CHAPTER III

EXPERIMENTAL

1. *Typha elephantina* Roxb. Fruits

1.1 Source of Plant Materials

The fruits of *Typha elephantina* Roxb. were collected from Salaya District, Nakhonpathom Province, Thailand, in August 1984 and authenticated by comparison with herbarium specimens at Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. A voucher specimen of plant material has been deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

1.2 General Techniques

1.2.1 Thin-layer Chromatography (TLC)

Analytical

Technique	:	one way, ascending
Adsorbent	:	silica gel G (E. Merck) 30 gm/60 ml of distilled water
Plate size	:	10 cm x 20 cm and 20 cm x 20 cm
Layer thickness	:	250 μ
Activation	:	air dried for 15 minutes and then at 110°C for 1 hour.

Solvent system : a) silica gel G/diethyl ether:petroleum ether(5:95)

b) silica gel G/diethyl ether:petroleum ether(2:3)

c) silica gel G/chloroform

d) silica gel G/chloroform : methanol (95:5)

Distance : 15 cm

Laboratory temperature : 24-30℃

Detection on chromatographic plate

: chromogenic reagent employed was Liebermann - Burchard spray reagent (acetic anhydride - sulfuric acid)

spray reagent :-

5 ml acetic anhydride are carefully mixed with 5 ml conc. sulfuric acid. This mixture is added cautiously to 50 ml absolute ethanol.

colours developed :-

Plate after spraying, was warmed in hot air oven. The colour reaction is blue green or blue spots indicated the presence of steroidal compound.

1.2.2 Column Chromatography (CC)

Adsorbent : silica gel 0.040-0.063 mm (E. Merck)

Packing of column: dry packing

Sample loading : the portion of crude extract was dissolved in small amount of organic solvent, mixed with small quantity of adsorbent, are dried, triturated and added onto the top of a dry column.

Solvent : a) chloroform

b) chloroform : methanol (95:5)

Detection : Liebermann-Burchard Test (For Steroid or triterpenoid Compound)

To dissolve 1-2 mg of a compound in 0.5 ml of chloroform, 2 drops of acetic anhydride were added and followed by 1 drop of conc. sulfuric acid. The colour change from blue to green within a few minutes indicated the presence of steroid compound and pink to pink-red indicated the presence of triterpenoid compound.

1.2.3 Physical Constants

Melting Points : Melting points were determined by Gallenkamp Melting Point Apparatus with digital thermometer. Model MFB-595. Results were uncorrected.

1.2.4 Spectroscopy

Infrared (IR) Absorption Spectra

Infrared absorption spectra were obtained on a Perkin-Elmer 1330 Spectrophotometer for TE-1 and TE-2, and a Shimadzu Model IR 440 Spectrophotometer for TE-3 and TE-4, absorption bands are reported in wave number (cm^{-1})

Nuclear Magnetic Resonance (NMR) Spectra

Proton (^{1}H) and Carbon-13 (^{13}C) nmr spectra were taken on a Bruker WH 400 and AM 250 (^{1}H-NMR at 400 MHz and ^{13}C-NMR at 100 MHz) and Jeol GX-270

(^{1}H-NMR at 270 MHz and ^{13}C-NMR at 67.80 MHz) spectrometers respectively, with tetramethylsilane (TMS) as internal standard and deuterochloroform ($CDCl_3$) as solvent except for TE-4 which the solvent was pyridine-d_5 (C_5D_5N). The chemical shifts were reported on the δ value (ppm.)

Mass Spectra

Mass spectra were recorded on a Varian MAT CH7 or VG Micromass 7070 F for TE-1 and TE-2, a Mass Spectrometer Model DX 300 (Jeol) operating at 70 eV for TE-3, and a Hitachi High Resolution mass spectrometer operating at 70 eV with inlet temperature 130°C for TE-4.

1.2.5 Authentic Samples

1.2.5.1 β-sitosterol obtained from *Cissus quadrangularis* Linn. kindly supplied by Dr. Ekarin Saifah.

1.2.5.2 β-sitosteryl 3-O-β-D-glucopyranoside obtained from *Mitragyna hirsuta* Havil. kindly supplied by Mr. Kittisak Likhitwitayawuid.

1.3 Extraction and Isolation of TE-1 to TE-4 from *Typha elphantina* Roxb. Fruits

1.3.1 Extraction

The fresh plant (10 kg) was blended with 95% ethanol (36 litres) and macerated twice for 3 day-periods and filtered.

After combination, the ethanolic extract was evaporated under reduced pressure to dryness. The residue was suspended in petroleum ether and filtered. The remain residue was washed several times with petroleum ether till no traces of steroid could be detected. The combined filtrate was concentrated under reduced pressure to dryness. It was yielded 18.5 g of greenish mass and was designated as crude TE.

1.3.2 Isolation

The crude TE was divided into 18 equal portions and each one was treated in the same manner. Each portion was chromatographed on a silica gel column (2.5 x 16 cm). Elution with chloroform afforded 30 fractions and chloroform : methanol (95:5) afforded 15 fractions respectively. Twenty five ml of each fraction was collected and compared by TLC. Those fractions of similar pattern were combined and evaporated to dryness

a) fraction 1 was designated as TE-1 (42 mg, 0.00042%)

b) fracitons 13-14 were designated as TE-2 (35 mg, 0.00035%)

c) fractions 17-22 were designated as TE-3 (180 mg, 0.0018%)

c) fractions 35-39 were designated as TE-4 (92 mg, 0.00092%)

1.4 Identification of Isolated Compounds

The isolated compound were identified by comparison of hRf values, melting points, infrared absorption spectra, nuclear magnetic resonance spectra and mass spectra with authentic samples.

1.4.1 Identificaiton of TE-1 as Pentacosane

TE-1 was crystallised from absolute ethanol as white needle. It was soluble in chloroform, diethyl ether, petroleum ether and acetone

hRf value

The hRf values given are obtained with the following systems : -

a) silica gel G/diethyl ether : petroleum ether (5:95) = 93

b) silica gel G/diethyl ether : petroleum ether (2:3) = 98

c) silica gel G/chloroform = 100

d) silica gel G/chloroform : methanol (95:5) = 100

The thin layer chromatograms of pentacosane (TE-1) are shown in Figures 8-11 (pp. 130-133).

Melting Point

50.5-52℃

Molecular Weight

$(M^{\cdot} + H^{+})$ = 352 (C.I. mass spectrometry)

Infrared Absorption Spectrum

ν_{max} (cm^{-1})

2920, 2843 and 1468

(Figure 19, p. 141)

Proton NMR Spectrum (in $CDCl_3$, 400 MHz)(Figure 20, p. 142)

Chemical Shift(δ)	Proton	Multiplicity	Coupling Constant
0.88	$-CH_3$(6H)	t	J = 6.0 Hz
1.26	$-CH_2-$(46H)	s	

Carbon-13 NMR Spectrum (in $CDCl_3$, 100 MHz)(Figure 21, p. 143)

Carbon Position	Chemical Shift(δ)
C-1, C-25	14.1
C-2, C-24	22.7
C-3, C-23	32.0
C-4, C-22	29.4
C-5 to C-21	29.8

Mass Spectrum (EIMS)

m/z (%, relative intensity)(Figure 22A, p. 144)

352 (M^+, 5.5), 323 (2.9), 309 (4.8), 295 (5.0)
281 (5.9), 267 (7.5), 253 (9.4), 239 (10.3),
225 (11.1), 211 (13.9), 197 (17.5), 183 (16.6),
169 (20.2), 155 (36.9), 141 (39.4), 127 (47.1),
113 (72.8), 99 (100), 85 (100), 69 (100), 57 (100),
55 (100), 43 (100), 41 (100) and 29 (100)

Mass Spectrum (CIMS)

m/z (%, relative intensity)(Figure 22B, p. 145)

352 (M^+, 18.4), 351 (M-1, 66.9), 323 (100),
295 (9.4), 285 (15.5), 281 (12.3), 267 (17.6),
253 (21.3), 239 (23.4), 225 (26.1), 211 (25.4),
197 (27.3), 182.9 (31.4), 169 (30.9), 155 (32.9),
141 (33.57), 127 (37.6), 113 (39.4), 99 (47.8),
97 (20.8), 85 (98.6), 83.9 (20.1), 71 (100) and
57 (100)

From the above data, TE-1 was identified as a long chain hydrocarbon. It is in complete agreement with published value of pentacosane (53-56). Therefore it is concluded that TE-1 is a pentacosane. The structure of which is shown below.

TE-1

PENTACOSANE

1.4.2 Identification of TE-2 as 1-Triacontanol

TE-2 was crystallised from methanol as white amorphous solid. It is soluble in chloroform, ether and benzene.

hRf value

The hRf values given are obtained with the following system :-

a) silica gel G/diethyl ether : petroleum ether (5:95) = 85

b) silica gel G/diethyl ether : petroleum ether (2:3) = 53

c) silica gel G/chloroform = 64

d) silica gel G/chloroform : methanol (95:5) = 90

The thin layer chromatograms of 1-Triacontanol (TE-2) are shown in Figures 8-11 (pp. 130-133).

Melting Point

82-83°C

Molecular Weight

438 (C.I. mass spectrometry)

Infrared Absorption Spectrum

ν_{max} (cm^{-1})

3250 (broad), 2920, 2840 and 1460

(Figure 23, p. 146)

Proton NMR Spectrum (in $CDCl_3$, 400 MHz)(Figure 24, p. 147)

Chemical Shift(δ)	Proton	Multiplicity	Coupling Constants
0.88	$-CH_3$(3H)	t	J = 6.6 Hz
1.26	$-CH_2$(56H)	s	
2.16	-OH(1H)	s	
3.63	$-CH_2$-OH(2H)	t	J = 6.0 Hz

Carbon-13 NMR Spectrum (in $CDCl_3$, 100 MHz)(Figure 25, p. 148)

Carbon Position	Chemical Shift(δ) ppm
C-1	63.1
C-2	32.9
C-3	25.8
C-4	29.4
C-5 to C-27	29.7
C-22	32.0
C-29	22.7
C-30	14.1

Mass Spectrum (EIMS)(Figure 26A, p. 149)

m/z (%, relative intensity)(Figure 26A, p. 149)

no M^+ observed, 421 (M^+-OH, 1.63), 420 (M^+-H_2O, 5.23) 392 (M^+-46, 343), 252 (1.47), 238 (1.72), 237 (1.89), 236 (1.47), 224 (2.0), 210 (2.5), 196 (2.7), 182 (3.9), 181 (4.8), 167 (6.7), 154 (5.23), 153 (10.13), 139 (14.71), 125 (29.58), 111 (53.69), 97 (100), 85 (42.5), 83 (93.4), 71 (61.2), 69 (59.7), 57 (100), 55 (35.3), 43 (36.4), 41 (5.9) and 29 (1.9)

Mass Spectrum (CIMS)(Figure 26B, p. 150)

m/z (%, releative intensity)(Figure 26B, p. 150)

439 (M+1, 4.03), 438 (M^+, 8.27), 437 (M-1, 25.06), 435 (4.89), 423 (6.64), 422 (32.58), 422 (100), 420 (9.00), 419 (6.49), 410 (6.49), 409 (4.83), 407 (6.73), 394 (5.04), 393 (17.39), 323 (3.99), 309 (4.95), 295 (4.83), 281 (5.34), and 266 (5.68)

The E.I. mass spectrum does not show a parent peak because of the easy loss of H_2O but the cracking pattern is just that expected for a long-chain linear alcohol.

The physical and spectral data of this compound are in complete agreement with the structure of 1-triacontanol (58). It is therefore concluded that TE-2 is 1-triacontanol and the structure of which is shown below.

$$\overset{30}{C}H_3-\overset{29}{C}H_2-\overset{28}{C}H_2-(CH_2)_{23}-\overset{4}{C}H_2-\overset{3}{C}H_2-\overset{2}{C}H_2-\overset{1}{C}H_2-OH$$

TE-2

1-TRIACONTANOL

1.4.3 Identification of TE-3 as β-sitosterol

TE-3 was obtained as white needles from absolute ethanol. It was soluble in chloroform, acetone and ethyl acetate. It gave a pink color which immediatly changed to blue and finally to green when treated with Liebermann-Burchard's reagent. This color reaction was suggested that TE-3 might be steroid in nature.

hRf value

The hRf values given are obtained with the following systems :-

a) silica gel G/diethyl ether : petroleum ether (5:95) = 27

b) silica gel G/diethyl ether : petroleum ether (2:3) = 40

c) silica gel G/chloroform = 55

d) silica gel G/chloroform : methanol (95:5) = 77

The thin layer chromatograms of β-sitosterol (TE-3) are shown in Figures 8-11 (pp. 130-133).

Melting Point

137-140°C

Molecular Weight

M^+ = 414 (E.I. mass spectrometry)

Infrared Absorption Spectrum (KBr disc)(Figure 27, p. 151)

ν_{max} (cm^{-1})

3520 (broad), 2950, 2850, 1650, 1450, 1390, 1380

1060, 1020 and 800

Proton NMR Spectrum (in $CDCl_3$, 270 MHz)(Figure 28, p. 152)

Chemical Shift(δ) ppm	Proton	Multiplicity
0.68	18-CH_3(3H)	s
0.78	29-CH_3(3H)	t
0.82	26,27-CH_3(6H)	d
0.92	21-CH_3(3H)	d
1.01	19-CH_3(3H)	s
3.5	3-H(1H)	m
5.3	6-H(1H)	t

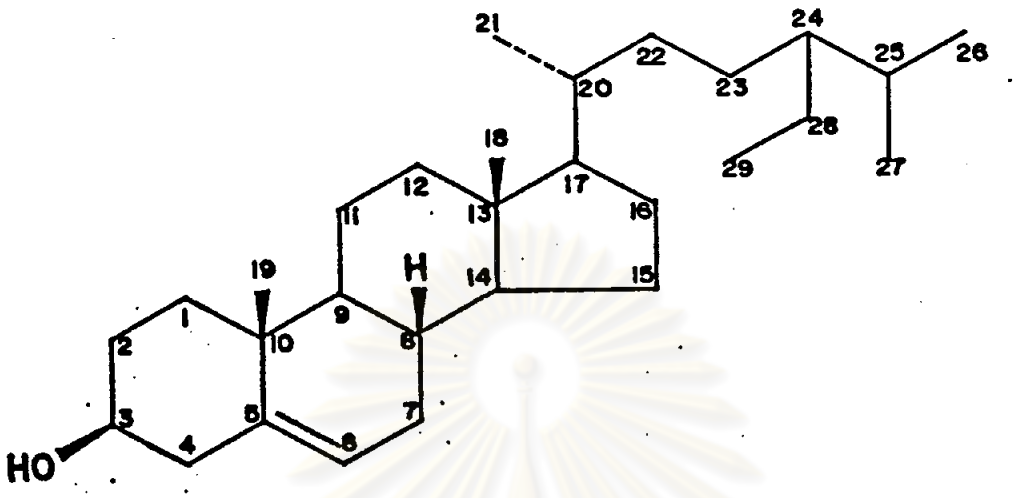
Carbon-13 NMR spectrum (in $CDCl_3$, 67.8 MHz)(Table 3, p. 77 and Figure 29, p. 153).

Mass Spectrum (EIMS)(Figure 30, p. 154)

m/z (%, relative intensity)

414 (M^+, 84.9), 399.2 (20.7), 396.3 (100), 381.3 (35.7), 329.2 (29.1), 273.2 (24.8), 255 (61.1), 231.1 (19.3), 213.1 (40.8), 173.1 (21.4), 163.1 (37.0), 161.1 (47.3), 159.1 (58.0), 147 (66.1), 145 (84.7), 135 (40.8), 133 (54.8), 131.0 (42.8), 121.0 (53.3), 119 (50.9), 109 (47.8), 107 (75.1), 104.9 (78.8), 95 (74.8), 93.0 (63.7), 90.9 (65.3), 83.0 (50.1), 81.0 (100), and 43 (91.8)

These data are in complete agreement with published values of β-sitosterol (57,59-64) and the structure was confirmed by comparison (mmp., TLC) with an authentic sample. It is therefore concluded that TE-3 is β-sitosterol unambiguously. The structure of which is shown on page 73.



TE-3

β-SITOSTEROL

1.4.4 Identification of TE-4 as β-sitosteryl 3-O-β-D glucopyranoside

TE-4 was crystallized from chloroform : petroleum ether as white amorphous solid. It was soluble in chloroform, ethyl acetate and slightly soluble in ethanol. It gave a green violet color with Liebermann-Burchard's reagent. This colour reaction is indicated that TE-4 might be a steroidal compound.

hRf value

The hRf values given are obtained with the following

systems :-

a) silica gel G/diethyl ether : petroleum ether (5:95) = 0

b) silica gel G/diethyl ether : petroleum ether (2:3) = 5

c) silica gel G/chloroform = 16

d) silica gel G/chloroform : methanol (95:5) = 27

The thin layer chromatograms of β-sitosteryl 3-O-β-D-glucopyranoside (TE-4) are shown in Figures 8-11 (pp. 130-133).

Melting Point

255°C

Molecular Weight

566

Infrared Absorption Spectrum (KBr disc)(Figure 31, p. 155)

ν_{max} (cm^{-1})

3400 (broad), 2950, 2850, 1640, 1460, 1380, 1360, 1160, 1100, 1070, 1020 and 800

Proton NMR Spectrum (in pyridine-d_5, 270 MHz)(Figure 32, p. 156)

Chemical Shift(δ) ppm	Proton	Multiplicity
0.68	18-CH_3(3H)	s
0.86	29-CH_3(3H)	t
0.88	27,26-CH_3(3H)	d,d
0.91	21-CH_3(3H)	d
1.01	19-CH_3(3H)	s
4.01	3-H(1H)	m
4.1	6'-CH_2(2H)	t

Chemical Shift(δ) ppm	Proton	Multiplicity
4.25-4.35	3'-H, 4'-H(1H, 1H)	t,t
4.4 -4.45	2'-H(1H)	d,d
4.55-4.6	5'-H(1H)	d,t
5.18	1'-H	d
5.35	6-H(1H)	t

Carbon-13 NMR Spectrum (in pyridine-d_5, 67.8 MHz)(Table 3, p. 77 and Figure 33, p. 157).

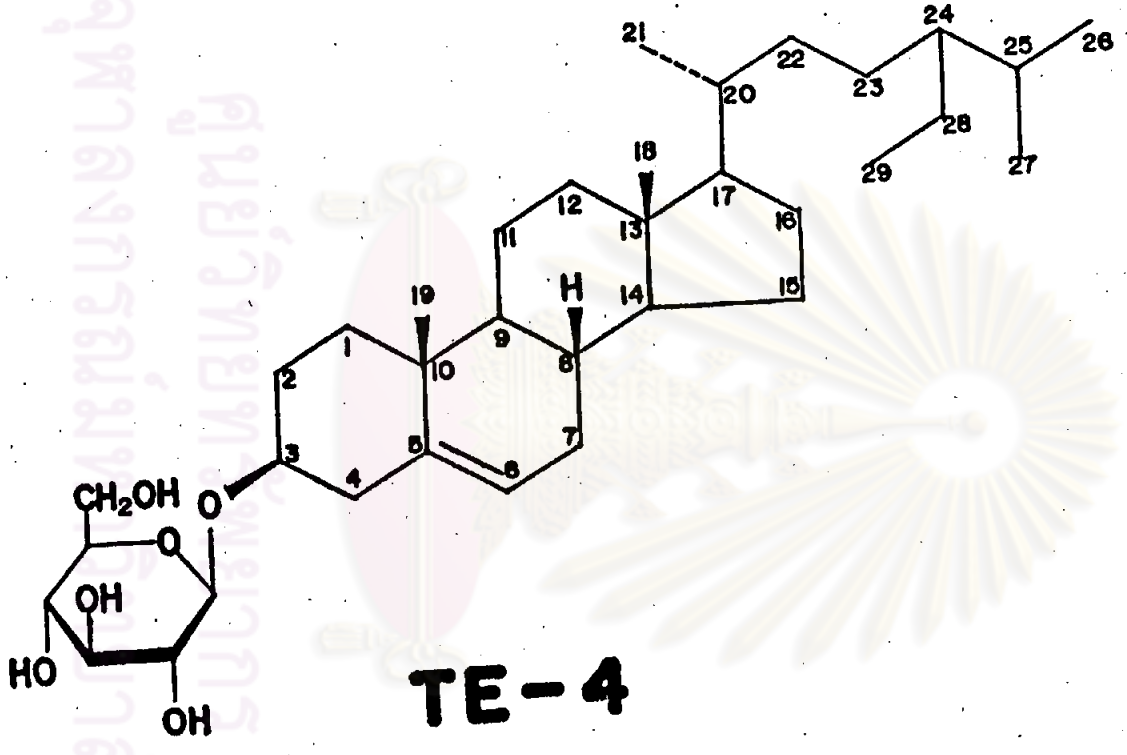
Mass Spectrum (EIMS)

m/z (%, relative intensity)(Figure 34, p. 158)

396 (M^+ -$C_6H_{12}O_6$, 100), 369 (2.9), 297 (4.6), 275 (6.7), 255 (11.4), 213 (8.2), 173 (6.9), 147 (20.6), 125 (3.5), 107 (15.1), 83 (26.7) and 43 (25.7)

The MS showed diagnostically important peaks which were similar to those of TE-3. This immediately suggested that TE-4 was identical to or very similar to β-sitosterol. However melting point and the spectroscopic data of TE-4 were compared with those of β-sitosterol, it was concluded that TE-4 was not β-sitosterol.

These data are in agreement with the structure of β-sitosteryl 3-O-β-D-glucopyranoside (64,65) for this isolated compound. The structure was confirmed by comparison (mp, mmp, tlc) with an authentic sample. It is therefore concluded that TE-4 is β-sitosteryl 3-O-β-D-glucopyranoside, the structure of which is shown on page 76.



TE-4

β-SITOSTERYL 3-O-β-D GLUCOPYRANOSIDE

Table 3 ^{13}C-Chemical shift(δ) of TE-3 and TE-4

Carbon Position	Chemical Shift(δ) ppm	
	TE-3	TE-4
C-1	37.288	37.489
C-2	31.702	30.262
C-3	71.812	78.118
C-4	42.327	39.361
C-5	140.774	140.918
C-6	121.712	121.914
C-7	31.932	32.191
C-8	31.932	32.076
C-9	50.188	50.360
C-10	36.539	36.942
C-11	21.250	21.307
C-12	39.822	39.966
C-13	42.327	42.500
C-14	56.810	56.868
C-15	24.330	24.532
C-16	28.275	28.563
C-17	56.090	56.263
C-18	12.007	12.007
C-19	19.061 *	19.435 **
C-20	36.194	36.395
C-21	18.802 *	19.032 **
C-22	32.977	34.236
C-23	26.145	26.432
C-24	45.868	46.076
C-25	29.197	29.485
C-26	19.435 *	19.983 **
C-27	19.839 *	19.234 **
C-28	23.121	23.409
C-29	11.891	12.179
1'	-	102.593
2'	-	75.325
3'	-	78.435
4'	-	71.693
5'	-	78.579
6'	-	62.857

* and ** these assignments may be interchanged.

2. *Randia siamensis* Craib Fruits

2.1 Source of Plant Materials

The fruits of *Randia siamensis* Craib were collected from Nakhonpathom Province, Thailand, in July, 1984. Authentication was achieved by comparison with herbarium specimens at Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. A voucher specimen of plant material has been deposited in the Herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2.2 General Techniques

2.2.1 Thin-layer Chromatography (TLC)

Analytical

Technique : one way, ascending

Adsorbents : silica gel G(E. Merck), 30 gm/60 ml of distilled water

Plate size : 10 cm x 20 cm and 20 cm x 20 cm

Layer thickness : 250 µ

Activation : air dried for 15 minutes and then at 110°C for 1 hour

Solvent systems : for triterpenoids :-

a) silica gel G/chloroform

b) silica gel G/chloroform : methanol (95:5)

c) silica gel G/chloroform : methanol : water (65:35:10)

for sugar :-

i) silica gel G/methyl ethyl ketone : glacial acetic acid : 2-methyl propan-2-ol (60:20:20)

ii) silica gel G/n-butanol : glacial acetic acid : diethyl ether : water (9:6:3:1)

iii) silica gel G/n-butanol : glacial acetic acid : water (60:30:10)

iv) silica gel G/chloroform : methanol : water (60:40:10)

Distance : 15 cm

Laboratory temperature : 24-30℃

Detection on chromatographic plate

: for triterpenoid :-

Liebermann-Burchard spray reagent (p. 61)

for sugar : -

a) Anisaldehyde-sulfuric acid spray reagent

Spray reagent :-

1 ml conc. sulfuric acid is added to a solution of 0.5 ml anisaldehyde in 50 ml acetic acid

Colors developed :-

After spraying the plate was heated at 100-105℃ until the spots attain maximum color intensity. The pink background can be bleached by exposure to steam.

b) α-Naphthol-sulfuric acid spray reagent

Spray reagent :-

A mixture is composed of 10.5 ml 15% ethanolic α-naphthol, 6.5 ml conc. sulfuric acid, 40.5 ml ethanol and 4 ml water

Colors developed :-

After spraying the plate was heated at 100℃ for 3-6 min.

2.2.2 Column Chromatography (CC)

Adsorbent : silica gel, 0.040-0.063 mm (E. Merck)

Packing of Column : dry packing

Sample loading : the portion of crude extract was dissolved in small amount of organic solvent, mixed with small quantity of adsorbent, air dried, triturated and added onto the top of a silica gel column.

Solvent systems : a) chloroform : methanol, (95:5)

b) chloroform : methanol : water, (65:35:10)

2.2.3 Testing for Compounds

Liebermann-Burchard test :-

To a solution of solid or dry extract 1-2 mg in 0.5 ml of chloroform, 2 drops of acetic anhydride were added followed by 1 drop of concentrated sulfuric acid. The colour reaction obtained as blue green or blue is indicated the presence of steroid compound and that obtained as pink or pink-red is indicated the presence of triterpenoid compound.

Molish's test :-

A sample (2-3 mg) was placed in a test tube containing 0.5 ml of water and mixed with 2 drops of a 10% solution of α-naphthol in ethyl alcohol. Then 1 ml of concentrated sulfuric acid was carefully dropped down to the side of the inclined tube so that the acid formed a layer beneath the aqueous solution. In the pressence of carbohydrate, a brown ring was appeared at the conjunction

of the two liquids, the color quickly change on standing or shaking, resulting in a reddish violet solution.

2.2.4 Lyophilization

The pure solution was dried by Termovac Lyophilizer.

2.2.5 Sugar Identificaiton of RS-1, RS-2 and RS-3

High Performance Liquid Chromatography (HPLC)

Machine : Jasco HPLC, model TRI ROTAR II

Column : Ionpak S-801

Eluaent : water

Flow rate : 1.0 ml/min

Pressure : 67 kg/cm^2

Chart speed : 5 min/cm

Detector : Refractive Index (RI)

Temperature : 40℃

Sample size : 4 µl

Gas Liquid Chromatography (GLC)

Machine : on a Shimadzu GC-6A gas chromatography

Glass column : 2% SE-30 on Chromosorb W (AW-DMCS) 2.6 mm x 2 m

Detector : FID

Injection temperature : 200℃

Column temperature : 180℃

Carrier Gas : N_2 (40 ml/min)

2.2.6 Physical Constants

Melting Points : Melting points were determined on a Gallenkamp Melting Point Apparatus with digital thermometer Model MFB-594. Results were uncorrected.

2.2.7 Spectroscopy

Infrared (IR) Absorption Spectra

Infrared absorption spectra were obtained with a Shimadzu Model IR 440 Spectrophotometer, absorption band were reported in wave number (cm^{-1})

Nuclear Magnetic Resonance (NMR) Spectra

a) Proton nmr spectra were taken at 270 MHz with Jeol spectrometer in pyridine-d_5 using tetramethylsilane (TMS) as an internal standard and chemical shift are given as δ(ppm).

b) Carbon-13 nmr spectra were taken at 67.8 MHz with a Jeol spectrometer in pyridine-d_5 using TMS as an internal standard and chemical shift are given as δ(ppm).

Mass Spectra

Mass spectra were recorded on a Hitachi High Resolution Mass Spectrometer operating at 70 eV with inlet temperature 130°C

2.2.8 Authentic Samples

a) Oleanolic acid obtained from *Schefflera* sp. (Hanumann-Prasangai) kindly supplied by Dr. Pittaya Tuntiwachwuttikul.

b) Pseudoginsenoside-RP_1 and RT_1 obtained from *Panax pseudoginseng* Wall. kindly supplied by Professor Osamu Tanaka.

2.3 Extraction and Isolation of Triterpenoid(s) from *Randia siamensis* Craib Fruits

2.3.1 Extraction

The dried and coarsely powdered fruits (250 gm) were macerated with 95% ethyl alcohol (1L) twice for three day-periods and filtered. The combined ethanolic extract was evaporated under reduced pressure to dryness. The residue was washed with petroleum ether (300 ml) and filtered, the filtrate was evaporated under reduced pressure to greenish viscous mass. TLC showed the presence of triterpenoid traces and no further study of this portion has been made. The petroleum ether insoluble portion was designated as residue C (19.64 gm)

2.3.2 Isolation of RS-1', RS-1, RS-2 and RS-3

The residue C was divided into four equal portion. Each portion was chromatographed on a silica gel column (4 cm x 16 cm). Elution with chloroform : methanol (95:5) afforded 20 fractions and then washed with methanol until no traces of triterpenoid could be detected. Twenty five ml of each fraction was collected and examined by thin layer chromatography (TLC). Those fractions of similar pattern were combined. Fraction 8-11 showed a similar pattern on TLC, hence it was combined. The combined fraction was evaporated under reduced pressure to yield white amorphous. It was crystallized in methanol yielding white needle crystals 62 mg (0.025%)

and was designated as RS-1'.

The methanol eluate was evaporated under reduced pressure to dryness. The residue (8.19 gm) was divided into 8 equal portions. Each portion was chromatographed on a silica gel column (2.5 cm x 16 cm) and eluted with chloroform : methanol : water (65: 35:10). Fractions of 25 ml each were collected and compared by TLC. The homogeneous fractions 8-10, 25-30 and 41-45 were combined, evaporated and lyophilized to dryness. The resulting of lyophylization of these fractions were designated as RS-1 (21 mg, 0.0084%) RS-2 (312 mg, 0.1248%) and RS-3 (67 mg, 0.0268%).

2.4 Identification of the Isolated Compounds

The isolated triterpenoids, (RS-1', RS-1, RS-2 and RS-3) were identified by comparison of hRf value, melting points, infrared, nuclear magnetic resonance and mass spectra with the authenthic samples

The hRf values given are those obtained with the following solvent systems :-

a) silica gel G/chloroform

b) silica gel G/chloroform : methanol (95:5)

c) silica gel G/chloroform : methanol : water (65:35:10)

2.4.1 Identification of RS-1' as Ursolic acid

RS-1' was obtained as white needle crystals from methanol. It was soluble in chloroform, acetone and ethanol. It gave a pink color with Liebermann-Burchard's test. This color reaction indicated that RS-1' might be a triterpenoid compound.

hRf values

The hRf values given are those obtained with the following systems :-

a) silica gel G/chloroform = 25

b) silica gel G/chloroform : methanol (95:5) = 54

c) silica gel G/chloroform : methanol : water (65:35:10) = 90

The thin layer chromatograms of ursolic acid (RS-1') are shown in Figures 12-14 (pp. 134-136).

Melting Point : 285°C

Infrared Absorption Spectrum (KBr disc)(Figure 35, p. 159)

ν_{max} (cm^{-1})

3420, 2950-2870, 1695, 1600, 1460, 1395, 1040 and 835

Proton NMR Spectrum (in pyridine-d_5, 270 MHz)(Figure 36, p. 160)

Chemical shifts(δ) ppm	Proton	Multiplicity
0.89	26-CH_3(3H)	s
0.95	24,25-CH_3(6H)	s
1.02	23-CH_3(3H)	s
1.07	27-CH_3(3H)	s
1.25	29,30-CH_3(6H)	d
1.95-2.0	5,9-CH(2H)	m
2.1	20-CH(1H)	m
2.35	19-CH(1H)	m
2.65	18-CH(1H)	d
3.5	3-CH(1H)	t,d
5.5	12-CH(1H)	t

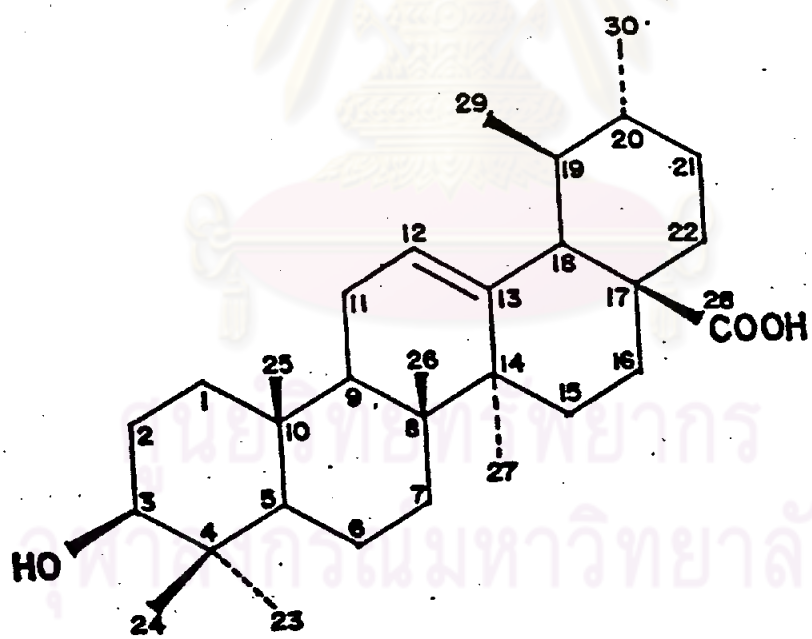
Carbon-13 NMR Spectrum (in pyridine-d_5, 67.8 MHz)(Table 4, pp. 87-88 and Figure 37, p. 161).

Mass Spectrum (EIMS)

m/z (%, relative intensity)(Figure 38, p. 162)

456 (8), 411 (1.7), 248 (100), 233 (4), 220 (9), 207 (25), 203 (49), 189 (14), 175 (8), 163 (4), 147 (9), 133 (42)

These data are in agreement with the published values of ursolic acid (66,67). It is therefore concluded that RS-1 is ursolic acid. The structure of which is shown below.



RS-1

URSOLIC ACID

Table 4 ^{13}C-Chemical Shifts(δ) ppm of RS-1', RS-2A, RS-1, RS-2 and RS-3 in C_5D_5N

	RS-1'	RS-2A	RS-1	RS-2	RS-3
C-1	39.1	39.0	38.6	38.7	39.0
C-2	28.1	28.1	26.5	26.6	26.5
C-3	78.1	78.2	89.2	89.2	89.9
C-4	39.4	39.4	39.6	39.5	39.7
C-5	55.8	55.9	55.9	55.8	56.1
C-6	18.8	19.0	18.5	18.5	18.8
C-7	33.6	33.5	33.3	33.1	33.3
C-8	40.0	40.1	39.7	39.8	40.1
C-9	48.1	48.2	48.0	48.0	48.2
C-10	37.3	37.5	36.9	36.9	37.1
C-11	24.9	23.3	23.7	23.6	23.7
C-12	125.7	123.0	122.5	122.8	123.0
C-13	139.2	145.0	144.8	144.1	144.1
C-14	42.5	42.2	42.1	42.1	42.3
C-15	28.7	28.4	28.3	28.1	28.3
C-16	23.6	23.8	23.7	23.7	23.7
C-17	48.1	46.7	46.6	46.9	47.1
C-18	53.6	42.0	41.9	41.7	41.9
C-19	39.4	46.6	46.6	46.2	46.4
C-20	39.5	31.0	30.9	30.7	30.8
C-21	31.1	34.3	34.3	34.0	34.2
C-22	37.4	33.3	33.3	32.5	32.6
C-23	28.8	28.8	28.3	27.8	28.3
C-24	16.6	16.6	16.3	16.3	16.7
C-25	15.7	15.6	15.4	15.5	15.5
C-26	17.5	17.4	17.4	17.4	17.5
C-27	23.9	26.0	26.2	26.1	26.1
C-28	180.0	180.3	180.2	176.3	176.3
C-29	17.5	33.3	33.3	33.1	33.1
C-30	21.4	23.8	23.7	23.6	23.7

Table 4 (Continued)

	RS-1'	RS-2A	RS-1	RS-2	RS-3
3-GlcUa-1	-	-	105.2	105.2	105.3
2	-	-	83.5	83.5	79.1[a)]
3	-	-	77.7[a)]	78.0[a)]	78.1[a)]
4	-	-	73.1	73.1	73.3
5	-	-	78.1[a)]	78.0[a)]	78.5[a)]
6	-	-	172.8	172.5	173.0
Xyl 1	-	-	106.9	106.9	102.8
2	-	-	76.5	76.5	78.9[a)]
3	-	-	77.2[a)]	77.7[a)]	77.2
4	-	-	71.0	71.0	71.5
5	-	-	67.5	67.5	66.5
Rha 1	-	-	-	-	101.9
2	-	-	-	-	72.2
3	-	-	-	-	72.6
4	-	-	-	-	74.2
5	-	-	-	-	69.6
6	-	-	-	-	18.8
28-Glc 1	-	-	-	95.6	95.7
2	-	-	-	74.0	74.1
3	-	-	-	78.8[b)]	78.5[a)]
4	-	-	-	71.0	71.1
5	-	-	-	79.2[b)]	78.9[a)]
6	-	-	-	62.1	62.2

a), b) These assignments may be reversed.

2.4.2 Identification of RS-1 as Pseudoginsenoside-RP_1 [3-O-β-GlcUA-(2-1)-β-Xyl of oleanolic acid]

RS-1 was crystallized from methanol to give colorless needle. It give pink color with Liebermann-Burchard's test and produce a positive Molish's test which indicated that it is a triterpenoid saponin.

Hydrolyzation of RS-1 and Identification of its sugar moieties by GLC :-

RS-1 (a few mg) was heated with 10% HCl in water : dioxane (1:1) in a sealed micro-tube at 80°C for 2 hours. The reaction mixture was evaporated to dryness by blowing N_2-gas over it at room temperature. The residue was trimethylsilylated by treatment with diazomethane (CH_2N_2) and N-trimethylsilylimidazole (5 drops) in a sealed micro-tube and heated at 80°C for 2 hours. The reaction mixture was diluted with distilled water and then extracted with n-cyclohexane. The cyclohexane layer was washed with water and evaporated to dryness by blowing N_2-gas over it at room temperature. The residue was subjected for GLC analysis. The GLC data showed the presence of two sugars which were identified as glucuronic acid and xylose by comparison in GLC with authentic samples of trimethylsilyl derivative of these sugars.

Alkaline Hydrolysis of RS-1 :-

RS-1 (a few mg) was heated with 5% potassium hydroxide in methanol : water (1:1) 5 ml at 80°C for 3 hours. Water was added to the reaction mixture and the whole was acidified to pH 5 with 10% aqueous acetic acid and then extracted with n-butanol saturated with water to give prosapogenin solution. The prosapogenin solution was

concentrated to dryness under reduced pressure. The residue was chromatographed on silica gel G plate under various solvent systems by comparison with RS-1. TLC examination showed that RS-1 was unchanged on alkaline hydrolysis. The negative result of the alkaline hydrolysis is indicated that sugars were not present in ester combination with the carboxylic group at C-28 of RS-1. This led to the conclusion that all the sugar units were linked with hydroxyl group at C-3 of aglycone.

hRf value

The hRf values given are those obtained with the following systems :-

a) silica gel G/chloroform = 2.7

b) silica gel G/chloroform : methanol (95:5) = 12.6

c) silica gel G/chloroform : methanol : water (65:35:10) = 50.7

The thin layer chromatograms of RS-1 are shown in Figure 12-14 (pp. 134-136).

Melting Point

230-232°C

Infrared Absorption Spectrum (KBr disc)

ν_{max} (cm^{-1})

3430, 2940, 1620, 1385, 1020-1040 (broad)

(Figure 39, p. 163)

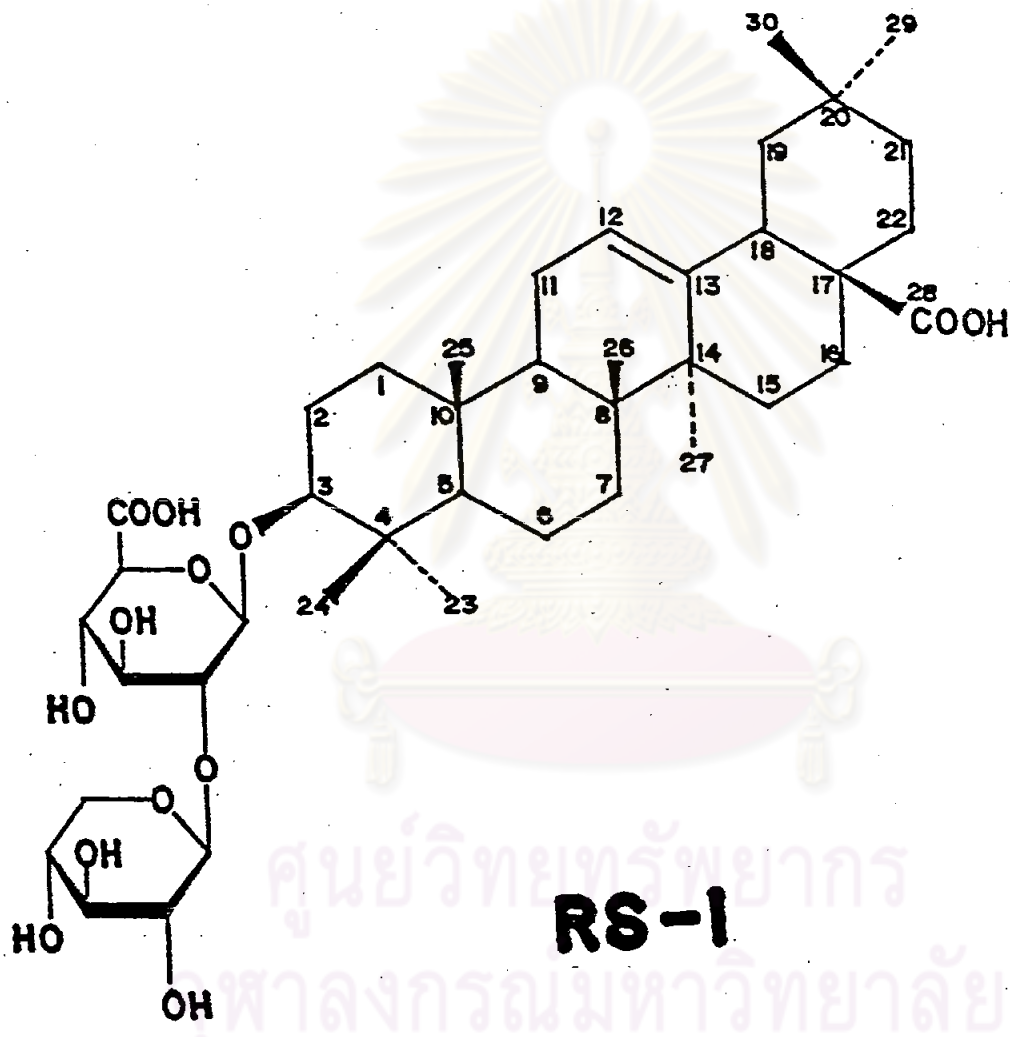
Proton NMR Spectrum (in pyridine-d_5, 270 MHz)(Figure 40, p. 164)

Chemical Shift(δ) ppm	Proton	Multiplicity
0.85	26-CH_3(3H)	s
0.89	25-CH_3(3H)	s
0.92	29,30-CH_3(6H)	s
0.99	24-CH_3(3H)	s
1.04	23-CH_3(3H)	s
1.24	23-CH_3(3H)	s
1.28	27-CH_3(3H)	s
4.85 - 4.91	GLcUA-H_1(1H)	d
5.1 - 5.15	Xyl-H_1(1H)	d
5.4	12-CH(1H)	t

Carbon-13 NMR Spectrum (in pyridine-d_5, 67.8 MHz)(Table 4, pp. 87-88 and Figure 41, p. 165)

The ^{13}C-NMR spectrum of RS-1 showed carbon signals due to an aglycone moiety at almost the same positions as those of RS-2A, indicated that RS-1 has oleanolic acid as sapogenin.

According to all spectral data and direct comparison with an authentic sample in both mixed m.p. and Co-TLC it is clearly proved that RS-1 is 3-O-β-GlcUA-(2-1)-β-Xyl of oleanolic acid which its name is pseudoginsenoside-RP_1 (71). The structure of which is shown on page 92.



RS-1

PSEUDOGINSENOSIDE–RP$_1$

2.4.3 Identification of RS-2 as Pseudoginsenoside-RT_1 (3-O-β-GlcUA-(2-1)-β-Xyl of Glucosyl oleanolate)

RS-2 was crystallized from methanol to give white amorphous. It gave pink color with Liebermann-Burchard's test and produced a positive Molish's test which indicated that it was a triterpenoid saponin.

Hydrolyzation of RS-2 and Identification of its sugar moieties by TLC and HPLC :-

RS-2 (50 mg) was hydrolyzed by refluxing with 25% HCl in ethanol for 5 hours. Ethanolic solution was evaporated under reduced pressure. The residue was diluted with water and extracted with diethyl ether. Ethereal solution was dried over anhydrous sodium sulfate and evaporated in *vacuo* to dryness. The residue was crystallized from methanol yielding aglycone (18 mg) as white needle crystals and was designated as RS-2A.

The aqueous acid phase was neutralized with 3.5% ammonium hydroxide solution and evaporated on water-bath. The residue was identified for the sugar moieties by TLC and HPLC.

On TLC plates, the sugars were identified by comparison with authentic glucose, galactose, xylose, arabinose, rhamnose and D-mannose. The hRf values given are those obtained from four solvent systems. Chromatographic plates were detected by spraying with anisaldehyde-sulfuric acid and α-naphthol-sulfuric acid spray reagent. The results are shown on Table 5.

Table 5 Identification of Sugars by TLC under various Solvent Systems

Solvent and Spray Reagent / Sugar	a	b	I	II	III	IV	Remark
Sugars of RS-2	-	blue	5	8	12	0	may be glucuronic acid
	light blue gray	blue-violet	42	46.0	53.1	46.2	glucose
	gray	light-blue	53.6	54.2	60.8	55.1	xylose
Reference sugars							
D(+) - glucose	light blue-gray	blue-violet	42.1	46.1	53.6	45.9	
D(+) - galactose	gray-green	green-violet	38.9	41.8	47.1	44.4	
D(+) - xylose	gray	light blue	54.3	54.6	60.7	54.8	
L(+) - arabinose	yellow-green	blue-green	45.7	48.9	50.7	51.1	
L(+) - rhamnose	yellow,yellow-green	green	58.6	55.3	62.8	61.5	
D(+) - mannose	green	light blue	38.6	45.4	52.9	50.4	

a = anisaldehyde - sulfuric acid spray reagent

b = α-naphthol - sulfuric acid spray reagent

I = silica gel G/methyl ethyl ketone : glacial acetic acid : 2-methyl propan-2-ol (60:20:20)

II = silica gel G/n-butanol : glacial acetic acid : diethyl ether : water (9:6:3:1)

III = silica gel G/n-butanol : glacial acetic acid : water (60:30:10)

IV = silica gel G/chloroform : methanol : water (60:40:10)

The TLC data showed hRf values and colours on chromatographic plates, indicating that the sugars in acid hydrolysate of RS-2 are glucose, xylose and one unknown sugar which was confirmed by HPLC by comparison with the authentic sugars. The qualitative analysis by HPLC found that the retention time of 12.94 and 13.74 were glucose and xylose respectively.

Hydrolyzation of RS-2 and Identification of its sugar moieties by GLC :-

RS-2 (a few mg) was heated with 10% HCl in water : dioxane (1:1) in a sealed micro-tube at 80°C for 2 hours. The reaction mixture was evaporated to dryness by blowing N_2 gas over it at room temperature. The residue was trimethylsilylated by treatment with diazomethane (CH_2N_2) and N-trimethylsilylimidazole (5 drops) in a sealed micro-tube and heated at 80°C for 2 hours. The reaction mixture was diluted with distilled water and then extracted with n-cyclohexane. The cyclohexane layer was washed with water and evaporated to dryness by blowing N_2 gas over it at room temperature. The residue was subjected for GLC analysis. The GLC data showed the presence of three sugars which were identified as glucose, glucuronic acid and xylose trimethylsilyl derivative of these sugars.

Alkaline Hydrolysis of RS-2 :-

RS-2 (50 mg) was heated with 5% potassium hydroxide in methanol : water (1:1) 5 ml at 80°C for 3 hours. Water was added to the reaction mixture and the whole was acidified to pH 5 with 10% aqueous acetic acid and then extracted with n-butanol saturated with water to give a prosapogenin solution. The aqueous solution was neutralized with Amberlite MB-3 and the presence of 1,6 anhydroglucose was further confirmed as follows. The aqueous solution was concentrated

under reduced pressure to dryness and the residue was treated with 5% H_2SO_4 at 80℃ for 2.5 hours. The reaction mixture was neutralized with Amberite MB-3 and concentrated to dryness under reduced pressure. The residual glucose was identified by TLC and GLC. GLC was carried out under the same conditions as performed for hydrolysis of RS-1.

The prosapogenin solution (Butanolic layer) was concentrated to dryness under reduced pressure. The residue was chromatographed on silica gel G plate (solvent :- chloroform : methanol : water, 6:4:1) by comparison with RS-2. TLC examination showed the changed of RS-2. The positive result of the alkaline hydrolysis is indicated that glucose is glycosidically linked with carboxylic group at C-28 of RS-2A (oleanolic acid)

Identificaiton of RS-2A as Oleanolic Acid by comparison with a saponin RS-2

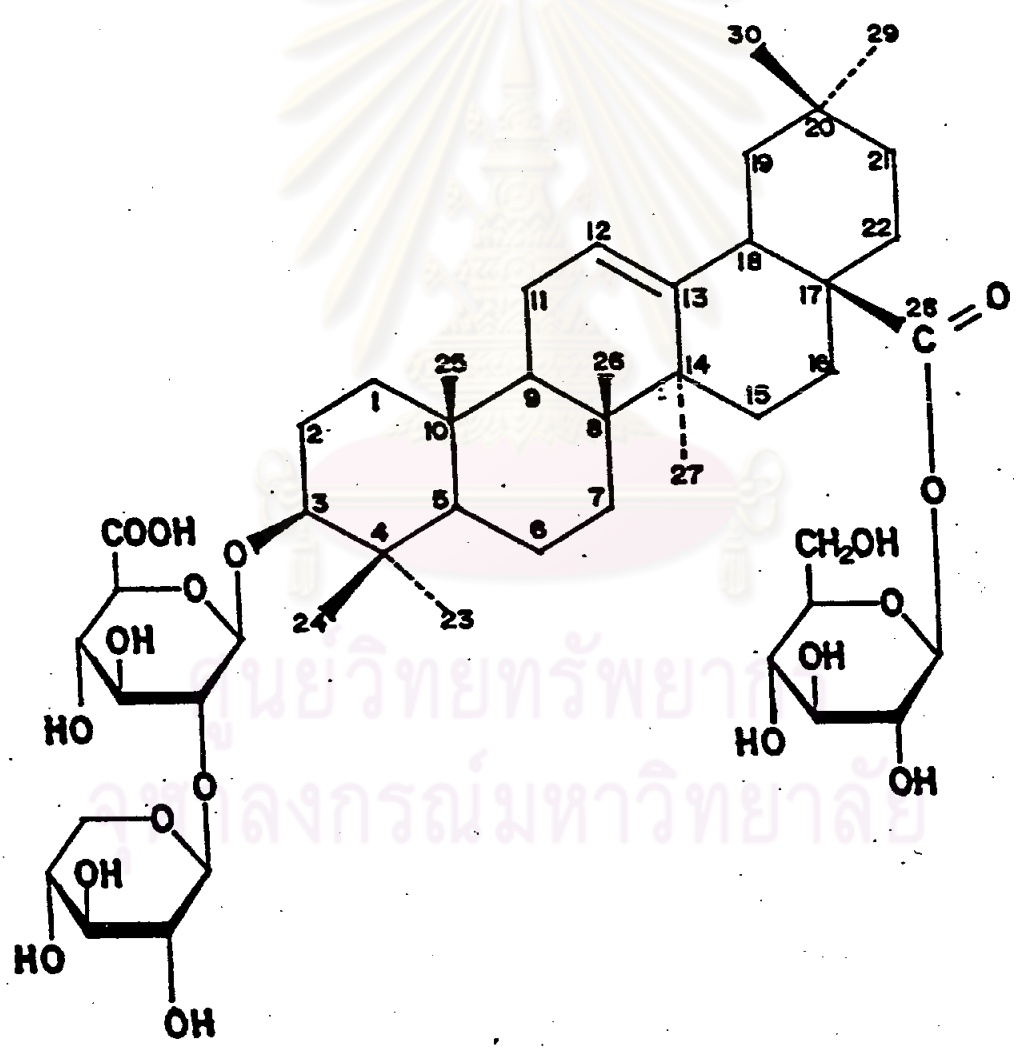
The characterization for RS-2 and RS-2A was by comparison of hRf values, melting points, infrared, nuclear magnetic resonance and mass spectra with the authentic sample. These data are shown on the Table 6.

Table 6 Characterization Data of RS-2 and RS-2A

Characterization	RS-2	RS-2A
1. Crystallization	white amorphous (from methanol)	white needles (from methanol)
2. hRf Values	see Figures 12-14, pp. 134-136	see Figures 12-14, pp. 134-136
a) silica gel G/chloroform	a) 0	a) 27
b) silica gel G/chloroform : methanol (95:5)	b) 7	b) 59
c) silica gel G/chloroform : methanol : water (65:35:10)	c) 36	c) 96
3. Melting Points	235 - 238°C (decomposed)	310°C
4. Infrared Absorption Spectrum ν_{max} (cm^{-1})	see Figure 46, p. 170 3410, 2920, 1740, 1720, 1620, 1460-1360, 1140-1030 (broad) 800	see Figure 42, p. 166 3420, 2920, 2840, 1700,1464, 1390, 1366, 1347, 1325, 1305, 1264, 1180, 1035, 1000, 828, 818, 804
5. Proton NMR Spectrum (in pyridine-d_5, 270 MHz) Chemical shift(δ) ppm (proton, multiplicity, carbon position)	see Figure 47, p. 171 0.90 (6H, s, 25,26-CH_3) 1.04 (9H, s, 24,29,30-CH_3) 1.24 (6H, s, 23,27-CH_3) 4.84 (1H, d, GlcUA-H_1) 5.15 (1H, d, Xyl-H_1) 5.40 (1H, t, 12-H) 6.15 (1H, d, Glc-H_1)	see Figure 43, p. 167 0.93 (3H, s, 26-CH_3) 0.97 (3H, s, 25-CH_3) 1.03 (9H, s, 24,29,30-CH_3) 1.29 (3H, s, 23-CH_3) 1.31 (3H, s, 27-CH_3) 3.33 (1H, d, 3-OH) 3.45 (1H, m, 3-CH-OH) 5.51 (1H, d, 12-CH=C<)
6. Carbon-13 NMR Spectrum	see Table 4, pp. 87-88 and Figure 41, p. 165	see Table 4, pp. 87-88 and Figure 44, p. 168
7. Mass spectrum m/z (%, relative intensity)	-	see Figure 45, p. 169 456 (1), 248 (100), 223 (11), 219 (6), 207 (20), 203 (97), 189 (17), 175 (11), 133 (20)

The data of RS-2A are in agreement with published value of oleanolic acid (68-70).

According to all spectral data and direct comparison with an authentic sample in both mixed m.p. and Co-TLC it is clearly proved that RS-2A is oleanolic acid and RS-2 is 3-O-β-GlcUA-(2-1)-β-Xyl of Glucosyl oleanolate which its name is pseudoginsenoside-RT_1 (71). The structure of which is shown below



RS-2

PSEUDOGINSENOSIDE-RT_1

2.4.4 Identification of RS-3 as a novel saponin (3-O-β GlcUA-(2-1)-β-Xyl-(2-1)-α-Rha of Glucosyl Oleanolate)

RS-3 was obtained as white amorphous powder which gave pink colour in Liebermann-Burchard's test and produced a positive with Molish's test. The resulting of these tests indicated that RS-3 was a triterpenoid saponin.

Hydrolyzation of RS-3 and Identification of its sugar moieties by GLC :-

RS-3 (a few mg) was prepared by the same procedure as described (on page 95) for GLC analysis of RS-2 residue. The GLC data showed the presence of four sugars which were identified as glucose, glucuronic acid, xylose and rhamnose by comparison in GLC with authentic samples of trimethylsilyl derivative of these sugars.

Alkaline Hydrolysis of RS-3 :-

RS-3 (50 mg) was hydrolyzed to give prosapogenin and sugar(s) by the same procedure as that used for RS-2 (on page 95). On the alkaline hydrolysis, glucose was also found in aqueous fraction of RS-3 by GLC analysis. The prosapogenin was examined by TLC examination. The result from alkaline hydrolysis is indicated that glucose is glycosidically linked with carboxylic group at C-28.

hRf values

The hRf values given are those obtained with the following systems :-

a) silica gel G/chloroform = 0

b) silica gel G/chloroform : methanol (95:5) = 0

c) silica gel G/chloroform : methanol : water (65:35:10) = 27

The thin layer chromatograms of RS-3 are shown in Figures 12-14 (pp. 134-136).

Melting Point : 215-220℃ (decomposed)

Infrared Absorption Spectrum (KBr disc)(Figure 49, p. 173)

ν_{max} (cm^{-1})

3400, 2910, 1720, 1620, 1460-1360, 1070, 1020, 890 and 810

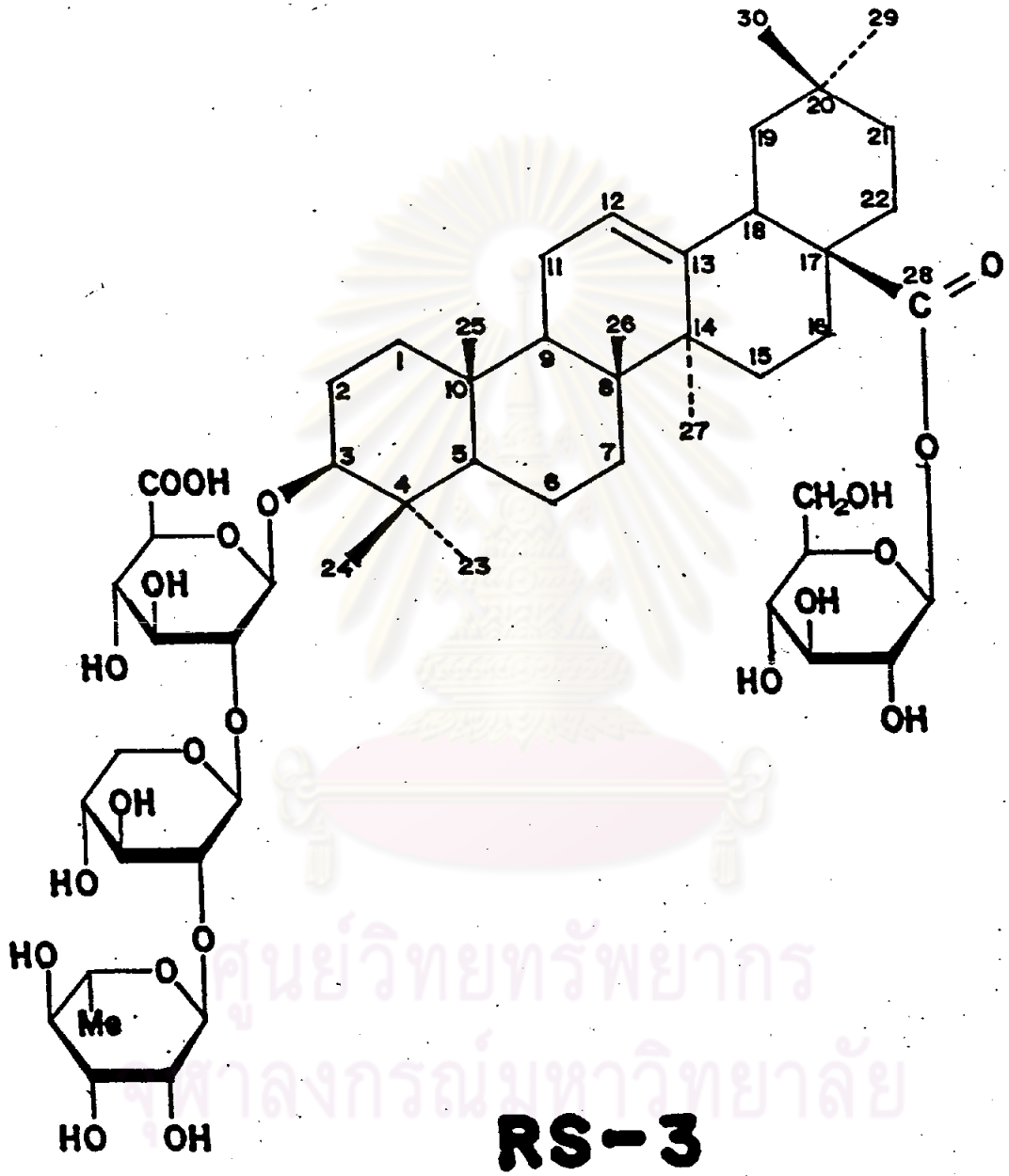
Proton NMR Spectrum (in pyridine-d_5, 270 MHz)(Figure 50, p. 174)

Chemical Shift(δ) ppm	Proton	Multiplicity
0.9	25,26-CH_3(6H)	s
1.02	24,29,30-CH_3(9H)	s
1.24	23-CH_3(3H)	s
1.28	27-CH_3(3H)	s
1.7	Rha-6-CH_3(3H)	d
4.8	GlcUA-H_1(1H)	d
5.4	12-CH(1H)	t
5.6	Xyl-H_1(1H)	d
6.15	Rha-H_1(1H)	d
6.22	Glc-H_1(1H)	d

Carbon-13 NMR Spectrum (in pyridine-d_5, 67.8 MHz)(Table 4, pp. 87-88 and Figure 51, p. 175).

The ^{13}C-NMR spectrum of a novel saponin (RS-3) showed carbon signals due to an aglycone moiety at almost the same positions as those of RS-2A, indicated that RS-3 has oleanolic acid as sapogenin. Glucose, glucuronic acid, xylose and rhamnose were identified in the

acid hydrolysate of RS-3 by GLC. On alkaline hydrolysis, RS-3 afforded 1,6-anhydroglucose and prosapogenin. By comparison with spectrum of RS-2, the ^{13}C-NMR signals of sugar moieties of RS-3 has another hexose linked at C-3 position. It is clearly shown for the structure of RS-3 as 3,28-bisdesmoside of oleanolic acid. Several pieces of evidence have shown that the additional hexose is rhamnose. The ^{1}H-NMR spectrum of RS-3 showed signals of free methyl group of rhamnose at δ 1.7 (3H, d, 6-CH_3) and δ 6.15 (1H, d) of ether-linkage. The ^{13}C-NMR spectrum of saponin which has rhamnose linked at terminal of sequence exhibits chemical shifts of carbon-rhamnose (C-2, C-3 and C-4) located at lower field than internal one (about 1 ppm). Unless it has substituent of those positions the carbon signals will shift to lower field about 5-10 ppm. The results were concluded for the structure of RS-3 as a novel saponin and the chemical structure was assigned as 3-O-β-GlcUA-(2-1)-β-Xyl-(2-1)-α-Rha of Glucosyl oleanolate and was named siamenoside (71-73). The structure of which is shown on page 102.



RS-3

SIAMENOSIDE