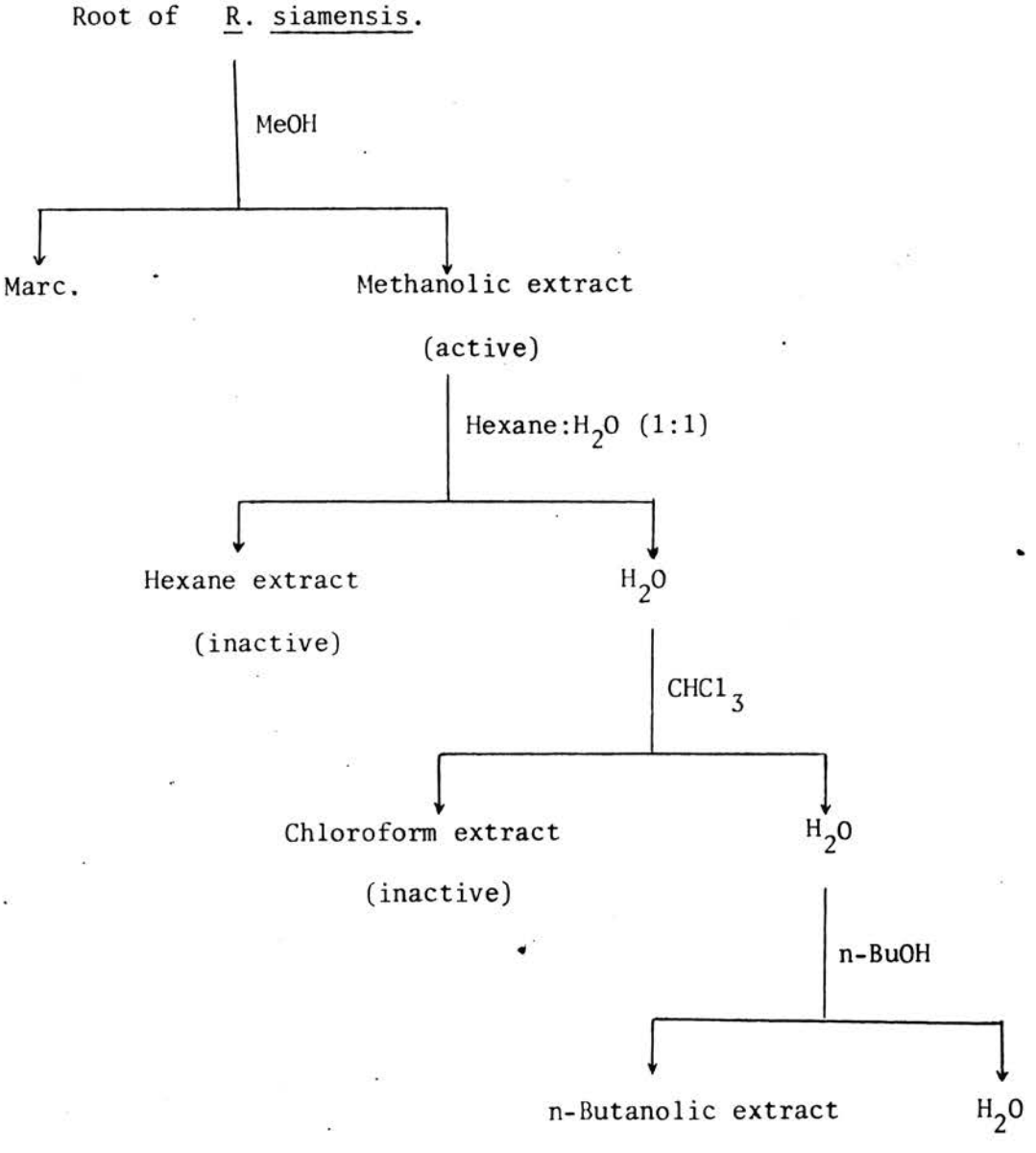
CHAPTER II

RESULTS AND DISCUSSION.

Pharmacological tests[3] of the total ethanolic extract of the dried root of R. siamensis done at the Faculty of Pharmaceutical Sciences of Chulalongkorn University scientifically confirmed the traditional used of R. siamensis for inducing abortion.

The hexane and chloroform soluble fractions of the methanolic extract obtained according to the procedure shown in Scheme 1 were tasted[12] at the Natural Products Research Institute of the Seoul National University in Seoul and was found to be inactive. Although the n-butanolic extract was not checked for such activity due to the limited amount of the available material one can predict that the pharmacologically active principles may be present in this fraction.

The lack of literature reports on chemical studies of R.siamensis encouraged us to isolate, purify and identify some of the chemical compounds from methanolic, n-butanolic and chloroform extracts despite the fact that the last fraction does not show any inducing abortion activity.



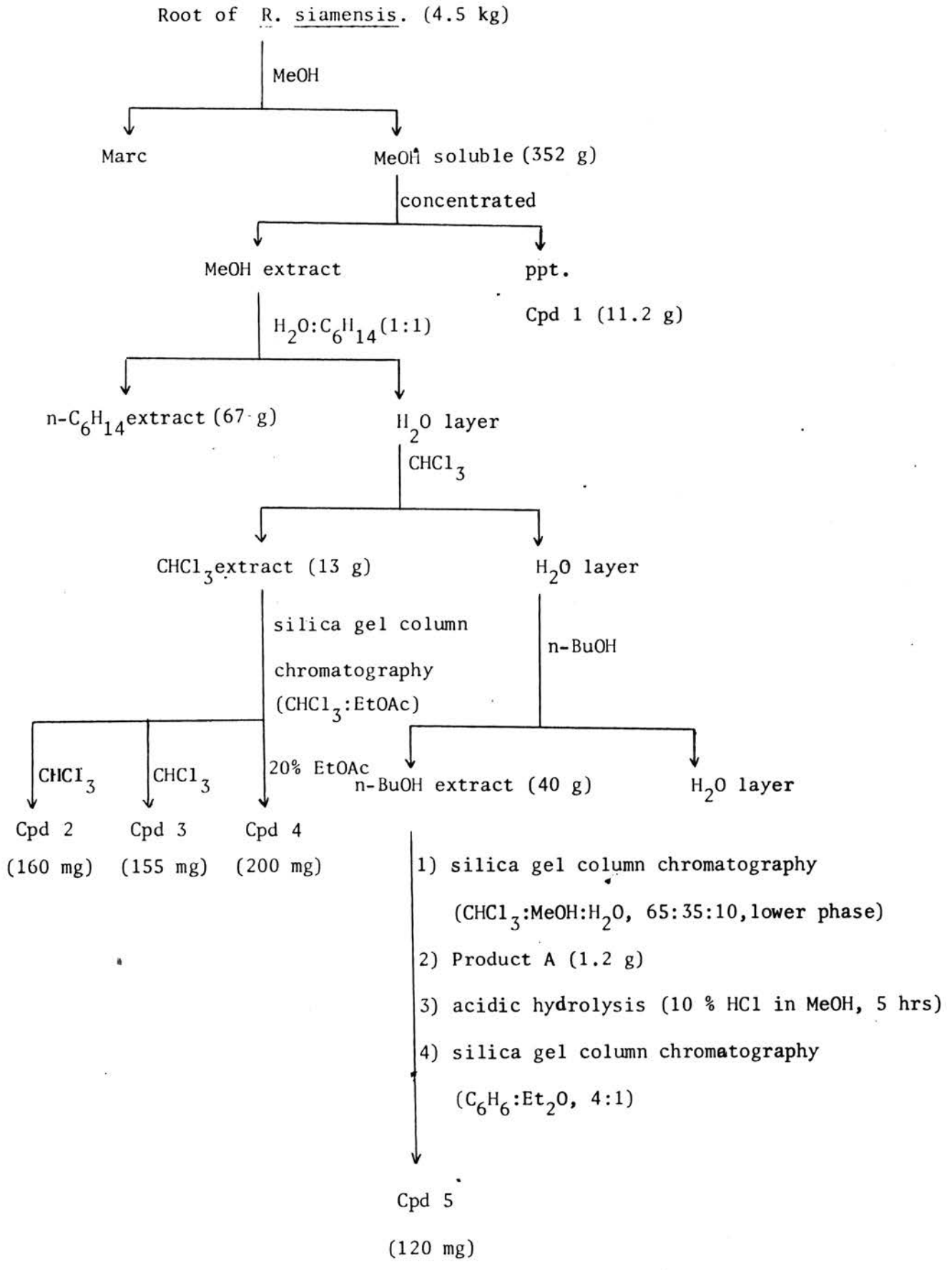
Scheme 1. Results of pharmocological tests.

Extraction and Isolation.

Finely ground root of R. siamensis (4.5 kg) was extracted with hot MeOH in Soxhlet apparatus. This methanolic extract was concentrated and kept in a refrigerator overnight to form a precipitate which was filtered off and was recrystallized from hot MeOH to yield Compound 1.

The concentrated filtrate was fractionated with aqueous hexane (C_6H_{14}: H_2O, 1:1) , $CHCl_3$ and n-BuOH successively. All fractions were concentrated separately under reduced pressure. The procedure for extraction and isolation is shown in Scheme 2.

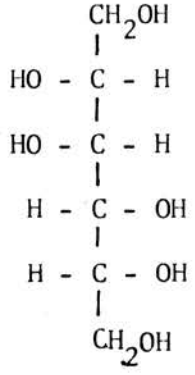
Compound 2, 3, and 4 were isolated from a chloroform soluble fraction by means of column chromatography using silica gel as an adsorbent. Product A was obtained in a similar way from the n-butanolic soluble fraction. Acidic hydrolysis of Product A followed by column chromatography gave Compound 5.



Scheme 2 The extraction and isolation procedure.

Structural Elucidation of Compound 1.

The methanolic extract gave much precipitate upon concontration. The precipitate was recrystallized from hot MeOH to afford Compound 1, 11.2g, m.p. 166° - 167° c, as white needles. Its IR spectrum shows strong absorption bands at 3300 and 1100 cm^{-1} corresponding to OH and C-O groups respectively. Optical rotation $(\alpha)_D^{20}$ -1.1° (c 2.07, H_2O) indicates that Compound 1 is optically active. The spectroscopic data as well as its sweet taste and negative test to Fehling reagent were in agreement with a structure of an alditol for Compound 1. The presence of hydroxyl groups was confirmed by acetylation of Compound 1. The resulted hexaacetate, m.p. 188° - 190° C, was crystallized from MeOH. Strong absorption bands in its IR spectrum at 1350 and 1750 cm^{-1} are characteristic to acetate groups. In addition there was a signal at δ 2.05 in the ^1H NMR spectrum with its integration of 18 protons corresponding to the 6 acetyl groups. Compound 1 was identified as D-mannitol by direct comparison with an authentic sample by both mixed m.p. and Co-TLC. The structure of Compound 1 is shown below.



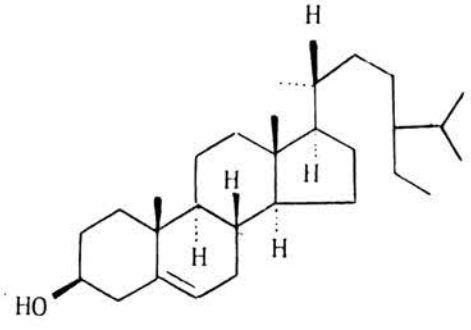
Compound 1 : D-mannitol.

D-mannitol has been found in many other species of this Rubiaceae family[7,13].

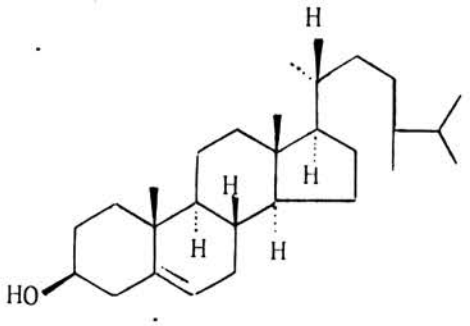
Structural Elucidation of Compound 2.

Chloroform soluble fraction (13 g) of the methanolic extract was subjected on silica gel column chromatography using $CHCl_3$ followed by $CHCl_3$- EtOAc mixtures of increasing polarity as an eluents. Two compounds assignated as Compound 2 (160 mg) and Compound 3 (155 mg) were obtained from the $CHCl_3$ fraction.

Compound 2,m.p. 138° - 140° ,C was crystallized from $CHCl_3$ to afford white needles. It gave blue colour in Liebermann-Burchard test which indicated that it is a steroidal compound. Its IR spectrum shows absorption bands at 3400 cm^{-1} which corresponds to a hydroxyl group and at 810, 840 cm^{-1} indicating a trisubstituted double bond. Mass spectrum shows its M^+ at 414 and other peaks at m/e 396 (M^+-H_2O), 273 [M^+-$C_{10}H_{21}$) 255 (M^+- $C_{10}H_{23}O$) and 214 (M^+- $C_{13}H_{28}O$). The fragmentation pattern of Compound 2 indicates that Compound 2 is a C_{29} steroidal Compound [14]. ^1H NMR of its acetate shows the presence of signals typical for steroid at δ 0.66 (CH_3 at C-18), 0.86 ($4CH_3$ at C-21,26,27,29), 1.0 (CH_3 at C-19), 2.02 ($COCH_3$), 4.6 (H at C-3) and 5.4 (H at C-6). All above spectroscopic results apparently agreed to the characteristic of β-sitosterol. However GLC analysis (see experimental conditions on page 36)shows the presence of two components (Rt 3.2 and 2.8 min)in the ratio of 3:2 corresponding to β-sitosterol and campesterol respectively by means of comparison with the authentic samples. Thus the assignated Compound 2 was in fact a mixture of these two compounds namely β-sitosterol and campesterol. Their co-occurence is well known [15,16] as a matter of fact that they have very similar chromatographic properties due to their similar structures as shown on the next page.



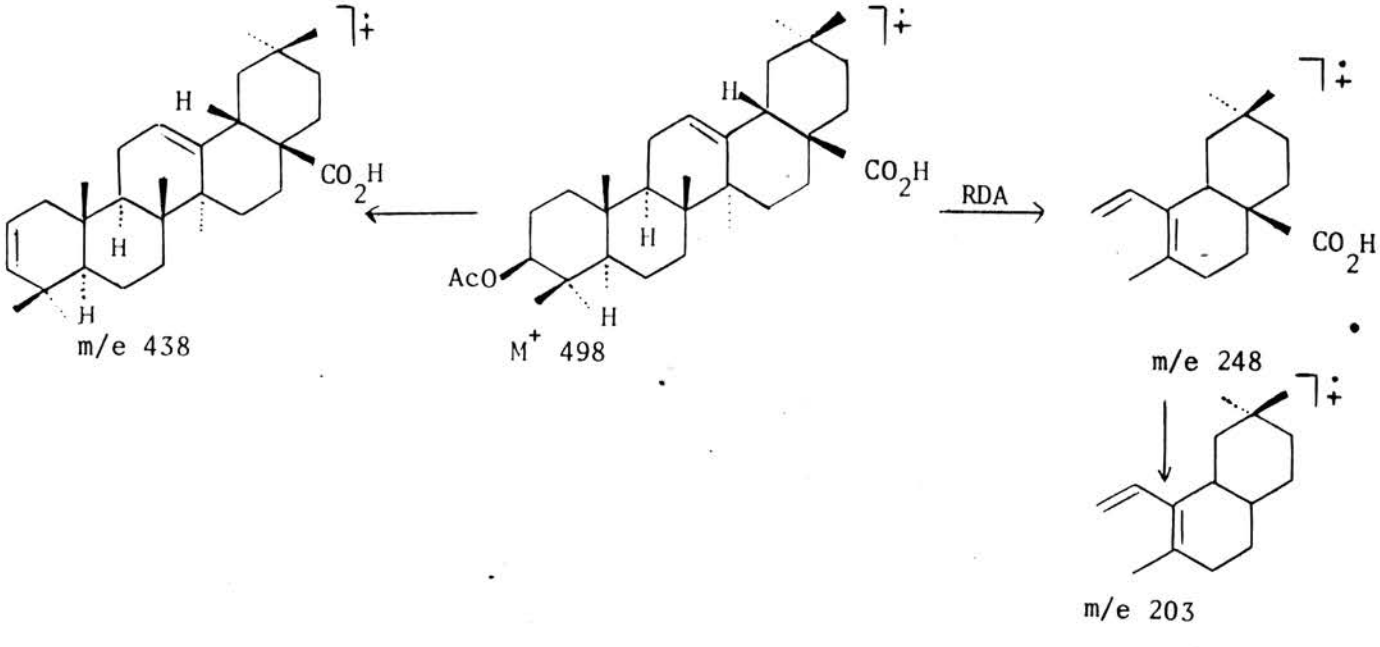
β-sitosterol



campesterol

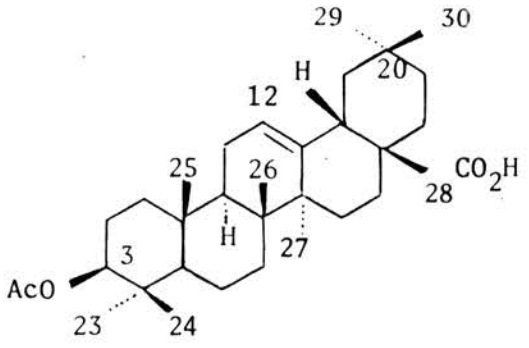
Structural Elucidation of Compound 3

Compound 3, m.p. 248° - 250° C, was crystallized from MeOH to give white needles. It gave pink colour in Lieberman-Burchard test which is characteristic to a triterpenoidal skeleton. IR spectrum shows strong absorption bands at 3200 (OH), 1700 (COOH), 1740 and 1260 cm^{-1} ($COCH_3$). Mass spectrum shows a M^+ of Compound 3 at 498 and other fragments at m/e 439 (M^+-OAc), 438 (M^+-HOAc), 248 (RDA), 203 (RDA-CO_2H) indicating the presence of acetyl and carboxyl groups in Compound 3. The fragmentation pattern[17,18] is shown below.



Due to the above retro Diels-Alder fragmentation as indicated in the mass spectrum it is in fact that Compound 3 has a olean-12-ene skeleton [19]. ^{1}H NMR spectrum of Compound 3 shows a singlet at δ 2.03 indicating the presence of the three protons of the acetoxy group at C-3 and a multiplet for proton on C-3 bearing the acetoxy group at δ 4.6 . The single olefinic proton[20] at C-12 appears at δ 5.3 as a complex multiplet.

The last step in the structural elucidation of Compound 3 is to establish the position of a carboxylic group. The analysis of the methyl resonances is very useful for this purpose. It is known for steroid and triterpenoid skeletons that the modifications in a substitution pattern[21,22] are normally accompanied by the systematic changes in ^{1}H NMR chemical shifts of the angular methyl groups. The calculated values of the chemical shifts of angular methyl groups to 3β-acetoxy-28-carboxy-olean-12-ene (3β-acetyl oleanolic acid) are in good agreement with experimental data for Compound 3 (see Table 3).



3β-acetoxy-28-carboxy-olean-12-ene (3β-acetyl oleanolic acid)

Table 3 The chemical shifts, calculated and observed values, of the angular methyl groups in Compound 3.

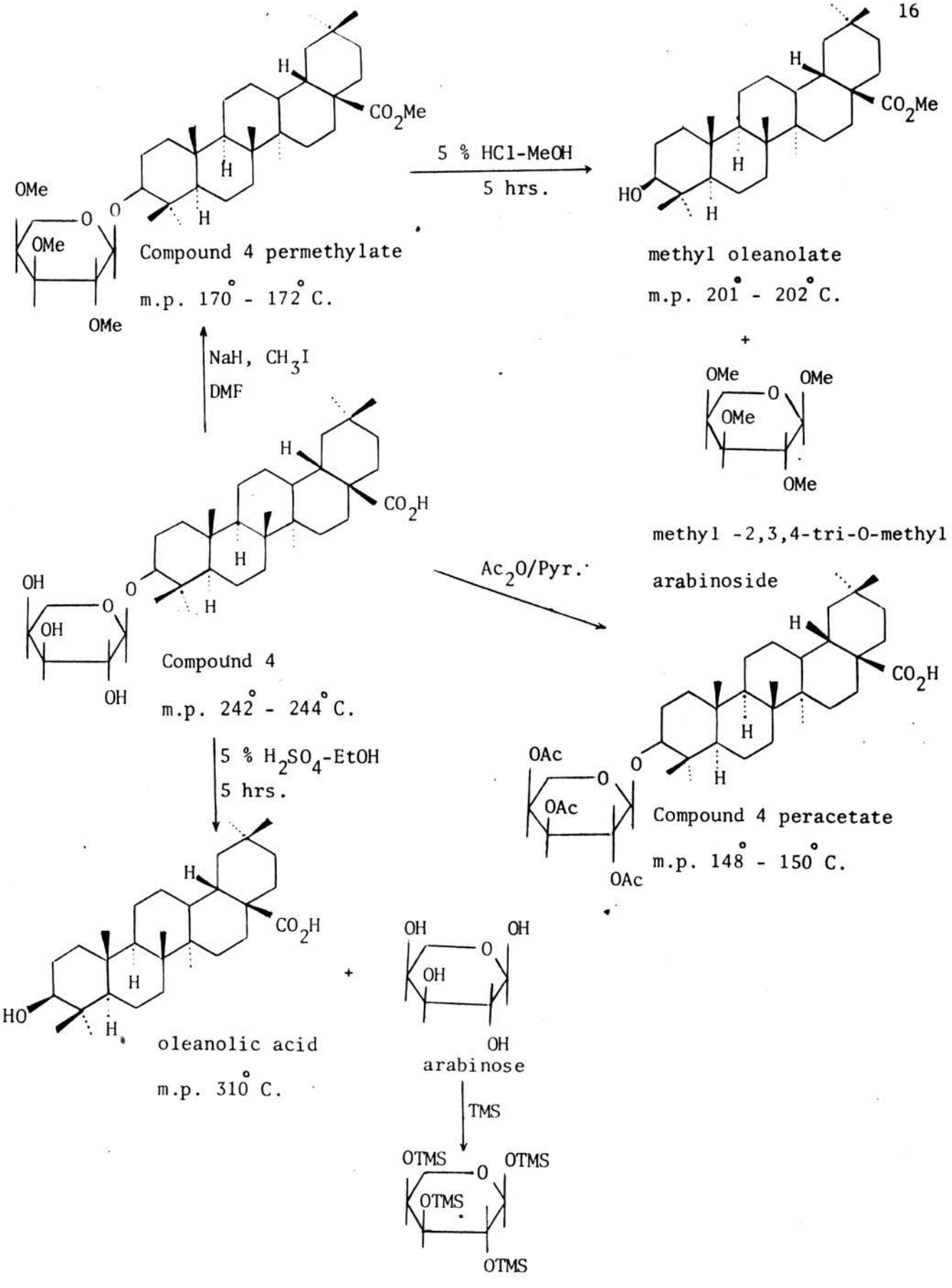
	Me-23	Me-24	Me-25	Me-26	Me-27	Me-29	Me-30
Oleanene[21,22]	53	50.5	56.5	59	69	53	53
3β-OAc	0	+3	+1	0	0	+0.5	+0.5
28-COOH	-1	-0.5	-1.5	-14	0	+2.5	+2.5
Cal.(cps)	52	53	56	45	69	56	56
ppm	0.87	0.88	0.93	0.75	1.15	0.93	0.93
Obs.	0.90	0.90	0.97	0.76	1.13	0.97	0.97
Diff.	0.03	0.02	0.04	0.01	0.02	0.04	0.04

Compound 3 was clearly proved to be 3β-acetyloleanolic acid which was further confirmed by deacetylation with 10 % NH_4OH in MeOH. The deacetylated product was identified as oleanolic acid by comparison with an authentic sample in both mixed m.p. and Co-TLC.

Structural Elucidation of Compound 4.

Chloroform soluble fraction (13 g) of methanolic extract was subjected on silica gel column chromatography using $CHCl_3$ and subsequently with $CHCl_3$-EtOAc mixtures of increasing polarity as eluents. Eventually, Compound 4 (200 mg) was eluted with $CHCl_3$ containing 20 % of EtOAc.

Compound 4, m.p. 242° - 244°C was crystallized from MeOH to give white needles. It gave pink colour in Liebermann-Burchard test and produced a positive Molish test which indicated that it was a triterpenoid with a sugar attached to it. IR spectrum shows strong absorption bands at 3400 (OH), 1700 (COOH) and 1000-1100 cm^{-1} (glycosidic linkage). Chemical reactions of Compound 4 are illustrated in Scheme 3.

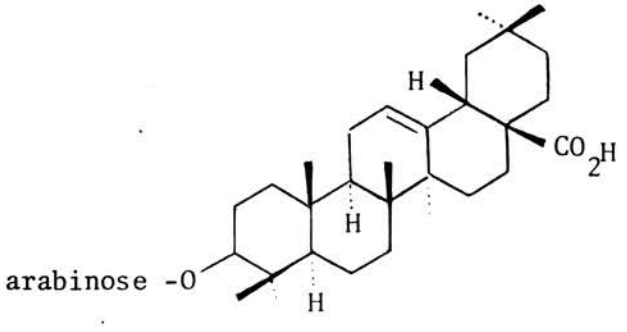


Scheme 3. Chemical reactions of Compound 4.

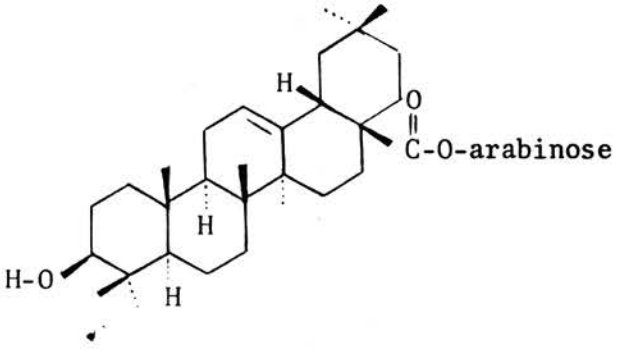
Acidic hydrolysis of Compound 4 gave a sapogenin as white needles m.p. 310° C. It was identified as oleanolic acid by direct comparison with an authentic sample in both mixed m.p. and Co-TLC. A carbohydrate was also found to be arabinose by GLC analysis (See experimental conditions on page 40).

An attempt to saponify Compound 4 by refluxing with 10 % KOH in EtOH for 5 hrs. was unsuccessful. TLC examination of the reaction mixture showed only the unchanged Compound 4.

There are two possible structures for Compound 4 since it can be formed by either an ether (I) or an ester (II) linkage as depicted below.



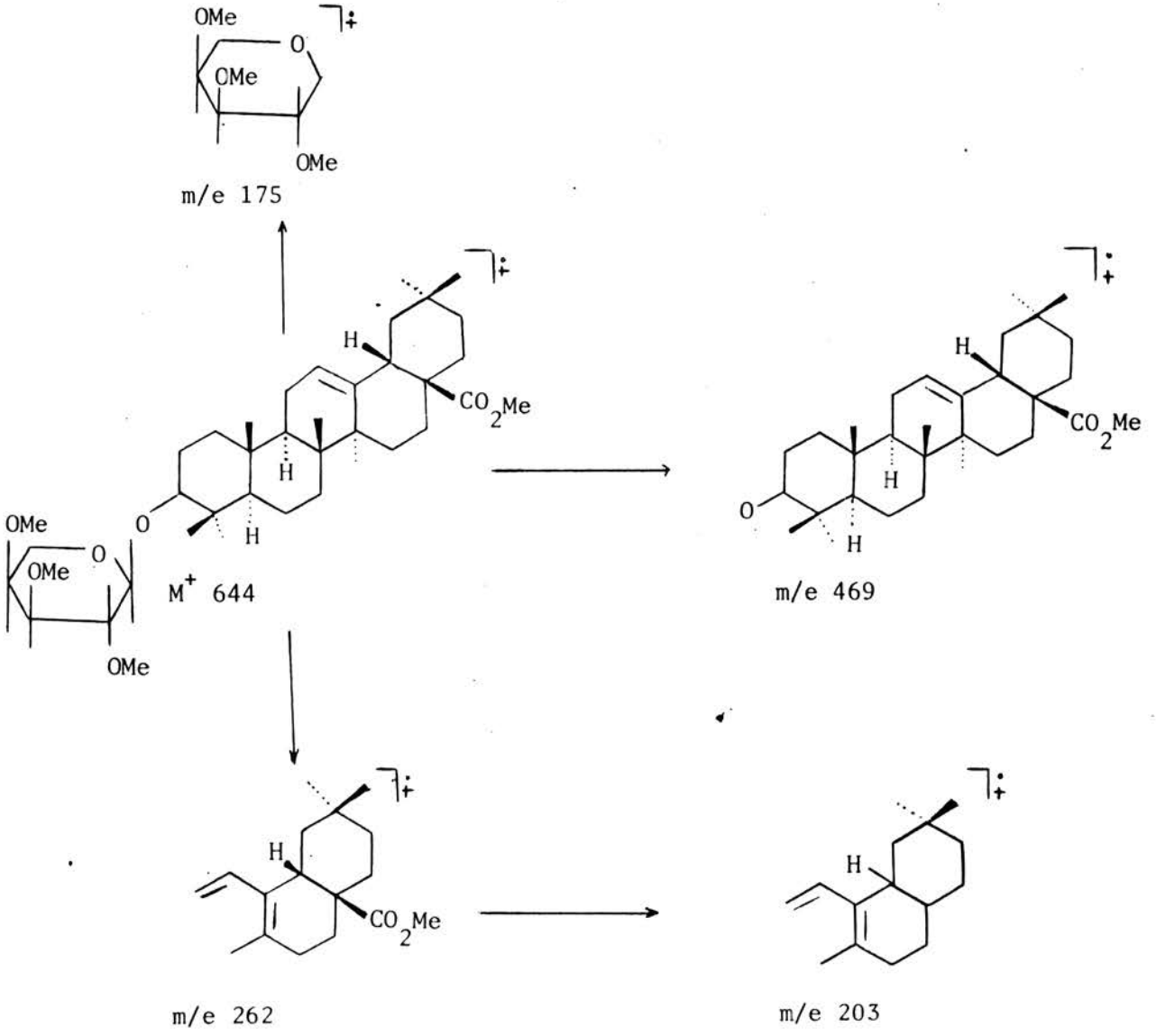
I



II

In general, glycoside are very stable under basic conditions which an esters are easily hydrolyzed. The negative result of the basic hydrolysis as mentioned above indicated the presence of Structure I rather than Structure II, in which arabinose is glycosidically linked with hydroxyl group at C-3 of oleanolic acid.

Compound 4 was permethylated by modified[23] Hakomori method[24]. The mass spectrum of permethylated derivative shows its peaks at M^+ 644 ($C_{39}H_{64}O_7$), m/e 469 ($M^+-C_8H_{15}O_4$), 262 ($M^+-C_{22}H_{58}O_5$), 203 (RDA-CO_2Me)[19] and m/e 175 ($M^+-C_{31}H_{44}O_3$). The fragmentation pattern is shown as follow.

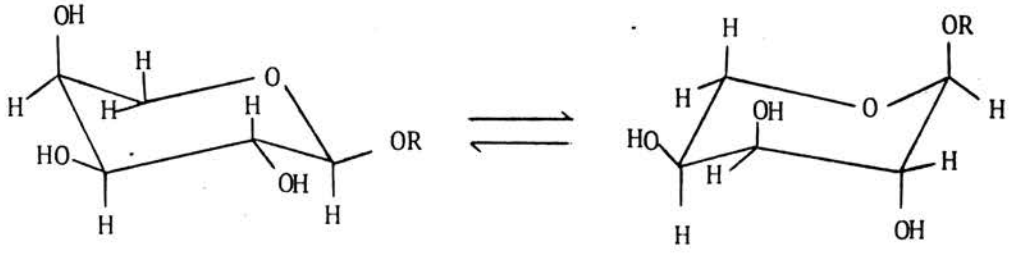


From the MS data[25], it was apparent that the molecular structure of Compound 4 was composed of one molecule of each arabinose and oleanolic acid. Permethylated derivative gave methyl -2,3,4-tri-O -methyl arabinoside and methyl oleanolate upon hydrolysis with 5 % methanolic HCl for 5 hrs. (see experimental details on page 41).

All the presented results are in consistence with the characteristic of Structure I and it is confirmed that Compound 4 is composed of an arabinopyranose linked to the C-3 hydroxyl group of oleanolic acid.

In order to establish the configuration of the glycosidic linkage, Compound 4 was peracetylated and identified by means of ^{1}H NMR which shows signals at δ 0.73 (CH_3 at C-26), 0.98 ($5CH_3$ at C-23,24,25,29,30), 1.15 (CH_3 at C-27), 2.05 (2 $COCH_3$), 2.08 ($COCH_3$), 5.4 (H at C-12) and 4.12 (d, J=6 Hz , anomeric proton). The last chemical shift and its coupling constant indicated the existing α - glycosidic linkage.

Conformational analysis of α -L-arabinopyranoside shows that two chair conformations, $^{4}C_1$ (L) and $^{1}C_4$ (L) are possible[26,27,28].



$^{4}C_1$ (L) $\rightleftharpoons$ $^{1}C_4$ (L)

In the $^4C_1(L)$ conformation, the anomeric proton and the proton at (C-2 are diaxial and so, according to Karplus equation [29], a vicinal coupling constant ^3J (H-1 , H-2) of 7-10 Hz can be predicted[26]. On the other hand, diequatorial arrangment of H -1 and H -2 in $^1C_4(L)$ conformation would produce a smaller vicinal coupling constant of 2-4 Hz[26]. Incidentally, the observed valued J (H -1, H -2) was 6 Hz which was rather small for the diaxial coupling constant. This might due to the considerable contribution of $^1C_4(L)$ conformation[27] in the molecular structure of compound 4 in which $^4C_1(L)$ conformation was predominantly adopted[26,27,28].

The presence of α-L-glycosidic linkage was further confirmed by applying the method of molecular rotation differences, $(M)_D$[30]. It was demonstrated that in steroid glycosides the optical rotation contribution of the carbohydrate component is almost independ on the nature of aglycone. The carbohydrate contribution is approximately equal to $(M)_D$ of the correspond α-and β- methyl glycopyranoside.

Thus the molecular rotation of sugar[31] can be estimated as a difference between the molecular rotation of Compound 4 and its sapogenin[32] namely oleanolic acid.

$$(M)_D \text{ of sugar} = (M)_D \text{ of saponin} - (M)_D \text{ of sapogenin}$$

$$= \left[\frac{(\alpha)_D^{23} \times MW.}{100}\right]_{\text{Saponin}} - \left[\frac{(\alpha)_D^{23} \times MW.}{100}\right]_{\text{sapogenin}}$$

Saponin $(\alpha)_D^{23} = +47.5^\circ$

$MW. = 588$

Sapogenin $(\alpha)_D^{23} = +58.65^\circ$

MW. = 456

$$(M)_D \text{ of sugar} = \frac{+47.5^\circ \times 588}{100} - \frac{+58.65^\circ \times 456}{100}$$

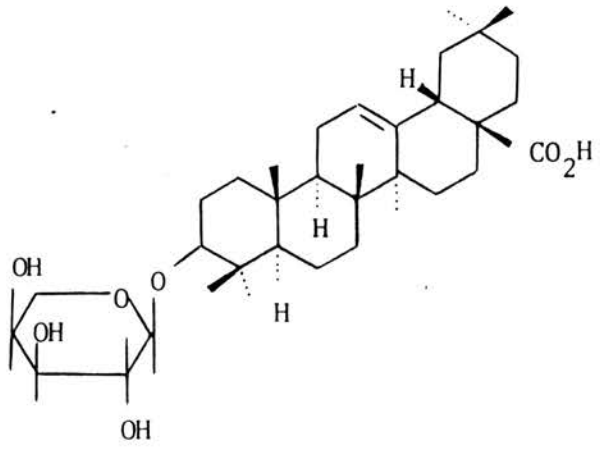
$$= +12.4^\circ$$

Literature data[33] for molecular rotation of methyl arabinosides in D-and L- series are shown below.

$(M)_D$ of methyl	-α -L-arabinopyranoside	+ 28.37
"	-α -D- "	- 29.86
"	-β -L- "	+402.62
"	-β -D- "	-401.8
$(M)_D$ of methyl	-α -L-arabinofuranoside	-209.92
"	-α -D- "	+164
"	-β -L- "	+193.5

The calculated value + 12.4° is obviously in the same range as that of the one for methyl-α,-L-arabinopyranoside.

According to all spectral and chemical evidences, it was clearly proved that Compound 4 was in fact 3-0- α -L-arabinopyranosyl oleanolic acid with its structure as shown on the next page.



Compound 4. 3-O- α -L-arabinopyranosyl oleanolic acid.

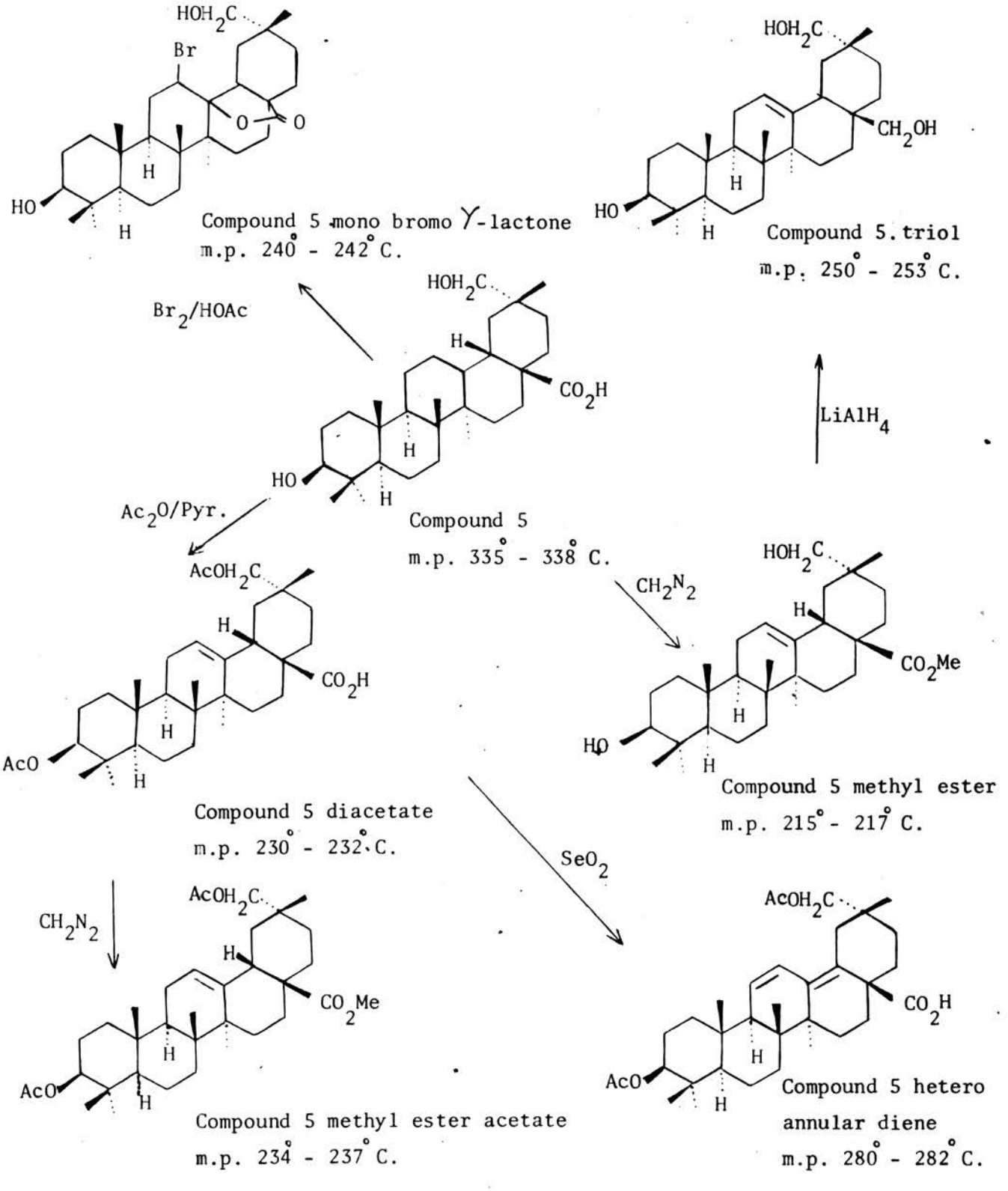
Incidentally, this particular compound was previously reported to be found in the flower buds of Fatsia japonica[34].

Structural Elucidation of Compound 5.

Column chromatography of n-butanol soluble fraction (40 g) on a silica gel column yielded Product A (1.2 g, R_f 0.33 in EtOAc :MeOH: H_2O , 600:99:81), m.p. 230° - 235° C, which was crystallized from MeOH:H_2O (1:1). Product A gave pink colour in Liebermann-Burchard test and positive result in Molish test. Its IR shows strong absorption bands at 3400 (OH) and 1050 cm^{-1} (glycosidic linkage).

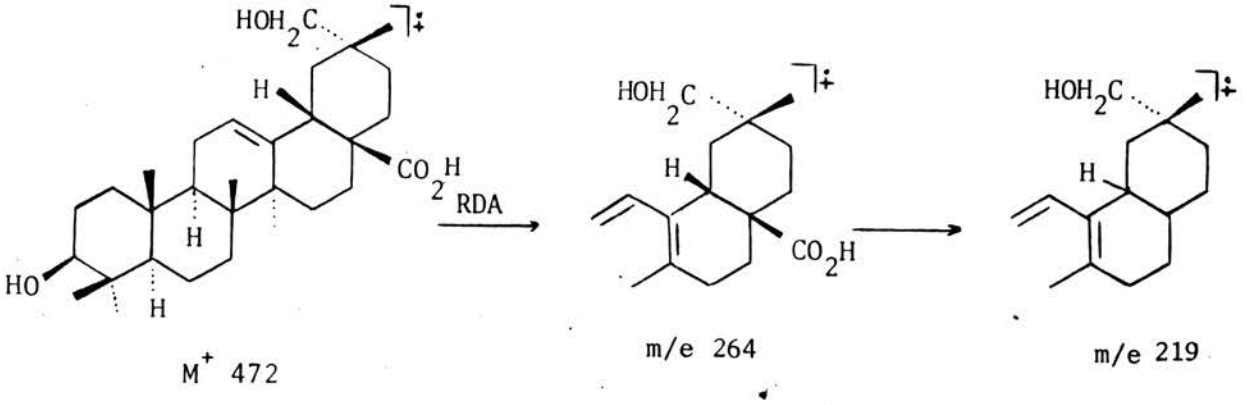
Acidic hydrolysis of Product A with 10 % HCl in MeOH gave a mixture of two sapogenins (R_f 0.40 and 0.32 in C_6H_6:Et_2O, 4:1, developed 3 times). They were chromatographed on a silica gel column and were eluted by a mixture of C_6H_6:Et_2O in the ratio of 4:1. Only one sapogenin (R_f 0.40 in C_6H_6: Et_2O, 4:1, developed 3 times) was isolated in chromatographic purity. Recrystallization from MeOH gave white needles, m.p. 335° - 338° C, which was assignated as Compound 5.

Its chemical reactions are shown in Scheme 4.



Scheme 4 Chemical reactions of Compound 5

Compound 5,m.p. 335° - 338° C,was crystallized from MeOH to give white needles. It gave pink colour in Liebermann Burchard test which indicated the presence of a triterpenoidal nucleus. The IR spectrum showed strong absorption bands at 3450 (OH) and 1700 cm^{-1} (COOH). In the mass spectrum of Compound 5,the relatively intense molecular ion peak at m/e 472 is present in addition to the other peaks at m/e 454 (M^+-H_2O), 262 (RDA) and 219 (RDA-COOH). Retro Diels-Alder reaction is the most characteristic fragmentation of all Δ^{12}-unsaturation oleanenes[19]. Mass spectrum data of Compound 5 are in agreement with the following fragmentation pattern.

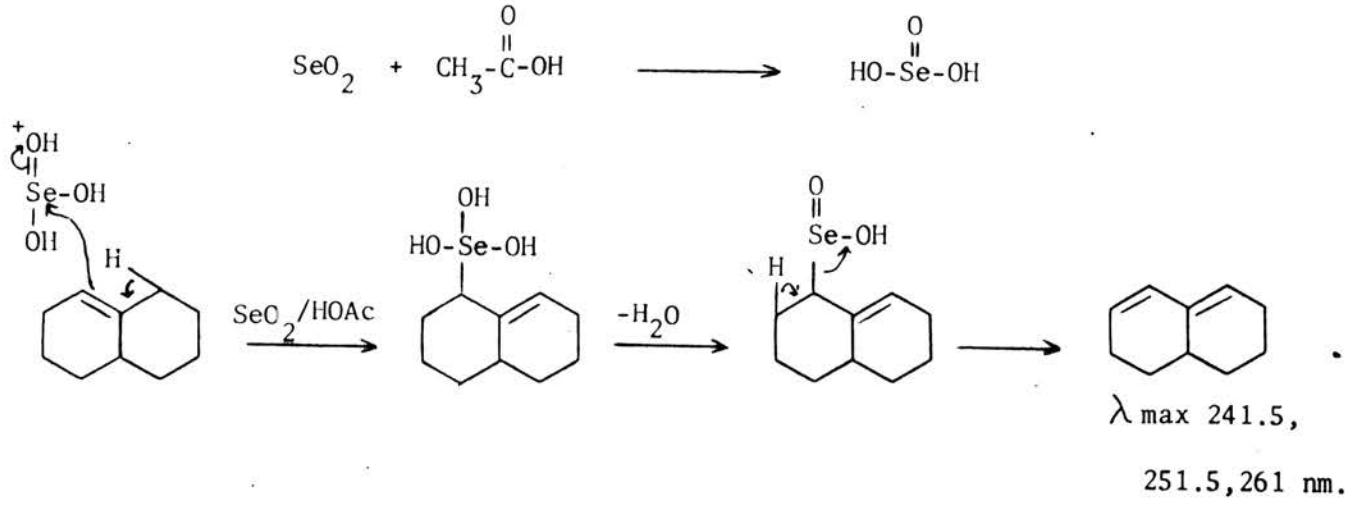


Acetylation of Compound 5 gave diacetate,m.p. 230° - 232° C, from MeOH (R_f 0.46 in C_6H_6:Et_2O, 4:1). The IR spectrum showed strong absorption bands at 1700 (COOH), 1725 cm^{-1} ($COCH_3$).

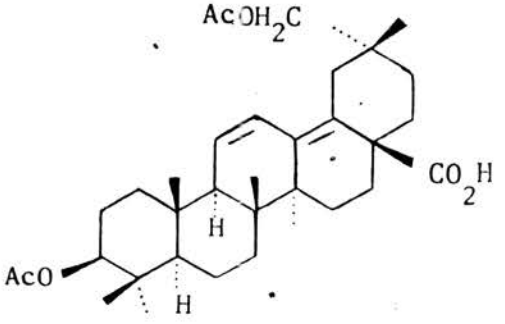
Compound 5 diacetate was allowed to react with SeO_2 in HOAC. The reaction product,m.p. 280° - 282° C,shows absorption in UV at λmax

241.5, 251.5 and 261 nm. which indicated the presence of Δ^{12}- unsaturated derivative. Generally, oxidation of such unsaturated compound with SeO_2 provides a transformation of the compound into a hetero annular diene[35] which can be easily detected by UV.

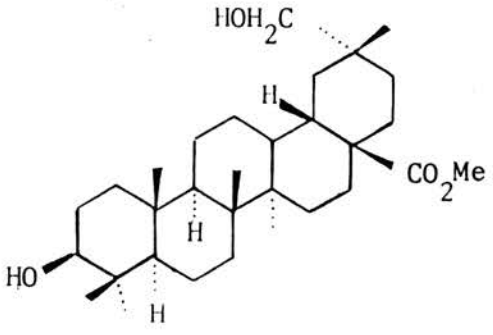
Mechanism[36] of the oxidation can be described as shown below.



Oxidation of Compound 5 diacetate with SeO_2 gave a hetero annular diene of Compound 5 indicating the structure as follow.

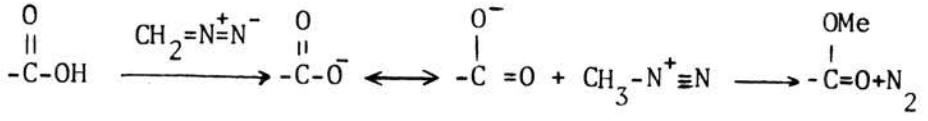


Methylation of Compound 5 with diazomethane gave Compound 5 methyl ester m.p. 215° - 217° C . It showed a characteristic absorption band of ester at 1725 cm^{-1} in its IR spectrum.



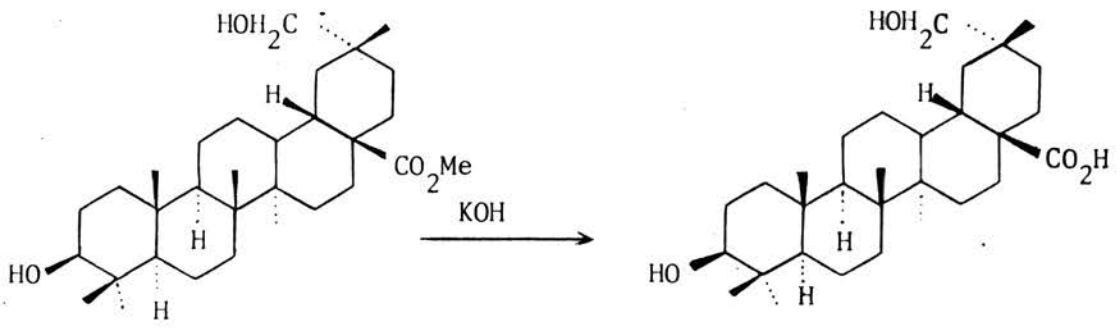
Compound 5 methyl ester

According to the mechanism[37] shown below an alcohol will not be methylated by diazomethane but an acid will be esterified by the same reagent.



Since a methyl ester was formed by the reaction of Compound 5 with diazomethane as mentioned above, it is obviously proved that a free carboxylic group is present in the structure of Compound 5.

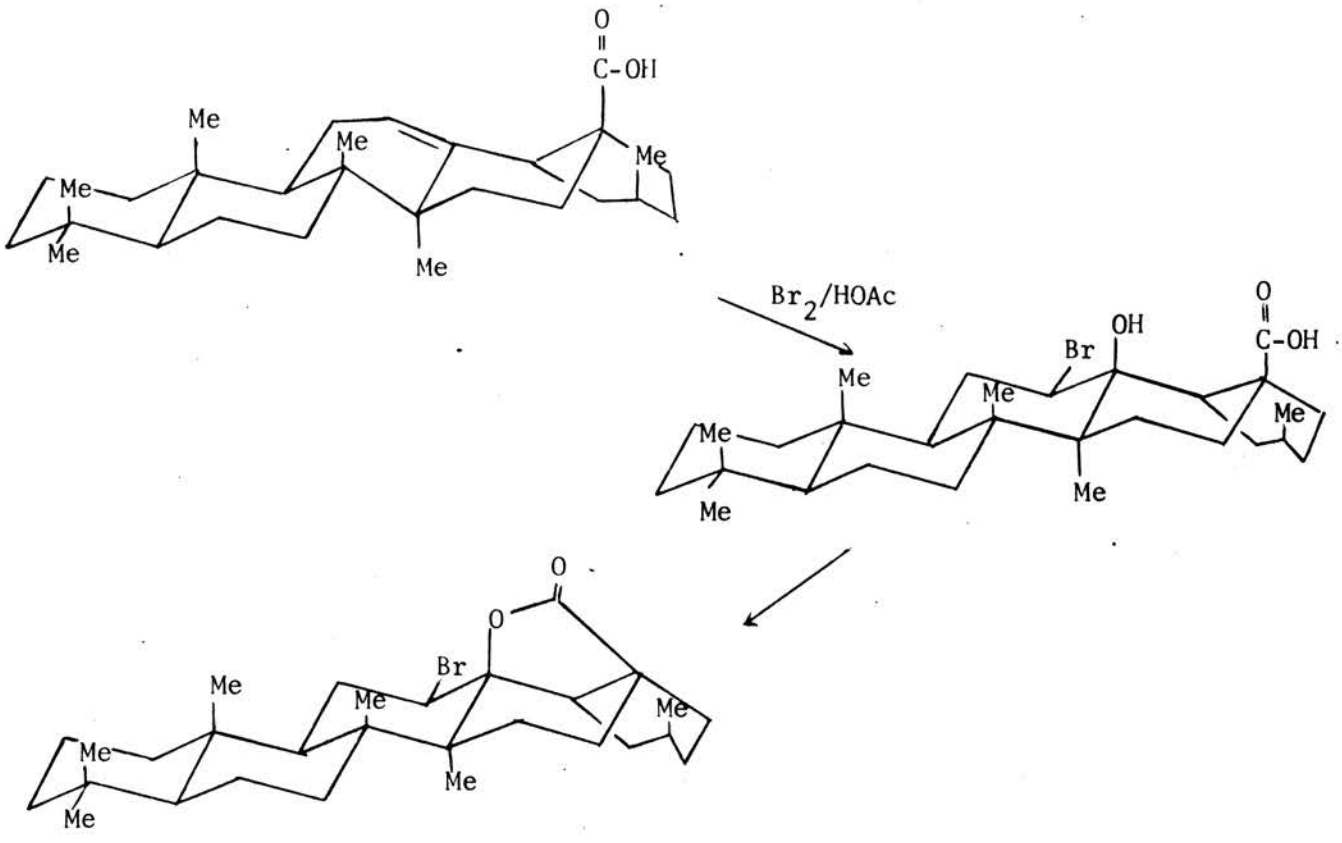
Saponifications of Compound 5 methyl ester with 5 % and 8 % KOH in EtOH for 8 hrs.each were unsuccessful.



Only one third of Compound 5 methyl ester was susceptible to hydrolysis after refluxing with 10 % KOH in EtOH for 8 hrs. The evidence of low hydrolysis rate may arise from a steric hindrance of the ester group.

After inspecting the models of three possible structures with an ester group at C-17, C-29 and C-30 respectively, it is convinced that only C-17 position shows a steric hindrance. In order to confirm this hypothesis, Compound 5 was allowed to react with Br_2 in HOAc containing NaOAc. The reaction product,m.p. 240° - 242° C,was identified as mono bromo γ-lactone. Absorption at 1790 cm^{-1} in its IR spectrum was due to a lactone ring which was particularly informative.

The following reaction mechanism shows an addition of the bromine into the double bond as a first step.

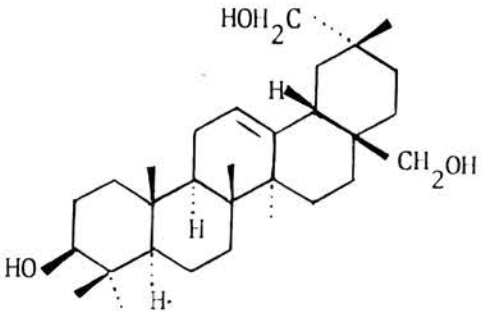


When a carboxylic acid group is attached to C-17, a lactone can be easily formed[38] due to the spacial proximity of hydroxyl and carboxylic group in which they are in a 1,3-diaxial configuration. Analogeous lactonizations with carboxylic substituent at C-29 or C-30 would be sterrically difficult[38].

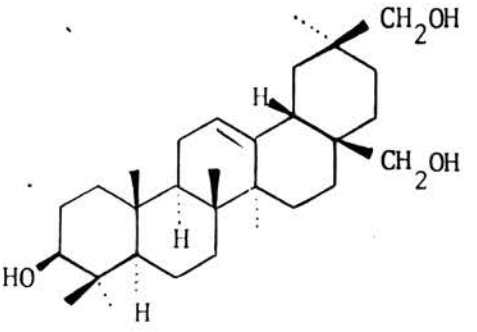
When Compound 5 methyl ester was reduced with $LiAlH_4$ to a triol m.p. 250° - 252° C, the strong absorption band at 3400 cm^{-1} which was dued to the OH group was appeared in the IR spectrum. However, the band at 1725 cm^{-1} (ester) was completely disappeared.

The reduction product was compared in TLC with an authentic samples of 3β,28,29-triol (III)[39] and 3β,28,30-triol (IV)[40]. The result of this comparison proved that the reduction product was identical with structure III. Thus it can be concluded that Compound 5

has the same absolute configuration at C-20 as the 3β, 28, 29-triol.

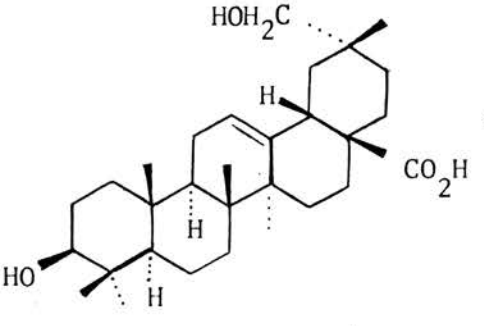


III



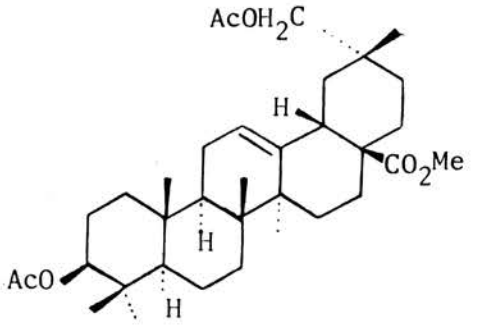
IV

Consequently, Compound 5 was elucidated as 3β, 29-dihydroxy-olean-12-ene-28-oic acid (V)



V

The structural elucidation of Compound 5 was further confirmed by the analysis of angular methyl group resonances in the ^{1}H NMR spectrum of its methyl ester diacetate (VI) m.p. 234°-237° C . The method has already been described as in the case of Compound 3 (see on page13). Calculated values of the chemical shifts of the angular methyl groups and experimental data shown in Table 4 are in good agreement with the purposed structure.



VI Compound 5 methyl ester diacetate

Table 4 The chemical shifts, calculated and observed values, of the angular methyl groups in Compound 5.

	Me-23	Me-24	Me-25	Me-26	Me-27	Me-30
Oleanene (21,22)	50	50.5	56.5	59	69	53
3B-OAc	0	+3	+1	0	0	+0.5
29-OAc	0	0	+1	-0.5	-0.5	+4
28-CO_2Me	-0.5	-0.5	-1	-15.5	.0	+3
Cal-(cps)	52.5	53	57.5	43	68.5	+60.5
ppm.	0.88	0.88	0.96	0.72	1.14	1.0
Obs.	0.85	0.85	0.96	0.72	1.10	0.92
Diff.	0.03	0.03	0	0	0.04	0.08

All data supported that Compound 5 was 3β,29-dihydroxy-olean-12-ene-28-oic acid or mesembryanthemoidigenic acid[41].