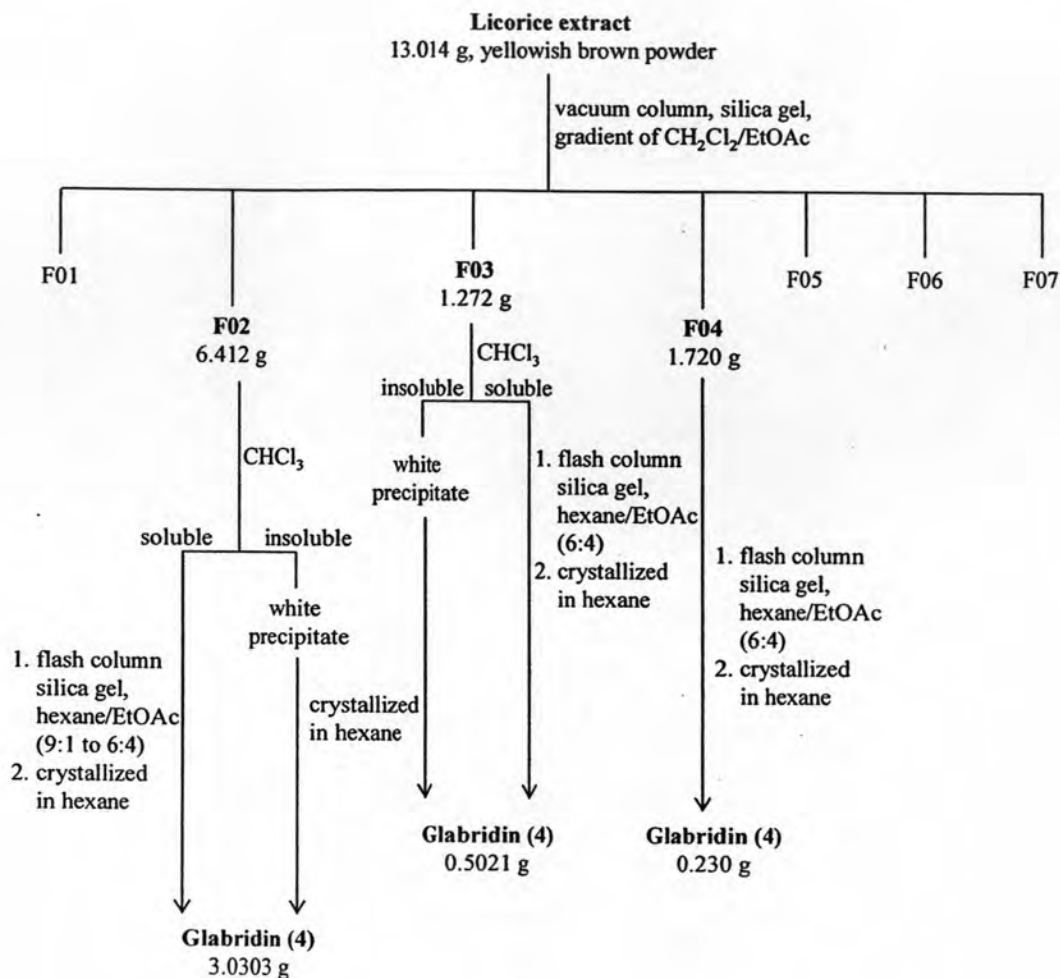
TOF-MS [M + H]$^+$ m/z: 325.1439 (found)

325.1440 (calcd for $C_{20}H_{20}O_4$ + H) (Figure 28)

Elemental analysis for $C_{24}H_{24}O_4$ calcd. C: 74.06   H: 6.21

found C: 73.92   H: 6.30

melting point: 238-240 °C (uncorrect).

Licorice extract
13.014 g, yellowish brown powder

vacuum column, silica gel,
gradient of CH_2Cl_2/EtOAc

F01

F03
1.272 g

F05   F06   F07

F02
6.412 g

F04
1.720 g

CHCl₃
insoluble | soluble

CHCl₃

soluble | insoluble

white
precipitate

1. flash column
silica gel,
hexane/EtOAc
(6:4)
2. crystallized
in hexane

1. flash column
silica gel,
hexane/EtOAc
(6:4)
2. crystallized
in hexane

white
precipitate

1. flash column
silica gel,
hexane/EtOAc
(9:1 to 6:4)
2. crystallized
in hexane

crystallized
in hexane

Glabridin (4)
0.5021 g

Glabridin (4)
0.230 g

Glabridin (4)
3.0303 g

Scheme 1. Isolation of glabridin (**4**) from licorice extract.

6. Structure Modification of Glabridin

6.1 Preparation of Glabridin Acyl Ester Derivatives

General Procedure for Acylation

To a solution of glabridin (0.068 g, 0.21 mmol) in anhydrous pyridine (2 ml), the acid anhydride (0.84 mmol) was added and stirred for 1-4 hr at room temperature, under a nitrogen atmosphere. The reaction mixture was poured into the ice water (5 ml) and extracted with appropriated solvents (CHCl₃ or hexane) (10 ml x 3). The combined organic phase was washed with brine (10 ml), dried and evaporated *in vacuo*. The residue was purified by silica gel column chromatography using appropriated eluant (CHCl₃ or hexane) to give the purified products of **16-20**.

Glabridin diacetate (16) (83.4 mg, 97.35 %)

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 29) δ: 1.40-1.42 (3H x 2, s, H-5″ and H-6″), 2.27 (3H, s, COCH$_3$), 2.30 (3H, s, COCH$_3$), 2.86 (2H, m, H-4), 3.29 (m, H-3), 3.96 (t, J = 10.4 Hz, H-2ax), 4.29 (d, J = 10.4 Hz, H-2eq), 5.56 (d, J = 9.9 Hz, H-3″), 6.36 (d, J = 8.2 Hz, H-6), 6.62 (d, J = 9.9 Hz, H-4″), 6.80 (d, J = 8.2 Hz, H-5), 6.90 (s, H-3′), 6.99 (d, J = 8.4 Hz, H-5′), 7.19 (d, J = 8.4 Hz, H-6′).

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 30) δ: 21.01 (CO$\underline{C}$H$_3$), 21.06 (CO$\underline{C}$H$_3$), 27.56 (C5″/C6″), 27.81 (C5″/C6″), 31.28 (C4), 31.91 (C3), 69.72 (C2), 75.65 (C2″), 108.96 (C6), 109.94 (C8), 113.62 (C10), 116.34 (C3′), 116.71 (C4″), 119.65 (C5′), 127.87 (C6′), 129.11 (C3″), 129.14 (C5), 130.86 (C1′), 148.93 (C4′), 149.49 (C2″),149.62 (C9), 152.06 (C7), 169.02 (CO), 169.16 (CO).

IR ν (cm^{-1}): 2987, 2944, 1765, 1477, 1195 (Figure 34).

Elemental analysis for C$_{24}$H$_{24}$O$_6$ calcd. C: 70.57   H: 5.92

                                                                 found C: 70.60   H: 5.86

melting point: 177-179 °C (uncorrect).

Glabridin dihexanoate (17) (104.4 mg, 95.60 %)

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 35) δ: 0.88 (3H x 2, m, H-5‴), 1.34 (2H x 4, m, H-3‴ and H-4‴), 1.39-1.41 (3H x 2, s, H-5″ and H-6″), 1.72 (2H x 2, m, H-2‴), 2.53 (2H x 2, m, H-1‴), 2.86 (2H, m, H-4), 3.28 (m, H-3), 3.95 (t, J = 10.3 Hz, H-2ax), 4.28 (d, J = 10.3 Hz, H-2eq), 5.55 (d, J = 9.9 Hz, H-3″), 6.36 (d, J = 8.2 Hz, H-6), 6.62 (d, J = 9.9 Hz, H-4″), 6.79 (d, J = 8.2 Hz, H-5), 6.88 (d, 2.1, H-3′), 6.97 (dd, J = 8.5, 2.1 Hz, H-5′), 7.18 (d, J = 8.5 Hz, H-6′).

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 36) δ: 13.80 (C5‴), 13.87 (C5‴), 22.27 (C3‴/C4‴), 24.53 (C2‴), 24.58 (C2‴), 27.58 (C5″/C6″), 27.81 (C5″/C6″), 31.22 (C4 and C3‴/C4‴), 31.99 (C3), 34.24 (C1‴), 34.31 (C1‴), 69.77 (C2), 75.63 (C2″), 108.93 (C6), 109.94 (C8), 113.66 (C10), 116.35 (C3′), 116.78 (C4″), 119.52 (C5′), 127.73 (C6′), 129.02 (C3″), 129.13 (C5), 130.67 (C1′), 149.07 (C4′), 149.57 (C2′), 149.75 (C9), 152.06 (C7), 171.88 (CO), 172.03 (CO).

IR ν (cm^{-1}): 2957, 2932, 1762, 1143, 1095 (Figure 41).

Elemental analysis for C$_{32}$H$_{40}$O$_6$ calcd. C: 73.82   H: 7.74

                                                                 found C: 73.85   H: 7.69

Glabridin didecanoate (18) (115.6 mg, 86.39 %)

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 42) δ: 0.86 (3H, m, CH$_3$), 0.87 (3H, m, CH$_3$), 1.23-1.26 (2H x 12, m, CH$_2$), 1.39-1.41 (3H x 2, s, H-5″ and H-6″), 1.71 (2H x 2, m, H-2‴), 2.53 (2H x 2, m, H-1‴), 2.87 (2H, m, H-4), 3.29 (m, H-3), 3.95 (t, J = 10.3 Hz, H-2ax), 4.28 (d, J = 10.3 Hz, H-2eq), 5.54 (d, J = 9.9 Hz, H-3″), 6.35 (d, J = 8.2 Hz, H-6), 6.61 (d, J = 9.9 Hz, H-4″), 6.79 (d, J = 8.2 Hz, H-5), 6.87 (d, 2.2, H-3′), 6.97 (dd, J = 8.5, 2.2 Hz, H-5′), 7.18 (d, J = 8.5 Hz, H-6′).

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 43) δ: 14.08 (CH$_3$ x 2), 22.64 (CH$_2$), 24.86 (C2‴), 24.89 (C2‴), 27.63 (C5″/C6″), 27.79 (C5″/C6″), 29.10 (CH$_2$), 29.22 (CH$_2$), 29.34 (CH$_2$), 29.39 (CH$_2$), 31.21 (C4), 31.82 (CH$_2$), 31.84, (CH$_2$), 31.98 (C3), 34.28 (C1‴), 34.35 (C1‴), 69.76 (C2), 75.64 (C2″), 108.94 (C6), 109.94 (C8), 113.64 (C10), 116.36 (C3′), 116.78 (C4″), 119.54 (C5′), 127.75 (C6′), 129.03 (C3″), 129.13 (C5), 130.72 (C1′), 149.06 (C4′), 149.54 (C2′), 149.75 (C9), 152.06 (C7), 171.91 (CO), 172.06 (CO).

IR ν (cm^{-1}): 2926, 2855, 1764, 1144, 1114, 1093 (Figure 47).

Elemental analysis for C$_{40}$H$_{56}$O$_6$　　calcd. C: 75.91　　H: 8.92

　　　　　　　　　　　　　　　　　　found C: 75.06　　H: 8.78

Glabridin dipalmitate (19) (142.1 mg, 84.58 %)

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 48) δ: 0.85 (3H x 2, m, CH$_3$), 1.24 (2H x 20, m, CH$_2$), 1.40-1.41 (3H x 2, s, H-5″ and H-6″), 1.71 (2H x 2, m, H-2‴), 2.53 (2H x 2, m, H-1‴), 2.87 (2H, m, H-4), 3.29 (m, H-3), 3.95 (t, J = 10.4 Hz, H-2ax), 4.29 (d, J = 10.4 Hz, H-2eq), 5.54 (d, J = 9.9 Hz, H-3″), 6.36 (d, J = 8.2 Hz, H-6), 6.62 (d, J = 9.9 Hz, H-4″), 6.79 (d, J = 8.2 Hz, H-5), 6.88 (d, 2.0, H-3′), 6.97 (dd, J = 8.5, 2.0 Hz, H-5′), 7.18 (d, J = 8.5 Hz, H-6′).

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 49) δ: 14.10 (2C, CH$_3$), 22.68 (CH$_2$), 24.87 (C2‴), 24.90 (C2‴), 27.64 (C5″/C6″), 27.74 (C5″/C6″), 29.09 (CH$_2$), 29.12 (CH$_2$), 29.24 (CH$_2$), 29.35 (CH$_2$), 29.41 (CH$_2$), 29.44 (CH$_2$), 29.59 (CH$_2$), 29.65 (CH$_2$), 29.69 (CH$_2$), 31.21 (C4), 31.92 (C3), 31.98 (CH$_2$), 34.28 (C1‴), 34.36 (C1‴), 69.77 (C2), 75.64 (C2″), 108.94 (C6), 109.94 (C8), 113.64 (C10), 116.37 (C3′), 116.79 (C4″), 119.54 (C5′), 127.75 (C6′), 129.03 (C3″), 129.14 (C5), 130.72 (C1′), 149.07 (C4′), 149.55 (C2′), 149.75 (C9), 152.06 (C7), 171.92 (CO), 172.06 (CO).

IR ν (cm^{-1}): 2920, 2851, 1761, 1151 (Figure 54).

Elemental analysis for C$_{52}$H$_{80}$O$_6$ calcd. C: 77.95   H: 10.06

                                                found C: 78.03   H: 10.17

melting point: 37-38 °C (uncorrect).

Glabridin distearate (20) (127.3 mg, 70.82 %)

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 55) δ: 0.86 (3H x 2, m, CH$_3$), 1.24 (2H x 24, m, CH$_2$), 1.39-1.41 (3H x 2, s, H-5″ and H-6″), 1.70 (2H x 2, m, H-2‴), 2.53 (2H x 2, m, H-1‴), 2.87 (2H, m, H-4), 3.28 (m, H-3), 3.95 (t, J = 10.4 Hz, H-2ax), 4.28 (d, J = 10.4 Hz, H-2eq), 5.54 (d, J = 9.9 Hz, H-3‴″), 6.35 (d, J = 8.2 Hz, H-6), 6.61 (d, J = 9.9 Hz, H-4‴″), 6.79 (d, J = 8.2 Hz, H-5), 6.87 (d, J = 2.0 Hz, H-3″), 6.97 (dd, J = 8.5, 2.0 Hz, H-5″), 7.18 (d, J = 8.5 Hz, H-6″).

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 56) δ: 14.10 (2C, CH$_3$), 22.68 (CH$_2$), 24.87 (C2‴), 24.90 (C2‴), 27.63 (C5″/C6″), 27.79 (C5″/C6″), 29.09 (CH$_2$), 29.12 (CH$_2$), 29.24 (CH$_2$), 29.35 (CH$_2$), 29.41(CH$_2$), 29.45 (CH$_2$), 29.59 (CH$_2$), 29.65 (CH$_2$), 29.69 (CH$_2$), 31.21 (C4), 31.92 (C3), 31.98 (CH$_2$), 34.29 (C1‴), 34.36 (C1‴), 69.77 (C2), 75.63 (C2″), 108.94 (C6), 109.94 (C8), 113.64 (C10), 116.37 (C3′), 116.79 (C4″), 119.54 (C5′), 127.75 (C6′), 129.02 (C3″), 129.14 (C5), 130.71 (C1′), 149.07 (C4′), 149.54 (C2′), 149.75 (C9), 152.06 (C7), 171.91 (CO), 172.06 (CO).

IR ν (cm^{-1}): 2917, 2850, 1754, 1471, 1151 (Figure61).

Elemental analysis for C$_{56}$H$_{88}$O$_6$ calcd. C: 78.46   H: 10.35

                                                found C: 78.45   H: 10.35

melting point: 44-45 °C (uncorrect).

6.2 Preparation of 3″,4″-Dihydroglabridin (21)

A mixture of 0.1008 g (0.31 mmol) of glabridin and 0.0497 mg (0.05 mmol) of 10% Pd/C in 10 ml of ethyl acetate was vigorous shaken under H$_2$ gas (46 lbs/inch^2) using Parr apparatus at room temperature for 1 hr. The catalyst was filtered off through celite, the filtrate was evaporated under reduced pressure and purified by silica gel column chromatography, eluted with a mixture of hexane/EtOAc (7:3) and re-fractionated on a silica gel column (CHCl$_3$/EtOAc 10:0 to 6:4) to give pink-brown flake-like compound **21**.

3″,4″-dihydroglabridin (21) (93.9 mg, 93%)

^{1}H-NMR (300 MHz, DMSO-d$_6$, Figure 62) δ: 1.22 (3H x 2, s, H-5″ and H-6″), 1.69 (2H, m, H-3″), 2.50 (2H, m, H-4″), 2.66 (br d, J = 15.0 Hz, H-4eq), 2.89 (t, J= 15.0 Hz, H-4ax), 3.27 (m, H-3), 3.89 (t, J = 10.1 Hz, H-2ax), 4.22 (br d, J = 10.1 Hz, H-2eq), 6.17 (d, J = 8.2 Hz, H-5′), 6.22 (d, J = 8.2 Hz, H-6), 6.31 (s, H-3′), 6.76 (d, J = 8.2 Hz, H-5), 6.84 (d, 8.2, H-6′), 9.09 (br s, OH), 9.35 (br s, OH).

^{13}C-NMR (75 MHz, DMSO-d$_6$, Figure 63) δ: 16.89 (C4″), 26.32 (C5″/C6″), 26.51 (C5″/C6″), 30.21 (C4), 31.07 (C3), 31.79 (C3″), 69.85 (C2), 73.28 (C2″), 102.60 (C3′), 106.37 (C5′), 108.54 (C6), 108.68 (C8), 112.95 (C10), 117.75 (C1′), 127.41 (C6′), 127.60 (C5), 151.77 (C9), 152.38 (C7), 155.91 (C2′), 156.89 (C4′).

IR v (cm^{-1}): 3534, 3321, 2969, 1618, 1598, 1487, 1163, 1092. (Figure 67)

TOF-MS [M + H]$^+$ m/z: 327.1589 (found)

327.1596 (calcd for C$_{20}$H$_{22}$O$_4$ + H) (Figure 68)

melting point: 223-224 °C (uncorrect).

6.3 Preparation of Glabridin Benzoate Analogues.
General Procedure for Benzoylation.

Glabridin (0.0324 g, 0.1 mmol) was dissolved in 1 ml of anhydrous pyridine, benzoyl chloride derivatives (0.4-1.0 mmol) was added dropwise over a period of 10 min at 0 °C. The reaction mixture was stirred at room temperature for 30-60 min. The resulting solution was diluted with 5 ml of water and extracted with CH$_2$Cl$_2$ (10 ml x 3), washed with brine, dried and evaporated *in vacuo*. The residue was purified by column chromatography using silica gel (gradient elution of hexane/EtOAc or CHCl$_3$/EtOAc) and followed by Sephadex LH-20 column (CHCl$_3$/MeOH 1:1) to give as **22-28**.

Glabridin-2′,4′-O-dibenzoate (22) (47.6 mg, 89.47 %).

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 69) δ: 1.36-1.39 (3H x 2, s, H-5″ and H-6″), 2.95 (2H, m, H-4), 3.43 (m, H-3), 4.05 (t, J = 10.2 Hz, H-2ax), 4.37 (d, J = 10.2 Hz, H-2eq), 5.52 (d, J = 9.9 Hz, H-3″), 6.34 (d, J = 8.2, H-5), 6.59 (d, J = 9.9 Hz, H-4″), 7.16 (br s, H-3′), 7.18 (dd, J = 8.2, 2.2, Hz, H-5′), 7.31 (br d, J = 8.2 Hz, H-6′),

7.49 (H x 4, br t, J = 7.6 Hz, H-3''' and H-5'''), 7.62 (H x 2, br t, J = 7.6 Hz, H-4'''), 8.17 (H x 4, d, J = 7.6 Hz, H-2''' and H-6''').

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 70) δ: 27.59 (C5''/C6''), 27.83 (C5''/C6''), 31.28 (C4), 32.00 (C3), 69.77 (C2), 75.64 (C2''), 108.94 (C6), 109.92 (C8), 113.58 (C10), 116.67(C3'), 116.74 (C4''), 119.93 (C5'), 128.10 (C6'), 128.62-128.76 (4C, C3''' and C5'''), 129.01 (C3''), 129.14 (C5), 129.23 (2C, C1'''), 130.22-130.30 (4C, C2''' and C6'''), 131.38 (C1'), 133.75-133.96 (2C, C4'''), 149.31 (C4'), 149.55 (C2'),150.02 (C9), 152.03 (C7), 164.83 (CO), 164.97 (CO).

IR ν (cm^{-1}): 2974, 1739, 1243, 1092 (Figure 73).

TOF-MS [M + Na]$^+$ m/z: 555.1798

555.1778 (calcd for C$_{34}$H$_{28}$O$_6$Na (Figure 74).

Elemental analysis for C$_{34}$H$_{28}$O$_6$ calcd. C: 76.69   H: 5.26

found C: 76.62   H: 5.30

melting point: 70-73 °C (uncorrect).

Glabridin-2',4'-O-di-3'''-bromobenzoate (23) (70.70 mg, 100 %).

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 75) δ: 1.36-1.40 (3H x 2, s, H5'' and H-6''), 2.95 (2H, m, H-4), 3.38 (m, H-3), 4.40 (t, J = 10.3 Hz, H-2ax), 4.36 (dd, J = 10.2, 2.3 Hz, H-2eq), 5.53 (d, J = 9.9 Hz, H-3''), 6.35 (d, J = 8.3 Hz, H-6), 6.59 (d, J = 9.9 Hz, H-4''), 6.80 (d, J = 8.3 Hz, H-5), 7.16 (2H, m, H-3' and H-5'), 7.32 (d, J = 8.5 Hz, H-6'), 7.38 (H x 2, t, J =8.0 Hz, H-5'''), 7.75 (H x 2, d, J =8.0 Hz, H-4'''), 8.10 (H x 2, d, J = 8.0 Hz, H-6'''), 8.30 (H x 2, t, J = 1.5 Hz, H-2'''),

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 76) δ: 27.58 (C5''/C6''), 27.83 (C5''/C6''), 31.28 (C4), 31.96 (C3), 69.79 (C2), 70.21 (C2''), 109.02 (C6), 109.94 (C8), 113.42 (C10), 116.41 (C3'), 116.69 (C4''), 120.02 (C5'), 122.71-122.85 (C3'''), 128.27 (C6'), 128.77-128.82 (C6'''), 129.08 (C3''), 129.34 (C5), 130.27-130.33 (2C, C5'''), 130.65-131.09 (C1'''), 131.62 (C1'), 133.20-133.25 (C2'''), 136.76-136.98 (C4'''), 149.05 (C4'), 149.48 (C2'),149.74 (C9), 152.06 (C7), 163.47 (CO), 163.70 (CO).

IR ν (cm^{-1}): 2971, 1742, 1237, 1091 (Figure 79).

melting point: 76-78 °C (uncorrect).

Glabridin-2',4'-O-di-4'''-bromobenzoate (24) (41.3 mg, 61.03 %).

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 80) δ: 1.37-1.40 (3H x 2, s, H-5'' and H-6''), 2.94 (2H, m, H-4), 3.37 (m, H-3), 4.03 (t, J = 10.2 Hz, H-2ax), 4.35 (dd, J = 10.2, 1.9 Hz, H-2eq), 5.53 (d, J = 9.9 Hz, H-3''), 6.34 (d, J = 8.3 Hz, H-6), 6.58 (d, J = 9.9 Hz, H-4''), 6.79 (d, J = 8.3 Hz, H-5), 7.17 (2H, m, H-3' and H-5'), 7.31 (d, J = 8.2 Hz, H-6'), 7.64 (H x 4, d, J = 8.4 Hz, H-3''' and H-5'''), 8.02 (H x 2, d, J = 8.4 Hz, H2''' and H-6''').

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 81) δ: 27.58 (C5''/C6''), 27.83 (C5''/C6''), 31.25 (C4), 32.02 (C3), 69.70 (C2), 75.67 (C2''), 109.02 (C6), 109.93 (C8), 113.42 (C10), 116.52 (C3'), 116.67 (C4''), 119.97 (C5'), 127.63 (C6'), 128.06 (C3''), 128.21 (C5), 129.11 (C1'), 129.39 (2C, C4'''), 131.48 (2C, C1'''), 131.66-131.72 (4C, C2''' and C6'''), 132.04-132.19 (4C, C3''' and C5'''), 149.07 (C4'), 149.48 (C2'), 149.78 (C9), 152.07 (C7), 164.12 (CO), 164.29 (CO).

IR ν (cm^{-1}): 2975, 2951, 1733, 1588, 1237, 1092 (Figure 84).

melting point: 129-131 °C (uncorrect).

Glabridin-2',4'-O-di-3'''-nitrobenzoate (25) (37.10 mg, 60.37 %).

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 85) δ: 1.35-1.40 (3H x 2, s, H-5'' and H-6''), 2.96 (2H, m, H-4), 3.38 (m, H-3), 4.06 (t, J = 10.2 Hz, H-2ax), 4.38 (m, H-2eq), 5.53 (d, J = 9.9 Hz, H-3''), 6.35 (d, J = 8.2 Hz, H-6), 6.58 (d, J = 9.9 Hz, H-4''), 6.80 (d, J = 8.2 Hz, H-5), 7.23 (2H, m, H-3'' and H-5''), 7.37 (d, J = 8.5 Hz, H-6''), 7.73 (H x 2, t, J = 8.0 Hz, H-5'''), 8.50 (H x 4, br d, J = 8 Hz, H-4''' and H-6'''), 9.01 (H x 2, br s, H-2''').

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 86) δ: 27.59 (C5''/C6''), 27.82 (C5''/C6''), 31.34 (C4), 32.07 (C3), 69.64 (C2), 76.00 (C2''), 109.12 (C6), 109.95 (C8), 113.25 (C10), 116.26 (C3'), 116.59 (C4''), 120.19 (C5'), 125.15 (C2'''), 125.30 (C2'''),128.24 (C6'), 128.44 (C4'''), 128.53 (C4'''), 129.13 (C3''), 129.19 (C5), 130.00 (C5'''), 130.15 (C5'''), 130.46 (C1'''), 130.94 (C1'''), 131.32 (C1'), 135.76 (2C, C6'''), 148.47 (C4'), 148.53 (C2'), 148.84 (C9), 149.43 (C3'''), 149.55 (C3''), 152.12 (C7), 162.70 (CO), 163.00 (CO).

IR ν (cm^{-1}): 2972, 1746, 1534, 1350, 1245, 1115 (Figure 89).

TOF-MS [M + Na]$^{+}$ *m/z*: 645.1492

645.1480 (calcd for C$_{34}$H$_{26}$N$_2$O$_{10}$Na) (Figure 90)

Elemental analysis for C$_{34}$H$_{26}$O$_{10}$N$_2$ calcd. C: 65.59   H: 4.18   N: 4.50

found C: 65.58   H: 4.20   N: 4.55

melting point: 95-97 °C (uncorrect).

Glabridin-2',4'-O-di-4'''-nitrobenzoate (26) (27.20 mg, 43.86 %).

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 91) δ: 1.36-1.40 (3H x 2, s, H-5'' and H-6''), 2.95 (2H, m, H-4), 3.39 (m, H-3), 4.05 (t, *J* = 10.3 Hz, H-2ax), 4.36 (dd, *J* = 10.3, 2.0 Hz, H-2eq), 5.53 (d, *J* = 9.9 Hz, H-3''), 6.35 (d, *J* = 8.2 Hz, H-6), 6.57 (d, *J* = 9.9 Hz, H-4''), 6.80 (d, *J* = 8.2 Hz, H-5), 7.23 (2H, m, H-3' and H-5'), 7.37 (d, *J* = 8.3 Hz, H-6'), 8.35 (H x 8, s, H-2''', H-3''', H-5''' and H-6''').

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 92) δ: 27.58 (C5''/C6''), 27.84 (C5''/C6''), 31.28 (C4), 32.10 (C3), 69.64 (C2), 75.72 (C2''), 109.17 (C6), 109.96 (C8), 113.17 (C10), 116.25 (C3'), 116.54 (C4''), 120.15 (C5'), 123.72-123.96 (4C, C3''' and C5'''), 128.53 (C6'), 129.09 (C3''), 129.26 (C5), 131.11-131.45 (4C, C2''' and C6'''), 131.84 (C1'), 133.97-134.44 (2C, C1'''), 148.84 (C4'), 149.41 (C2'), 149.54 (C9), 151.09 (C4'''),151.18 (C4'''), 152.16 (C7), 162.94 (CO), 163.16 (CO).

IR ν (cm^{-1}): 2973, 1745, 1528, 1253, 1092 (Figure 95).

TOF-MS [M + Na]$^{+}$ *m/z*: 645.1488

645.1480 (calcd for C$_{34}$H$_{26}$N$_2$O$_{10}$Na) (Figure 96)

Elemental analysis for C$_{34}$H$_{26}$O$_{10}$N$_2$ calcd. C: 65.59   H: 4.18   N: 4.50

found C: 65.64   H: 4.19   N: 4.53

melting point: 115-118 °C (uncorrect).

Glabridin-2',4'-O-di-2'''-methoxybenzoate (27) (55.4 mg, 63.75 %).

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 97) δ: 1.38-1.40 (3H x 2, s, H-5'' and H-6''), 2.94 (2H, m, H-4), 3.57 (m, H-3), 3.82 (3H, s, OCH$_3$), 3.92 (3H, s, OCH$_3$), 4.03 (t, *J* = 10.4 Hz, H-2ax), 4.40 (dd, *J* = 10.4 Hz, H-2eq), 5.53 (d, *J* = 9.9 Hz, H-3''), 6.35

(d, J = 8.2 Hz, H-6), 6.61 (d, J = 9.9 Hz, H-4″), 6.81 (d, J = 8.2 Hz, H-5), 7.01 (H x 4, m, H-5‴ and H-3‴), 7.17-7.27 (H x 3, m, H-3′, H-5′ and H-6′), 7,52 (H x 2, m, H-4‴), 7.96 (H x 2, dd, J = 7.5, 1.3 Hz, H-6‴).

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 98) δ: 27.63 (C5″/C6″), 27.72 (C5″/C6″), 31.17 (C4), 31.96 (C3), 55.76 (OCH$_3$), 56.25 (OCH$_3$), 69.93 (C2), 75.60 (C2″), 108.82 (C6), 109.89 (C8), 112.07 (C3‴), 112.26 (C3‴), 113.97 (C10), 116.85 (C3'), 116.90 (C4″), 118.87 (C1‴),118.92 (C1‴), 119.85 (C5′), 120.23 (C5‴), 120.31 (C5‴), 127.70 (C6′), 128.98 (C3″), 129.20 (C5), 130.95 (C1′), 132.18 (C6‴), 132.26 (C6‴), 134.39 (C4‴), 134.43 (C4‴), 149.39 (C4′), 149.60 (C2′), 150.00 (C9), 151.97 (C7), 159.58 (C2‴), 159.98 (C2‴), 164.01 (CO), 164.81 (CO).

IR ν (cm^{-1}): 2971, 1747, 1490, 1230, 1090 (Figure 101).

Elemental analysis for C$_{34}$H$_{32}$O$_8$ calcd. C: 71.83 H: 5.63
found C: 71.82 H: 5.65

melting point: 72-74°C (uncorrect).

Glabridin-2′,4′-O-di-3‴-methoxybenzoate (28) (47.2 mg, 86.02 %).

^{1}H-NMR (300 MHz, CDCl$_3$, Figure 102) δ: 1.37-1.40 (3H x 2, s, H-5″ and H-6″), 2.95 (2H, m, H-4), 3.42 (m, H-3), 3.84 (3H, s, OCH$_3$), 3.86 (3H, s, OCH$_3$), 4.05 (t, J = 10.3 Hz, H-2ax), 4.38 (dd, J = 10.3, 2.3 Hz, H-2eq), 5.52 (d, J = 9.9 Hz, H-3″), 6.34 (d, J = 8.3 Hz, H-6), 6.59 (d, J = 9.9 Hz, H-4″), 6.80 (d, J = 8.3 Hz, H-5), 7.16-7.31 (H x 5, m, H-3′, 5′, 6′ and H-4‴), 7.40 (H x 2, td, J = 7.3, 1.1 Hz, H-5‴), 7.67 (H x ,2 br s, H-2‴), 7.77 (H x 2, br d, J = 7.3 Hz, H-6‴).

^{13}C-NMR (75 MHz, CDCl$_3$, Figure 103) δ: 27.57 (C5″/C6″), 27.83 (C5″/C6″), 31.22 (C4), 32.09 (C3), 55.52 (OCH$_3$ x 2), 69.78 (C2), 75.64 (C2″), 108.96 (C6), 109.93 (C8), 113.56 (C10), 114.51 (2C, C2‴), 116.62 (C3′), 116.74 (C4″), 119.91 (C5′), 120.40-120.69 (2C, C4‴), 122.65 (C6‴), 122.72 (2C, C6‴), 128.09 (C6′), 128.97 (C3″),129.15 (C5), 129.65 (C5‴), 129.79 (2C, C5‴), 130.08 (C1‴), 130.49 (C1‴), 131.32 (C1′), 149.31 (C4′), 149.54 (C2′), 150.01 (C9), 152.04 (C7), 159.74 (C3‴), 159.80 (C3‴), 164.82 (CO), 164.71 (CO).

IR ν (cm^{-1}): 2971, 1738, 1270, 1211, 1091 (Figure 106).

Elemental analysis for $C_{34}H_{32}O_8$ calcd.   C: 71.83   H: 5.63

found   C: 71.80   H: 5.67

melting point: 67-69 °C (uncorrect).

7. Determination of Tyrosinase Inhibitory Activity

Tyrosinase inhibitory activity was determined by the dopachrome method using L-DOPA as the substrate (Iida *et al.*, 1995). The red color of dopachrome, one of the intermediates in the melanin biosynthesis, can be detected by visible light. A microplate reader (Perkin Elmer-Victor[3]) with a 490 nm interference filter was used for detection in this investigation. The potential tyrosinase inhibitor could show minimal dopachrome absorption. This enzyme-inhibitor pre-incubation method was modified from the methods of Iida (Iida *et al.*, 1995), Shin (Shin *et al.*, 1998) and Likhitayawuid and Sritularak (Likhitayawuid and Sritularak , 2001)

7.1 Preparation of the Reaction Mixture

7.1.1. Preparation of 20 mM Phosphate Buffer (pH6.8)

Monobasic sodium phosphate stock solution (solution A): $NaH_2PO_4.H_2O$ (275.98 mg) was dissolved in 100 ml of distilled water.

Dibasic sodium phosphate stock solution (solution B): Na_2HPO_4 (283.92 mg) was dissolved in 100 ml of distilled water.

Fifty-one ml of solution A was mixed with 49 ml of solution B and adjusted to pH 7.4 with either solution A or B.

7.1.2. Preparation of 0.85 mM L-DOPA

L-DOPA (0.84 mg) was dissolved in 5 ml of 20 mM phosphate buffer (pH6.8).

7.1.3. Preparation of Mushroom Tyrosinase Solution (480 unit/ml)

Mushroom tyrosinase enzyme (1.1 mg) was dissolved in 5 ml of 20 mM phosphate buffer (pH6.8).

7.1.4. Preparation of Test Sample

For preliminary evaluation, 1 mg of the test compound was dissolved in 3 ml of methanol (or suitable solvent). For determination of IC_{50}, this solution was diluted with methanol until a suitable range of concentration was obtained by the serial dilution method. The final concentration of each test sample was expressed as μM.

7.2 Measurement of Activity

The reaction mixture (200 μl) was measured in four wells (A, B, C and D). In each well, the substance was added in the order of mixing as follows;

A (control)	80 μl of 20 mM phosphate buffer (pH6.8)
	40 μl of methanol
	40 μl of mushroom tyrosinase solution (480 unit/ml)
B (blank of A)	120 μl of 20 mM phosphate buffer (pH6.8)
	40 μl of methanol
C (test sample)	80 μl of 20 mM phosphate buffer (pH6.8)
	40 μl of test sample in methanol
	40 μl of mushroom tyrosinase solution (480 unit/ml)
D (blank of C)	120 μl of 20 mM phosphate buffer (pH6.8)
	40 μl of test sample in methanol

After mixing and preincubating at 25 °C for 10 minutes, 40 μl of 0.85 mM of L-DOPA was added, and the mixture was incubated at 25 °C for 10 min. The absorbance of each well was measured at 490 nm with the microplate reader before and after incubation. All tests were performed in triplicate.

7.3 Calculation of the Percent inhibition of Tyrosinase Activity

The percent of inhibition of tyrosinase reaction was calculated as follows.

$$\% \text{ Tyrosinase inhibition} = \frac{(A-B)-(C-D)}{A-B} \times 100$$

A : The difference of optical density before and after incubation at 490 nm with enzyme, but without test sample.

B : The difference of optical density before and after incubation at 490 nm without test sample and enzyme.

C : The difference of optical density before and after incubation at 490 nm with test sample and enzyme.

D : The difference of optical density before and after incubation at 490 nm with test sample, but without enzyme.

7.4 Preliminary Evaluation of Tyrosinase Activity

The enzyme-inhibitor pre-incubation method was employed. The final concentration of the test sample in each well was 0.33 mg/ml. Activity was measured and expressed as percentage of inhibition. Samples showing more than 50% inhibition at this concentration will be further analyzed for the IC_{50} value.

7.5 Determination of IC_{50}

The IC_{50} value is the concentration of the test compound that can cause 50% inhibition of the enzyme activity. This was performed on a set of sample solutions with various concentrations prepared by the serial dilution method. An IC_{50} value was obtained by plotting % tyrosinase inhibitions versus concentrations of the inhibitor.

8. Determination of Free Radical Scavenging Activity

The free radical scavenging activity was performed by DPPH (1,1-diphenyl-2-picrylhydrazyl radical) assay which measured hydrogen atom (or one electron) donating activity and used to provide an evaluation of antioxidant activity. The experimental procedure was carried out as previously described (Son and Lewis, 2002).

8.1 Preparation of Reaction Mixture

8.1.1 Preparation of Test Sample

Test samples were prepared as previous described in section 7.1.4.

8.1.2 Preparation of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) Solution (500 μM)

Twenty mg of DPPH was dissolved in 100 ml of methanol.

8.2 Measurement of Activity

One ml of test sample was mixed with the equal volume of DPPH solution (500 μM). The reaction mixture was shaken and then incubated in the dark for 30 min, at room temperature (25 °C). The absorbance of each sample was measured at 517 nm on Thermo Spectronic Bionate 5 Spectrophotometer. The DPPH solution was used as negative control and quercetin as reference compound. All tests were performed in triplicate.

8.3 Calculation of Percentage of Free Radical Scavenging Activity

The free radical scavenging activity of the sample was expressed in terms of % reduction of DPPH, calculated as follows.

$$\% \text{ DPPH reduction} = \frac{A-B}{A} \times 100$$

A : The absorbance of DPPH solution after incubation at 517 nm

B : The absorbance of the reaction mixture after incubation at 517 nm

8.4 Preliminary Evaluation of Free Radical Scavenging Activity

The final concentration of 0.33 mg/ml of test sample was measured for free radical scavenging activity as the method described above. Samples containing more than 50% DPPH reduction will be further analyzed for the IC_{50} value.

8.5 Determination of IC_{50}

The IC_{50} value is the concentration in μM required for a 50% decrease in absorbance of DPPH radical. The experiment was performed on a set of sample solutions with various concentrations prepared by the serial dilution method. An IC_{50} value was calculated from the graph obtained by plotting % DPPH reduction versus concentrations of the inhibitor.

9. Determination of Partition Coeffient (log P)

The n-octanol/phosphate buffer partition coefficient was measured at room temperature (30 °C) using the shake-flask method (Bundgaard, Hansen and Larsen, 1979; Inagi et al., 1981; Beall, Prankerd and Sloan, 1994).

9.1 Preparation of Reaction Mixture

9.1.1 Preparation of 0.05 M Phosphate Buffer pH 5.5 with an Ionic Strength of 0.15

Monobasic sodium phosphate stock solution (solution A): $NaH_2PO_4.H_2O$ (690.0 mg) was dissolved in 100 ml of distilled water.

Dibasic sodium phosphate stock solution (solution B): Na_2HPO_4 (709.8 mg) was dissolved in 100 ml of distilled water.

Sodium chloride (292.2 mg) was added to the mixture of 97.0 ml of solution A and 3.0 ml of solution B. The prepared solution were mixed well and adjusted to pH 5.5 with either solution A or B.

The prepared buffer solution was saturated with *n*-octanol by vigorously stirring at room temperature for 24 h and stood for phase separation in a separatory funnel before using.

9.1.2. Preparation of Test Solution

The test solutions were prepared by separately dissolving 5 mg of glabridin (4), 1 mg of glabridin diacetate (16), 8 mg of glabridin dihexanoate (17), 10 mg of glabridin didecanoate (18), 3 mg of glabridin dipalmitate (19) and 3 mg of glabridin distearate (20) in buffer-saturated *n*-octanol in an individual 5-ml volumetric flask and diluting to volume with the same solvent.

9.2 Preparation of Standard Curve

Stock solution of glabridin (4) and its prodrugs were prepared by dissolving 5 mg of glabridin (4), 1 mg of glabridin diacetate (16), 8 mg of glabridin dihexanoate (17), 10 mg of glabridin didecanoate (18), 3 mg of glabridin dipalmitate (19) and 3 mg of glabridin distearate (20) in 5 ml of acetonotrile. The following volumes of 50, 100, 200, 300 and 400 µl of each stock solution were individually transferred into 5-ml volumetric flask and adjusted to volume with acetonitrile. The quatitative determination of each compound was analyzed on a Milton Roy Spectronic 3000 Array Spectrophotometer at the wavelength of 280 nm in a triplicated experiment. A standard curve was obtained by plotting absorbance against the concentrations of each compound (Figure 107-112).

9.3 Determination of log P

One ml of the test solution in buffer-saturated *n*-octanol was transferred into a screw-capped test tube, 10 ml of presaturated buffer was added. After shaken vigorously, the mixture was kept in a shaking water bath at room temperature for 12 hour. The mixture was centrifuged at 4000 rpm for 5 min to achieve complete separation. The 200 µl portion of *n*-octanol layer was diluted to 5 ml with acetonitrile

and analyzed by UV spectrophotometry at 280 nm, both before and after partition. The apparent partition coefficient (P) of each test compound was calculated as follows:

$$P = \frac{Co}{(Ci-Co)} \times \frac{Vw}{Vo}$$

Ci   = concentration of test compound in *n*-octanol phase before partition ($\mu g/ml$).

Co   = concentration of test compound in *n*-octanol phase after partition ($\mu g/ml$).

Ci-Co = concentration of test compound in aqueous phase after partition ($\mu g/ml$).

Vw   = volume of aqueous phase (buffer) in ml.

Vo   = volume of oil phase (*n*-octanol) in ml.

10. Enzymatic and Chemical Hydrolysis Studies

In this investigation, glabridin diacetate (16) was chosen as a representative prodrug for the study of its enzymatic and chemical hydrolysis reactions.

10.1 Preparation of Reaction Mixture

10.1.1 Preparation of 0.05 M Phosphate Buffer pH 7.4 and pH 5.5, with an Ionic Strength of 0.15

Monobasic sodium phosphate stock solution (solution A): $NaH_2PO_4.H_2O$ (690.0 mg) was dissolved in 100 ml of distilled water.

Dibasic sodium phosphate stock solution (solution B): Na_2HPO_4 (709.8 mg) was dissolved in 100 ml of distilled water.

Sodium chloride (292.2 mg) was added to the mixture of 19.0 ml of solution A and 81.0 ml of solution B. The prepared solution was mixed well and adjusted to pH 7.4 with either solution A or B.

Phosphate buffer pH 5.5 was prepared as previously described in section 9.1.1.

10.1.2. Preparation of 10 mM Prodrug Solution

Glabridin diacetate (16) (4.1 mg) was dissolved in 1 ml of dimethylsulfoxide.

10.1.3. Preparation of Internal Standard (IS) Solution

Betamethasone valerate was selected to use as internal standard for HPLC analysis. The IS solution was prepared by dissolving 8.4 mg, accurately weighed, of

betamethasone valerate in 100 ml of acetonitrile to give the final accurate concentration of 84 μg/ml.

10.1.4. Preparation of Porcine Liver Esterase

Porcine liver esterase (24 units/ mg protein) was accurately weighed, 4.2 mg and dissolved in 1 ml of 0.05 M Phosphate Buffer (pH 7.4).

10.2 Porcine Liver Esterase Hydrolysis

Hydrolysis of ester prodrug was performed using procedure described by Wang and Xu (Wang *et al.*, 1998; Xu *et al.*, 2003).

Eighty μl of porcine liver esterase solution was diluted with 7.84 ml of phosphate buffer solution (0.05M, pH 7.4, ionic strength = 0.15). Prodrug solution (80 μl) was added to enzyme-buffer mixture, mixed well and then kept in a water-bath at 37 ± 0.1 °C. At appropriate time intervals, 500 μl aliquots were withdrawn and 500 μl of methanol was added to quench the reaction. The extract was performed by adding 3 ml of chloroform. After 10 sec vortexing, the mixture was centrifuged at 5000 rpm for 5 min. The aqueous layer was removed and the organic phase was transferred to a new test tube. Two ml of organic phase was evaporated to dryness and reconstituted in 1 ml of internal standard solution and analyzed by HPLC.

10.3 Chemical Hydrolysis

The chemical hydrolysis of the prodrug was investigated in aqueous phosphate buffer solutions of pH 7.4 and 5.5 at 37 °C.

Two hundred and fifty μl of prodrug solution was added to 24.75 ml of phosphate buffer solutions. The mixtures were placed in a thermostatic incubator at 37 ± 0.5 °C. At appropriate time intervals, the sample was taken, extracted as the method described above (section 10.2) and analyzed by HPLC.

10.4 Quantitative Determination of Glabridin (4) and Its Prodrug (16)

The quantity of glabridin (4) and its prodrug, glabridin diacetate (16) were analyzed by high performance liquid chromatography using a Shimadzu HPLC system as following conditions:

Column (Analytical): Thermo Hypersil-Keystone, Part No. 77505-254630, C18, 4.6 x 250 mm, 5 micron, with guard column

Flow rate: 1 ml/min

Mobile phase: Acetonitrile : water (76:24)

Sample preparation: The sample was dissolved in 1 ml of the internal standard solution and filtered through 0.45µm nylon filter before inject.

Injection volume: 20 µl

Temperature Room temperature

Pump: LC-10ADvp

Autosampler: SIL-10ADvp

Detector: Photo-diode array at 280 nm

Controller System controller SCL-10ADvp

Software: CLASS VP

10.4.1 Preparation of Standard Curve

Stock solution glabridin (4) and glabridin diacetate (16) were prepared by dissolving 1.5 mg of glabridin (4) and 2.0 mg of glabridin diacetate (16), accurately weighed, in 10 ml of acetonotrile to give a standard solution of glabridin (4) and glabridin diacetate (16) with an accurate concentration of 0.15 and 0.20 mg/ml, respectively. The stock solution of betamethasone valerate were prepared by dissolving 10.3 mg, accurately weighed, in 25 ml of acetonotrile to give the concentration of and 0.41 mg/ml. The following volumes 2.5, 300, 500, 700 and 900 µl of glabridin (4) as well as 2.5, 100, 300, 500 and 700 µl of glabridin diacetate (16) stock solutions were individually transferred into 10-ml volumetric flask, 2 ml of betamethasone valerate solution was added and adjusted to volume with acetonitrile. Introduction of each standard solution to the HPLC was performed by a triplicate experiment. For each standard solution (G1–G5), the area under curve (AUC) of glabridin (AUC G) and the AUC of betametasone valerate (AUC IS) in each injection were measured and their ratio (AUC G/AUC IS) was determined. For each standard solution, an average value of the ratio (AUC G/AUC IS) was calculated based on the data from three injections. A standard curve was then obtained by plotting (AUC G/AUC IS) ratios against the concentrations of glabridin as illustrated in Figure 113.

The standard curve of glabridin diacetate (16) was obtained in a same manner and shown in Figure 114.