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Triterpenes in the Callus Culture of *Vitex negundo* L.

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A callus line of *Vitex negundo* was established using explants from the leaves. The calli showed stable morphology and thin layer chromatographic (TLC) profiles over several subcultures and in a variety of culture conditions. Seven oleanane-type triterpenes were identified from the callus culture extracts. The major triterpenes (2α,3α-dihydroxyolean-12-en-28-oic acid, 2α,3α,23-trihydroxyolean-12-en-28-oic acid, and oleanolic acid) were identified using ^{1}H and ^{13}C NMR, MS and IR, while the minor triterpenes (2α,3α,23-trihydroxyolean-12-en-28-oic acid methyl ester, 11-oxo-olean-12-en-28-oic acid propyl ester, 11-oxo-olean-12-en-28-oic acid butyl ester, and β-amyrin) were identified through their EIMS fragmentations alone. A biosynthetic scheme for the formation of oleanane-type triterpenes in *V. negundo* is proposed.

Key words: oleanane-type triterpenes, callus culture, *Vitex negundo*

INTRODUCTION

Vitex negundo L. is a pantropic herb which can be found in South and Southeast Asia. Known locally in the Philippines as "lagundi", *V. negundo* is a popular medicinal plant that is used as an antitussive, antiasthma and antipyretic (Quisumbing 1978, Pardo de Tavera 2000). A number of compounds have been isolated and identified from the leaves, including sesquiterpenes (β-eudesmol) (Dayrit & Trono 1995), flavonoids (casticin, chrysoplenol D, luteolin and isoorientin) (Dayrit et al.1987), iridoids (2'-para-hydroxybenzoyl mussaenosidic acid, 6'-para-hydroxybenzoyl mussaenosidic acid, agnuside and lagundinin) (Dayrit & Lagurin 1994), and minor amounts of the plant steroid β-sitosterol (Joshi et al. 1974). Many other monoterpenes and sesquiterpenes had also been detected in smaller amounts by GC-MS (Mallavarapu et al. 1994).

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The seeds, on the other hand, have yielded different flavonoids (3',5,7-trihydroxy-4',6,8-trimethoxyflavone and 3',5,7-trihydroxy-4',6,8-trimethoxyflavone-5-O-β-D-glucoside) (Bhargava 1984, 1986), steroids (β-sitosterol and daucosterol) and triterpenes (3 β-acetoxyolean-12-en-27-oic acid; 2α,3α-dihydroxyolean-5,12-dien-28-oic acid; 2α, 3β-diacetoxy-18-hydroxyolean-5,12-dien-28-oic acid; 2 β, 3α-diacetoxyolean-5,12-dien-28-oic acid; and lanosta-8,25-dien-3β-ol) (Chawla et al. 1991, 1992). β-Sitosterol is the only steroid that has been reported to occur in both the leaf and the seed.

Plant tissue culture is a potentially useful technique for the study of the biosynthesis of secondary metabolites and for the production of commercially important plant natural products (Verpoorte et al. 2002). However, in a number of cases, plant tissue cultures have also produced novel substances that are not observed in the intact plant.

A callus line of *Vitex negundo* L. was established using explants from the leaves to determine the secondary metabolites, which would be produced in the tissue culture. The calli showed stable morphology and thin layer chromatographic (TLC) profiles over several subcultures and in a variety of cultural conditions. Here, we describe the detection and identification of a number of triterpenes isolated from the callus culture.

MATERIALS AND METHODS

Plant material and callus culture

V. negundo leaves were collected from a plant grown from stem cuttings provided by Prof. Ernesta Quintana of the University of the Philippines Los Baños. Young leaves (< 3 months) were collected from the unfertilized plant and used as explant material for the initiation of callus cultures. The leaves were surface-sterilized using 2% detergent solution and 2.5% solution of commercial bleach, which contained 0.01% of sodium hypochlorite, then washed with 70% ethanol and sterile distilled water.

The callus cultures were established on Murashige and Skoog basal medium containing 0.5 ppm 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 ppm benzyladenine (BA) using surface-sterilized leaf sections. The cultures were maintained as callus cultures for over two years with continuous subculture every four weeks.

Extraction, isolation, and purification of secondary metabolites

All solvents used for extraction were technical grade, distilled, and dried prior to use. Solvents used for column chromatography, and analytical and preparative TLC were analytical grade (AR). Sorbents for conventional column chromatography and flash column chromatography (FCC) were silica gel 60 (0.063 – 0.200 mm) and silica gel (0.040-0.063 mm) (merck), respectively.

The calli were collected, freeze-dried, and extracted with 80% ethanol. The ethanol extract was concentrated *in vacuo* and then partitioned in hexane, ethyl acetate, and n-butanol. The ethyl acetate extract was concentrated *in vacuo* and the components of the residue were separated by vacuum liquid chromatography (VLC), followed by gravity and flash column chromatography, and preparative TLC. The ethyl acetate extract was concentrated *in vacuo* and the components of the residue were separated by vacuum liquid chromatography (VLC) with gradient elution using hexane-ethyl acetate mixtures. Each fraction was subsequently rechromatographed by gravity and flash chromatography, and preparative TLC (Figure 1).

Acetylation

A chromatographic fraction from the ethyl acetate extract was acetylated by reaction with acetic anhydride in freshly distilled pyridine under dry conditions. The reaction mixture was allowed to stand overnight at room temperature, and then the mixture was concentrated to dryness *in vacuo*.

Analytical methods

TLC was carried out on silica gel F_{254} pre-coated (Merck) plates using mixtures of hexane and ethyl acetate. Spots were visualized under UV light and by the use of vanillin-sulfuric acid spray reagent. IR spectra were recorded with a Nicolet Magna-IR 550 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a JEOL LA 400 spectrometer using TMS as internal standard. Analysis by gas chromatography-mass spectroscopy (GC-MS) was done with a GC HP 5890 Series II model (DB-5 column, 25 m x 0.25 mm i.d. x 0.25 μm film thickness) linked to a mass spectrometer (MAT 95S) operated in EI mode with electron energy at 70 eV. ESI-MS and FAB-MS were carried out using a MAT 95S and VG Masslab 12-250.

RESULTS

Callus lines were established for *V. negundo* on a variety of media. The callus grown in Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid and benzyl adenine at 0.5 ppm levels was flesh-colored, friable and fast-growing. It appeared to be morphologically stable and exhibited a characteristic metabolite profile that remained unchanged over two years. TLC analysis of extracts showed that under all culture conditions,s the secondary metabolites that were present in the ethyl acetate extract of the intact plant could not be detected in the corresponding extract from the tissue cultures.

A total of 475 g of fresh callus was obtained, freeze-dried, and extracted with 80% ethanol. Concentration of the ethanol extract yielded 534 mg of crude extract. Preliminary separation by vacuum liquid chromatography yielded six fractions, each of which was rechromatographed to obtain reasonably pure isolates (Figure 1).

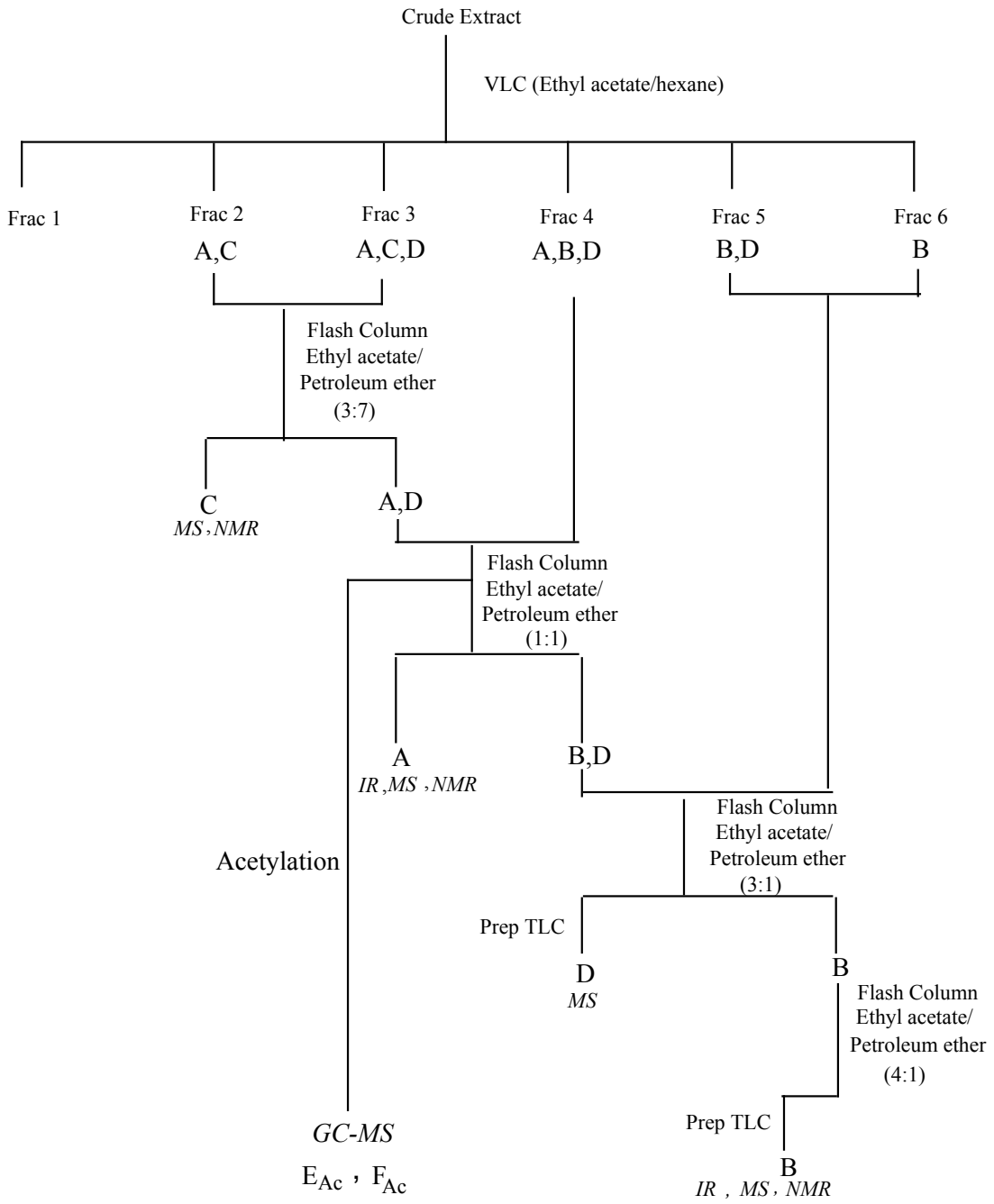

Figure 1. Isolation of components from the ethyl acetate extract from the callus of *Vitex negundo*

Three compounds were obtained in sufficient amount for structural elucidation of ^{1}H and ^{13}C, NMR, IR, and MS. These compounds are A, B, and C.

Compound A

Compound A was obtained by purification of the pooled fraction. The ^{1}H and ^{13}C NMR, DEPT 135, HMQC and HMBC spectra are given in Table 1. Data from IR, and EIMS analysis are summarized as follows:

IR (cm^{-1}): 3415, 2925, 2856, 1733, 1691, 1379, 1270, 1161.

EIMS (m/z (% relative intensity)): 472 (8), 470 (10), 454 (8), 439 (19), 426 (50), 408 (40), 393 (28), 250 (100), 248 (99), 224 (2), 223 (10), 203 (75), 189 (12), 133 (55).

The IR spectrum of compound A showed hydroxyl and carbonyl groups (3415 and 1733 cm^{-1}, respectively),

along with C–O (1270 and 1161 cm^{-1}), olefin (1691 cm^{-1}), and hydrocarbon C-H absorptions (2925, 2856, and 1379 cm^{-1}).

The ^1H NMR of A showed olefinic resonances at δ_H 5.29 and 3.99, and two oxygenated methine hydrogens at 3.43 and 2.82. The rest of the protons were overlapping aliphatic CH, CH_2 and CH_3 groups. Seven methyl singlets were observed at δ_H 0.75, 0.85, 0.91, 0.93, 0.97, 1.02 and 1.14 (Table 1).

The ^{13}C NMR spectrum shows 30 carbon resonances. The quaternary carbon (Cq) at δ_C 186.9 ppm is characteristic of a free carboxylic acid carbon; while the signals at 143.7

Table 1. Summary of ^{13}C, DEPT 135, HMQC and HMBC data for compound A (400 MHz ^1H and 100MHz ^{13}C, $CDCl_3$) and comparison with literature

C position[a]	^{13}C chemical shift, ppm	Carbon type	Literature comparison of ^{13}C data[b]	HMQC Correlations (^1H ppm)	HMBC Correlations (^1H ppm)
1	41.7	CH_2	41.7	1.62	0.97
2	66.5	CH	66.5	3.99	3.43
3	78.9	CH	78.9	3.43	1.02, 0.85
4	38.3	Cq	38.5		
5	48.1	CH	48.1	1.22	3.43, 1.02, 0.85
6	18.0	CH_2	18.1	1.47	
7	32.4	CH_2	32.5	1.56	0.75
8	39.5	Cq	39.7		1.14
9	47.3	CH	47.4	1.18	0.97, 0.75
10	38.2	Cq	38.3		
11	22.9	CH_2	23.0	1.94	
12	122.5	CH	122.1	5.29	2.82
13	143.7	Cq	143.8		1.14
14	42.1	Cq	41.9		1.14, 0.75
15	27.6	CH_2	27.7	1.02	1.14
16	23.4	CH_2	23.2	1.09	
17	46.5	Cq	46.8		0.91
18	41.0	CH	41.3	2.82	
19	45.9	CH_2	46.0	1.59	0.93, 0.91
20	30.7	Cq	30.7		0.91
21	36.7	CH_2	34.0	1.74	
22	33.8	CH_2	32.5	1.34	
23	28.5	CH_3	28.5	1.02	0.85
24	21.8	CH_3	21.9	0.85	1.47, 1.02
25	16.5	CH_3	16.4	0.97	
26	17.1	CH_3	17.0	0.75	
27	26.1	CH_3	26.2	1.14	
28	186.9	Cq	178.1[c]		
29	33.1	CH_3	33.2	0.91	0.93
30	23.6	CH_3	23.6	0.93	0.91

[a] See Figure 9
[b] 2α, 3α-dihydoxyolean-12-en-28-oate methyl ester, $CDCl_3$ at 15 MHz (Seo et al. 1981).
[c] The difference in ^{13}C chemical shift is due to carboxylic acid versus ester

and 122.5 ppm are assigned to Cq and CH alkene carbons. There are two oxygenated alkyl CH carbons at δ_C 66.5 and 78.9 ppm, and seven methyl carbons at δ_C 33.1, 28.5, 26.1, 23.6, 21.8, 17.1, and 16.5 (Table 1).

Structural fragments were deduced from homonuclear (^{1}H-^{1}H) correlations, as well as directly bonded and long-range heteronuclear (^{1}H-^{13}C) correlations. The oxygenated carbon at δ_C 66.5 is bound to the methine proton at δ_H 3.99, which is coupled to another methine proton at 3.43 and a methylene proton at 1.62. HMQC indicates that these protons are bonded to carbons at δ_C 78.9 and 41.7, respectively. The methyl hydrogens at δ_H 1.02 and 0.85 are long-range coupled

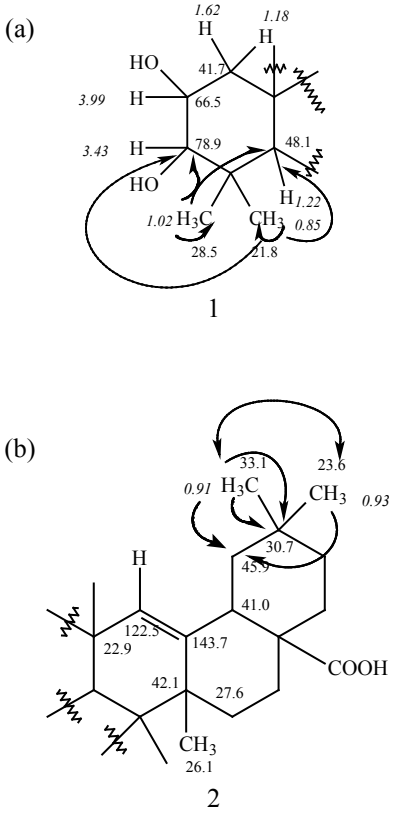

Figure 2. Structure elucidation of compound A from ^{1}H and ^{13}C NMR data. (a) Correlations from partial structure 1. (b) Correlations from partial structure 2

to the carbons at δ_C 78.9 and 48.1. These give a partial structure 1 (Figure 2a).

The partial structure surrounding the double bond was determined by COSY and HMBC. A one-proton signal due to an allylic H is observed as a doublet of doublets at δ_H 2.82. This is long-range coupled to the olefinic C at δ_C 122.5, as well as the methylene proton at δ_H 1.59. The COSY spectrum also reveals correlations between the one-proton multiplet at δ_H 5.29 assigned to the olefinic C and the methylene H at δ_H 1.94. The HMBC spectrum further shows that the methyl protons at δ_H 1.14 (δ_C 26.1) are long-range coupled to the quaternary carbon at δ_C 42.1, the methylene C at δ_C 26.1, and the quaternary olefinic C at δ_C 143.7. These data give the partial structure 2 (Figure 2b).

Partial structures 1 and 2 were joined using the HMBC correlations between the methyl protons at δ_H 0.97 (δ_C 16.5) and the methylene C at δ_C 41.7, as well as the methine C at δ_C 47.3 which in turn is coupled to the methyl protons at δ_H 0.75 (δ_C 17.1).

The NMR data yield the structure 2α,3α-dihydroxyolean-12-en-28-oic acid (A). The configurational assignment for the 2α,3α-dihydroxy group is based on the difference between the chemical shifts of H-2 and H-3, a difference of 0.56 ppm being indicative of the 2α,3α-dihydroxy isomer (Kojima & Ogura 1986). The ^{13}C NMR spectrum matched with the corresponding methyl ester derivative reported by Seo et al. (1981).

From the EIMS, the parent ion at m/z 472 gives a molecular formula of $C_{30}H_{48}O_4$, which is consistent with the proposed structure. A characteristic fragmentation mechanism of 12-ene triterpenes is a retro-Diels-Alder cleavage of ring C, which yields two fragments: the ene and diene (Figure 3). Fragmentation of A produces ions at m/z 248 (base peak, $C_{16}H_{24}O_2$) and m/z 223 ($C_{14}H_{23}O_2$). Loss of the –COOH group from the primary fragment at m/z 248 yields m/z 203 ($C_{15}H_{23}$) and m/z 189 ($C_{14}H_{21}$). Rearrangement of the m/z 203 ion and loss of a neutral 5-carbon fragment give rise to another relatively stable ion at m/z 133 ($C_{10}H_{13}$).

Compound B

Repeated column chromatography using gradients of ethyl acetate-petroleum ether and preparative TLC using acetone-chloroform yielded 3 mg of compound B.

IR: 3450, 2928, 2859, 1737, 1699, 1273.

The ^{1}H and ^{13}C NMR, DEPT 135, HMQC and HMBC spectra are given in Table 2.

FAB-MS: 511, 479, 413.

EIMS: 248 (72), 203 (58), 189 (17), 133 (50).

Compound B contains the same IR absorbances as those of A: a hydroxyl stretching band centered around 3450 cm^{-1} (hydrogen-bonded -OH), C=O and C–O stretching vibrations at 1737 and 1273 cm^{-1}, respectively, an olefinic stretch at 1699 cm^{-1} and

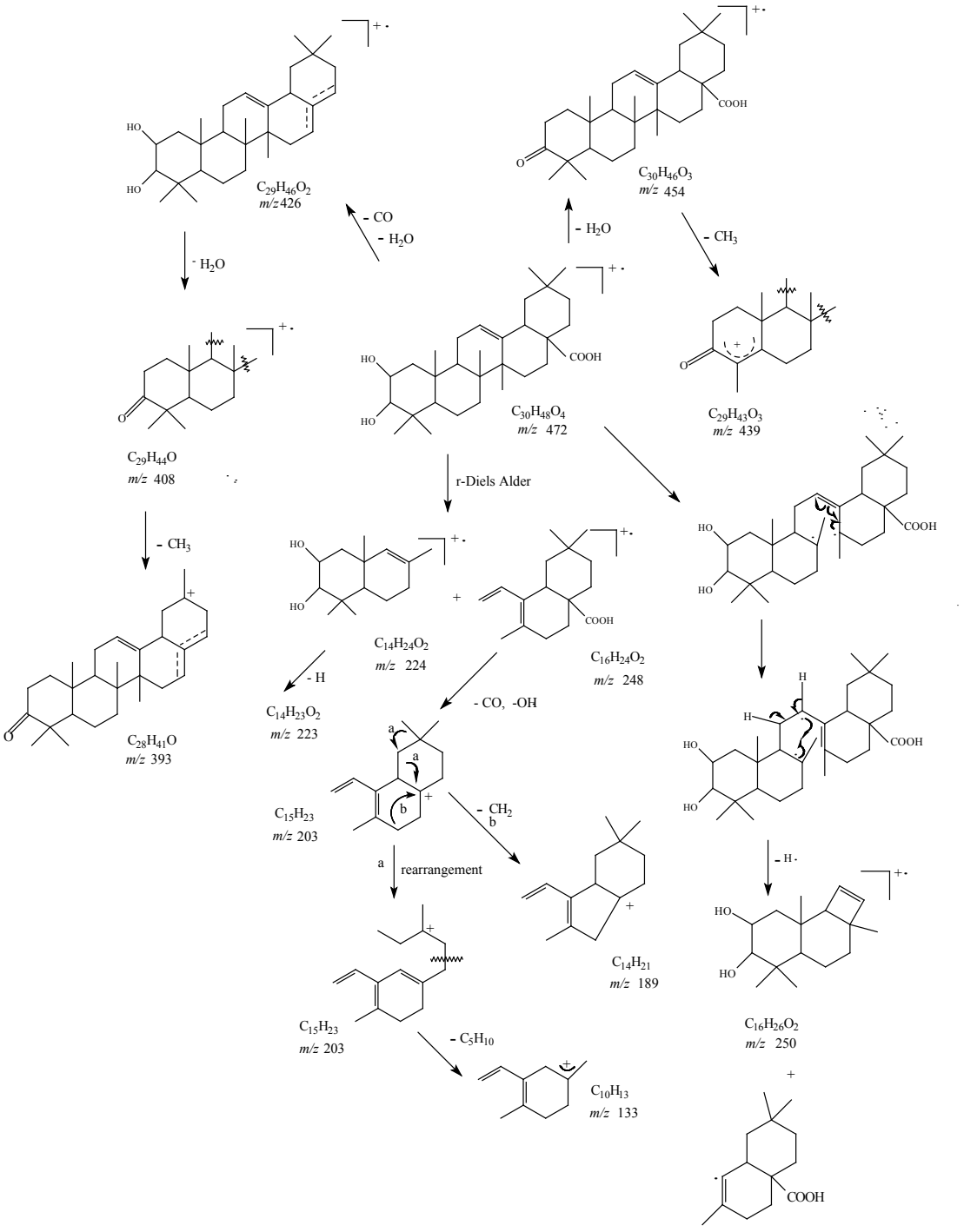

Figure 3. Proposed fragmentation of compound A from EIMS analysis

strong hydrocarbon absorptions at 2928 and 2859 cm^{-1}.

The structural similarity of compound B with A can also be inferred from the ^{1}H and ^{13}C NMR spectra (Table 2). ^{13}C NMR indicates 30 carbon atoms with generally similar DEPT assignments. The main differences of B are the presence of only 6 methyl groups and an additional oxygenated methylene carbon at δ 71.4 ppm.

Table 2. Summary of ^{13}C, DEPT 135, HMQC and HMBC data for compound B (400 MHz ^{1}H and 100 MHz ^{13}C, CD_3OD) and comparison with literature

C position[a]	^{13}C chemical shift, ppm	Carbon type	Literature comparison of ^{13}C data[b]	HMQC Correlations (^{1}H ppm)	HMBC Correlations (^{1}H ppm)
1	42.2	CH_2	41.7	1.6	3.60, 1.02
2	67.3	CH	66.7	3.85	3.6
3	78.8	CH	78.8	3.6	3.52, 3.37
4	43.1	Cq	41.1		3.60, 1.53, 0.78
5	44.2	CH	42.6	1.53	3.60, 3.37, 0.78
6	19.0	CH_2	18.0	1.4	
7	33.56	CH_2	32.6	1.29	
8	40.7	Cq	39.7		1.13, 0.83
9	49.3	CH	47.7		1.92, 1.02, 0.83
10	39.2	Cq	38.2		1.02
11	23.8	CH_2	23.2	1.92	
12	123.4	CH	122.4	5.25	1.92
13	145.6	Cq	144.4		1.13
14	42.5	Cq	42.0		1.13
15	28.2	CH_2	27.9		1.13
16	24.1	CH_2	23.6	1.95	
17	48.6	Cq	47.0		1.13
18	42.9	CH	41.6	2.85	
19	47.4	CH_2	46.2	1.73	0.91
20	30.9	Cq	30.8		0.91
21	33.9	CH_2	34.1		0.91
22	33.1	CH_2	32.4		
23	71.4	CH_2	71.5	3.52, 3.37	1.53, 0.78
24	17.9	CH_3	17.5	0.78	3.60, 3.52
25	17.3	CH_3	16.8	1.02	
26	17.6	CH_3	17.1	0.83	
27	26.6	CH_3	26.2	1.13	
28	182	Cq	178.4[c]		
29	33.6	CH_3	33.2	0.91	
30	24.07	CH_3	23.8	0.94	

[a] See Figure 9
[b] 2α, 3α, 23-trihydoxyolean-12-en-28-oate methyl ester, $CDCl_3$ at 100 MHz (Kojima & Ogura 1986)
[c] The difference in ^{13}C chemical shift is due to carboxylic acid versus ester

A partial structure (3) for ring A of an olean-12-ene skeleton can be deduced from the COSY, HMQC, and HMBC spectra (Figure 4). HMBC shows long-range couplings between the methyl hydrogens at δ_H 0.78 and the -CH_2OH group at δ_C 71.4, a CH at δ 44.2, and the quaternary carbon at δ_C 43.1, which in turn is coupled to the methine proton at δ_H 1.53. This proton is bonded to the carbon at δ_C 44.2, which also shows long-range coupling with the hydrogens at δ_H 3.60 and 3.37.

^{1}H and ^{13}C NMR data yield the structure 2α,3α,23-trihydroxyolean-12-en-28-oic acid (B). Our ^{13}C NMR data match closely with two previous literature reports (Bowden et al. 1975, Kojima & Ogura 1986).

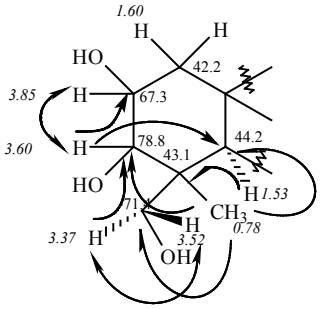

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Figure 4. Structural elucidation of compound B from ^{1}H and ^{13}C NMR data. Correlations from partial structure 3.

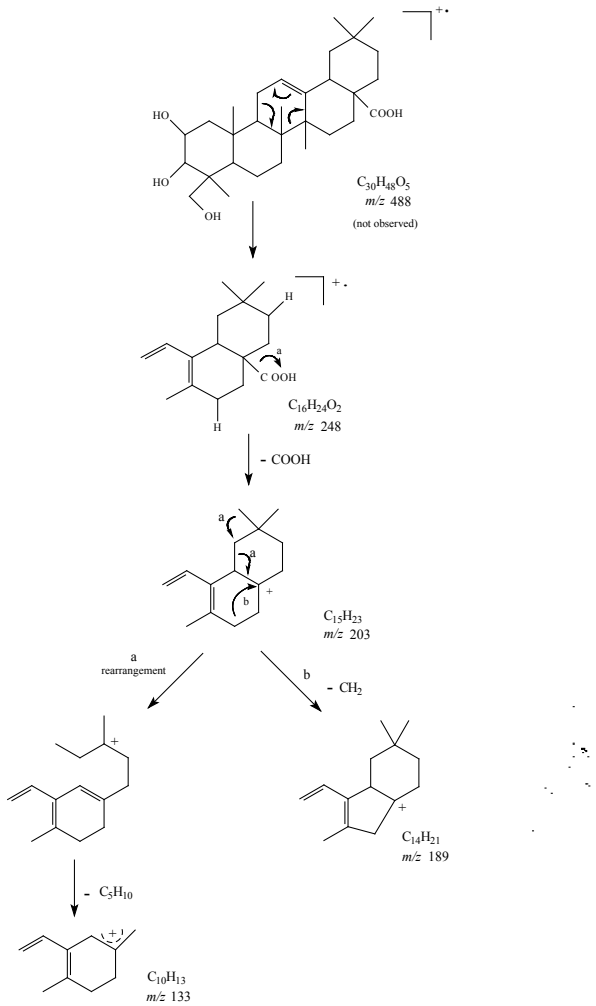


Figure 5. Proposed fragmentation of compound B from EIMS analysis

FAB-MS of compound B gives a parent ion at m/z 511 $[M + Na]^{+}$, for a molecular mass of 488 amu, in agreement with the proposed molecular formula, $C_{30}H_{48}O_5$. The strong peaks at m/z 479 and 413 are assigned to [(M - 3H2O - H) + 2Na]+ and $[(M – COO - 3H_2O) + Na]^{+}$, respectively, which are consistent with the presence of three hydroxyl groups.

EIMS of compound **B** does not yield the molecular ion but gives a base peak at m/z 248 ($C_{16}H_{24}O_2$), which is assigned to a cation obtained from the retro-Diels-Alder cleavage of the parent ion (Figure 5). Loss of a –COOH group accounts for the prominent peak at m/z 203 ($C_{15}H_{23}$), which in turn fragments to yield m/z 133 ($C_{10}H_{13}$). These results are similar to those obtained for compound A.

Compound C

Compound C was obtained in small amounts which were chromatographically pure on TLC.

The ^{13}C NMR spectrum is summarized in Table 3.

EIMS: 456 (28), 439 (32), 423 (21), 411 (23), 395 (20), 248 (100), 208 (27), 203 (62), 189 (15), 133 (57).

Table 3. ^{13}C NMR assignments for compound C. (100 MHz, $CDCl_3$)

C position[a]	^{13}C chemical shift, ppm	Literature comparison of ^{13}C data[b]
1	38.8	38.5
2	27.1	27.1
3	79.0	78.7
4	38.7	38.7
5	55.2	55.2
6	18.3	18.3
7	32.4	32.6
8	39.2	39.3
9	47.5	47.6
10	38.4	37.0
11	22.9	23.4
12	122.6	122.1
13	143.5	143.4
14	41.6	41.6
15	27.6	27.7
16	23.4	23.1
17	46.5	26.6[d]
18	41.0	41.3
19	45.8	45.8
20	30.6	30.6
21	36.7	33.8
22	33.8	32.3
23	28.1	28.1
24	15.6	15.6
25	15.3	15.3
26	17.0	16.8
27	25.9	26.0
28	190.8	177.9[c]
29	31.9	33.1
30	23.6	23.6

[a] See Figure 9
[b] methyl oleanoate, in $CDCl_3$ at 15 MHz (Seo et al. 1981)
[c] ^{13}C chemical shift of carboxylic acid versus ester
[d] as reported in Seo et al. (1981). May be a typographical error and should be “46.6”

The ^{13}C NMR data of compound C were found to be in close agreement with literature data for oleanolic acid (Table 3) (Seo et al. 1981).

The parent ion of C at m/z 456 is consistent with the molecular formula $C_{30}H_{48}O_3$ (Figure 6). Similar to A and B, compound C has a base peak at m/z 248 and prominent peaks at m/z 203, 189 and 133.

Compound D*

It was obtained as a minor component from preparative TLC (ethyl acetate–petroleum ether, 3:1). Because of the small quantity isolated, NMR analysis was not feasible and structural elucidation was carried out purely by mass spectrometry.

EIMS: 486 (7), 468 (10), 453 (17), 442 (20), 426 (40), 408 (27), 303 (67), 262 (100), 250 (87), 248 (70), 219 (9), 203 (47), 189 (12), 133 (68).

EIMS analysis did not give the parent ion but gave the ions characteristic of a retro-Diels-Alder fragmentation of a pentacyclic triterpene at m/z 262, 219, 203, 189 and 133. The loss of 59 amu from m/z 262 is consistent with

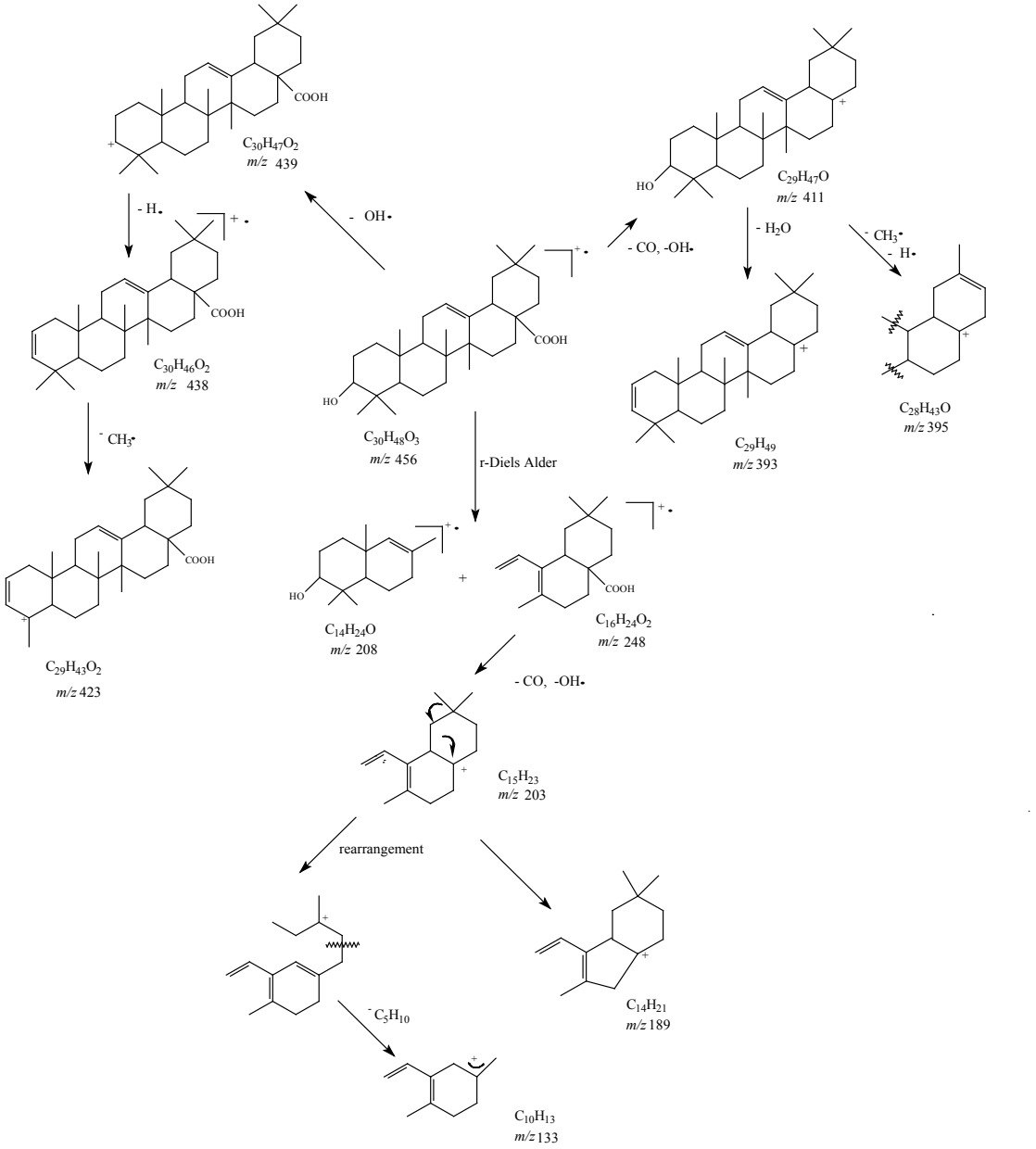


Figure 6. Proposed fragmentation of compound C from EIMS analysis

*compound D, which was isolated in an amount insufficient for NMR analysis, was identified by comparison of its EIMS fragmentation

the presence of a carboxymethyl group at C-17 (Figure 7). On the basis of its EIMS fragmentation and similarity with the EIMS spectrum of B, compound D is assigned the structure 2α,3α,23-trihydroxyolean-12-en-28-oic acid methyl ester with a molecular weight of 502 amu and molecular formula $C_{31}H_{50}O_5$. Compound D is thus the methyl ester derivative of B. These results are comparable with those reported in the literature (Bowden et al. 1975).

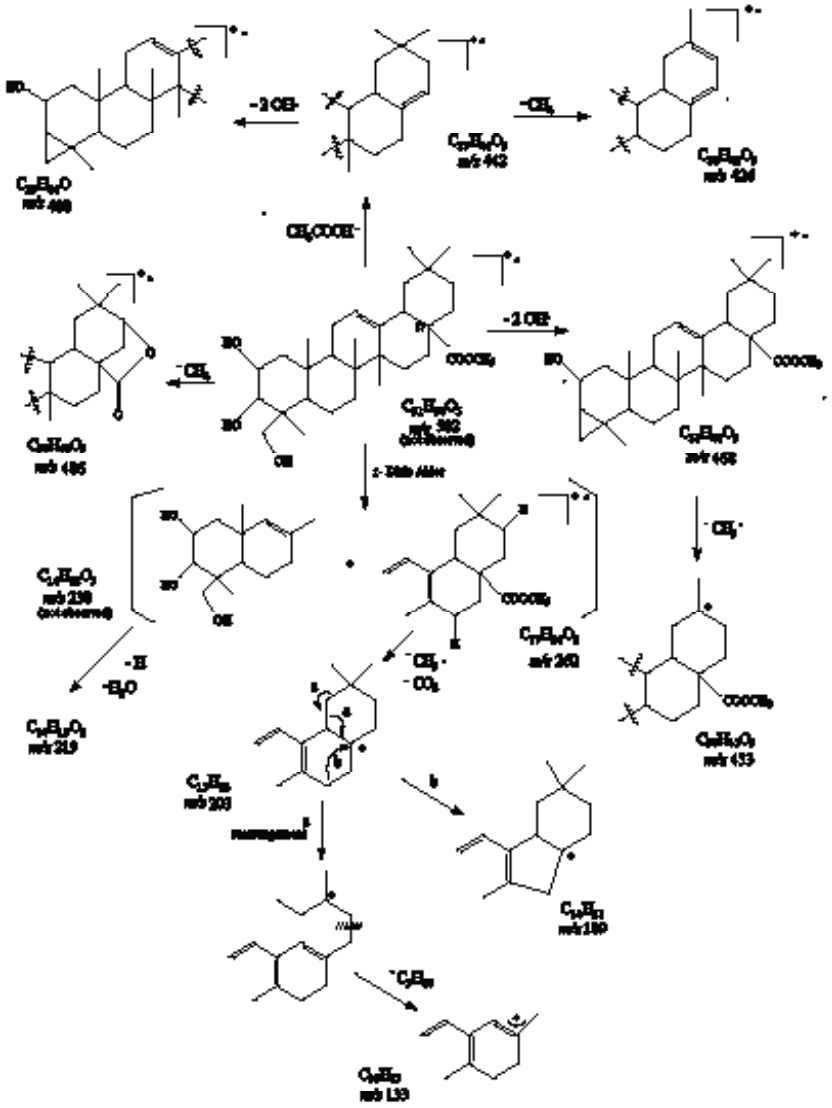


Figure 7. Proposed fragmentation of compound D from EIMS analysis

The stereochemistry proposed for compound D is based on its co-occurrence with compound B.

Compounds E and F*

A chromatographic fraction from the ethyl acetate extract of the callus culture which could not be further purified by silica gel chromatography was acetylated by reaction with acetic anhydride-pyridine and then analyzed by GC-MS. The EIMS of two acetylated compounds E_{Ac} and F_{Ac} from the mixture show similar fragmentation patterns which are analogous to the EIMS of compounds A - D.

EIMS of E_{Ac}: 554 (1), 511 (2), 466 (20), 216 (100), 204 (17), 203 (95), 190 (44), 189 (38), 133 (26).

EIMS of F_{Ac}: 568 (1), 510 (2), 466 (10), 216 (50), 204 (18), 203 (100), 190 (15), 189 (22), 133 (26).

E_{Ac} and **F_{Ac}** differ in their molecular ions (m/z 554 and 568, respectively), but otherwise show very similar EIMS

*compound E and F, were identified as their acetylated derivatives (E_{Ac} and F_{Ac}) BY GC – MS analysis of an ethyl acetate fraction

spectra. Sequential losses of the alkyl ester and (CO_2 + H) groups are observed as follows: for E_{Ac}: m/z 511 $[M-C_3H_7]+$ and m/z 466 $[M-C_3H_7-COO-H]^+$; and for F_{Ac}: m/z 510 $[M-C_4H_9-H]^+$ and m/z 466 $[M-C_4H_9-COO-H]^+$ (Figure 8). The prominent peak at m/z 216 found in both compounds is a characteristic fragment of oleanane-type compounds with a keto group at C-11 (Budzikiewicz et al. 1963).

The following structures are proposed for these acetylated compounds: 3-acetyloxy-11-oxo-olean-12-en-28-oic acid propyl ester (E_{Ac}), and 3-acetyloxy-11-oxo-olean-12-en-28-oic acid butyl ester (F_{Ac}). The original unacetylated compounds are therefore identified as compounds E and F (Figure 2). There is insufficient evidence to assign the stereochemistry of the hydroxy group at the 3-position.

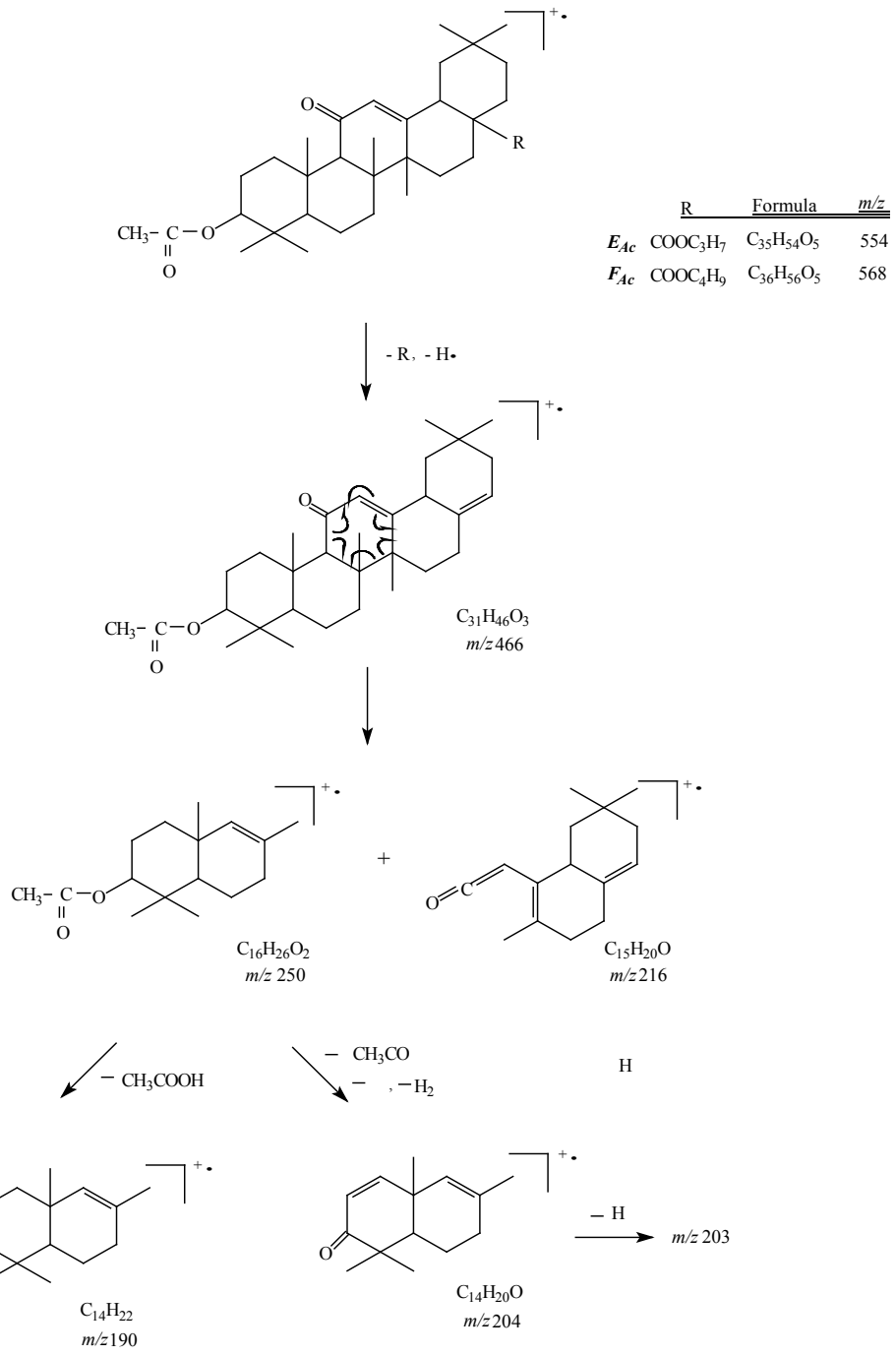
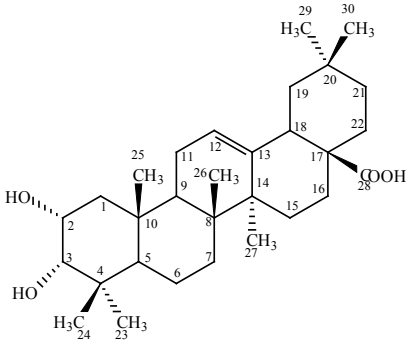


Figure 8. Proposed fragmentation mechanism of acetylated compounds E_{Ac} and F_{Ac} from EIMS analysis

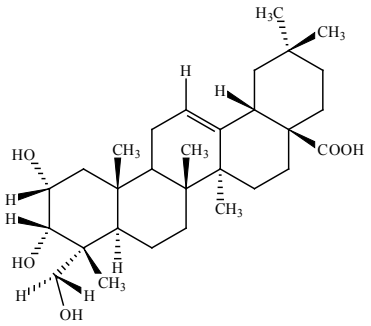
Compound G*

The hexane extract was fractionated and analyzed by GC-MS. One of the major GC peaks in the mixture was compound G.

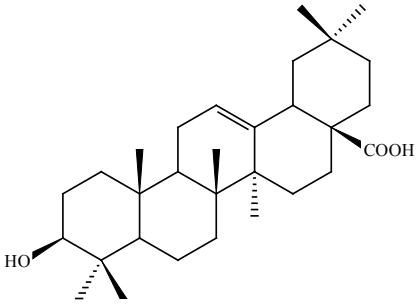
EIMS: 426 (4), 410 (8), 218 (100), 203 (48), 189 (18), 133 (6). Comparison with the MS library and analysis of the EIMS fragmentation pattern yielded a match for the triterpene β-amyrin.



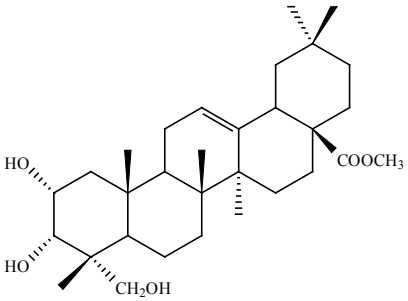
A: 2α, 3α-dihydroxyolean-12-en-28-oic-acid



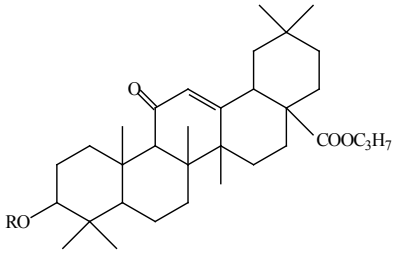
B: 2α, 3α, 23-trihydroxyolean-12-en-28-oic acid



C: oleanolic acid

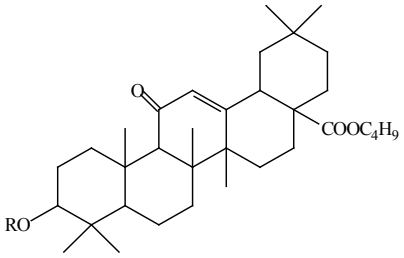


D: 2α, 3α, 23-trihydroxyolean-12-en-28-oic acid methyl ester



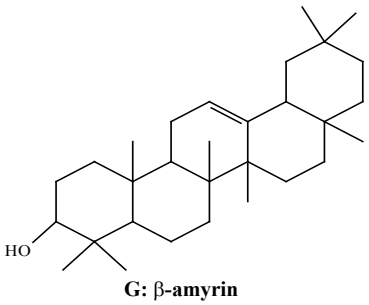
E: 3-acetyloxy-11-en-28-oic acid, prophyl ester. R = H

E_{Ac}: R = CH_3CO



F: 3-acetyloxy-11-oxo-olean-12-en-28-oic acid, butyl ester. R = H

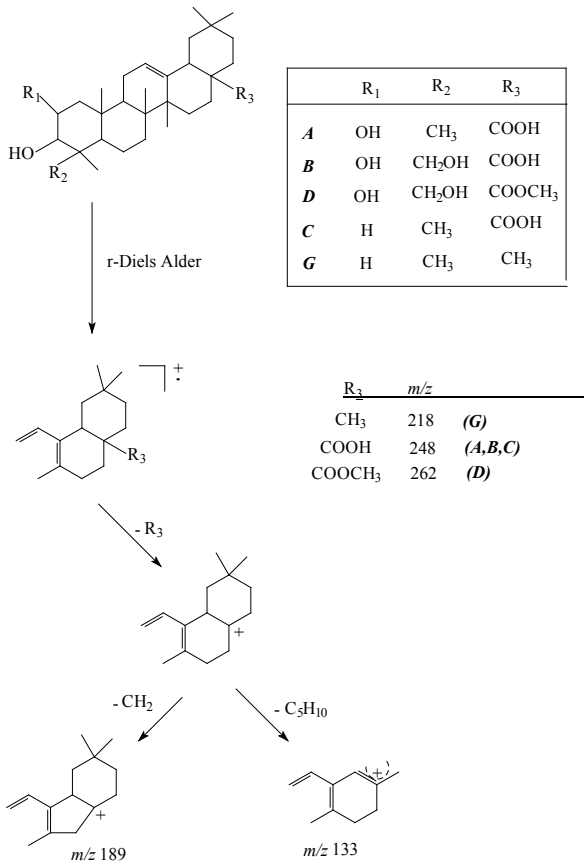
F_{Ac}: R = CH_3CO



G: β-amyrin

Figure 9. Triterpenes identified in the leaf callus culture of *Vitex negundo*

*compound G was identified by GC – MS analysis of the hexane fraction (see Figure 9)



	R_1	R_2	R_3
A	OH	CH_3	COOH
B	OH	CH_2OH	COOH
D	OH	CH_2OH	$COOCH_3$
C	H	CH_3	COOH
G	H	CH_3	CH_3

R_3	*m/z*	
CH_3	218	***(G)***
COOH	248	***(A,B,C)***
$COOCH_3$	262	***(D)***

Figure 10. Common fragmentation pattern of oleanane-type triterpenes

DISCUSSION

Seven oleanane-type triterpenes were identified from the extract of the callus culture from the leaves of *V. negundo* (Figure 9). Interestingly, these oleananes have not been previously observed in the leaves, although other oleanane-type triterpenes have been reported in the intact seeds. They could either be absent in the leaves of *V. negundo* or produced in very low quantities only.

Under the conditions used in the tissue culture of *V. negundo,* the callus did not accumulate detectable quantities of compounds characteristic of the parent plant, such as the flavonoids. The failure of plant tissue cultures to accumulate secondary metabolites characteristic of the intact plant has been reported in literature. It has been suggested that the presence of differentiated tissue is important for the biosynthesis of certain metabolites in the intact plant. On the other hand, the accumulation in the tissue culture of compounds that are not observed in the parent plant is also known (Banthorpe & Brown 1989).

This work also demonstrates the effective use of mass spectrometry for the structural determination of small amounts of structurally related triterpenes. The initial step involved establishing the structure of the more abundant compounds (A, B, and C) using all of the spectroscopic methods (^{1}H and ^{13}C NMR, IR, MS), along with comparison with literature. The use of mass spectrometry

Table 4. Oleanane triterpenes display similar MS fragmentation patterns which are useful for their identification: m/z (% relative abundance)

Type of ion	A	B	C	D	EAc	FAc	G
Parent ion, M^+	472 (8)	488 (0)	456 (28)	502 (0)	554 (1)	568 (1)	426 (4)
$[M - H2O]^{+\cdot}$ or [M - OH]+	454 (8)		439 (32)	NA*			
$[M-H2O-CO]^{+\cdot}$ or [M-OH-CO]+	426 (50)		411 (23)	NA			
Primary retro-Diels-Alder fragments	224 (2)		208 (27)				
	248 (99)	248 (72)	248 (100)	248 (70)	216 (100)	216 (50)	218 (100)
	223 (10)			219 (9)	204 (17)	204 (18)	
Secondary fragments from retro-Diels-Alder fragments	203 (75)	203 (58)	203 (62)	203 (47)	203 (95)	203 (100)	203 (48)
	189 (12)	189 (17)	189 (15)	189 (12)	190 (44)	190 (15)	189 (18)
	133 (55)	133 (50)	133 (57)	133 (68)	133 (26)	133 (26)	133 (6)

* NA: not applicable based on proposed structure

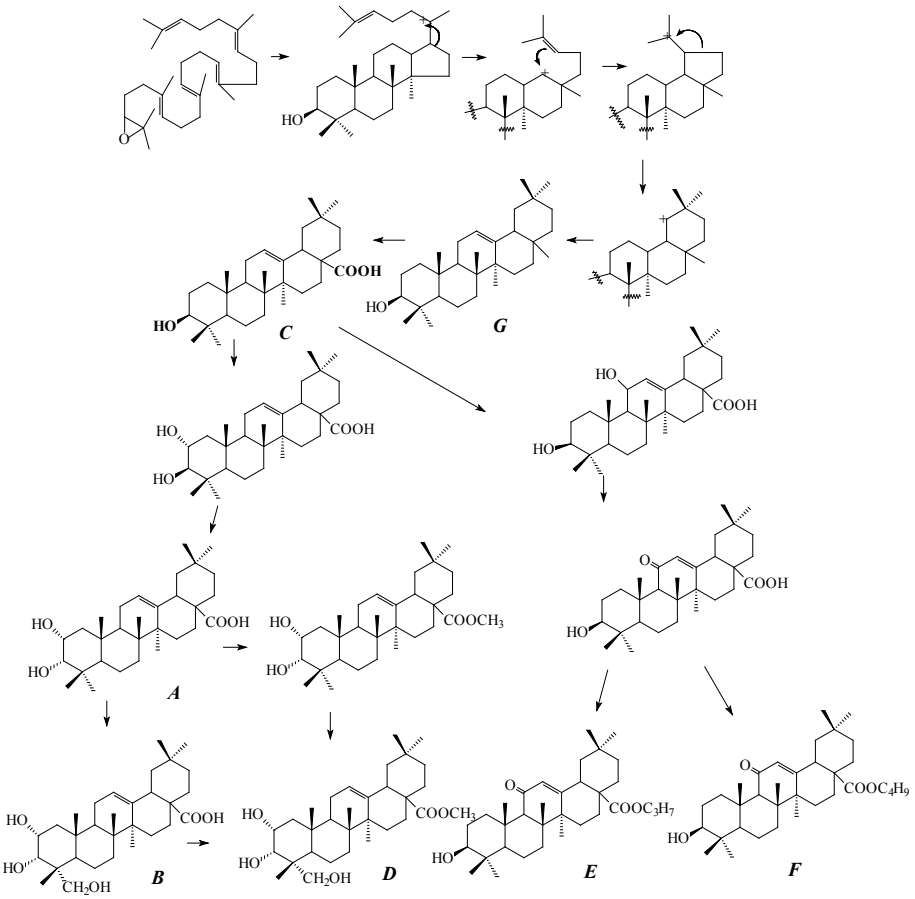


Figure 11. Proposed pathway for the biosynthesis of oleanane triterpenes in *Vitex negundo* callus culture

for the identification of the minor components (**D**, **E**, **F**, and **G**) was made possible by the similar fragmentation mechanisms that these compounds undergo. The ions at m/z 203, 189, and 133 are characteristic skeletal fragments produced by oleanane-type triterpenes. This fragmentation mechanism is typical of pentacyclic oleanane triterpenes that have a double bond at C12(13) and a carboxylic group at C-17. From a comparison of the MS data (Figure 10 and Table 4), it is evident that the oleanane-type triterpenes display a characteristic fragmentation pattern that are derived from rings C, D, and E. The structure of rings A and B yields other MS ions that can be used for their identification. If ring A contains a 3-hydroxy or 2,3-dihydroxy group, the parent ion is usually observable, and rings A and B produce fragments that may be used to elucidate the structure.

The occurrence of oleanane-type triterpenes in other plant species both from the intact plant and from tissue culture has been previously reported (Seo et al.1988, Banthorpe & Brown 1989, Bolta et al. 2000, Taniguchi et al. 2002). Earlier, Seo et al. (1981) proposed a biosynthetic route starting from 2,3-oxidosqualene to the 2,3-dihydroxyoleanane derivative (compounds A, C and G). Based on the triterpenes from the tissue culture of *V. negundo*, we propose a scheme which links other triterpenes (compounds B, D, E, and F) in the biosynthetic scheme (Figure 11).

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