CHAPTER 2

RESEARCH DESIGNS AND METHODS

2.1 Isolation of Solasodine from Dried Fruits and Leaves of *Solanum laciniatum* Ait.

2.1.1 Materials and chemicals

Fruits (code No. 160695-3P) and leaves (code No.G1090395) samples of *S. laciniatum* at the optimal age of 120 days (Manosroi *et al.*, 1996) grown in Bann Huay Sai District, Chiang Mai, Thailand were from Pharmaceutical-Cosmetic Raw Materials and Natural Products Research and Development Center (PCRNC), Institute for Science and Technology Research and Development (IST), Chiang Mai University, Thailand. These plants were collected, dried in a hot-air oven at 60°C and ground into powder by a simple grooved-disc mill. The reference standard of solasodine (solasod-5-en-3β-ol) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solvents used for chromatographic purposes were of HPLC grade. The remaining chemicals were procured from standard sources.

2.1.2 Effects of 2-propanol concentrations on crude glycoside extraction

The dried leaf powder (500 g) was extracted 4-5 times by 0, 50, 70, 80, 85 and 100% v/v of 2-propanol (1,500 ml each) at 70°C in an Erlenmeyer flask. All filtrates were collected and 2-propanol was vacuum evaporated using the rotary evaporator (Buchi, Switzerland). The filtrate was heated to 80°C and stirred while adding 30% aqueous ammonia solution until the pH reached 9-10. The formed precipitate was collected by filtration. Dried crude glycosides were hydrolyzed by 1 N hydrochloric acid in 2-propanol in a round-bottom flask at 80°C for 3 h. The 20% sodium hydroxide solution was added to precipitate the crude solasodine. The pure solasodine was further obtained by crystallization in methanol.

2.1.3 Comparison of various methods for hydrolysis of glycosides

The dried leaf powder (1,000 g) was extracted by the same procedure as section 2.1.2, but using distilled water instead of 2-propanol. The dried crude glycosides were divided into four portions and hydrolyzed by four methods: (1) electrolysis; (2) 1 N hydrochloric acid; (3) 1 N hydrochloric acid in ethanol; and (4) 1 N hydrochloric acid in 2-propanol. For electrolysis method, the aqueous extract (1,500 ml) in 0.02 N hydrochloric acid was applied with a DC current (30 A) for 2 h using 2 pairs of aluminum plate electrodes (10×10 cm^2) in a 3 l-tank.

2.1.4 Comparison of solasodine contents in fruits and leaves of *S. laciniatum*

The dried fruit (60-750 g) or leaf (40-750 g) powder was dispersed in 70% 2-propanol and sonicated for 2 h before extraction. The suspension was put into the thimble and the alcoholic solution (volume adjusted to 300 or 3,000 ml) was added to the Soxhlet receiving flask. The solution was refluxed until exhaust and 2-propanol was vacuum evaporated using the rotary evaporator. Boiling water was added to the residue while being stirred. The solution was filtered and the dark brown-oil materials were discarded. The filtrate was heated to 80°C and stirred while adding 30% aqueous ammonia solution until the pH reached 9-10. The precipitate was filtered, and repeatedly washed with distilled water and dried in a hot-air oven at 60°C.

The above crude glycosides, 2-propanol and 37.6% hydrochloric acid in the ratio of 16.4:76:7.6 by weight were placed in a round-bottom flask. The solution was refluxed for 3 h and was then filtered by vacuum pump. The collected precipitate was dissolved in 2-propanol and 20% (w/v) sodium hydroxide solution. A large amount of water was added into the solution to obtain the crystalline solasodine. Solasodine was filtered through the filter paper and dried in a hot-air oven at 60°C. Recrystallisation of solasodine from methanol gave colorless hexagonal plates of crystalline solasodine which was further used as a precursor for 16-dehydropregnenolone acetate synthesis.

2.1.5 Analysis of solasodine

2.1.5.1 Qualitative analysis

The isolated solasodine was preliminary determined by thin layer chromatographic (TLC) method comparing to the authentic sample using silica gel 60 GF_{254} aluminum sheet (Merck KGaA, Darmstadt, Germany) developed with mobile

phase consisting of chloroform/methanol (9:1). The spots were visualized by spraying 30% v/v sulfuric acid solution on TLC plate before heating on a hot-air oven.

2.1.5.2 Quantitative analysis

Solasodine was analyzed by high performance liquid chromatography (HPLC, Thermo Separation Products Inc., California, USA) at 205 nm using a Lichrosorb C-18 column (250×4.6 mm i.d.; 10 μm particle diameter, HPLC Technology, UK) and 0.01 M TRIS HCl buffer/acetonitrile (20:80 by volume) as the mobile phase at a flow rate of 2.00 ml min^{-1} with injection volume of 20 μl. The purity of product was measured by using the comparison of peak height between the product and the reference standard of solasodine. Solasodiene was also analyzed at the same procedure by HPLC at 236 nm.

2.1.5.3 Identification

The melting points of solasodine products were determined by melting point apparatus (Stuart Scientific, UK). Infrared (IR) spectra were recorded using KBr discs on a FT-IR spectrometer (Jasco FT/IR-5000, Japan) and nuclear magnetic resonance (NMR) spectra were obtained using ^{1}H-NMR spectrometer (Avance, Bucker, Germany) at 400 MHz, with tetramethylsilane (TMS) as internal standard in $CDCl_3$. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS and coupling constants (*J*) are given in Hertz (Hz). All of data were compared to the authentic sample.

2.2 Synthesis of 16-Dehydropregnenolone Acetate from Solasodine by Using Phase-Transfer Catalysis

2.2.1 Chemicals

Solasodine was isolated from dried fruits and leaves of *S. laciniatum*. The reference standard 16-dehydropregnenolone acetate (3β-acetoxy-5,16-pregnadiene-20-one) was purchased from Sigma Chemical Co. The oxidizing agents were from various sources. All solvents for chromatographic purposes were HPLC grade. Other solvents were reagent grade.

2.2.2 Acetylation and isomerisation

A mixture of solasodine (1 g), pyridine (10 ml) and acetic anhydride (5 ml) was refluxed for 2 h and then cooled. The mixture was poured on ice plus aqueous

ammonia solution. The precipitate was filtered and dried. The product was refluxed in glacial acetic acid (10 ml) for 1 h. Glacial acetic acid was distilled off under vacuum giving a yellow residue.

2.2.3 Oxidation and hydrolysis

2.2.3.1 Conventional technique

For hydrogen peroxide as an oxidizing agent, the residue from section 2.2.2. was refluxed with glacial acetic acid (10 ml) and hydrogen peroxide (30 mol % excess of solasodine) at 70°C for 5 h, and then water was added to destroy excess oxidizing agent. The reaction mixture was extracted with ethyl acetate and the solvent was evaporated. The residue was refluxed in glacial acetic acid (10 ml).

For potassium permanganate, potassium dichromate and chromic trioxide as oxidizing agents, the solution obtained after isomerisation in glacial acetic acid (section 2.2.2) was cooled to 0-15°C and a solution of potassium permanganate, potassium dichromate or chromic trioxide (30 mol % excess of solasodine) in glacial acetic acid (10 ml) was added dropwise with stirring for 3 h. Then, methanol was added to destroy the excess oxidizing agent. The reaction mixture was refluxed for 2 h and the solvent was distilled off under vacuum.

2.2.3.2 Phase-transfer catalysis technique

The residue from section 2.2.2 was dissolved in methylene chloride (10 ml). The oxidizing agent (30 mol % excess of solasodine) and phase-transfer catalyst (10 mol % of oxidizing agent) were dissolved in water (10 ml) and added to the reaction mixture dropwise. The mixture was vigorously stirred while maintaining the pH at 0-1 by adding sulfuric acid. The bath temperature was maintained at 0-15°C for 3 h. The organic phase was separated from the system and washed with water several times. Then, the solvent was removed under vacuum until gummy material was obtained. The product was refluxed in glacial acetic acid (10 ml) for 2 h and distilled off under vacuum.

2.2.4 Purification

The gummy material of the product (section 2.2.3) was packed on a column of silica gel 60 (Merck KgaA) and eluted with petroleum ether and ethyl acetate with slowly increasing polarity. The eluent gave pure 16-dehydropregnenolone acetate.

Recrystallisation from methanol gave a rod-like crystal of 16-dehydropregnenolone acetate.

2.2.5 Analysis of 16-dehydropregnenolone acetate

2.2.5.1 Qualitative analysis

16-Dehydropregnenolone acetate was preliminary determined by TLC comparing to the authentic sample using silica gel 60 GF_{254} aluminum sheet developed with mobile phase consisting of ethyl acetate/petroleum ether (3:7). The spots were detected under UV cabinet at 254 nm.

2.2.5.2 Quantitative analysis

16-dehydropregnenolone acetate was analyzed by HPLC at 254 nm using a Hypersil C-18 column (250×4.6 mm i.d.; 10 µm particle diameter; 250°A average pore size, Hichrom, UK) and 100% methanol was used as the mobile phase at a flow rate of 1.00 ml min^{-1} with injection volume of 5 µl. The purity of product was measured by using the comparison of peak height between the product and the reference standard of 16-dehydropregnenolone acetate.

2.2.5.3 Identification

16-Dehydropregnenolone acetate was determined by melting point apparatus, infrared spectrometer and nuclear magnetic resonance spectrometer, comparing to the authentic sample.

2.3 Factors Affecting the Biotransformation of Chlormadinone Acetate to Delmadinone Acetate

2.3.1 Chemicals

Chlormadinone acetate (17α-acetoxy-6-chloro-4,6-pregnadiene-3,20-dione), hydrocortisone (11β,17α,21-trihydroxy-4-pregnene-3,20-dione) and prednisolone (11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione) were reference grade from Sigma Chemical Co. Delmadinone acetate (17α-acetoxy-6-chloro-1,4,6-pregnatriene-3,20-dione) was synthesized from chlormadinone acetate as previously described by Shibata *et al.* (1992). Menadione, yeast extract and TRIS HCl (tris (hydroxymethyl) amino-methane hydrochloride) were from Fluka Chemie GmbH (Buchs, Switzerland). D-(+)-

glucose was from Riedel-de Haen Laborchemikalien GmbH (Seelze, Germany). All solvents for chromatographic purposes were HPLC grade. Other solvents were reagent grade. All other chemicals were procured from standard sources.

2.3.2 Microorganisms

The standard bacterial cells, *A. simplex* ATCC 6946 and *B. sphaericus* ATCC 13805 were provided by Pharmaceutical-Cosmetic Raw Materials and Natural Products Research and Development Center (PCRNC), Institute for Science and Technology Research and Development (IST), Chiang Mai University, Thailand. The organisms were maintained on tryptic soy agar, TSA (Difco Laboratories, Detriot, MI, USA) slants over-layered by mineral oil and stored at -20°C in low temperature incubator (Eyela LTI-100SD, Tokyo, Japan).

2.3.3 Cultivation of bacterial cells

Stock cultures of *A. simplex* and *B. sphaericus* kept on TSA slants were subcultured at 35-37°C in an incubator (Eyela SLI-600ND, Tokyo, Japan) for 48 h. Each strain was grown in 100 ml of medium containing (g l^{-1}): yeast extract, 5; ammonium sulfate, 3; and magnesium chloride, 0.1 in 50 mM TRIS HCl buffer pH 7.8 in 250-ml shake flasks held at 25±2°C, 200 rpm on the orbital shaker (Eyela S102, Tokyo, Japan) for 48 h. All of aseptic techniques were done in laminar air flow cabinet (CytAir 125, Wissous, France).

2.3.4 Bacterial counts

Numbers of bacterial cell in colony forming unit (CFU) were determined by the plate count method. Samples were prepared by serial dilutions (1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$ and $1:10^7$, respectively). The volume of each dilution was 1.00 ml and diluted by sterile tryptic soy borth, TSB (Difco Laboratories, Detriot, MI, USA). A 0.10 ml of each dilution ($1:10^4$, $1:10^5$, $1:10^6$ and $1:10^7$) was spread over the surface of TSA petri-dish in duplicates and then incubated at 35-37°C for 24-48 h. The number of bacteria colonies that appear on each of the plates were counted.

2.3.5 Biotransformations

A 5.0-ml bacterial cell suspension of *A. simplex* (4×10^7 CFU ml^{-1}) or *B. sphaericus* (2×10^6 CFU ml^{-1}) was inoculated in 45.0 ml of the earlier specified medium (Section 2.3.3) in 250-ml shake flasks held at 25±2°C, 200 rpm on the rotary shaker for 48 h. Then, various concentrations of menadione, chlormadinone acetate, Tween 80, D-(+)-glucose in DMF were added. For the steroid inducer, hydrocortisone was added as a 2% solution in ethanol and then, after 24-h, the other compounds were added. Samples (0.5 ml each) were collected for HPLC analysis at 0, 4, 24, 48, 72 and 96 h. The optimum condition of each factor was selected for further study.

2.3.5.1 Effect of menadione as an exogenous electron carrier

The medium was added with chlormadinone acetate and various concentrations of menadione (0, 0.3, 0.6, 0.9 and 1.2 mM) dissolved in 5% (v/v) DMF.

2.3.5.2 Effect of the substrate and DMF concentrations

The medium was added with the optimum concentration of menadione (section 2.3.5.1) and various concentrations of chlormadinone acetate (0.12, 0.25 and 0.50 mM) and DMF (5 and 10% (v/v)).

2.3.5.3 Effect of hydrocortisone as an enzyme inducer

The medium was added with hydrocortisone (0, 0.14, 0.28, 0.41 and 0.55 mM) in ethanol. The optimum concentrations of menadione (2.3.5.1), chlormadinone acetate and DMF (2.3.5.2) were added after a 24-h incubation with hydrocortisone.

2.3.5.4 Effect of Tween 80 as a surfactant

The medium was added with the optimum concentrations of menadione (2.3.5.1), chlormadinone acetate and DMF (2.3.5.2), and various concentrations of Tween 80 (0, 0.25, 0.50, 0.75 and 1.00% (w/v)) after a 24-h incubation with the selected concentration of hydrocortisone (2.3.5.3).

2.3.4.5 Effect of D-(+)-glucose as a carbon source

Various concentrations of D-(+)-glucose (0, 2.5, 5.0, 7.5 and 10.0 g l^{-1}) were added to the second-stage cultivation medium. The medium was added with the

optimum concentrations of menadione (2.3.4.1), chlormadinone acetate, DMF (2.3.4.2) and Tween 80 (2.3.4.4) after a 24-h incubation with the selected concentration of hydrocortisone (2.3.4.3).

2.3.4 Analysis of delmadinone acetate

2.3.4.1 HPLC analysis

The sample (0.5 ml) taken from the biotransformation flask was extracted with 2.0 ml chloroform by vortex mixing for 2 min. A portion (0.5 ml) of chloroform phase was transferred to a sampling vial. Samples (5 µl) were analyzed for delmadinone acetate and chlormadinone acetate by HPLC on a Hypersil C-18 column using methanol/water (70:30 by volume) as the mobile phase at a flow rate of 1.0 ml $min.^{-1}$ A UV-Visible detector (282 nm) was used.

2.3.4.2 Identification

The biotransformation media were collected after the reaction was finished. The mixture was centrifuged at 5,000 rpm by Avanti 30 centrifuge (Beckman Instrument, Inc., CA, USA) and then the supernatant was extracted with chloroform. The mixture was concentrated to dryness and the residue was then chromatographed on alumina column using methylene chloride as an eluent. The clear eluent was collected and then evaporated to give delmadinone acetate. The product was recrystallised from ethyl acetate. Delmadinone acetate was identified by melting point apparatus, nuclear magnetic resonance spectrometer as well as chromatographic methods (TLC and HPLC), in comparing to its chemically synthesized.

2.4 Biotransformation of Chlormadinone Acetate to Delmadinone Acetate by Free and Immobilized *Arthrobacter simplex* ATCC 6946 and *Bacillus sphaericus* ATCC 13805

2.4.1 Chemicals

Delmadinone acetate, a reference compound, was synthesized from chlormadinone acetate as previously described (Shibata *et al.*, 1992). Yeast extract, TRIS hydrochloride, sodium alginate and menadione were purchased from Fluka Chemie GmbH. L-α-phosphatidylcholine, dipalmitoyl (PC, 99% purity), dipalmitoyl phosphatidylcholine (DPPC, 95% purity) and hydrogenated phosphatidylcholine (Epikuron® 200SH minimum 95% purity) were obtained from Sigma Chemical Co., Nikko

Co. (Japan) and Lucas Mayer GmbH & Co. (Hamburg, Germany), respectively. Cholesterol was sourced from Sigma Chemical Co. Chloroform, butyl acetate, *n*-octanol, and *n*-decane were purchased from Merck KGaA.

2.4.2 Microorganisms

See section 2.3.2.

2.4.3 Cultivation of bacterial cells

See section 2.3.3.

2.4.4 Bacterial counts

See section 2.3.4.

2.4.5 Preparation of liposomes

Various liposomal formulations containing entrapped steroids were prepared from phosphatidylcholine and cholesterol together with 5 mg of chlormadinone acetate as a substrate. The mixtures were dissolved in chloroform (20 ml) and the solution was then evaporated in a rotary evaporator at 40±5°C to obtain a dried lipid film. Residual traces of chloroform were removed by an overnight vacuum drying at room temperature. The lipid film was rehydrated with 50 mM TRIS HCl buffer pH 7.8 using glass beads in the rotary evaporator at 60°C for 30 min and ultrasonicated with a probe sonicator (Sonics & Materials, Inc., CT, USA) for 30 min. The resulting liposomal medium was filtered through a 0.45 μ membrane filter.

2.4.5 Immobilization

A 5.0-ml bacterial cell suspension of *A. simplex* (4×10^7 CFU ml^{-1}) or *B. sphaericus* (2×10^6 CFU ml^{-1}) was homogenized with sodium alginate (4% (w/v) in aqueous suspension) The mixture was then pumped through a syringe needle dropwise into 100 ml of 0.2 M $CaCl_2$ in 50 mM TRIS HCl buffer, pH 7.8. The gel beads (3-mm diameter) were allowed to harden in buffered $CaCl_2$ for 1-2 h prior to use.

2.4.6 Biotransformations

2.4.6.1 Biotransformation in aqueous medium

Portions of the free and immobilized cells containing 2×10^8 CFU of *A. simplex* or 1×10^7 CFU of *B. sphaericus* in the 50.0-ml medium (section 2.4.3) were grown in 250-ml shake flasks, 25±2°C, 200 rpm (second-stage cultivation) for 48 h. Hydrocortisone powder (5 mg) was then added to each flask as an enzyme inducer.

After 24-h cultivation, a 5.0-ml portion of dimethylformamide (DMF) containing 5 mg of chlormadinone acetate and 5 mg of menadione were added to each flask. The culture conditions were maintained as described above. Samples (0.5 ml each) were collected for HPLC analysis at 0, 4, 24, 48, 72 and 96 h.

2.4.6.2 Biotransformation in liquid-liquid biphasic systems

After 24-h cultivation, the selected water-immiscible organic solvents (*n*-decane, *n*-octanol, chloroform and butyl acetate) containing 5 mg of chlormadinone acetate and 5 mg of menadione were added to each flask. The culture conditions were maintained as described in section 2.4.6.1.

2.4.6.3 Biotransformation in liposomal medium

The 40.0-ml milky liposomal medium (section 2.4.5) was transferred to a 250-ml Erlenmeyer flask. The free bacterial cell suspension (5.0 ml) containing 4×10^7 CFU ml^{-1} of *A. simplex* or 2×10^6 CFU ml^{-1} of *B. sphaericus* was added. The final volume was adjusted to 50.0 ml with 50 mM TRIS HCl buffer, pH 7.8. The flask was incubated on an orbital shaker at 200 rpm, 25±2°C, for 72 h. The culture conditions were maintained as described in section 2.4.6.1.

2.4.7 Analysis of delmadinone acetate

2.4.6.1 HPLC analysis

See section 2.3.4.1.

2.4.6.2 Identification

See section 2.3.4.2.

2.5 Chemical Synthesis of Delmadinone Acetate from Chlormadinone Acetate

2.5.1 Chemicals

Chlormadinone acetate was purchased from Sigma Chemical Co. 2,3-dichloro-4,5-dicyanobezoquinone was from Aldrich Chemical Co. (Milwaukee, WI, USA). Dioxane was from Fluka Chemie GmbH and other solvents were reagent grade.

2.5.2 Synthesis of delmadinone acetate

A solution of chlormadinone acetate (1,000 mg) and 2,3-dichloro-4,5-dicyanobenzoquinone (DDQ) (800 mg) in dioxane (12.5 ml) was refluxed under oil bath for 3 h and then cooled. The precipitate was filtered and discarded. The filtrate was concentrated to dryness and the residue was then column chromatographed on aluminium oxide 90 (70-230 mesh) from Merck KGaA column (60 g) using methylene chloride as an eluent. The clear eluent was collected and then evaporated to give delmadinone acetate.

2.4.3 Analysis of delmadinone acetate

2.4.3.1 Qualitative analysis

Delmadinone acetate was preliminary determined by thin-layer chromatographic method comparing to its substrate using silica gel 60 GF_{254} aluminum sheet developed with mobile phase consisting of ethyl acetate/petroleum ether (7:3). The spots were detected under UV lamp at 254 nm. The product was also analyzed by HPLC at 282 nm using a Hypersil C-18 column and 70% methanol was used as the mobile phase at a flow rate of 1.00 ml min^{-1} with injection volume of 5 µl.

2.4.3.2 Identification

Delmadinone acetate was determined by melting point apparatus and nuclear magnetic resonance spectrometer, comparing to its substrate.

2.5 Chemical Synthesis of Cyproterone Acetate from Delmadinone Acetate

2.5.1 Chemicals

Delmadinone acetate was produced by biotransformation (2.3 and 2.4) and chemical synthesis (2.5) methods as mentioned above. The standard cyproterone acetate (17α-acetoxy-6-chloro-1β,2β-methylenepregna-4,6-diene-3,20-dione) and

trimethylsulfoxonium iodide (TMSI) were obtained from Aldrich Chemical Co. Sodium hydride was purchased from Fluka Chemie GmbH. All solvents were reagent grade from various sources.

2.5.2 Synthesis of cyproterone acetate

Trimethylsulfoxonium iodide (2,400 mg) and pulverized sodium hydride (103 mg) were dissolved in 30 ml dimethylsulfoxide (DMSO) and stirred for 45 min at room temperature under nitrogen gas. Until sodium hydride was completely dissolved, delmadinone acetate (727.2 mg) was added into the reaction mixture and stirred 5 h at room temperature. When the reaction was not complete after 5 h, the reaction time should be longer. Then, the reaction mixture was poured into a mixture of acetic acid and ice water. The precipitate was filtered, then repeatedly washed with water and dried in hot-air oven. Chromatography on silica gel column by using hexane and acetone as an eluent and recrystallization from ethyl acetate gave cyproterone acetate

2.5.3 Analysis of cyproterone acetate

2.5.3.1 Qualitative analysis

Cyproterone acetate was preliminary determined by thin-layer chromatographic method comparing to the reference standard using silica gel 60 GF_{254} aluminum sheet developed with mobile phase consisting of ethyl acetate/petroleum ether (7:3). The spots were detected under UV lamp at 254 nm.

2.5.3.2 Quantitative analysis

Cyproterone acetate was analyzed by HPLC at 282 nm using a Hypersil C-18 column and 70% methanol was used as the mobile phase at a flow rate of 1.00 ml min^{-1} with injection volume of 5 µl. The purity and quantity of products was measured by using the peak height compared to the reference standard.

2.5.3.3 Identification

Cyproterone acetate was determined by melting point apparatus and nuclear magnetic resonance spectrometer, comparing to the reference standard.