CHAPTER II

EXPERIMENT

2.1 Instruments and Equipment

2.1.1 Infrared Spectra (IR)

The IR spectra were recorded on Shimadzu spectrophotometer Model IR-440. The spectra of solid were obtained by incorporating the sample into a pellet of potassium bromide.

2.1.2 Elemental Analyses

The elemental analyses were made using a Loven, Kemiske Tabrik equipment, DK-2750 Ballerup (Micro-analytical Laboratory, Denmark).

2.1.3 Ultraviolet Spectra (UV.)

The ultraviolet spectra were determined by double beam spectrophotometer Model 220 A, Hitachi, Japan.

2.1.4 Melting Point (m.p.)

The melting points were obtained on Fisher-Jones apparatus and were uncorrected.

2.1.5 Optical Rotation

The optical rotation was determined by an Autopol III automatic polarimeter Rudolph research Hainfield, New Jersey.

2.1.6 Gas Chromatography (GC)

The GC was carried out using Pye-Unicam Pyes Series 104 Chromatography Phillips.

2.1.7 Atomic Absorption Data

Atomic Absorption data were determined by Varian Techtron AA-5 spectrophotometer.

2.1.8 Mass Spectra (MS)

Qualitative mass spectra were obtained on a Micromass 707 F spectrophotometer at 70 eV.

2.1.9 Proton and Carbon Nuclear Magnetic Resonance (^{1}H NMR and ^{13}C NMR)

The ^{1}H NMR and ^{13}C NMR spectra were performed using Fourier Transform NMR Spectrophotometer Model FX902, Jeal Japan.

2.2 Chemical Reagents

All solvents used in this research were redistilled commercial grade.

The alumina and silica gel using as adsorpbent in the chromatographies were obtained from E. Merck, Darmstadt., Ltd.

Art 7731 Silica gel 60.

Art 7734 Silica gel 60.

Art 1097 Aluminium oxide 90 (70-230 mesh ASTM)

2.3 Physical Separations

2.3.1 Quick Column Chromatography

This method[12] is especially useful for separating large quantities of complex mixtures which were obtained from natural sources into fraction. Because of its speed and separating power, it can also be used for separating the reaction mixtures.

Packing the Column

The column was a glass column of 7 cm. diameter with sintered glass frit. Silica gel was added to the column and distributed evenly over the surface. The vacuum was then applied (water pump) and the silica gel was allowed to settle. Any cracks which developed were pressed with a glass rod and more silica was added to give a packed bed of 4 cm. or less. When the bed was compressed, the application of vacuum was continued and the column was ready to be charged with the extract.

Separating the Extract on the Column

The extract was dissolved in petroleum ether and added directly on the column. It was preferred to add 50 ml of petroleum ether before adding the extract to ensure that the surface was wetted evenly to ensure smooth flowing of the solution in the column. When the column was about to go dry, added the extract quickly but gently in one portion to the top of the column, and 50 ml fractions were collected. Polarity of solvents was changed as shown in Table II.

Table II The polarity of solvents as eluents.

Solvents	Ratio of solvents
Petroleum ether	100 99 98 95 92.5 90 80...50...0
Chloroform	0 1 2 5 7.5 10 20 50...100 95 90 80...50...0
Methanol	0 5 10 20...50...100

2.3.2 Column Chromatography

Silica gel was placed in a large container and the solvent was added in small increments, swirling the container to ensure good

mixing after each addition.

Packing the Column

Pre-adjust the stopcock so that it was almost closed, but still allow the solvents to drip through. Allowed approximately 0.5 cm. of solvent to remain in the column when the slurry of silica gel was poured. After the bed has settled, a paper disk, prepared from 3 mm. paper was inserted in the column. The sample was dissolved in the solvent as a 10% (w/v) solution and it was introduced when the solvent level was just above the paper disk in the column. If the material was not soluble in the developing solvent, one could always dissolve the sample in a solvent in which it was soluble. The solution was next added to a minimum quantity of adsorbent in a boiling flask and then removed the solvent in vacuo. and the dried powder was sprinkled on top of the column and the disk. Washed sea sand was usually added to the top of the column prior to the addition of the eluent.

2.3.3 Thin Layer Chromatography (TLC)

The thin layer chromatography was performed using silica gel 60 Art 7731 to coat the glass plates chromatoplates with thickness of 0.5 mm. for identification. The plates were activated at 110^{o} for 1 hr and cooled. The plates were developed by a suitable solvent system and the sample spots on the plates were detected by using UV, I_2 and 20% sulfuric acid.

2.4 Colour Tests

2.4.1 Molisch's Test

This was a general test for carbohydrates. A sample (5 mg) was placed in a test-tube containing 0.5 ml of water and then mixed with 2 drops 10% solution of β-naphthol in alcohol. 1 ml of concentrated sulphuric acid was dropped down the side of the inclined

tube so that the acid formed a layer beneath the aqueous solution without mixing with it. If a carbohydrate was present, a red ring appeared at the common surface of the liquids, the colour quickly changed on standing or shaking, resulting in a dark purple solution.

2.4.2 Liebermann-Burchard Test

To a solution of the sample to be tested (3 mg) in chloroform (0.5 ml) was added a few drops of acetic anhydride, followed by one drop of concentrated sulphuric acid. Development of the colour after a few minutes suggests the presence of steroid or triterpenoid.

2.4.3 Dragendorff's Reagent Test

This was a test for alkaloid nucleus. The sample solution was added a few drops of Dragendorff's reagent yielded orange precipitation. The test suggested the presence of alkaloid nucleus.

2.4.4 Kraut's Reagent Test

This was a test for alkaloid nucleus. Add a few drops of Kraut's reagent into the sample solution yielded brown precipitation. This exhibited the presence of alkaloid nucleus.

2.5 Plant Material

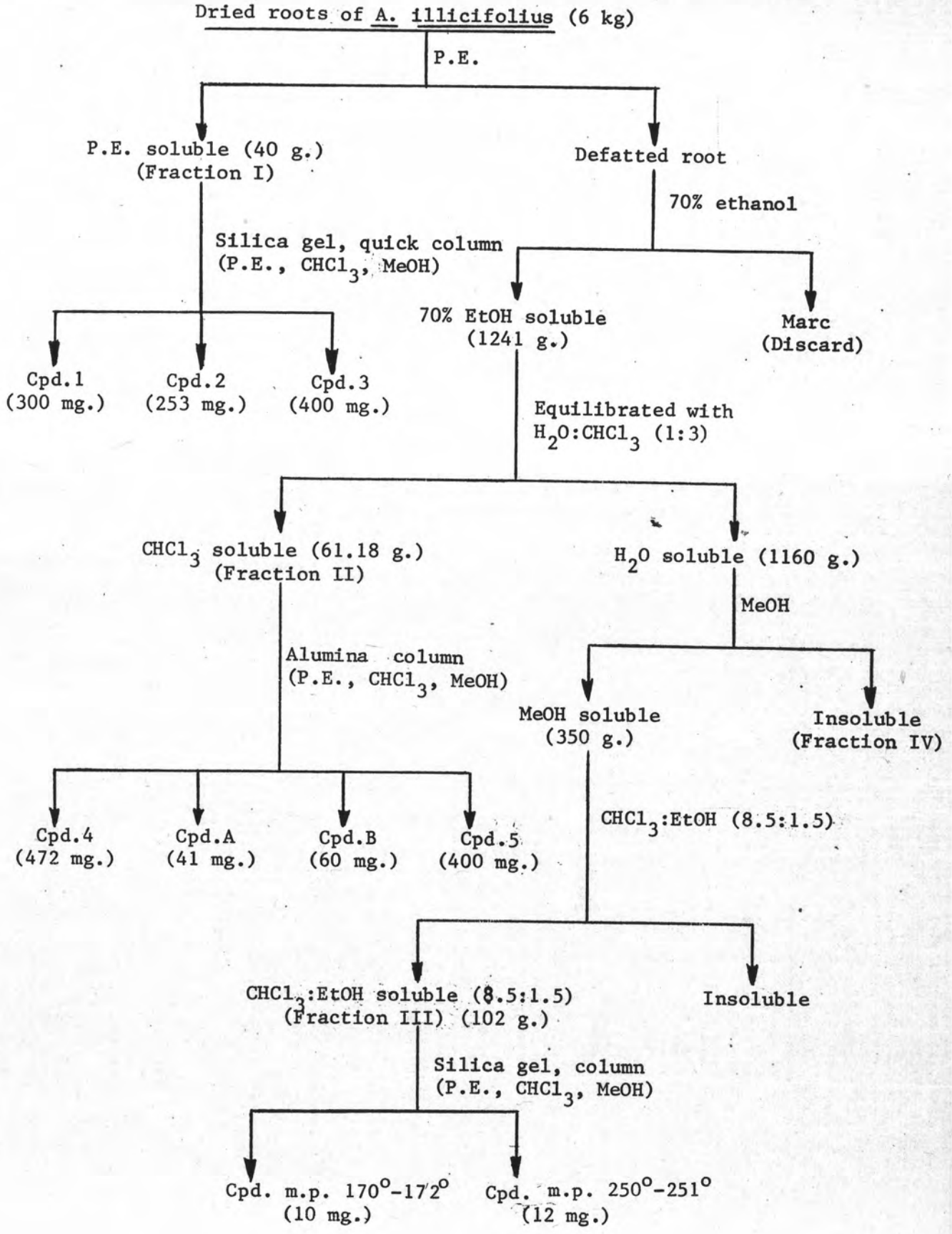
Roots of A. illicifolius were collected from Samutrsakhon province, in May 1982. The material was air-dried and milled to coarse powder.

2.6 Extraction

The dried powder material (6 Kg) was defatted by continuous extraction with petroleum ether (20 l) for seven days at room temperature. The extraction was repleated for four times. The extracted solution was collected and removed the solvent in vacuo, yielding a crude petroleum ether extracted 40 g (Fraction I). After the plant material was extracted with petroleum ether. This material was reextracted with 70% ethanol at

room temperature in the same manner. The crude ethanol extract, 1241 g, (24.8% wt) was obtained after the extracted solutions were collected and the solvent were removed in vacuo. The ethanol crude extract was then equilibrated between chloroform and water (3:1) by liquid-liquid extractor. Evaporation, in vacuo, of the chloroform soluble fraction gave 61.18 g (1.21% ot) of the crude material (Fraction II). The aqueous fraction was concentrated, in vacuo, yielded a water soluble fraction 1160 gel (23.2% wt) which was then extracted with methanol gave 350 g (7% wt) of methanol soluble fraction and 621 g (12.40% wt) of the insoluble fraction (Fraction III). The methanol soluble fraction was reextracted with chloroform-ethanol (8.5:1.5) yielded Fraction IV 102 g (2.04% wt) (see Scheme I).

Scheme I Extraction and isolation procedure for the roots of A. illicifolius.



2.7 Separation of Fraction I

The petroleum ether extract was concentrated under reduce pressure. It gave a brownish stickly liquid of 40 g (0.8% wt). Thin layer chromatography (solvent, P.E.: $CHCl_3$ = 2:8) of the concentrated extract revealed that there were two major components in this crude. The portion of the concentrated extracts (20 g) was chromatographed on silica gel using the quick column chromatography technique. The elution was initiated with petroleum ether and changed to chloroform by gradual introduction of the latter. Finally, the column was eluted with chloroform-methanol (1:1). The eluted solutions were collected in 50 ml fractions and the solvent was removed *in vacuo*. The separation in each fraction was checked by TLC and then those equivalent fractions were combined as shown in Table III.

Table III Quick column chromatography of Fraction I

Eluents	Fraction No. (50 ml)	Remarks
Petroleum Ether	1-3	yellow oil
1% Chloroform-Petroleum Ether	4-16	yellow oil + yellow ppt. (Fraction A)
5% Chloroform-Petroleum Ether	17-20	yellow oil
10% Chloroform-Petroleum Ether	21-25	yellow oil
20% Chloroform-Petroleum Ether	26-28	yellow oil
25% Chloroform-Petroleum Ether	29-32	yellow oil
30% Chloroform-Petroleum Ether	33-35	yellow oil
40% Chloroform-Petroleum Ether	36-40	yellow oil + white ppt. (Fraction B)
44% Chloroform-Petroleum Ether	41-60	yellow oil + white ppt. (Fraction C)
50% Chloroform-Petroleum Ether	61-70	yellow oil
Chloroform	71-75	yellow oil
20% Methanol-Chloroform	76-78	yellow oil
50% Methanol-Chloroform	79-80	yellow oil

2.7.1 Purification and Properties of Compound 1

The crystal product was obtained from the concentration of Fraction A (see Table III) which was purified by recrystallization from petroleum ether for several times and then followed by methanol to afford the Compound 1 as yellow rhombics, 300 mg, m.p. 115°-116° (Rf 0.527, solvent: $n\text{-}C_6H_{14}$). Sodium fusion test showed that sulfur was the only element presented.

Elemental analysis found: S 97.28%, H 0.17%, N 0.11%, C 0.12%

calcd. for S_8 : S 100%

The mass spectrum gave a molecular ion peak, M^+, at 256 (calcd. for S_8: MW = 256.7) and other fragments at m/e 244 (256-S), 192 (244-S), 160 (192-S), 128 (160-S), 96 (128-S), 64 (96-S) and at m/e 32 (S). The ^1H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) spectrum did not show any signal because this compound was not soluble in this solvent. Compound 1 was identical with S_8 by both of IR spectrum and mixed m.p. with authentic S_8.

2.7.2 Purification and Properties of Compound 2

Fraction B was obtained from the combination of fractions No. 36 to No. 40 in 40% chloroform-petroleum ether, which contained a mixture of yellow oil and white crystalline solid. The yellow oil was removed by washing with hot methanol and the remaining material was recrystallized from hot petroleum ether for several times into Compound 2 (253 mg.) as white amorphous m.p. 77°-78° (Rf 0.26, solvent: 80% $CHCl_3$-P.E.).

The IR spectrum showed absorption peaks at ν_{max}^{KBr} cm^{-1}: 3300-3200 (OH), 2960-2850 (C-H), 1100-1000 (C-O) and 800 (C-C).

The ^1H NMR ($CDCl_3$) spectrum exhibited signals at δ 1.27 (1H, s) and 3.63 (1H, t, J = 4 Hz).

The ^{13}C NMR ($CDCl_3$) spectrum gave signals at 14.09 (C_1), 22.70 (C_2), 25.79 (C_{26}), 29.47 (C_4), 29.75 (C_5-C_{26}), 31.97 (C_3), 32.89 (C_{27}) and 63.12 (C_{28}) ppm.

The MS showed mass fragments at m/e 392 (410-H_2O) 364 (392-$(CH_2)_2$), 336 (364-$(CH_2)_2$), 167 (336-$(CH_2)_{11}$-CH_3) and at m/e 97 (167-$(CH_2)_5$).

Elemental analysis found: C 81.77%, H 14.19%

calcd for $C_{28}H_{56}O$:MW 410: C 81.87%, H 14.23%

Acetylation of Compound 2

Compound 2 was treated with acetic anhydride (1 ml) and a few drops of dry pyridine in the usual manner and kept at room temperature overnight. The reaction mixture was poured onto ice water with vigorous stirring to give precipitate which was filtered off, followed by washing with water. The acetate derivative of Compound 2 was purified from petroleum ether and further by methanol yielded white crystals (15 mg), m.p. 66°-67° (Rf 0.545), solvent: 40% ($CHCl_3$-P.E.).

The IR spectrum gave absorption bands at ν_{max}^{KBr} cm^{-1}: 1725 and 1205 ($COCH_3$).

2.7.3 Purification and Properties of Compound 3

Fraction C was eluted from Fraction I by using 44% Chloroform-petroleum ether as an eluent (see Table III) to afford Compound 3 (Rf 0.23, solvent: $CHCl_3$) which was recrystallized from petroleum ether and further by methanol as white needles (400 mg) m.p. 167°-168°.

The IR spectrum revealed absorption bands at ν_{max}^{KBr} cm^{-1}: 3300-3500 (OH), 2850-2950 (C-H), 1390 (C-H), 800, 820 (trisubstituted double bond).

The ^1H NMR ($CDCl_3$) spectrum showed signals at δ5.33 (olefinic proton, 1H), 5.08 (2H, d of d), 3.46 (1H, m), 1.54 ($2CH_3$, s).

The ^{13}C NMR ($CDCl_3$) spectrum displayed signals at 140.81, 138.32, 129.38, 121.74, 71.84 and 56.94 ppm.

The UV (EtOH) spectrum showed λmax at 203 nm (Σ 6012).

The MS gave a molecular ion peak, M^+, at 412 (calcd. for $C_{29}H_{48}O$: MW = 412) and other fragments at m/e 397, 369, 314, 283, 257 and 83.

The GC analysis (conditions; column OV-107, column temp. 260°C, carrier gas He) revealed a sole component Rt 27.99 min and identified as stigmasterol by comparison with authentic sample.

All evidences were clearly showed that Compound 3 is a stigmasterol which was confirmed by both mixed m.p. and Co-TLC with an authentic stigmasterol.

Acetylation of Compound 3

Compound 3 was reacted with acetic anhydride (2 ml) and a few drops of dry pyridine in the usual manner yielded the acetyl derivative of Compound 3. The product was recrystallized from petroleum ether to give plate crystals (46 mg), m.p. 141°-142° (Rf 0.80, solvent: $CHCL_3$).

The IR spectrum of the acetyl derivative of Compound 3 was completely agree with the spectrum of an authentic stigmasteryl acetate which showed strong absorption bands at 2950 (C-H), 1740 and 1020 cm^{-1} (acetate).

2.8 Separation of Fraction II.

Fraction II, chloroform soluble fraction, was obtained by equilibrating the 70% ethanol soluble fraction with chloroform-water (3:1). Fraction II was chromatographed on alumina 90 column eluted with 10% chloroform-petroleum ether and then increasing polarity by chloroform and methanol. All fractions were monitored by thin layer chromatography, then Fraction D, E and F were obtained. The results from column chromatography of Fraction II were shown in Table IV.

Table IV The results from column chromatography of Fraction II

Eluents	Fraction No. (250 ml)	Remarks
10% Chloroform-Petroleum ether	1-3	yellow oil
20% Chloroform-Petroleum ether	4-6	yellow oil
25% Chloroform-Petroleum ether	7-8	yellow oil
40% Chloroform-Petroleum ether	9-16	yellow oil + white crystals (stigmasterol)
60% Chloroform-Petroleum ether	17-22	dark tarry oil
90% Chloroform-Petroleum ether	23-30	dark tarry oil
Chloroform	31-35	dark tarry oil
10% Methanol-Chloroform	36-42	yellow oil + 3 spots the major one, Rf 0.314 (Fraction D).
	43-50	yellow oil + 2 spots Rf 0.485, Rf 0.675 (Fraction E).
20% Methanol-Chloroform	51-54	yellow oil + white crystals (Fraction F).
30% Methanol-Chloroform	55-58	oil and tar
40% Mathanol-Chloroform	59-62	tar
50% Methanol-Chloroform	63-64	tar
70% Methanol-Chloroform	65-70	brown crude
Methanol	71-78	dark-brown crude

2.8.1 Purification and Properties of Compound 4

Fraction D (1.5 g) was obtained by eluting Fraction II with chloroform containing 10% methanol (see Table IV) which was then subjected to silica gel column chromatography using 50% chloroform-petroleum ether

as an eluting solvents (see Table V). From fraction 19 to 40, a mixture of yellow oil and colourless crystals was obtained. Removal of yellow oil by washing with 5% chloroform-petroleum ether for several times yielding colourless rhombic which was assigned as Compound 4 (472 mg, Rf 0.314, Solvent: 2% MeOH-$CHCl_3$) m.p. 137°-138°. The presence of nitrogen in Compound 4 was detected by sodium fusion test as well as the positive test on Dragendorff's and Kraut's reagents.

The IR spectrum showed absorption bands at ν_{max}^{KBr} cm^{-1}. 3450 (N-H), 3220 (O-H), 1780 (lactone), 1730 (ester) and 1150 (C-O).

The ^1H NMR ($CDCl_3$) spectrum exhibited signals at δ 9.92 (1H, broad, NH), 7.14 (4 H, S, H of benzene ring).

The ^{13}C NMR ($CDCl_3$) spectrum showed signals at 178.54 (-C=O), 143.94 (C-O), 129.53 (C-N), 124.20 (C-C-C-O), 122.74 (C-C-N), 110.32 (C-C-C-N) and 110.15 ppm. (C-C-O).

The UV (EtOH) spectrum displayed λmax at 202 nm.

The MS spectrum gave a molecular ion peak at 135 and other fragments at m/e 106 (M^+-COH), 91 (M^+-CO_2), 79 (M^+-C_2H_2NO), 63 (M^+-$C_2H_2NO_2$) and 52 (M^+-$C_2H_2NO_2$-CH_2).

Elemental analysis found : C 62.06%, N 10.35%, H 3.75%

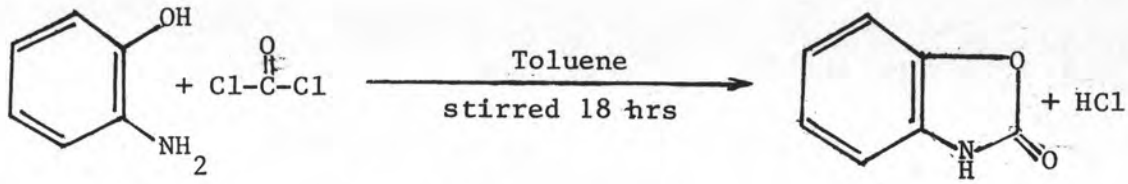
calcd. for $C_7H_5NO_2$; MW 135 : C 62.22%, N 10.37%, H 3.73%

Table V The results from column chromatography of Fraction D

Eluents	Fraction No. (25 ml)	Remarks
50% Chloroform-Petroleum Ether	1-18	yellow oil
	19-40	yellow oil + colorless crystals (Rf 0.314)
	41-44	yellow oil
	45-53	yellow oil + white crystals (Rf 0.67, Rf 0.48) (Fraction D_1)
	54-58	yellow oil

Synthesis of Benzoxazoline-2-one[13]

O-Aminophenol (1.09 g) was dissolved in toluene (20 ml) and mixed with carbonyl dichloride (2.97 g). The mixture was continuously stirred at room temperature for 18 hrs., stripped off the solvent toluene yielded colorless solid. This compound was dissolved in hot dichloromethane, cooled and added petroleum ether, giving colorless rhombic crystals 0.85 g (77.98% yield) which was recrystallized from petroleum ether for several times. The product showed the m.p. of 137°-138° (Rf = 0.313, solvent: 2%, MeOH-$CHCl_3$). The equation of the reaction was shown in the next page.



O-aminophenol Carbonyl dichloride

The IR spectrum of benzoxazoline-2-one showed ν_{max}^{KBr} cm^{-1}: 3450 (N-H), 3220 (OH), 1780 (lactone), 1730 (ester) and 1150 (C-O).

The ^{1}H NMR)$CDCl_3$) spectrum gave two signals at δ 7.10 (4 H, s, H of benzene ring) and 9.77 (1 H, broad, NH).

The m.p. of this synthetic benzoxazoline-2-one was underpress on admixture with the isolated Compound 4.

2.8.2 Purification and Properties of Compound 5

Compound 5, m.p. 292° (decomp.), was eluted by chloroform containing 20% methanol and recrystallized from 10% methanol chloroform to give a white amorphous crystals (400 mg.), $[\alpha]_D^{23}$ - 48.5° (in $CHCl_3$: MeOH = 2:1), and Rf 0.769 (solvent: 15% MeOH-$CHCl_3$). It gave violet colour with Liebermann-Burchard's reagent and gave positive test with Molish's reagents.

The IR spectrum showed ν_{max}^{KBr} cm^{-1}: 3400 (O-H) and 1020-1080 (glycosidic linkage).

The ^{1}H NMR (DMSO + $CDCl_3$) spectrum exhibited signals typical to steroid at δ 0.66-2.03 and other peaks at 5.2 (3 H, m, olefinic proton).

The ^{13}C NMR ($CDCl_3$ + CD_3OD) spectrum displayed at 139.8, 121.3, 137.6, 128.6 ppm (4 carbons double bond), 106.8, 73.2, 69.9, 61.4 56.3 and 56.2 ppm (6 carbons adjacent to oxygen) and other peaks with respect to steroid compound.

The UV (EtOH) spectrum gave λmax 203 nm (Σ 6089).

The MS data revealed mass fragments at m/e 412 (M^+), 395 (M^+-OH), 394 (M^+-H_2O), 366 (394-C_2H_4), 283 (336-C_6H_{11}) and 255 (283-C_2H_3).

Acid Hydrolysis of Compound 5

Compound 5 (100 mg.) was hydrolyzed by refluxing with 10% hydrochloric acid in ethanol for 8 hrs. Solvent was removed under reduced pressure. The residue was diluted with water and extracted with diethyl ether which was separated into two layers as aglycone in diethyl ether and sugar component in water.

Study of Aglycone

Ethereal solution, that described above was washed with 5% sodium bicarbonate, water, dried over anhydrous sodium sulfate and evaporated, the residue was recrystallized from petroleum ether and further by methanol to yield white needles (75 mg) m.p. 167°-118°, $[\alpha]_D^{23}$- 51.3° (in $CHCl_3$-MeOH, 2:1) and Rf 0.23 (solvent: $CHCl_3$). It gave blue colour on Liebermann-Burchard test.

The IR spectrum gave absorption peaks at ν_{max}^{KBr} cm^{-1}: 3300-3500 (OH), 1640, 820 and 820 (C=CH-), 1060 (C-O).

The UV (EtOH) showed λmax 203 nm (Σ 6018).

The mass spectrum displayed mass fragments at m/e 412 (M^+), 394 (412-H_2O), 273 ($M^+-C_{10}H_{19}$).

The GC analysis (condition: column, OV-I 3% 4 mm x 1.5 m, column temp. 290°, carrier gas N_2 45 ml/min) gave only one component at Rt 3.7 min which was identical to an authentic stigmasterol.

Elemental analysis found : C 84.41%, H 11.86%

calcd. for ($C_{29}H_{48}O$: MW = 412) : C 84.40%, H 11.72%

The mixed m.p. of this aglycone with stigmasterol did not show any depression.

Acetylation of Aglycone

Aglycone of Compound 5 (20 mg) was acetylated as it was described for Compound 2 to yield white needles (15 mg), m.p. 142° (Rf 0.81, solvent: $CHCl_3$) from petroleum ether and followed by methanol.

The IR spectrum of aglycone acetate showed strong absorption band at 1740 cm^{-1} (acetate).

Hydrogenation of Aglycone

The aglycone (80 mg.) in absolute ethyl acetate (10 ml) was reacted with 1 g of 10% palladium-charcoal and then placed in hydrogenator vessel. The hydrogen gas was introduced into the reaction mixture, stirred for 6 hrs, filtered off, and washed with ethyl acetate yielded the white plate crystals. It was recrystallized from petroleum ether giving hydrogenated aglycone (75 mg.), m.p. 138° (Rf 0.714, solvent: 80% $CHCl_3$).

The IR spectrum showed absorption bands ν_{max}^{KBr} cm^{-1}: 3400–3500 (OH) and 1060 (C-O).

The ^1H NMR ($CDCl_3$) spectrum exhibited signals at δ 3.59 (1 H at C-3, m).

The ^{13}C NMR ($CDCl_3$) spectrum showed chemical shifts at 71.41 (C-OH), 56.28, 56.57, 56.28 and 54.46 ppm.

The GC analysis of hydrogenated aglycone of Compound 5 (condition: column, OV-107, column temp. 260°, carrier gas He) gave Rt 30.62 min which similar to stigmastanol.

The MS gave mass fragments at 416 (M^+), 399 (M^+-OH), 398 (M^+-H_2O) and 275 ($M^+-C_{10}H_{21}$).

Determination of Carbohydrate Component of Compound 5

After removing the aglycone from the hydrolysis mixture of Compound 5 as described above, the aqueous phase was neutrallized

with silver carbonate, filtered off, and concentrated in vacuum oven at room temperature to white powder (3 mg). The solution of this carbohydrate (0.2 ml in H_2O 1 ml) was spoted on appropriate sheet (30 x 40 cm) of Watmann No. 1 chromatrography paper with various authentic sugars. The chromatogram was developed by desending technique with n-butanol-benzene-pyridine-water (5:1:3:3) for 24 hrs, dried at room temperature and detected with aniline hydrogen phthalate, further by heating at 110^{o}-120^{o} for 15 min. It gave one spot (Rf 0.43) which aas identical to authentic glucose.

TLC of the component of neutral aqueous phase of glycoside hydrolysate was achived on plastic sheet silica gel 60 (F_{254}) compared with various authentic sugars (solvent: $CHCl_3$-MeOH-H_2O = 6:5:1). The sugar was detectid by spraying with 20% sulphuric acid, and dried at 110^{o}-120^{o} to develop colour spot of the resolved sugar (Rf 0.416).

Trimethylsilylation of Carbohydrate Component of Compound 5[14]

The sugar component obtained as described above was further evaporated in high vacuum oven at room temperature. Dried sample was allowed to react with trimethylsilylether (0.5 ml) for 20 min at room temperature. The water was added and the product was extracted twice with petroleum ether. Petroleum ether extract was dried over anhydrous sodium sulfate and filtered. The filtrate was evaporated to afford the sample which was subjected to GC analyses. (condition: column OV-2 3%, column temperature 160^{o}, column length 4 mm x 1.5 m, carrier gas (N_2) 45 ml/min, chart speed 1 cm/min.).

The GC data showed the presence of one sugar (Rt 14.2 min) which was identical with authentic trimethylsililated glucose.

Peracetylation of Compound 5

Compound 5 (100 mg) was acetylated as described for the acetylation of Compound 2 to yield colourless crystals, m.p. 145°-146° (Rf 0.26, solvent: $CHCl_3$) from hexane and methanol.

The IR spectrum of Compound 5 peracetate showed absorption bands at ν_{mas}^{KBr} cm^{-1}: 2900 (C-H), 1740, 1240 (acetate).

The ^1H NMR ($CDCl_3$) spectrum showed signals which typical of steroid at 0.66-1.80 and 2.03 (12 H of acetate).

The ^{13}C NMR ($CDCl_3$) spectrum exhibited signals type of 4 carbonyl groups at 170.6, 170.3, 169.3 and 169.2, 140.3, 138.2, 129.3 and 122.1 (2 C=C) ppm.

The mass spectrum showed mass fragments at m/e 412 (M^+), 394 (M^+-H_2O), 347 ($C_{14}H_{19}O_{10}$), 273 ($M^+-C_{10}H_{19}$).

Elemental analysis found : C 69.90%, H 9.13%

calcd. for $C_{43}H_{66}O_{10}$: MW 742 : C 69.54%, H 8.89%

Permethylation of Compound 5[15]

The Compound 5 (20 mg) and dissolved in DMF (8 ml) containing NaH (66 mg) with stirring, followed by addition of CH_3I (8 ml). The reaction mixture was left at room temperature for 24 hrs and extracted with aqueous chloroform. The chloroform layer was washed with water, dried over anhydrous $Na_2S_2O_3$, concentrated and crystallized from hexan to afford colorless needles (17 mg), m.p. 135° (Rf 0.69, solvent: 80% $CHCl_3$-Hexane).

Methanolysis of Permethylated Compound 5

Permethylated Compound 5 (15 mg) was hydrolyzed by refluxing with 5% hydrochloric acid in methanol for 4 hrs. The reaction mixture was concentrated, water was added and extracted with chloroform. The chloroform layer was dried over anhydrous sodium sulfate. The

solvent was evaporated to give white needles (6 mg), m.p. 167°-168° (Rf 0.235, solvent: $CHCl_3$) which was identified as stigmasterol by both mixed m.p. and Co-TLC.

The aqueous layer was neutralized with silver carbonate. The precipitate was filtered off, the aqueous layer was evaporated in high vacuum oven at room temperature. The residue was found to be methyl-2, 3, 4, 6-tetra-O-methyl glucopyranoside on the basic of GC analysis (Rt 6 min; condition: column 5% NPGS, column length 4 mm x 1.5 m, column temp. 160°, injector temp. 180°, carrier gas N_2 40 ml/min, chart speed 1 cm/min) by comparison with authentic methyl-2, 3, 4, 6-tetra-O-methyl-glucopyranoside.

2.8.3 Purification and Properties of Compound A

Fraction D_1 and Fraction E gave the same two Rf values (Rf 0.48 and 0.68, solvent: 5% MeOH-$CHCl_3$). These two fractions were combined to give a mixture (125 mg.). This mixture was separated by column chromatrography (2.5 x 40), silica gel 60, and eluted with benzene-ether-methanol (7.5:1.5:0.5). The fractions No. 20 to No. 27 from this column gave a mixture of yellow oil and white crystals (Table VI). The yellow oil was removed by washing with petroleum ether and further by methanol. The remaining material was recrystallized from 10% methanol-chloroform to give Compound A (41 mg.), m.p. 114°-115° as white amorphous (Rf 0.675, solvent: 5% MeOH-$CHCl_3$).

The IR spectrum showed peaks ν_{max}^{KBr} cm^{-1}, 3350 (OH), 3400 (N-H), 1675 (C=O).

The ^1H NMR ($CDCl_3$ + CD_3OD) spectrum revealed a single peak at δ 1.26.

The ^{13}C NMR ($CDCl_3$ + CD_3OD) spectrum exhibited signals at 186.87 (C=O), 108.96 (C-OH) and 34.62, 29.80, 29.47, 25.41, 22.81, 14.14 ppm. (CH_2-CH_2-CH_{2n}).

The MS showed mass fragments at m/e 492 (M^+), 464 (M^+-CO), 451 (464-CH_3), 423 (451-$(CH_2)_2$), 394 (423-C-OH), 339 (357-H_2O), 308 (339-CH_3-O), 278 (308-CH_2-NH_2).

Acetylation of Compound A

Compound A (6 mg.) was treated in the same manner for the acetylation of Compound 2 to give white amorphous, m.p. 88°-89° after purified by washing with methanol.

The IR spectrum showed ν_{max}^{KBr} cm^{-1}, 3450 (N-H), 1750 ($COCH_3$)

Table VI Column chromatography of Fraction D-E

Eluents	Fraction No. (25 ml)	Remakrs
Benzene-Ether-Methanol (7.5:1.5:0.5)	1-20	yellow oil
	20-27	yellow oil + white crystals (Compound A)
	28-34	yellow oil
	35-42	yellow oil + white crystals (Compound B)
	43-50	yellow oil

2.8.4 Purification and Properties of Compound B

Compound B, m.p. 146°-147° was obtained from fractions No. 35 to No. 42 in column chromatography. It was a mixture of yellow oil and white crystal (Table VI). The yellow oil was removed by washing with methanol several times yielding white amorphous solid (60 mg., Rf 0.485, solvent: 5% MeOH-$CHCl_3$).

The IR spectrum gave ν_{max}^{KBr} cm^{-1}: 3350 (N-H), 3200 (O-H) and 1620 (C=O).

The ^{1}H NMR ($CDCl_3$ + CD_3OD) spectrum revealed signals at δ 1.27 (s), 3.53 and 3.80.

The ^{13}C NMR ($CDCl_3$ + CD_3OD) spectrum showed 14.14 (CH_3-CH_2), 22.92, 25.47, 26.11, 29.58, 29.96, 32.18, 33.16, 34.78, 61.39 (C-OH), 72.65 (C-OH), 72 (C-OH), 176 (C=O) and 202.09 ppm (C=O).

The MS data gave molecular ion M^+ = 482 and other fragments at m/e 453 (M^+-COH), 437 (M^+-OCOH), 425 (439-CH_2), 408 (425-OH), 339 (357-H_2O), 308 (326-H_2O), 278 (308-CH-OH), 111 ($C_{11}H_{23}$).

Acetylation of Compound B

Compound B (17 mg.) was acetylated as described for acetylation of Compound 2 yielding white needles (15 mg.), m.p. 61°-62° after recrystallization from methanol.

The IR spectrum showed ν_{max}^{KBr} cm^{-1}: 1730 ($COCH_3$).

The ^{1}H NMR ($CDCl_3$) spectrum revealed signals at δ 2.80, 2.01 and 2.01 (H of acetate), 1.53 and 1.27.

2.9 Separation of Fraction III

The Fraction III 50 g (crude $CHCl_3$-EtOH = 8.5:1.5) was dissolved in chloroform 100 ml and subjected to silica gel column chromatography which was eluted with 60% chloroform-petroleum ether and increasing polarity by adding chloroform and methanol (see Table VII). The fraction No. 27 to No. 32 gave a white crystals in yellow oil solution. Removing the yellow oil by washing with 15% methanol-chloroform yielded a white crystals (10 mg), m.p. 170°-172° and the fraction No. 41 to 47 gave white crystals and yellow oil which were recrystallized from methanol and followed by petroleum ether to afford white amorphous solid (12 mg), m.p. 250°-251°.

Table VII Column chromatography of Fraction III.

Dluents	Fraction No. (250 ml)	Remarks
60% Chloroform-Petroleum ether	1-10	yellow oil
80% Chloroform-Petroleum ether	11-26	yellow oil
Chloroform	27-32	white crystals + yellow oil
10% Methanol-Chloroform	33-35	red brown oil
30% Methanol-Chloroform	36-40	red brown oil
50% Methoanol-Chloroform	41-47	white crystals + yellow oil
70% Methanol-Chloroform	48-57	red brown oil

2.10 Separation and Identification of Fraction IV (Inorganic Salts)

Insoluble inorganic salts separated from ethanol (1241 g) in Fraction IV. This insoluble salts were purified by washing with methanol for several times, dissolved in water, decolourised with charcoal and filtered off. After the solvent was evaporated giving the salts as white crystals (558 g). The chemical tests for these salts displayed that the major components were chloride salts. The atomic absorption spectra suggested that they composed of sodium 50%, potassium 28%, calcium 0.012%, magnesium 0.011%, manganese 0.0102% and zinc 0.00052%.