CHAPTER III

RESEARCH METHODOLOGY

General experiment procedures

A silica gel column (0.040-0.063 mm granule size; Merck, Germany) was used for the chromatographic isolation of the extract components. Preparative thin layer chromatography (TLC) analyses were performed on in-house TLC plates coated with silica gel 60 F_{254} (0.2 mm thick, Merck, Dramstadt, Germany). TLC analysis was performed on TLC silica gel 60 F_{254} aluminium sheet (20 x 20 cm, Merck, Germany). Testosterone remaining from the enzymatic reaction was measured using high performance liquid chromatography (HPLC) (Shimadzu, Japan). HPLC analysis was equipped with an SPD-20A UV/Vis detector, an LC-10AT prominence liquid chromatography pump, CTO-10AS VP column oven and an injector with a 20 μL loop. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker AV400 (USA) spectrometer at 400 MHz for proton and 100 MHz for carbon with tetramethylsilane (TMS) as the internal standard. Mass spectra were obtained using electrospray ionization-mass spectrometer (ESI-MS) in full scan mode (API 4000, Applied Biosystem, Fostercity, CA, USA) and electron impact ionization-mass spectrometer (EI-MS) (Thermo Finnigan Polaris Q, NY USA and Agilent Technologies, Palo Alto, CA, USA). The absorbance in MTT assay and polarization were measured using DTX 880 multimode detector (Beckman coulter, Fullerton, CA, USA). Quantitative analysis of chemical constituents was performed on both HPLC and gas chromatography (GC) system. HPLC analysis was conducted on a Shimadzu HPLC system equipped with (i) an SPD-20A UV/Vis detector or (ii) with an SPD-M10AVP photodiode array detector (PDA), an LC-10ATVP pump, CTO-10AS VP column oven and an injector with a 20 μL loop. The phenomenex Luna 5u C-18 column (150 x 4.6 mm) was used for the HPLC separation. GC analysis was performed on a Hewlett Packard (Agilent Technologies, Palo Alto, CA, USA) model 6890 gas chromatograph equipped with a flame ionization detector (GC-FID). A fused silica capillary Hewlett Packard HP-5 (5% phenyl methyl siloxane) column (30 m x 0.25 mm i.d., 0.25 μm film thickness) was used for the GC separation.

Chemicals

Testosterone, 5α-dihydrotestosterone, ethinylestradiol, finasteride, flutamide, prednisolone, tris-(hydroxy methyl)-aminomethane and PEG-40 hydrogenated castor oil were purchased from Sigma-Aldrich (USA). Propylene glycol (PG) was obtained from Ajax Finechem (Australia), β-nicotinamide adenine dinucleotide phosphate (NADPH, tetrasodium salt) from Calbiochem (USA). Methanol (MeOH), ethanol (EtOH), ethyl acetate (EtOAc), chloroform ($CHCl_3$) dichloromethane (CH_2Cl_2), dimethyl sulphoxide (DMSO) and acetonitrile (CH_3CN) were bought from RCI Labscan Ltd (Thailand), and thiopental sodium from Abbott (Thailand). The androgen receptor competitor assay kit (part no. P3018) was purchased from Invitrogen Company (USA).

Androgen-sensitive human prostate cancer cells (LNCaP) (CRL-1740) were obtained from American Type Culture Collection (ATCC). Primary cell cultures of human foreskin fibroblasts (HF) were obtained from Buddhachinaraj Hospital, Phitsanulok, Thailand. Roswell Park Memorial Institute formulation 1640 (RPMI-1640) medium, Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Invitrogen Corporation (USA) and phenol red-free RPMI-1640 medium from Sigma-Aldrich (USA). Charcoal stripped fetal bovine serum was purchased from PAA laboratories GmbH (Austria). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for the MTT assay was obtained from Amresco (USA).

Plant materials

Fresh rhizomes of *C. aeruginosa* were collected from Khaokhor district, Phetchabun province, Thailand. The specimen (collection number: ganniga001) is kept at Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, and at PBM herbarium, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The plant materials were identified by Professor Wongsatit Chuakul, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

Extraction and isolation

The powdered rhizomes of *C. aeruginosa* (5 kg) were extracted with hexane (2.5 L) for 3 days at room temperature and filtered. The maceration procedure was repeated 3 times. The solvent was evaporated under reduced pressure to produce the hexane extract (30 g).

C. aeruginosa extract (21.5 g) was roughly separated by silica gel quick column chromatography (10 x 8 cm) and a gradient sequentially formed of hexane, CH_2Cl_2 and MeOH as the mobile phase. 17 fractions were obtained (CA1-CA17). These fractions were tested for anti-androgenic activity. Fractions CA6 (2.47 g) and CA8 (1.65 g) were further fractionated using a silica gel column and recrystallized to give colorless needle-shaped crystals which yielded **1** (138 mg) and **2** (42 mg), respectively (Figure 23). The remainder from fraction CA8 (CA8/4, 1.00 g) was further refractionated on a silica gel column using CH_2Cl_2 and MeOH as a mobile phase to yield 2 fractions (CA8/4/1 and CA8/4/2). Fraction CA 8/4/2 contained a yellow viscous oil of **3** (570 mg) (Figure 24). The remainder from CA8/4/2 (476 mg) was further fractionated on a Sephadex LH-20 column (2.2 x 120 cm) with MeOH to give 5 fractions (CA8/4/2/1-CA8/4/2/5). Fractions CA 8/4/2/3 (197 mg) and CA 8/4/2/4 (256 mg) were combined and repeatedly separated on a silica gel preparative TLC and developed with 9.5:0.5 CH_2Cl_2: MeOH mobile phase to provide **4** (30 mg). Compound **4** (57 mg) was also obtained from the fractionation of CA13/4 (195 mg) using a silica gel column chromatography eluted with a gradient of hexane, $CHCl_3$ and MeOH (Figure 24). CA15 (4.60 g) and CA16 (4.40 g) were combined and fractionated on a silica gel column eluted with gradient ratio of hexane: EtOAc: MeOH to give 15 fractions (CA15/1-CA15/15). CA15/12 (138 mg) was subjected to silica gel column eluted with gradient of hexane: EtOAc: MeOH to give **5** (34 mg) (Figure 25). Fraction CA9 (586 mg) was fractionated using the same procedures to that of CA13/4 to provide 10 fractions (CA9/1-CA9/10). CA9/4 (360 mg) was further fractionated by a silica column chromatography eluted with a step gradient of hexane: $CHCl_3$ and MeOHto give 12 fractions (CA9/4/1-CA9/4/12). CA9/4/2 was finally recrystallized in MeOH to provide **6** (7 mg) (Figure 25). The structures of these isolated compounds were elucidated using NMR and MS.

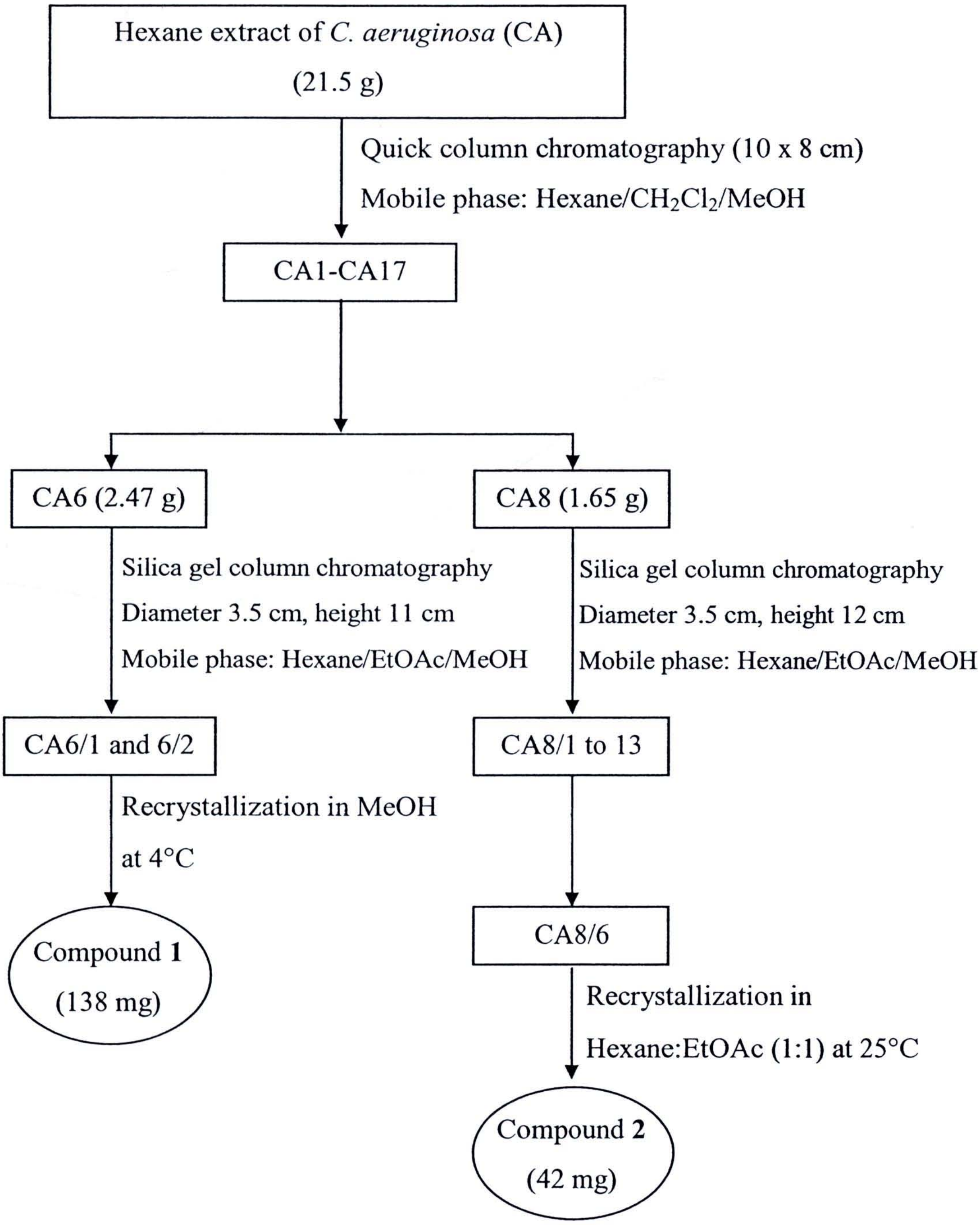

Figure 23 Isolation process of 1 and 2

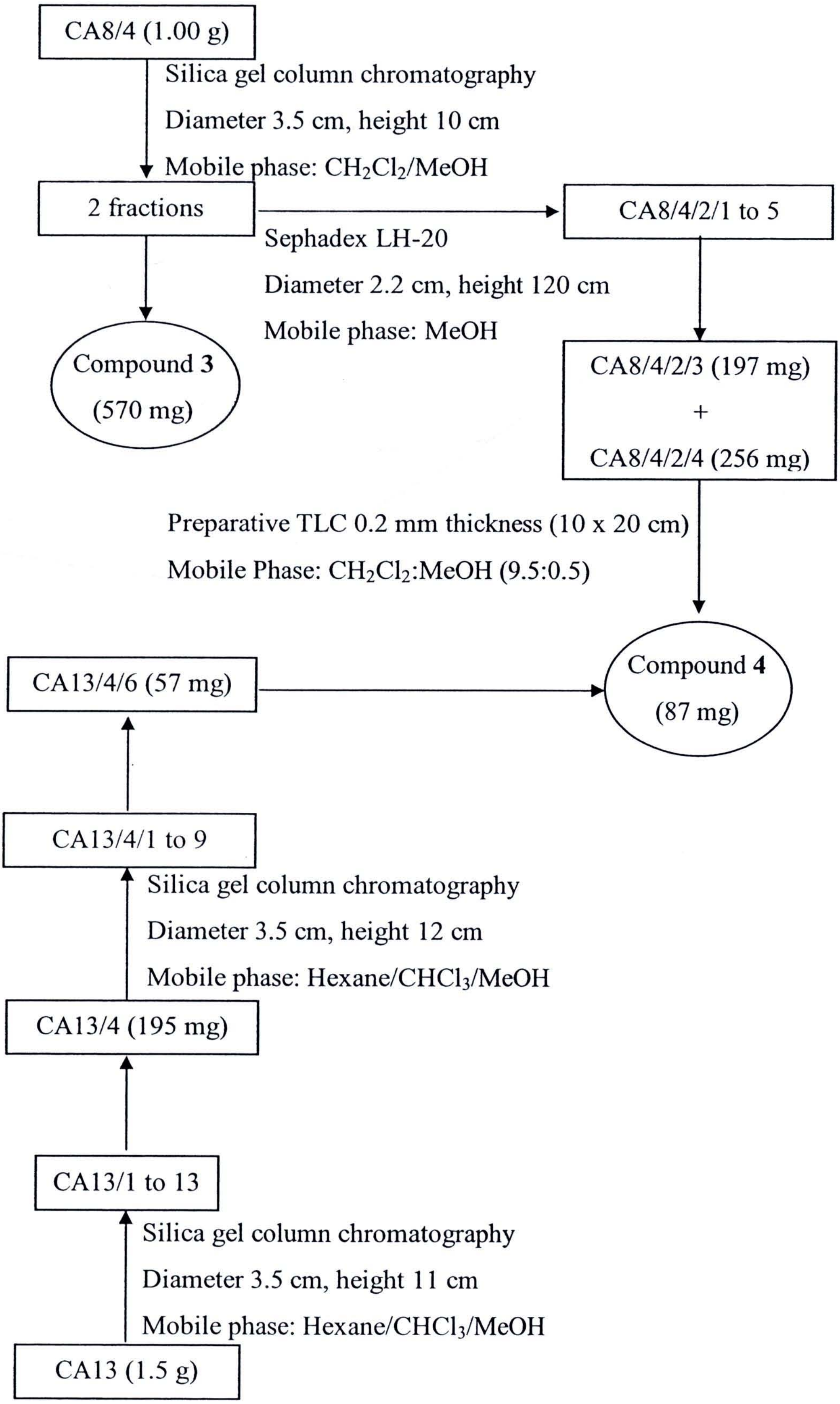

Figure 24 Isolation process of 3 and 4

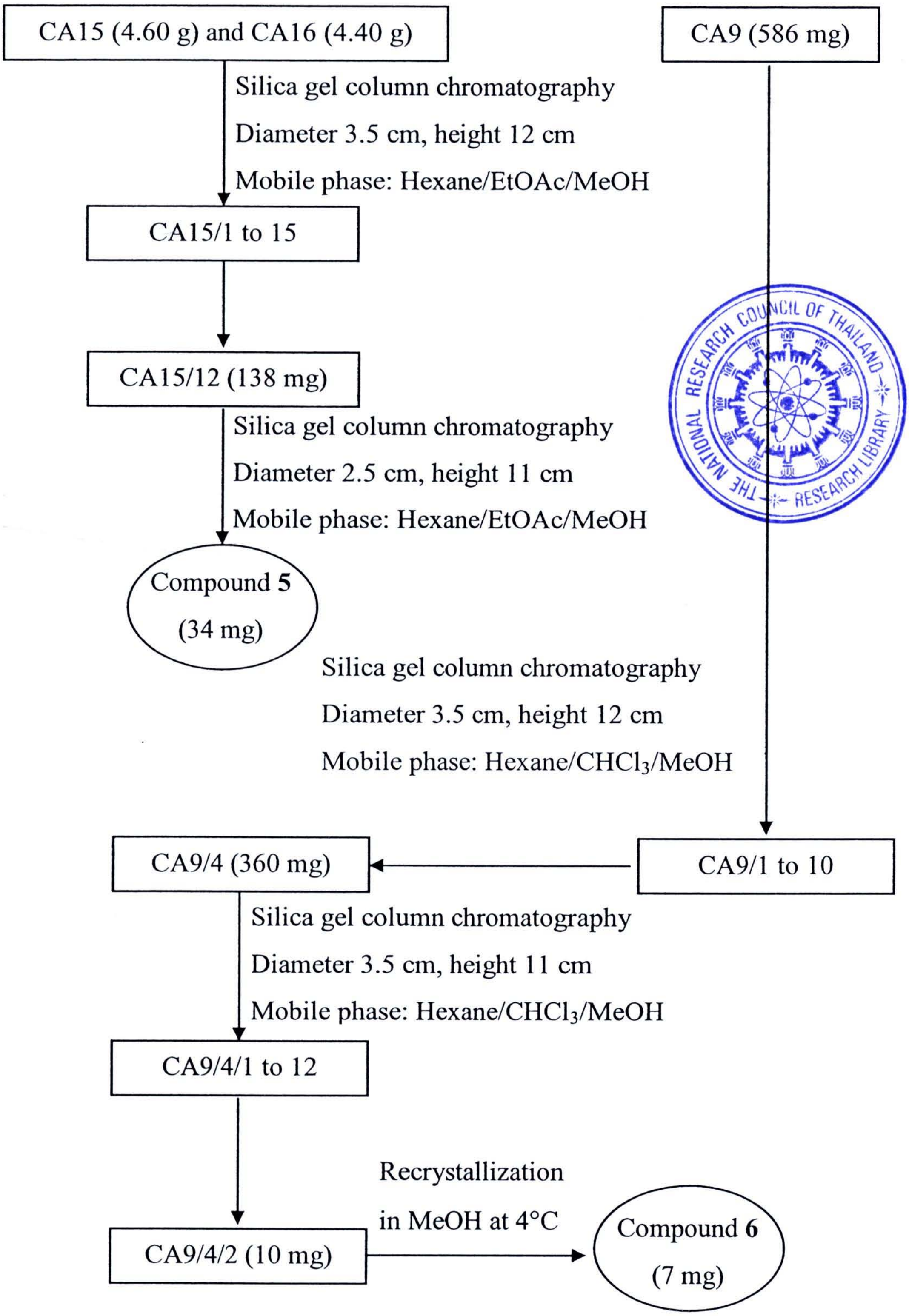

Figure 25 Isolation process of 5 and 6

Preparation of rat liver 5α-reductase

5α-reductase was prepared as a crude microsomal fraction from the livers of male sprague dawley rats (6 weeks of age) using a method of Matsuda et al. [36] with some modifications. The rats were anaesthetised with sodium thiopental and then killed. After that, the livers were removed. The blood was washed out with Krebs-ringer phosphate buffer pH 7.4 and the livers were homogenized in Tris-HCl buffer pH 7.4. The homogenate was sequentially centrifuged at 880, 4775 and 8595 x g to collect the supernatants. The final supernatant was stored at -80°C.

Measurement of inhibitory activity against conversion of testosterone

The inhibitory activity against the conversion of testosterone to DHT of the samples was tested *in vitro* using rat liver enzyme. Ethinylestradiol, which has been reported as 5α-reductase inhibitor, was used as a positive control [81]. The decrease of testosterone after the enzymatic reaction was determined using HPLC [16].

Briefly, 50 μL of test compound dissolved in DMSO, 350 μL of Tris-buffer pH 7.2, 100 μL of testosterone (0.5 mg/mL in the 1:1 v/v mixture of PG and Tris-buffer pH 7.2) and 350 μL of the thawed enzyme solution were mixed. The 150 μL of NADPH (5.13 mg/mL in Tris-buffer pH 7.2) was added to the mixture to start the reaction. The mixture was incubated at 37°C for 30 min and reaction was stopped by adding 3 mL of CH_2Cl_2. 150 μL of prednisolone as an internal standard (0.1 mg/mL in methanol) was added to the tube. The tube was shaken and centrifuged at 1450 x g (10 min, 4°C). The layer of CH_2Cl_2 was transferred to another tube and evaporated to dryness. The residue was dissolved in 2 mL of MeOH. In addition, control samples were prepared with all the solutions including the DMSO but no test compound. For the $\text{control}_{0\ \text{min}}$ the CH_2Cl_2 was added at time 0 and at 30 min of incubation for the $\text{control}_{30\ \text{min}}$. IC_{50} were calculated from the effects on samples with various concentrations i.e. *C. aeruginosa* extract (0.005-10 mg/mL), **1** (0.005-1 mg/mL) and ethinylestradiol (0.01-5 mg/mL).

The testosterone remaining after the enzymatic reaction was determined by HPLC. The phenomenex Luna 5 μm C-18 column (150 x 4.6 mm) was used as a stationary phase and 65% MeOH in water for the mobile phase except for measurement of **2** when 45% CH_3CN in water was the mobile phase. The flow rate was 0.8 mL/min and the injection volume was 20 μL. The separation was conducted at 40°C. The detector was set at 254 nm. Percent enzymatic inhibition was determined using peak height ratios (r = peak height of testosterone / peak height of prednisolone) as shown in the equation below.

$$\text{Enzymatic inhibition (\%)} = \left(\frac{(\text{r of test sample} - \text{r of control}_{30\text{ min}})}{(\text{r of control}_{0\text{ min}} - \text{r of control}_{30\text{ min}})}\right) \text{x } 100$$

Cell culture

LNCaP cells were cultured in phenol red RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% of 10,000 U/mL Penicilin G and 10,000 μg/mL Streptomycin (antibiotic mixture). The HF cells were maintained in DMEM F12 medium supplemented with 10% FBS, 1% of 2 mM L-glutamine and 1% antibiotic mixture. They were maintained in 75 cm^3 culture flask at 37°C under 5% CO_2 humidified atmosphere. The medium was changed every 3-4 days. The cells were detached using 0.25% trypsin (Ca^{2+}, Mg^{2+} free phosphate buffer (PBS)-0.2 g/L EDTA).

Cell viability test

The HF cell (normal cell) and LNCaP cell (cancer cell) were used to assess the cytotoxic effect of *C. aeruginosa* extract and isolated compounds. The LNCaP cell also was used to find a suitable concentration to assess testosterone-induced cell proliferation. Cell viability was determined using MTT assay. This assay based on the ability of viable cells to convert yellow water-soluble dye into insoluble dark blue formazan crystals [66]. The cells were seeded into a 96-well plate at 10^4 cells/well and incubated for 96 hr (HF cells) and 48 hr (LNCaP cells) at 37°C under 5% CO_2 humidified atmosphere. The medium was then changed to serum free RPMI-1640 medium (without phenol red which interferes with the spectrophotometry) and the test compounds for another 22 hr. Then, 10 μL of 5 mg/mL of MTT reagent (in phosphate buffer saline) was added and incubated for 2 hr. The medium was then removed and the formazan produced in the viable cells was solubilized by adding DMSO:EtOH (1:1

v/v). The absorbance at 595 nm was measured using microplate reader. The % cell viability was determined by comparing the absorbance with the control (non-treated).

The anti-androgenic effect of germacrone (1) on testosterone-induced cell proliferation of LNCaP cells

To examine the anti-androgenic effect of compound **1**, LNCaP cells were maintained in phenol red RPMI-1640 medium containing 10% charcoal stripped serum and cultured for 24 hr. After that, the cells were seeded at an initial density of 5,000 cells/well. 48 hr later, the cells were treated with either vehicle or testosterone in presence or absence of varying concentrations of test compounds for another 96 hr. Cell proliferation was quantified using MTT assay. Flutamide (androgen receptor antagonist) and finasteride (5α-reductase inhibitor) were used as positive controls. The cell growth (% of control) was plotted against each group of treatment.

Growth suppression of hamster flank glands by germacrone (1)

Germacrone (**1**) was tested for anti-androgenic activity using *in vivo* assay flank gland model in castrated Syrian hamster in comparison with finasteride. The assay for growth suppression of hamster flank gland was performed according to the method described by Liao and Liang [58,68].

1. Animals and treatments

Pre-pubertal male Syrian golden hamsters were obtained from the National Laboratory Animal Center, Mahidol University. Hamsters were castrated at 7 weeks of age. One week after castration, hamsters were divided into 9 groups with 5-8 animals per group. Hair on the lower back of each animal was removed using electric hair clippers to expose the flank glands. A treatment solution (5 μL) was applied topically to one of the flank glands of the animal daily while another gland was applied with vehicle only. The surface of the flank gland was wiped with an alcohol pad to remove compound residues before each treatment. The treatments were conducted for 4 weeks. The treatment solution contained either (a) vehicle alone (30%PG in ethanol) (b) an androgen (0.5 μg testosterone or DHT) and (c) a combination of the androgen and a test compound (**1**) or 100 μg finasteride. **1** was tested at the doses of 3, 30 and 100 μg. Finasteride was used as positive control. At the end of every week, flank glands from both sides were examined. The body weight of each animal was recorded before and after treatment. At the end of the experiment, animals were sacrificed by overdose injection of an anesthetic. This protocol was approved by the Animal Ethics Committee of Naresuan University.

2. Determination of the size of the flank glands and data analyses

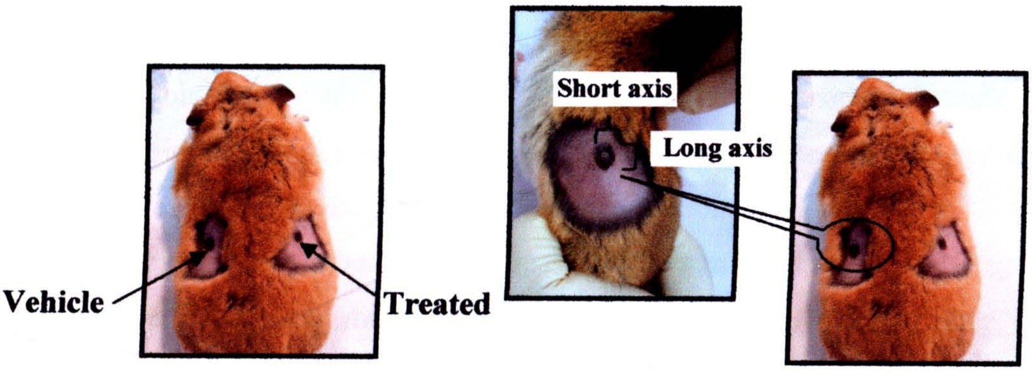

Figure 26 Treatment and measurement on flank glands

In this study, the growth of the flank gland was determined by measuring the length of the long axis and short axis of the pigmented spot with a caliper and compared with androgen treated control group (Figure 26). The paired t-test was used to examine differences between before and after the 4 week-treatment. *P-values* < 0.05 were considered significant. The equation for calculate % inhibition is shown below.

$$\text{Inhibition (\%)} = 100 - [(B \times 100) / A]$$

where A = the increase of the size of the flank gland treated with androgens after 4 weeks (mm^2)

B = the increase of the flank gland size treated with test compound after 4 weeks (mm^2)

Androgen receptor binding assay

The binding activity of **1** on androgen receptor was evaluated using the fluorescent polarization (FP) method [55]. DHT and flutamide were used as an androgen standard and a positive control, respectively. In the assay, the ability of the sample to displace a high affinity fluorescent ligand from androgen receptor-ligand complex at room temperature was measured. The concentration of solvent in this tested compounds preparation was 2% MeOH. Then, 20 μL of test compound was transferred to a black 384-well microplate. A 20 μL mixture of androgen receptor (25 nM final concentration) and fluorescent ligand (1 nM final concentration) was added to the diluted test compounds. The plate was then incubated in the dark for 5 hr at room temperature. The polarization was measured on a microplate reader using 485 nm excitation and 535 nm emission interference filters in polarization mode. The polarization values (mP) were plotted against the concentrations of the test compounds.

Analyses of chemical components of *C. aeruginosa* extract

In this study, three chromatographic methods i.e. TLC, HPLC and GC were optimized for determination of chemical components in *C. aeruginosa* extract.

1. TLC analyses

TLC analyses were performed on a precoated aluminium plate of silica gel 60 F_{254} using the different ratio of hexane:$CHCl_3$ or hexane:EtOAc or hexane:CH_2Cl_2 as the mobile phase. The developing distance was 3.5 cm. After the TLC plate was removed from the chamber, it was dried using an air dryer. Then, the plate was sprayed with anisaldehyde, followed by heating at 110 °C for 5-10 min. The plate was examined under UV cabinet/TLC plate reader at 254 and 366 nm. The R_f value of the component was determined using following the equation below.

$$R_f = \frac{\text{distance from the origin to the spot}}{\text{distance from the origin to the solvent front}}$$

2. HPLC analysis

The separation was performed using Shimadzu HPLC system, equipped with (i) UV detector and (ii) photodiode array detector (PDA). The phenomenex Luna 5u C-18 column (150 x 4.6 mm) was used as a stationary phase. Both isocratic and gradient elution system were optimized. The flow rate was 1.0 mL/min and the injection volume was 20 μL. The UV detector and PDA were set at 235 nm and 214 nm, respectively.

HPLC system 1 (isocratic system); CH_3CN and 0.5 mM phosphate buffer pH 3.0 (73:27) was used. The column was operated at 40°C.

HPLC system 2 (gradient system); a mixture of CH_3CN (A) and 0.5 mM phosphate buffer pH 3.0 (B) was used as a mobile phase. The gradient elution conditions applied were: 0-15 min, linear gradient 30-35% A; 15-25 min, isocratic 40% A; 25-30 min, gradient 40-45% A; 30-40 min, gradient 45-55% A; 50-55 min, isocratic 55% A; and finally washing up column with gradient 65% A for 15 min before reconditioning the column with 30% A. The column was operated at 25°C.

3. GC analysis

GC was performed with a gas chromatography equipped with FID detector (GC-FID). High purity helium was used as carrier gas with flow rate 1.0 mL/min. The other GC conditions; inlet mode, injection temperature and separation temperature program were optimized using 8 compounds (compounds **1-6**, furanodiene **(7)** and germacrene B **(8)**) as references. All compounds were isolated from *C. aeruginosa* extract by ourselves except **7** and **8** which were isolated by our colleague, Mr. Jukkarin Srivilai [82].

GC-FID system 1; the injector was set at 150°C and splitless mode (1.0 μL) was used. The FID detection was set at 250°C. The following GC temperature program was applied: The initial temperature was set at 50°C and held for 2 min, then at the temperature was increased with the rate of 10°C/min until 150°C. Then, the rate was changed to 2°C/min until 190°C and 15°C/min to 250°C and held for 7 min.

GC-FID system 2; the column temperature program was set at 100°C and held for 2 min, then programmed at 15°C/min to 160°, and finally at 3°C/min to 180°C and held for 4 min at 180°C.

The identification of the components was based on the comparison of their retention time with those of reference compounds under the same conditions.

Method validation for determination the chemical components in *C. aeruginosa* extract using GC-FID (system 1)

The GC method for analysis of chemical components in *C. aeruginosa* extract was validated for linearity, limit of detection, precision and accuracy. *C. aeruginosa* extract was used as a sample for the method of validation. Four sesquiterpenes (**1**, **2**, **4**, and **6**) were used as reference compounds.

1. Linearity

Methanol stock solutions containing four compounds were prepared and diluted to 18.75-250 μg/mL. The calibration curves were constructed by plotting the peak areas versus the concentration of the reference compounds.

2. Limit of detection (LOD)and quantification (LOQ)

Methanol stock solutions containing four references compounds were diluted to a series of appropriate concentrations and injected to GC system. The LOD and LOQ under optimized conditions were determined at the ratio of signal to noise (S/N) equal to 3 and 10, respectively.

3. Precision and accuracy

The precision of the developed method was determined by intra-day and inter-day variations. Certain concentration solutions of four reference compounds (**1**, **2**, **4** and **6**) were tested. For intra-day, three different concentrations of four reference compounds (25, 100, 200 μg/mL) were analyzed 3 times within 1 day, while for inter-day variability; the samples were examined in triplicate for consecutive 3 days. Variations were expressed by RSD.

The accuracy of the method was tested by performing recovery studies of 2.0 mg/mL of *C. aeruginosa* extract spike with four reference compounds (10, 50 and 100 μg/mL). The samples were analyzed by the GC-FID method. The percentage recovery as well as the average percentage recovery was calculated. Three determinations were carried out for each level of concentration. The recovery was calculated as follows:

Recovery (%) = (found amount/expected amount) x 100

Method validation for determination germacrone (1) isolated from *C. aeruginosa* extract using GC-FID (system 2)

The GC method was also developed for determination **1** in stability studies. The parameters for method validation were tested in the same fashion as mentioned previously.

1. Linearity

Methanol stock solutions containing **1** were prepared and diluted to 15.625-250 μg/mL. The calibration curve was obtained by plotting the peak area versus the concentration of **1**.

2. LOD and LOQ

The LOD and LOQ were evaluated by serial dilutions of **1** stock solution in order to obtain signal to noise ratios of 3:1 for LOD and 10:1 for LOQ.

3. Precision and accuracy

Intra- and inter-day precisions were determined by the analysis of three concentrations of **1** (25, 100 and 200 μg/mL) and expressed as RSD (%). The recovery test was performed by adding known amount of **1** into a sample solution of **1** (27.40 μg/mL). Three replicates were performed for the test. The recovery was calculated as the mentioned equation above.

Qualitative and quantitative analyses of *C. aeruginosa* from different sources

To study the variation of *C. aeruginosa*, the samples from different sources were tested. In this study, the fresh rhizomes of *C. aeruginosa* were collected from the Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok province in November 2011 and purchased from the market at Pra Sri Rattana Mahathat Vora Maha Vihar temple, Muang district, Phitsanulok province, Thailand. One dried sample was purchased from Si Satchanalai district, Sukhothai province, Thailand.

The powdered of fresh and dried rhizomes of *C. aeruginosa* were extracted with different solvents: CH_2Cl_2 and hexane for 2 days at room temperature and filtered. The maceration procedure was repeated 3 times. The solvent was evaporated under reduced pressure to produce the hexane and CH_2Cl_2 extract. The fingerprints of samples were analyzed using TLC and GC-FID system 1.

In addition, to analyze the content of marker compounds in dried rhizomes, each sample (1000 mg) was accurately weighed and extracted with hexane. The sample was extracted with the same procedure 3 times. Then, extract was transferred to a 25 mL volumetric flask which was made up to its volume with hexane to provide a final concentration 40 mg/mL and filtered through a 0.45 μm. The samples were injected into GC-FID system 1. Four components (**1**, **2**, **4** and **6**) were used as the marker for quality control of *C. aeruginosa*.

Stability study of *C. aeruginosa* extract and its anti-androgenic components

For the stability study, *C. aeruginosa* extract and **1** (which was studied as pure compound) were tested. The samples were prepared and transferred to microcentrifuge tubes and then kept at designed conditions. The 75% relative humidity was achieved by equilibration of the samples over a saturated solution of sodium chloride [83]. Three sample tubes were randomly collected at the various interval times and determined the remaining of marker compounds.

1. **Effect of pH**

Acid-base degradation studies were carried out to determine the pH at which stability of *C. aeruginosa* was maximum. Four different pH; 2.0, 5.5, 7.4 and 9.0 were tested. The buffer solution systems were prepared as follows; HCl/KCl buffer (pH 2.0), acetate buffer (pH 5.5), and phosphate buffer (pH 7.4 and 9.0). The 5 mg/mL of *C. aeruginosa* extract or 0.1 mg/mL of compound **1** were prepared in the mixture solution of PEG-40 hydrogenated castor oil and buffer solution (15:85 v/v). The mixture solutions of sample was transferred to microcentrifuge tubes and stored at room temperature (25°C) for 14 days. The samples were analyzed in various time intervals. All of samples were kept at -80°C before analyses. The remaining of marker compounds was determined using HPLC system 1.

2. **Effect of temperature**

C. aeruginosa extract and **1** were tested both in solid and solution forms. For solid form testing, 5 mg of *C. aeruginosa* extract and 0.1 mg of **1** were prepared and aliquoted in microcentrifuge tubes. For solution form testing, 5 mg/mL of *C. aeruginosa* extract or 0.1 mg/mL of compound **1** were prepared in the mixture solution of PEG-40 and optimized buffer solution (15:85 v/v). The stability of samples was conducted at different temperatures (4, 25 and 45°C) for 6 months. The samples were analyzed in various time intervals (0, 3, 7, 14, 30, 60, 90, 120, 150 and 180 days).

3. Effect of light

Both solid and solution forms of samples were exposed to direct fluorescence light for 7 days. After that, the exposed samples were analyzed for its remaining constituents.

Statistical analysis

The results were expressed as mean ± SD. Statistical comparisons were made by one-way analysis of variance (ANOVA) and independent t-test. Differences were considered to be significant when the p values were < 0.05.