CHAPTER II

DISCUSSION AND INTERPRETATION

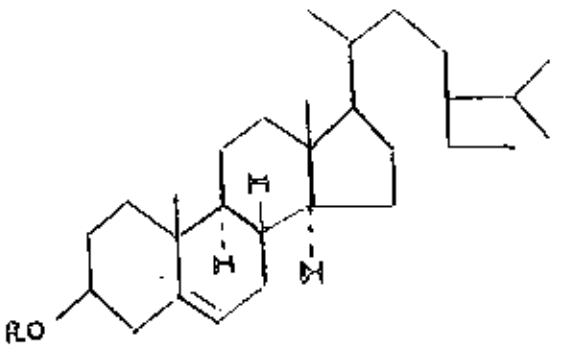
I. The constituents of leaf wax of Klue Namwa

Extraction of the dried leaves of the Thai banana plant Klue Namwa (Musa-cultivar Namwa) with light petroleum has afforded a wax which could be separated into two parts by treatment with light petroleum. The light petroleum insoluble wax was saponified by methanolic potassium hydroxide and the resulting product separated into acidic and neutral fractions. Chromatography of the neutral fraction on aluminium oxide using light petroleum and mixtures of light petroleum and chloroform as eluting solvents afforded a number of fractions; the fractions eluted by light petroleum and chloroform (3:7 and 2:3) and chloroform gave positive results in the Liebermann - Burchard test[9],[10](an immediate green colour was produced which later turned to brown)which indicated that these fractions contained steroids. The fractions were combined and added to the steroidal fraction isolated from the light petroleum soluble wax and worked up as described below.

The light petroleum soluble wax was saponified and worked up in a similar mannar to the light petroleum insoluble wax. The unsaponifiable matter was chromatographed on silica gel using light petroleum, mixtures of light

petroleum and benzene, and mixtures of benzene and chloroform as eluting solvents. Fractions containing steroids, as indicated by the Liebermann - Burchard test (green → brown), were obtained when the column was eluted with benzene / chloroform (1:1). Chromatography of these fractions on thin layers of aluminium oxide showed that they contained similar steroids to the steroid containing fractions obtained from the light petroleum insoluble wax. The combined steroidal fractions from the light petroleum soluble and insoluble waxes were chromatographed on aluminium oxide using mixtures of benzene and chloroform, and chloroform as eluting solvents. The fractions eluted by benzene / chloroform (1:1) afforded a compound $C_{29}H_{50}O$, m.p. 135° - 136°, $[\alpha]_D^{23} = -15°$ (c=5.02; $CHCl_3$), which gave a positive Liebermann - Burchard test (blue → green → brown). The infra-red spectrum of the compound suggested that it was a 3β-hydroxysteroid; strong absorption peaks were observed at 3400-3200 and 1050 $cm.^{-1}$ (O-H and C-O stretching frequencies of equatorial secondary alcohol).(11)-(14)

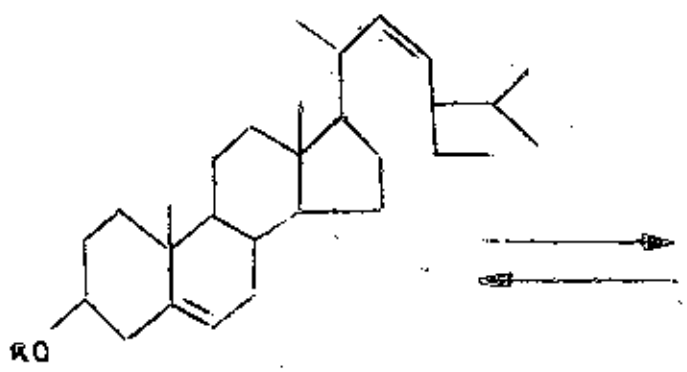
Acetylation of this compound with acetic anhydride and pyridine gave an acetate, $C_{31}H_{52}O$, as needles, m.p. 126° - 127°. The above evidence suggested that the sterol might be β-sitosterol (XII; R=H). This was confirmed by mixed melting point determinations and comparison of the I.R. spectra of the alcohol and its acetate with commercial β-sitosterol and its acetate.

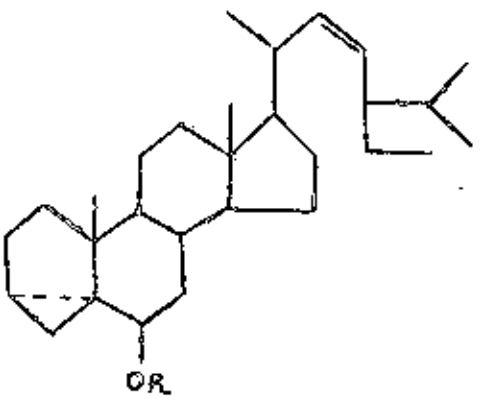


(XII)

The physical constants of β-sitosterol and its derivatives as recorded in the literature vary considerably. The alcohol has been reported to have a m.p. varying between 134° and 140°, and an $[\alpha]_D^{20}$ between -33° and -38°; the acetate a m.p. between 121° and 131°, and an $[\alpha]_D^{20}$ between -36° and -38°.(15)-(19)

J.A. Steele and E. Mosettig[19] have reported recently the preparation of pure β-sitosterol from stigmasterol as shown below.-





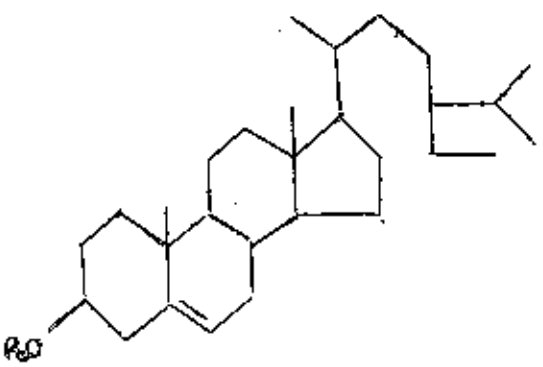
(XIII) R=Tosyl

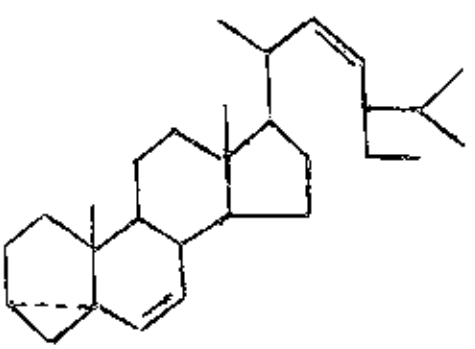
(XIV) R=CH_3

(XV) R=H

(XVI) R=CH_3

(XVII) R=H





(XIX) R=CH_3CO

(XX) R=H

(XVIII)

Solvolysis of stigmasteryl tosylate (XIII) in methanol afforded 5-stigmasteryl methyl ether (XVI) and, as minor components, stigmasteryl methyl ether (XIV),

stigmasterol (XV) and a hydrocarbon (XVIII). Hydrogenation of i - stigmasteryl methyl ether followed by rearrangement with zinc acetate in boiling acetic acid gave, after one crystallization, 22,23 - dihydrostigmasteryl acetate (β-sitosteryl acetate, XIX), m.p. 121° - 122°, $[\alpha]_D^{20}$ = -36.8°. Hydrolysis of this acetate furnished pure 22,23 - dihydrostigmasterol (β-sitosterol; XX), m.p. 137.5° - 138°, $[\alpha]_D^{20}$ = -33°.

J.W. Rowe[20] has reported a procedure for the separation of β-sitosterol from the other sterols of pine bark by precipitation of the 3β-hydroxysteroids with digitonin, followed by chromatography on aluminium oxide. He has found, however, that the resulting β-sitosterol was admixed with a small amount of campesterol, and that this could not be removed by fractional crystallization.

It is clear from the results of these workers that many of the samples of β-sitosterol which have been isolated from natural sources were contaminated with small quantities of other sterols.

The specific rotation of the β-sitosterol obtained from the leaf wax of Klue Namwa was more dextrorotatory than the pure β-sitosterol obtained by Steel and Mosettig. This suggested that it contained small quantities of more dextrorotatory compounds which could not be removed by column chromatography or fractional crystallization. Thin layer chromatography of the β-sitosterol on silica gel

using chloroform / light petroleum (1:1) as eluting solvent showed that the sterol contained small quantities of four components in addition to β-sitosterol; the thin layer chromatogram was similar to that of commercial β-sitosterol. Purification was achieved by chromatography on a 2 mm. thick layer of silica gel using chloroform / light petroleum (1:1) as eluting solvents. This afforded β-sitosterol m.p. 135° - 136°, $[\alpha]_D^{19}$ 17.6 (c=1.28; $CHCl_3$).

Further elution of the silica gel column, after the removal of β-sitosterol, with chloroform / benzene (3:1) and chloroform afforded a compound, $C_{20}H_{42}O_2$, m.p. 103° - 104°. The infra-red spectrum of this compound had a broad maximum at 3500-3200 $cm.^{-1}$ (OH stretching of a polymeric associated alcohol), and strong absorption peaks at 1050 $cm.^{-1}$ (C-O stretching of primary alcohol); 2900, 2840 and 1452 $cm.^{-1}$ (stretching and bending frequencies of methylene group); 720 and 710 $cm.^{-1}$ (rocking mode of CH_2). This information indicated that the compound was a long chain aliphatic diol; the absence of bands at 2950, 1450 and 1380 $cm.^{-1}$ showed that the OH groups were located at the ends of the chain, and that no branching existed.

Confirmation of the presence of hydroxyl groups in the compound (XXI) was obtained by the preparation of an acetate and a benzoate. Acetylation of the alcohol using acetic anhydride and pyridine gave an acetate, $C_{24}H_{46}O_4$, m.p. 60° - 61° (XXII), and benzoylation with benzoyl chloride

and pyridine yielded the benzoate, $C_{34}H_{50}O_4$, m.p. 60° - 61° (XXIII).

$CH_3.CO.O.H_2C.(CH_2)_{18}.CH_2.O.OC.CH_3$

(XXII)

$\uparrow Ac_2O + C_5H_5N$

$HO.H_2C.(CH_2)_{18}.CH_2.OH$

(XXI)

$\downarrow C_6H_5COCl + C_5H_5N$

$C_6H_5.CO.O.H_2C.(CH_2)_{18}.CH_2.O.OC.C_6H_5$

(XXIII)

Chuit and Hausser have reported[21] the preparation of 1,20 - Eicosanediol (XXVIII), m.p. 103° from 1,16 - Hexadecanediol using a series of malonic ester condensations as shown in the scheme below:-

$HO.H_2C.(CH_2)_{14}.CH_2.OH \xrightarrow{HBr} Br.CH_2.(CH_2)_{14}.CH_2Br$

(XXIV) (XXV)

$\downarrow CH_2.(CO_2Me)_2$

$MeO_2C.H_2C.(CH_2)_{16}.CH_2.CO_2Me \xleftarrow{MeOH} HO_2C.H_2C.(CH_2)_{16}.CH_2.CO_2H$

(XXVII) (XXVI)

$\downarrow$ Na / EtOH

$HO.H_2C.(CH_2)_{18}.CH_2.OH$

(XXVIII)

Bromination of 1,16 - Hexadecanediol (XXIV) using hydrobromic acid afforded the dibromocompound (XXV), which was condensed with dimethylmalonate, hydrolysed and decarboxylated to give 1,18 - Octadecanedicarboxylic acid (XXVI). Methylation of this acid furnished the dimethyl ester (XXVII), which on reduction with sodium and ethyl alcohol yielded 1,20 - Eicosanediol (XXVIII).

The evidence presented above indicates that the compound isolated from the leaf wax of Klue Namwa and having a m.p. 103° - 104° is 1,20 - Eicosanediol (XXVIII). Further support for this structure should be obtained from the mass spectra of this compound and its derivatives; a discussion of these results can be found in the appendix of this thesis.

II. The constituents of skin wax of Klue Namwa

A yellow wax has been isolated from the dried skins of the fruit of the Thai banana Klue Namwa (Musa cultivar Namwa) by extraction with light petroleum. A solution of the wax in hot chloroform and methanol deposited a solid, m.p. 75° - 78° (about 10 % by weight of the wax), on cooling, which was shown to be almost pure by chromatography on a thin layer of aluminium oxide. Purification of this compound was achieved by chromatography on a column of aluminium oxide using light petroleum and mixture of light petroleum and benzene as eluting solvents; the fractions eluted by light petroleum, benzene / light petroleum (1:3, 1:1 and 3:1), were found to be identical by thin layer chromatography and so they were combined. Crystallization of the solid from the combined fractions afforded a compound, $C_{52}H_{104}O_2$, m.p. 79° - 80°, whose infra-red spectrum showed ν_{max} at 1730 $cm.^{-1}$ (C=O stretching), at 735 and 720 $cm.^{-1}$ ($(CH_2)_n$ rocking mode, where n is greater than 4). The absence of absorption due to stretching and bending modes of an olefin or C-O-C asymmetric stretching of an ester indicated that it might be a long chain fatty acid ester of a long chain saturated alcohol.

Confirmation of the presence of an ester linkage in the compound was obtained by hydrolysis using 10 % ethanolic potassium hydroxide. This afforded an acid, $C_{26}H_{52}O_2$, m.p. 79° - 80° (XXX), and an alcohol, $C_{26}H_{54}O$,

m.p. 79° - 80° (XXXI).

Strong peaks in the infra-red spectrum of the acid (XXX) at 3400, 2700 and 1690 $cm.^{-1}$ (O-H and C-O stretching of a CO_2H); 2900 and 2835 $cm.^{-1}$, 1460 $cm.^{-1}$ (CH_2 stretching and bending vibrations); 1420 $cm.^{-1}$ (CH_2 adjacent to CO_2H); 920 $cm.^{-1}$ (O-H out of plane bending of acid dimer); 720 and 710 $cm.^{-1}$ ($(CH_2)_n$ rocking, where $n > 4$) suggested that the compound was a long chain monocarboxylic acid. The presence of bands (10-11 bands) in the region 1350-1180 $cm.^{-1}$ showed that the chain length of this acid must be greater than that of stearic acid[13], and possibly contained 24 methylene groups. The acid amide, prepared by treatment of the acid chloride with ammonia, was an amorphous solid, $C_{26}H_{53}ON$, m.p. 108° - 109°.

$$CH_3.(CH_2)_{24}.CO.O.CH_2.(CH_2)_{24}.CH_3$$

(XXIX)

$\downarrow$ hydrolyse

$$CH_3 - (CH_2)_{24}.CO_2H + CH_3.(CH_2)_{24}.CH_2.OH$$

(XXX) (XXXI)

The evidence presented above indicated that the acid was cerotic acid (XXX), which has been found as a constituent of bees wax[22] carnauba wax[23] and klep wax[24] and in chinese[25] and opium waxes[26] as it ceryl ester. Cerotic acid has been reported to have a m.p. 77° - 79°, and its amide a m.p. 108° - 109°.

The infra-red spectrum of the alcohol showed strong hydroxyl (3400-3300 and 1050 $cm.^{-1}$), methyl and methylene bending and stretching (2940, 2900, 2835, 1464 and 1455 $cm.^{-1}$)) and methylene rocking (725 and 715 $cm.^{-1}$) absorption, and suggested that the compound was a long chain alcohol.

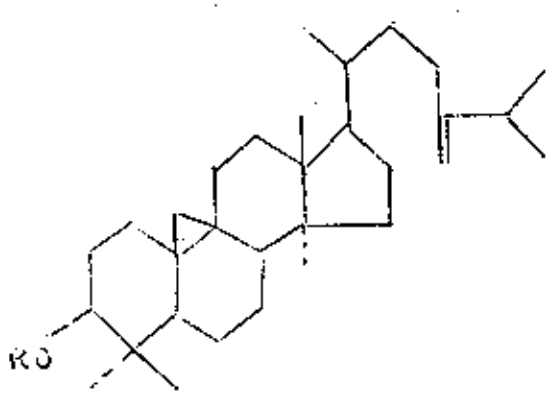
The presence of a hydroxyl group was confirmed by acetylation using acetic anhydride and pyridine. This gave an acetate, $C_{28}H_{56}O_2$, m.p. 60° - 61°.

Ceryl alcohol (XXXI), m.p. 79° - 83°, has been reported to be a constituent of a large number of plants including Ajuga bracteosa[28], Argemone mexicana[29], Apple cuticular wax[30], Colendula officinalis[31], Grindelia squarrosa flowers[32], Olive oil[33], Picea omorika - wood oil[34], Pine (maritime)[35], Vitex luceus bark[36], Citrus[37], Gamma - millet bran oil[38], Zizyphus jujuba[39], Xanthium strumarium[40].

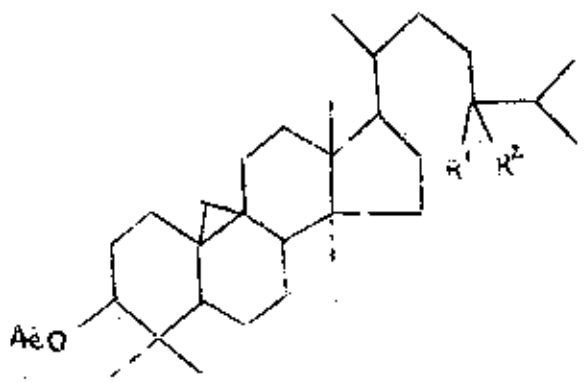
The evidence presented above showed that the ester derived from the skin wax of Klue Namwa was ceryl cerotate (XXIX). This compound has been found to be the main constituent of chinese[25] and opium waxes[26], and to have a m.p. 81° - 82°[27].

The remaining part of the wax after separation of the ester was an oil. Chromatography of the oil over silica gel using mixture of light petroleum and benzene, mixture of benzene and chloroform, and chloroform as eluting

solvents afforded a number of fractions. Fractions eluted by benzene / light petroleum (3:2) gave a positive Liebermann-Burchard test (yellowish green fluorescence → yellow → brown). These fractions were saponified using methanolic potassium hydroxide and the resulting mixture of alcohol was acetylated. The acetylated mixture was chromatographed on aluminium oxide using mixture of light petroleum and benzene, and benzene as eluting solvents. Fractions eluted by benzene / light petroleum (3:7) contained steroids and were rechromatographed on aluminium oxide. The fractions eluted by benzene / light petroleum (1:4) afforded a compound, $C_{33}H_{54}O_2$, m.p. 115° - 116°, $[\alpha]_D^{23}$ + 45.2° (c=2.68; $CHCl_3$). The infra-red spectrum of this compound showed strong absorption at 1725 $cm.^{-1}$ (C=O stretching of an acetate), 1640 and 885 $cm.^{-1}$ (C=C stretching and out of plane bending of terminal methylene), 1238 $cm.^{-1}$ (C-O-C stretching of acetate), and suggested that it might be a tetracyclic triterpenoid containing a vinylidene group in the side chain. Hydrolysis of the acetate using 10 % methanolic potassium hydroxide afforded an alcohol, $C_{31}H_{52}O$, m.p. 122° - 123°. This evidence suggested that the acetate might be 24-methylenecycloartanyl acetate (XXXII); R=CH_3CO. Confirmation of this assignment was obtained by a mixed m.p. determination and comparison of the infra-red spectra of the acetate with a sample of 24-methylenecycloartanyl acetate which was kindly supplied by Dr. G. Ohta.



(XXXII)



(XXXIII)

24-methylenecycloartanol has been reported[41]-[43] to have a m.p. 120° - 123°, $[\alpha]_D^{20}$ = 42° - 44° and to afforded an acetate a m.p. 114° - 117°, $[\alpha]_D^{20}$ = 53° - 54°. The alcohol was first isolated from rice bran oil by Ohta and Shinizu[8] and later from Populus tremuloides heartwood[41], Tristania conferta[42] (Myrtaceae), and Cephalosphaera usambarensis Warb[43]. Its structure was shown to be (XXXII; R=H) by Ohta and Shinizu[8] by relating it to cycloartanol. 24-methylenecycloartanyl acetate was ozonized to 24-oxocycloartanyl acetate (XXXIII ; $R^1=R^2=O$), which was reduced to cycloartanyl acetate (XXXIII; $R^1=R^2=H$) by the Wolff - Kishner method.

24-methylenecycloartanol always occurs together with other phytosteroids or triterpenoids and its separation from them is very difficult;Ohta has found that fractional crystallization of the ferulate derivative[8] affords pure 24-methylenecycloartanol, m.p. 121° - 122°, $[\alpha]_D^{20}$ + 43°. In a more recent paper he has reported[44] that gas - liquid

chromatographic separation of the triterpenoids mixture reveals the presence of compounds which could not be isolated by other chromatographic techniques or fractional crystallization. Attempts by Ohta to separate cycloartenol from 24-methylenecycloartanol by means of thin layer chromatography proved unsuccessful. However in our hands 24-methylenecycloartanol has been separated from the other constituents of banana skin wax by means of preparative layer chromatography using a multiple run technique. In this way, compounds having similar R_f values after one elution could be separated.