CHAPTER III

EXPERIMENTAL

1. GENERAL

Standard B5 media and various plant hormones were purchased from Gibco Laboratories. All other chemicals were reagent or analytical grade, as available. TLC plates: silica gel precoated Al sheets (Merck); Column chromatography: silica gel (40-63 μm, Merck) and Sephadex LH-20 (Pharmacia); MPLC: prepacked Lobar column (LiChroprep, Diol, 40-63 μm, Merck); TLC densitometer: Shimadzu Dual Wavelength Model CS-930; HPLC: constaMetric 4100 (Thermo Separation Products); UV: Shimadzu UV-160A; IR: Perkin Elmer 16 PC FT-IR or Jasco; MS: Finnigan MAT TSQ 70 triple stage quadrupole instrument and JEOL HX-110; NMR: 500 MHz (^{1}H) and 125 MHz (^{13}C); TLC-radioscanner: Automatic TLC-Liner Analyzer (Tracemaster 20); Liquid scintillation counter: Wallac 1409; FPLC: FPLC^{R} system (Pharmacia); Chromatographic material for enzyme purification: Superose 12 HR.

2. ROOT CULTURE OF *IMPATIENS BALSAMINA*

Root cultures of *I. balsamina* L. were initiated from young leaf explants on B5 solid medium containing 0.1 mgl^{-1} NAA, 0.1 mgl^{-1} kinetin, 1.0 mgl^{-1} BA, and 20 gl^{-1} sucrose (Pharkphoom Panichayupakaranant and Wanchai De-Eknamkul, 1992). The roots were formed after 2 weeks of incubation (25°C, continuous light 2,000 lux). After 3 weeks, the root culture was transferred into B5 liquid medium containing the same supplements as the solid medium (100 ml medium in 500 ml Erlenmeyer flask). The culture was maintained by transfer of 2.0 g fresh weight tissue into 100 ml fresh medium every 3 weeks and maintained at 25°C, 80 rpm, in continuous light.

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3. EXTRACTION AND ISOLATION

3.1 Extraction of *I. balsamina* root cultures

I. balsamina root cultures (3.5 kg, fresh weight) were harvested and dried at 60°C for 12 hr to yield 170 g dried roots. The dried ground root (170 g) was successively extracted under reflux with ethyl acetate and methanol. Both extracts were concentrated separately *in vacuo* to yield 2.0 g ethyl acetate extract and 26 g methanol extract.

3.2 Isolation and purification of secondary products

The ethyl acetate extract was separated into 9 fractions (200 ml each) on a silica gel (acidified by 0.5 N oxalic acid) column (3 x 20 cm) using stepwise gradient elution (chloroform-methanol, 100:0 to 19:1). Further purification of fraction 1 on an RP-18 Lobar column using stepwise gradient elution (methanol-water, 7:3 to 100:0) yielded **ET-1** (5.1 mg) as yellow needles crystallized from a mixture of ethanol and water, whereas further purification of fractions 2 and 3 using the same column and conditions yielded **ET-2** (3.4 mg) as yellow crystals crystallized from a mixture of methanol and water. Another portion of the ethyl acetate extract was fractionated into 16 fractions (10 ml each) on a silica gel (oxalic acid) column (5 x 40 cm) using stepwise gradient elution (benzene-acetone, 100:0 to 4:1). Crystallization of fraction 10 from a mixture of chloroform and methanol gave **ET-3** (8.5 mg) as colorless needles.

The methanolic extract was first partitioned between ethyl acetate and water. Further separation of the ethyl acetate fraction by gel filtration on a Sephadex LH-20 column (5 x 40 cm) using methanol as eluent yielded 4 fractions (11-14). Purification of fraction 13 by separated gel filtration on a Sephadex LH-20 column (methanol) afforded compound **ME-1** (25.7 mg) as yellowish crystals crystallized from methanol. Purification of fraction 12 on a Sephadex LH-20 column (ethanol) yielded 3 fractions (15-17). Fraction 16 was subjected to silica gel column chromatography using stepwise gradient elution (chloroform-acetone, 100:0 to 3:2) and 4 fractions were collected (18-21). Crystallization of fractions 19

and 20 from a mixture of chloroform and hexane yielded **ME-2** (3.4 mg) as white crystals and **ME-3** (9 mg) as red rosette aggregates. Separation of another ethyl acetate fraction on a silica gel column (5 x 22 cm) using stepwise gradient elution (chloroform-methanol, 100:0 to 9:1) yielded 24 fractions (22-45), 200 ml each. Purification of fractions 26-27 on a Sephadex LH-20 column (3 x 85 cm) using methanol as eluent yielded 9 fractions (46-54). Further purification of fractions 49-51 on a Sephadex LH-20 column (methanol) yielded 5 fractions (55-59). Crystallization of fractions 57-59 from a mixture of chloroform and methanol gave **ME-4** (6 mg) as yellow crystals.

4. CHARACTERIZATION OF THE ISOLATED COMPOUNDS

4.1 Characterization of ET-1

Solubility : soluble in chloroform, ethyl acetate, ethanol

Melting point : 182-183°C

UV λ_{Max} (EtOH): 243, 248, 277 and 330 nm

IR υ_{max}^{KBr} cm^{-1} : 1680 (C=O), 1645 (C=O), 1605 (C=C, Ar), 1240 (C-O)

EIMS : *m/z* 188 [M$^+$], 173 [M - Me]$^+$, 157 [M - OMe]$^+$

^{1}H NMR : See Table 5

^{13}C NMR : See Table 6

4.2 Characterization of ET-2

Solubility : soluble in methanol, ethanol, acetone

Melting point : 192 °C

UV λ_{Max} (EtOH): 244, 250 and 273 nm

IR υ_{max}^{KBr} cm^{-1} : 3160 (OH), 1674 (C=O), 1630 (C=C, Ar), 1220 (C-O)

EIMS : *m/z* 174 [M$^+$], 146 [M - CO]$^+$, 118 [M - 2 x CO]$^+$, 105 [C_6H_5CO]$^+$

^{1}H NMR : See Table 7

^{13}C NMR : See Table 8

4.3 Characterization of ET-3

Solubility : soluble in chloroform, ethyl acetate

Melting point : 169-170°C

IR υ_{min}^{KBr} cm^{-1} : 3420 (OH), 1625 (C=C), 1450 (C-H), 1380 (C-H)

EIMS : *m/z* 412 [M^+], 397 $[M - Me]^+$, 369 $[M - Me - H_2O]^+$, 300 $[C_{22}H_{46}]^+$

^{13}C NMR : See Table 9

4.4 Characterization of ME-1

Solubility : soluble in DMSO, a mixture of chloroform and methanol

Melting point : 229 °C

UV λ_{Max} (MeOH): 271.5 nm

IR υ_{min}^{KBr} cm^{-1} : 3420 (OH), 1674 (C=O), 1605, 1570 (C=C, Ar), 1455 (C=C), 1260, 1210 (C-O)

EIMS : *m/z* 360 [M^+], 342 $[M - H_2O]^+$, 314 $[M - H2O - CO]^+$, 188 $[C_{11}H_8O_3]^+$, 173 $[C_{10}H_5O_3]^+$

HRFAB-MS : *m/z* 360.0631 [M^+] (calc. 360.0628 for $C_{21}H_{12}O_6$), *m/z* 342.0497 $[M - H_2O]^+$ (calc. 342.0494 for $C_{21}H_{10}O_5$), *m/z* 314.0584 $[M - H_2O - CO]^+$ (calc. 314.0583 for $C_{20}H_{10}O_4$)

^{13}C NMR : See Table 10

^{1}H NMR : See Table 11

4.5 Characterization of ME-2

Solubility : soluble in methanol, chloroform, ethyl acetate

Melting point : 204 °C

UV λ_{Max} (EtOH): 230, 255, 300 and 345 nm

IR υ_{min}^{KBr} cm^{-1} : 3325 (OH), 1720 (C=O), 1610, 1560, 1510 (C=C, Ar), 1300, 1130 (C(O)-O)

EIMS : *m/z* 192 [M^+], 177 $[M - Me]^+$, 164 $[M - CO]^+$, 149 $[M - Me - CO]^+$, 121 $[M - Me - CO]^+$

4.6 Characterization of ME-3

Solubility : soluble in methanol, chloroform, ethyl acetate

Melting point : 148 °C

EIMS : *m/z* 222 [M^+], 207 [M - Me]$^+$, 194 [M - CO]$^+$, 179 [$C_9H_7O_4$]$^+$,

^{13}C NMR : See Table 12

^1H NMR : See Table 13

4.7 Characterization of ME-4

Solubility : soluble in DMSO, a mixture of chloroform and methanol

Melting point : 303°C (decomposition)

UV λ_{Max} (EtOH): 390 nm

IR υ_{min}^{KBr} cm^{-1} : 3410 (OH), 1706 (C=O), 1608, 1576, 1500 (C=C, Ar), 1460 (C=C), 1232, 1106 (C(O)-O)

EIMS : *m/z* 442 [M^+], 414 [M - CO]$^+$, 399 [M - CO - Me]$^+$

HRFAB-MS : *m/z* 442.0900 [M^+] (calc. 442.0891 for $C_{22}H_{18}O_{10}$)

^{13}C NMR : see Table 14

^1H NMR : see Table 15

5. CHEMICAL PATTERN AND CONTENT OF SECONDARY PRODUCTS PRODUCED BY THE CULTURED ROOTS AND THE INTACT PLANTS

5.1 Extraction and sample preparations

Each dried sample (0.2 g) including the cultured roots, the leaves and the roots of the intact plants was extracted as described below. The sample was ground and then sonicated with 10 ml petroleum ether for 1 hours (x2). The petroleum ether extract was discarded and the remaining residues were then refluxed with 20 ml methanol for 1 hour. The methanolic extract was dried *in vacuo* and then re-dissolved in methanol and the volume was adjusted to 5 ml. The obtained sample was then filtered through millipore filter (0.45 μm) before use in qualitative and quantitative analysis by HPLC.

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5.2 Qualitative and quantitative analysis of secondary products by HPLC

Each filtered sample preparation was subjected to HPLC analysis. The conditions of HPLC system were described below. For the qualitative analysis, the sample peaks were identified by comparison of their R_t values and UV absorption spectra with authentic compounds. The areas under peaks of each secondary product were used for the quantitative analysis by converting to concentrations using their calibration curves. The calibration curves were established from the authentic lawsone, 2-methoxy-1,4-naphthoquinone and scopoletin at the concentration in the range of 0.003-0.050 mg/ml. In this way, the semi-quantitative determination of diphthicol was based on the calibration curve of lawsone, while, those of isofraxidin and diisofraxidin were based on the calibration curve of scopoletin.

<u>HPLC conditions</u>

Column	:	Octyl-80Ts (4.6 x 150 mm)
Mobile phase	:	Solvent A : 0.15 M H_3PO_4 in methanol-water (1:9) Solvent B : 0.15 M H_3PO_4 in methanol-water (6:4)
Elution	:	0.15 M H_3PO_4 in methanol-water 1:9 to 6:4, in 50 min
Flow rate	:	0.7 ml/min
Detector	:	UV 275 nm for naphthoquinones; UV 365 nm for coumarin derivatives
Injection volume	:	20 µl

6. KINETIC STUDIES OF GROWTH AND THE FORMATION OF NAPHTHOQUINONE AND COUMARIN DERIVATIVES

Fresh roots (*ca* 2.0 g), subcultured on B5 medium, were transferred to fresh B5 medium and cultured for 30 days. The roots were harvested every 2 or 3 days. The harvested roots were, then, dried at 60°C for 12 hours and the dry weights were recorded. The dried roots were extracted by refluxing with methanol and the amounts of naphthoquinones and coumarin derivatives were determined by HPLC analysis as described in section 5.2. These data were then plotted to obtain growth and production curves.

7. BIOSYNTHETIC STUDIES OF LAWSONE AND 2-METHOXY-1,4-NAPHTHOQUINONE

7.1 Synthesis of OSB and [2,3-^{14}C]-OSB

OSB was synthesized according to the method of Roser (Roser, 1984). Succinic acid and phthalic acid (both 3.0 g) and sodium acetate (1.05 g) were mixed and heated at 240°C for 2 minutes. The mixture was then extracted with 800 ml of water (x4) on the steam bath and filtered. The dilactone formed was hydrolyzed with a small amount of sodium carbonate on the water bath for 45 minutes. In order to purify the hydrolyzed product, it was partitioned with ethyl acetate. The aqueous part was then acidified and OSB was extracted by ether. The ether part was evaporated *in vacuo* and applied on to a Sephadex LH-20 column, eluted with ethanol. Fraction 3 was evaporated and recrystallized from a mixture of ether and petroleum ether to yield 184 mg of OSB. In the synthesis of [2,3-^{14}C] OSB, [2,3-^{14}C]-succinic acid was used instead of succinic acid.

7.2 Identification of OSB

OSB was converted into the form of dimethyl ester by refluxed with methanol in the presence of sulfuric acid. The obtained OSB dimethyl ester was then identified by ^{1}H NMR and ^{13}C NMR data as follows: ^{1}H NMR (500 MHz): δ 3.71 (3H, s), 3.88 (3H, s), 2.80 (2H, t, *J* = 6.8 Hz), 3.14 (2H, t, *J* = 6.8 Hz), 7.43 (1H, d, *J* = 7.5 Hz), 7.49 (1H, td, *J* = 7.51, 1.0 Hz), 7.58 (1H, td, *J* = 7.51, 1.0 Hz), 7.90 (1H, d, *J* = 8.0 Hz); ^{13}C NMR (125 MHz): δ 28.2, 37.5, 51.8, 52.6, 126.3, 128.2, 129.8, 129.9, 132.3, 143.0, 167.0, 173.3, 203.8

7.3 Preparation of cell-free extract for OSB-CoA ligase assay

The root culture of one-week old (25 g) were ground in liquid nitrogen. The root powder was then added into 20 ml of 0.1 M potassium phosphate buffer (pH 7.5), containing 0.2 mM DTT, 10 % PVP and 0.1 % protamine sulphate. After homogenized for 30 seconds (2 min interval, 5 times) and passed through 4-layer cheese-cloth, the obtained enzyme extract was centrifuged at 10,000g, for 20 min at 4°C. Thereafter, the supernatant was

passed through a PD-10 column, eluted with the same buffer containing 0.2 mM DTT. The desalted enzyme solution was used for the assay of OSB-CoA ligase activity.

7.4 Assay for OSB-CoA ligase activity

The routine assay mixture contained, in a total volume of 140 µl: 0.7 mM OSB or 23,000 cpm [^{14}C]-OSB, 7.1 mM ATP, 1.8 mM CoASH, 7.1 mM $MgCl_2$ and 100 µl cell-free extract. The incubation mixture was incubated at 30°C for 30 minutes. The reaction was stopped by adding 20 µl formic acid and then analyzed by HPLC and TLC radioscanner.

<u>HPLC conditions</u>

Column	:	ODS-120T (4.6 x 150 mm)
Mobile phase	:	Solvent A : 0.15 M H_3PO_4 in methanol-water (1:9) Solvent B : 0.15 M H_3PO_4 in methanol-water (3:7)
Elution	:	0.15 M H_3PO_4 in methanol-water 1:9 to 3:7, in 35 min
Flow rate	:	1.0 ml/min
Detector	:	UV 257 nm
Temperature	:	40°C
Injection volume	:	50 µl

<u>TLC conditions</u>

Technique	:	one way, ascending, double development
Absorbent	:	aluminium sheet silica gel 60 F254
solvent system	:	Chloroform-methanol-water (65:35:10-lower phase)
Sample size	:	5 µl
Distance	:	10 cm
Detection	:	Automatic TLC-Liner Analyzer

7.5 [^{14}C-U]α-Ketoglutarate *in vivo* feeding experiment

Sodium salt of [^{14}C-U]α-ketoglutaric acid (3.5 mCi) was fed into one-week and four-week-old *I. balsamina* root cultures. After further incubated for 3 days, the root culture was

harvested and refluxed with 50 ml methanol. The methanol extract was then concentrated *in vacuo* before partitioned between ethyl acetate and water. The ethyl acetate part was adjusted to 1 ml, and 100 ml aliquot was subjected to TLC using double development solvent system of petroleum ether-chloroform (20:80) and benzene-acetic acid (98:2). The TLC plates were then scanned to produce radiochromatograms by TLC-radioscanner.

7.6 [^{13}C]-Methionine *in vivo* feeding experiment

Sterile [^{13}C]-methionine (50 mg/ml) was administered to the three-week old root culture by aseptic technique. After 3-day incubation, the root culture was harvested and extracted by refluxing with 30 ml methanol for 1 hour. The crude extract, after concentrated in vacuo, was partitioned between ethyl acetate and water. The ethyl acetate fraction was then concentrated and subjected to preparative TLC (silica gel 60 F254). Double development using the solvent systems of (I) chloroform and (II) chloroform-methanol-water (65:35:10-lower phase) was performed for the separation of 2-methoxy-1,4-naphthoquinone, scopoletin, isofraxidin and diphthicol. These compounds were eluted with chloroform and subjected to ^{13}C NMR measurement.

7.7 Preparation of cell-free extract for the enzyme complex assay

I. balsamina root culture (4-week-old) was ground in liquid nitrogen. Ten ml of 0.1 M potassium phosphate buffer pH 7.0 containing 0.2 mM DTT was added to extract proteins. The crude protein extract was filtered through 4-layer chese-cloth. The filtrate was centrifuged at 100,000g for 30 min. The supernatant was separated and used for the enzyme assay.

7.8 Superose 12 column chromatography

A superose 12 HR 16/50 column (1.6 x 50 cm) was equilibrated with elution buffer, 0.1M potassium phosphate buffer (pH 7.0), containing 0.2 mM DTT. The supernatant fraction, after concentrated with Centricon-30 to 1 ml was applied to a Superose 12 column

in the FPLC^R system. The enzyme was eluted with elution buffer at the flow rate of 0.5 ml/min. The eluates were collected into 1 ml fractions. After the determination of enzyme activity, the active fractions were pooled.

7.9 Assay for enzyme complex activity

The routine assay mixture contained, in a total volume of 250 μl: 8.8 mM ATP, 2.0 mM CoASH, 8.8 mM $MgCl_2$, 5.8 mM [^{14}C-U]α-ketoglutarate and 200 μl cell-free extract. Boiled enzyme was used for the control experiment. The mixture was incubated for 1 hour at 30 °C. The reaction was terminated by the addition of 700 μl ethyl acetate, and then vortex for 1 min. A 500 μl aliquot was concentrated to 20 μl and subjected to TLC using the TLC system as described in section 7.5. The TLC plates were then scanned to produce radiochromatograms by TLC-radioscanner. The radioactivity of a 100 μl aliquot was also measured by scintillation counter.

7.10 Determination of protein concentration

Protein concentration was estimated by the dye-binding method (Bradford, 1976), with bovine serum albumin as standard. Five dilutions of the standard BSA in the concentration range of 100-500 μg/ml were used for the calibration curve establishment. A 160 μl portion of each fraction was mixed with 40 μl of dye reagent. After incubation for at least 5 min at room temperature, the absorbance at 595 nm was measured.

7.11 Identification of enzymatic product

For the identification of the enzymatic product, TLC technique was used with two different solvent systems: (I) petroleum ether-chloroform (2:8), then benzene-acetic acid (98:2), (II) chloroform. The Rf value and UV absorption spectrum of the product were compared with those of the authentic 2-methoxy-1,4-naphthoquinone. The product was further identified by recrystallization method as described below. Spots of the product on TLC were cut off and eluted with chloroform. The chloroform fraction was, then, diluted with

unlabelled 2-methoxy-1,4-naphthoquinone, and recrystallized from a mixture of chloroform and methanol to a constant specific activity.

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