CHAPTER III

EXPERIMENTAL

Plant Material

Root of R. siamensis was collected in February 1981, in Singburi province in central Thailand. It was investigated by taxanomists at the Department of Forestry of the Ministry of Agriculture and at the Department of Botany of Chulalongkorn University in order to prove that it is authentic and free of any contamination.

Chemical Reagents

Commercial grade solvents were distilled before using except ethyl acetate and diethyl ether that were reagent grade.

Equipment.

Precoated silica gel 60F-254 TLC plates from E.Merck, Damstadt Co., Ltd, were used for one dimension thin layer chromatography.

Silica gel Art 7730 Kieselgel H type 60 were used for column chromatography.

Precoated cellulose plates were used for sugar detection.

Melting points were determined by using Mitamura Riken Co., Ltd., and are uncorrected.

The Ultraviolet (UV) spectram were obtained in MeOH on a Shimadzu Model MPS-50L spectrometer.

The Infrared (IR) spectra were recorded in KBr pellets on a Jasco Model IR.S. Spectrophotometer.

The proton magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Model T-60A instrument operating at 60 MHz with a Nicolet, Model TT-7, Fourier Transform attachment. $CDCl_3$ was used as solvent and Tetramethylsilane as the internal standard.

The Mass Spectra were determined by a Varian MAT 112S double focusing mass spectrometer operating at 70 eV.

Gas Liquid Chromatography (GLC) was carried out with a Pye Unicam Pyes series 104. Philips Chromatograph.

Optical rotation, $(\alpha)_D$, was determined by Autopol III automatic polarimeter, Rudolph research Hairfield New Jersey.

Colomn Chromatography.

Silica gel for column chromatography were activated at 110° - 120° C for 2-3 hours prior to use. The calculated amount of activated silica gel was prepared as a 25 % (w/v). Slurry with a predeterminated solvent system. Suitable glass column was chosen for the amount of absorbent to maintain a maximum height to diameter ratio of 10:1. Chromatographed sample, usually 1-4 % wt/wt of the absorbent, was dissolved in smallest amount of a solvent system and carefully added on the top of the column. The column was developed by the suitable solvent systems. An eluent was monitored by thin layer chromatography.

Fehling's Test.

This is a test for reducing sugar. To a solution of 0.2 g of sample in 5 ml of water add 5 ml. of Fehling's solution and heat the mixture to boiling. In the presence of a ketose or aldose. The precipitate occured.

Liebermann-Burchard test.

This test is for steroidal or triterpenoidal compounds. To a compound 1-2 mg in 0.5 ml of chloroform, 2 drops of acetic anhydride were added followed by 1 drop of concentrated sulphuric acid. The color change from blue to green within a few minutes suggested the presence a steroidal or triterpenoidal nucleus in a compound.

Molish's Test.

This is a test for carbohydrate. A sample (2-3mg) placed in a test tube containing 0.5 ml of water was mixed with 2 drops of a 10 % solution of β-naphthol in alcohol. Then 1 ml of concentrated sulphuric acid was carefully dropped down the side of the inclined tube so that the acid formed a layer beneath the aqueous solution. In the presence of a carbohydrate, a red ring appeared at the conjunction of the two liquids, the color quickly change on standing or shaking, resulting in a reddish violet solution.

Extraction.

The air dried root of R.siamensis (4.5 kg) was finely ground and extracted with hot MeOH (15 l, 3 times) in Soxhlet apparatus. The methanolic extract was concentrated on rotatory evaporator and kept in a refrigerator overnight. The precipitate was filtered off. The filtrate was concentrated and then fractionated with C_6H_{14}: H_2O (1:1), $CHCl_3$ and n-BuOH successively. All fractions were concentrated separately under reduced pressure on rotatory evaporator. The yields of extractions with solvents are shown in Table 5.

Table 5 Yields of the Extracted Products.

solvent	extracted weight (g)	% of crude product.
MeOH	352	7.9
n-C_6H_{14}	67	1.5
$CHCl_3$	74	1.64
n-BuOH	146	3.23

Isolation.

Isolation procedure is outlined in Scheme 2 at page 9 . Concentration of methanolic extract of dried root gave much precipitate which was crystallized from hot MeOH to afford Compound 1 (11.2 g), m.p. 166° - 167° C, as white needles.

From $CHCl_3$ soluble fraction (13 g) of methanolic extract,

Compound 2,3 and 4 were obtained by column chromatography on silica gel using $CHCl_3$ and then $CHCl_3$-EtOAc mixture of increasing polarity. $CHCl_3$ fractions gave Compound 2 (160 mg) and Compound 3 (155 mg). Both compounds were further recrystallized from MeOH as white needles m.p. 138° - 140°C and 248° - 250° C respectively. Compound 4 (200 mg) was eluted with $CHCl_3$ containing 20 % of EtOAc and then recrystallized from MeOH as white needles m.p. 242° - 244°C.

n-BuOH soluble fraction (40 g) was chromatographed on silica gel column eluted with the lower phase of the solvent system $CHCl_3$: MeOH : H_2O in the ratio of 65:35:10 yielding Product A (1.2 g) which was crystallized from MeOH : H_2O in the ratio of 1:1 as a pale yellow powder m.p. 230° - 235° C . Acidic hydrolysis of Product A (1.0 g) followed by column chromatography on silica gel using C_6H_6: Et_2O in the ratio of 4:1 and recrystallized from MeOH yielded Compound 5 (120 mg) as white needles m.p. 335° - 338° C .

Purification and Structural Elucidation.

Compound 1, m.p. 166° - 167° C, was crystallized as white needles from hot MeOH (R_f 0.13 in $CHCl_3$:MeOH:H_2O, 65:35:10, lower phase) $(\alpha)_D^{23}$ -1.1° (c 2.07,H_2O). IR (KBr): ν max 3300 (OH), 1100 cm^{-1} (C-O). Its hexaacetate, m.p. 188° - 190° C, gave white needles from MeOH (R_f 0.31 in C_6H_6:Et_2O:MeOH, 8:2:0.5). IR (KBr): ν max 1750, 1350 cm^{-1} ($COCH_3$). ^1H NMR (60 MHz, $CDCl_3$): δ 2.0 (18H,s,$6CH_3$), 4.12 (4H,t, J=4Hz,$2CH_2OAc$) and 4.9-5.5 (4H,m,4CH). Compound 1 was identified as D-mannitol by direct comparison with an authentic sample of D-mannitol (mixed m.p., Co-TLC.).

Compound 2, m.p. 138° - 140° C, was crystallized from $CHCl_3$ as white needles. It gave positive blue colour in Liebermann-Burchard test. IR (KBr) : νmax 3400 (OH), 840,810 (trisubstituted double bond). $C_{29}H_{50}O$. MS : m/e 414 (M^+), 273 (M^+-$C_{10}H_{18}$), 255 (M^+- $C_{10}H_{20}O$). Its acetate, m.p. 118°- 120° C, was crystallized from $CHCl_3$ as white needles. ^1H NMR (60 MHz, $CDCl_3$) : δ 0.66-1.0 (18 H, $6CH_3$), 2.02 (3H, s, $COCH_3$), 4.60 (1H, broad singlet, H at C-3); 5.40 (1H, m, H at C-6). Compound 2 was elucidated as β-sitosterol by direct comparison with authentic sample (mixed m.p., Co-TLC). GLC analysis (condition : column OV-1 3 %, 60-80 mesh, column length 4 mm x 1.5 m, column temp 270° C, FID 300° C chart speed 1cm/min, carrier gas (N_2) 45 ml/min) revealed that compound 2 was in fact a mixture of β-sitosterol and campesterol (R_t 3.2 and 2.8 min, respectively) in the ratio of 3:2. These two steroids could not be separated by TLC in different solvent systems.

Compound 3, m.p. 248° - 250° C, was crystallized from MeOH as white needles (R_f 0.35 in $CHCl_3$: MeOH : 7 % HOAc, 250:80:50, lower phase). It gave pink colour in Liebernann-Burchard test. IR (KBr): νmax 3200 (OH), 1740, 1260 ($COCH_3$), 1695 cm^{-1} (COOH). $C_{32}H_{50}O_4$. MS: m/e 498 (M^+) 438 (M^+-$C_2H_4O_2$), 248 (M^+-$C_{16}H_{26}O_2$), 203 (M^+-$C_{17}H_{27}O_4$). ^1H NMR (60 MHz, $CDCl_3$): δ 0.88-1.13 (21H, $7CH_3$), 2.03 (3H, s, $COCH_3$), 4.60 (1H, m, H at C-3), 5.30 (1H, m, H at C-12). Deacetylation of Compound 3 followed by crystallization from MeOH gave oleanolic acid, m.p. 310° C, which was identified by direct comparison with an authentic sample (mixed m.p., Co-TLC). Compound 3 was elucidated as 3β-acetyl oleanolic acid.

Compound 4, m.p. 242° - 244°C, was crystallized from MeOH as white needles (R_f 0.58 in $CHCl_3$: MeOH : 7 % HOAc, 250:80:50, lower phase) $(\alpha)_D^{23}$ +47.5°. Gave pink colour in Liebermann-Burchard test and positive result in Molish test. IR (KBr): νmax 3400 (OH), 1700 (COOH) and 1100-1000 (glycosidic linkage). Permethylated Compound 4 was crystallized from $CHCl_3$ as white needles m.p. 170° - 172° C (R_f 0.60 in C_6H_6: Dioxane : HOAc, 90:25:4). $C_{39}H_{64}O_7$. MS : m/e 644 (M^+), 469 ($M^+-C_8H_{15}O_4$), 262 ($M^+-C_{22}H_{38}O_5$), 203 (RDA-CO_2Me), 175 ($M^+-C_{31}H_{49}O_3$). Methanolysis of permethylated derivative of Compound 4 gave methyl oleanolate m.p. 201° - 202° C which was identified by direct comparison with an authentic sample (mixed m.p., Co-TLC). The sugar was found to be methyl -2,3,4-tri-O-methyl arabinoside by GLC analysis (see page 41). Peracetylated derivative of Compound 4, m.p. 148° - 150° C, was crystallized from MeOH as white needles. ^{1}H NMR (60 MHz, $CDCl_3$): δ 0.73-1.15 (21 H, 7 CH_3), 2.05 (6H, s, 2$COCH_3$), 2.08 (3H, s, $COCH_3$), 4.21 (1H, d, J=6Hz, anomeric proton). The specific rotation of compound 4 and oleanolic acid are +47.5° and +58.65° respectively. The molecular rotation difference of Compound 4 and oleanolic acid equal to +12.1° which indicated that the orientation of arabinose was α -L-form. Compound 4 was elucidated as 3-O- [α -L-arainopyranosyl] oleanolic acid.

Compound 5 n-BuOH soluble fraction (40 g) was chromatographed on a silica gel column eluted with the lower phase of the solvent system $CHCl_3$: MeOH : H_2O in the ratio of 65:35:10 yielding Product A (1.2 g, R_f 0.33 in EtOAc:MeOH:H_2O, 600:99:81).

Product A, m.p. 230° - 235° C, was crystallized from MeOH:H_2O (1:1)

as a pale yellow powder (R_f 0.33 in EtOAc:MeOH:H_2O, 600:99:81) which gave pink colour in Liebermann-Burchard test and positive result in Molish test. IR (KBr): ν max 3400 (OH), 1050 cm^{-1} (glycosidic linkage). Acidic hydrolysis of Product A (see page 42).gave a mixture of 2 sapogenins. (R_f 0.40,0.32 in C_6H_6:Et_2O, 4:1,developed 3 times). Column chromatography on silica gel eluted with C_6H_6:Et_2O in the ratio of 4:1 gave one sapogenin (120 mg, R_f 0.40 in C_6H_6:Et_2O, 4:1,developed 3 times) which was assignated as Compound **5**, m.p. 335° - 338°C, was crystallized from MeOH as white needles (R_f 0.40 in C_6H_6:Et_2O, 4:1, developed 3 times). IR (KBr): νmax 3400 (OH), 1700 cm^{-1}(COOH). $C_{30}H_{48}O_4$. MS:m/e 472 (M^+) 454 (M^+-H_2O),264 (RDA), 219 (RDA-CO_2H). Its acetate,m.p. 230° - 232° C,was crystallized from MeOH as white needles (R_f 0.46 in C_6H_6:Et_2O, 4:1). IR (KBr):ν max 1700 (COOH), 1725 ($COCH_3$). Oxidation of its acetate (see page 42)gave a hetero annular diene,m.p. 280° - 282° C,as white needles from MeOH. λ max:241.5, 251.5 and 261 nm. Methyl ester of Compound 5 gave white needles from MeOH (R_f 0.22 in C_6H_6:Et_2O, 4:1, developed 3 times) m.p. 215° - 217°C . IR (KBr): νmax 1725 cm^{-1}($OCOCH_3$). Reduction of Compound 5 methyl ester gave triol of Compound 5,m.p. 250° - 252° C,as white powder from MeOH (R_f 0.38 in Tol:Ethyl formate: Formic acid 5:4:1): IR (KBr): νmax 3400 cm^{-1} (OH). Mono bromo γ-lactone of Compound 5,m.p. 240° - 242° C,was crystallized as white needles from MeOH (R_f 0.26 in Tol : Ethyl formate : Formic acid, 5:4;1). IR (KBr): ν max 1790 cm^{-1} (lactone ring). Its methyl ester acetate,m.p. 234° - 237° C,gave white needles from MeOH (R_f 0.70 in C_6H_6:Et_2O, 4:1). ^1H NMR (60MHz, $CDCl_3$) : δ 0.72-1.10 (18 H, 6 CH_3), 2.0 (3H,s,$COCH_3$), 2.03 (3H,s,$COCH_3$), 3.6 (3H,s,OCH_3), 5.25

(1H,broad singlet,H at C-12), 4.45 (1H,m,H at C-3).

Compound 5 was elucidated as 3β, 29-dihydroxy-olean-12-ene-28-oic acid known also as mesembryanthemoidigenic acid.

Chemical Reactions:

Acetylation of Compound 1.

To Compound 1 (1.1 g) in dry pyridine (10 ml) acetic anhydride (20 ml) was added and the reaction mixture was left at room temperature for 48 hrs. Then it was pour onto ice water, precipitate was filtered off washed with water and crystallized from MeOH to yield hexaacetate of Compound 1 (1.08 g) m.p. 188° - 190° C.

Acetylation of Compound 2.

Compound 2 (5 mg) was acetylated as described above. The acetate of Compound 2,m.p. 118° - 120° C,was recrystallized from MeOH. Resulted product (3 mg) was further subjected for ^{1}H NMR.

Deacetylation of Compound 3.

Compound 3 (10 mg) was refluxed with 10 % NH_4OH in MeOH for 3 hrs.then the reaction mixture was concentrated under reduced pressure and the precipitate was recrystallized from MeOH yielding oleanolic acid (7 mg),m.p. 310° C,which was identified by direct comparison with an authentic sample (mixed m.p. Co-TLC).

Hydrolysis of Compound 4.

Compound 4 (8 mg) was hydrolyzed by refluxing with 5 % H_2SO_4 in EtOH for 5 hrs. EtOH was evaporated under reduced pressure. The residue was diluted with water and extracted with Et_2O. Ethereal solution was evaporate and the residue was recrystallized from MeOH yielding aglycone (3 mg) which was found to be identical with oleanolic acid m.p. 310° C (mixed m.p, Co-TLC).

Trimethysilylation of Carbohydrate Component of Compound 4.

Acidic hydrolysis of Compound 4 as described above, yielded aqueous layer which was neutralized with $BaCO_3$. The precipitate was filtered off, washed with water and the combined aqueous extracts were further evaporated in high vacuum oven at room temperature. Dried sample was allowed to react with trimethylsilylether (0.5 ml) for 15 min at room temperature. The water was added and the product was extracted three times with petroleum ether. Petroleum ether extract was dried over anhydrous Na_2SO_4 and filtered. The filtrate evaporation yielded the sample which was subjected for GLC analysis. (Condition : column OV-1 5 % , column temperature 140° - 180° C, carrier gas (N_2) 30 ml/min, chart speed 0.5 cm/min.

The GLC data showed the presence a sole carbohydrate (R_t 1.6 min) which was identified as arabinose by comparision in GLC with an authentic sample of the trimethylsilyl derivative of this carbohydrate.

Permethylation of Compound 4.[23]

To Compound 4 (30 mg) in DMF (3ml) containing NaH (40 mg) was added with stirring, followed by CH_3I (2 ml). The reaction mixture was left at room temperature for 24 hrs, then extracted with H_2O:$CHCl_3$ (1:1). Organic layer was washed with water, dried over anhydrous $Na_2S_2O_3$, concentrated and crystallized from MeOH to yield white needles (23 mg) m.p. 170° - 172° C.

Methanolysis of Permethylated Compound 4.

Permethylated Compound 4 (10 mg) in 5 % HCl-MeOH (3ml) was refluxed for 5 hrs.then reaction mixture was concentrated. H_2O was added, the precipitate was filtered off and recrystallized from MeOH to yield methyl oleanolate (4 mg),m.p. 201° - 202° C, identified by direct comparison with an authentic sample (mixed m.p, Co-TLC)

The filtrate was extracted with $CHCl_3$, the extract was evaporated to dryness. The residue was found to be methyl -2,3,4-tri-O-methyl arabinopyranoside on the basic of GLC data (R_t 3.4 min; condition:column 5 % NPGS, column length 4 mm/1.5min, column temp 180° C, injector temp.200° C, carrier gas N_2 4.5 ml/min) and by direct comparisen with an authentic sample (mixed m.p, Co-TLC).

Acetylation of Compound 4.

Compound 4 (30 mg) was acetylated as described for acetylation of Compound 1 yielding white needles (26 mg),m.p. 148° - 150° C,after

crystallization from MeOH.

Acid Hydrolysis of Product A.

Product A (1 g) was hydrolyzed by refluxing with 10 % HCl in MeOH for 6 hrs. Solvent removal under reduced pressure left the residue which was diluted with water and extracted with Et_2O. Ethereal solution was evaporated and subjected for TLC. (R_f 0.16 in C_6H_6:Et_2O:MeOH, 8:2:0.5). TLC data indicated the presence of 2 sapogenins (R_f 0.40, 0.32 in C_6H_6:Et_2O, 4:1, developed 3 times). Silica gel column chromatography of the sapogenins by using C_6H_6:Et_2O, 4:1 as an eluting mixture gave Compound 5 (120 mg) m.p. 335° - 338° C.

Acetylation of Compound 5.

Compound 5 (10 mg) was acetylated as it was described above yielding its acetate (9 mg), m.p. 230° - 232° C, which was crystallized from MeOH.

Oxidation of Compound 5 Acetate with SeO_2.[35,43]

Compound 5 acetate (10 mg) and freshy prepared SeO_2 (10 mg) in HOAc (1 ml) were refluxed for 2 hrs. The solution was filtered, the filtrate was diluted with water and extracted with Et_2O. The ethereal solution was washed with 10 % $NaHCO_3$ and then with water, dried and evaporated, yielding the residue as a white powder of Compound 5 hetero annular diene (7 mg) m.p. 280° - 282° C.

Methylation of Compound 5.

Compound 5 (30 mg) was dissolved in MeOH and treated with ethereal diazomethane. The crude product was crystallized from MeOH as needles (30 mg) m.p. 215° - 217° C.

Saponification of Compound 5 Methyl Ester.

Compound 5 methyl ester (10 mg) was added to 5 and 8 % KOH in EtOH and refluxed for 8 hrs. They were examined by TLC that the saponification was not occured. Then reaction was repeated by 10 % KOH in EtOH and refluxed for 8 hrs. EtOH was evaporated under vacuum, the residue was diluted with water and extracted with Et_2O. Ethereal solution was evaporated and the residue was found to be a Compound 5 methyl ester (2.8 mg) by direct comparision with an authentic sample (mixed m.p, Co-TLC).

H_2O layer was neutralized with 5 % HCl, extracted with Et_2O filtered out then the filtrate was evaporated and identified as Compound 5 (5.1 mg) by direct comparision with an authentic sample (mixed m.p, Co-TLC).

Reduction of Compound 5 Methyl Ester.

Compound 5 methyl ester (10 mg) was dissolved in dry tetrahydrofuran (20 ml) and $LiAlH_4$ (50 mg) was added slowly with stirring. The mixture was refluxed for 3 hrs., then filtered and concontrated, yielding Compound 5-triol (5 mg), m.p. 250° - 252° C, as white powder from MeOH.

Monobromo γ-Lactone of Compound 5.[38]

Compound 5 (5 mg) was dissolved in HOAC containing NaOAc (2 mg) then 3 % solution of bromine in HOAc (1 ml) was added dropwise. The mixture was kept at room temperature for 3 hrs. and then poured onto water containing $Na_2S_2O_3$ (500 mg) to discharge excess of bromine. The precipitate was filtered, washed thoroughly with water, dried and crystallized from MeOH to give Compound 5 mono bromo γ-lactone (2 mg) m.p. 240° - 242°C.

Methylation of Compound 5 Acetate.

Compound 5 acetate (5 mg) was methylated as described for methylation of Compound 5 yielding white needles (5 mg), m.p. 234° - 237° C, from MeOH.