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Investigation on The Stem Bark of *Trema orientalis*

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ABSTRACT A chemical investigation was conducted on the ethyl acetate-soluble fraction of the stem bark of *Trema orientalis*. This led to the isolation of seven methyl ester of fatty acids hexadecanoic, (*Z,Z*)-9,12-octadecadienoic, (*Z*)-9-octadecenoic, octadecanoic, eicosanoic, docosanoic and tetracosanoic. Two triterpenoids identified as β–sitosterol and 3β–acetoxyurs-12-en-28-oic acid were isolated. The methyl ester of fatty acids were identified by GC-MS while the triterpenoid compounds were identified using NMR and infrared spectroscopy and mass spectrometry. The identification of 3β–acetoxyurs-12-en-28-oic acid was verified by comparing it to the authentic 3β–acetoxyurs-12-en-28-oic acid synthesized from the acetylation of ursolic acid.

ABSTRAK Kajian kimia terhadap fraksi larut-etil asetat dari kulit batang *Trema orientalis* telah dilakukan. Tujuh sebatian ester metil asid lemak heksadekanoik, (*Z,Z*)-9,12-oktadekadienoik, (*Z*)-9-oktadekenoik, oktadekanoik, eikosanoik, dokosanoik dan tetrakosanoik telah diperolehi. Selain daripada ester tersebut, dua sebatian triterpenoid juga diasingkan telah dikenalpasti sebagai β-sitosterol dan asid 3β–asetoksiurs-12-en-28-oik. Ester metil asid lemak telah dikenalpasti melalui kaedah KG-SJ sementara sebatian triterpenoid dikenalpasti melalui teknik spektroskopi NMR dan inframerah serta spektrometri jisim. Pengesahan sebatian asid 3β–asetoksiurs-12-en-28-oik telah juga dilakukan melalui perbandingan dengan sebatian autentik yang disintesis daripada asid ursolik melalui proses pengasetilan.

(*Trema orientalis*, methyl esters of fatty acids, triterpenoid)

INTRODUCTION

Trema orientalis or more locally known as 'mengkirai', which grows up to 15 feet tall, is a common uncultivated wild plant found in Malaysia [1]. This plant has long been used by the villagers in the east coast Peninsular Malaysia to treat mouth ulcers because of its ability to abolish ulcer pain and hasten the process of ulcer healing. Investigations showed that the plant extract possesses anti-inflammatory, anti-ulcerogenic and anaesthetic properties [2,3,4,5,6,7]. Several papers had reported components such as simiarenol, simiarenone, octacosanoic acid, trematol, scopoletine, 3,4-dihydroxybenzoic acid, lupeol and *p*-hydroxybenzoic acid from the stem bark of *Trema orientalis* [8,9,10,11,12,13]. Botanical samples were obtained from Tanah Merah, Kelantan on December 2002 and identified by the botanist of Universiti Kebangsaan Malaysia (SNM 12/02) (UKMB).

EXPERIMENTAL

All solvents used in this study were of AR grade. Thin layer chromatography was performed on Merck silica gel $60F_{254}$ TLC Plates. Melting points were measured on Electrothermal Melting Point and Gallenkamp Melting Point apparatus. IR spectra were recorded on a FTIR model Jasco 410 instrument. Mass spectra were recorded on a mass spectrometer model VG-70SE Electron Impact from United Kingdom. NMR spectra (in $CDCl_3$) were conducted on a Varian INOVA 500 from New Zealand. Gas chromatography analysis was performed using a Shimadzu 17A GC equipped with FID detector on a fused silica

AT-1 capillary column (25m length × 0.2mm; 0.25μm film thickness) and nitrogen as carrier gas (gas flow: 50cm^3 min^{-1}). Column temperature was programmed with initials temperature of 100°C for 10 minutes, then increased to 250°C at 3°C per minute and kept isothermally for 15 minutes. GC-MS analysis on the oily mixture was conducted using a Hewlett-Packard GC-MSD 5890 Series II mass spectrometry (MSD 5971A HP) on a fused silica ZB-1 capillary column (30m in length × 0.2mm; 0.25μm film thickness) and helium as carrier gas (gas flow: 50cm^3 min^{-1}). Initially the column temperature was programmed to start at 100°C for 10 minutes, then increased to 250°C at 3°C per minute and kept isothermally for 15 minutes.

Dried ground stem bark (500g) of *Trema orientalis* was soaked in methanol for three days at room temperature. The extracting solvent was removed under vacuum using a rotary evaporator to obtain a greenish-black crude extract (75.01 g, 15%). The crude extract was fractionated with ethyl acetate into ethyl acetate-soluble and ethyl acetate-insoluble fractions. The ethyl acetate-soluble fraction (1.95 g, 0.39%) was chromatographed over alumina II (deactivated with 6% of distilled water) and gave on elution with hexane containing increasing amount of ethyl acetate:

a) 2% ethyl acetate, an oily mixture (0.12g), which contained methyl hexadecanoate **1**, (Z,Z) methyl 9,12-octadecadienoate **2**, (Z) methyl 9-octadecenoate **3**, methyl octadecanoate **4**, methyl eicosanoate **5**, methyl docosanoate **6** and methyl tetracosanoate **7** (Table 1).

b) 15% ethyl acetate, β–sitosterol **8** (82.8 mg), after repeated column chromatography.

c) 100% methanol, 3β–acetoxyurs-12-en-28-oic acid **9** (0.11 g), after repeated column chromatography and recrystallisation.

The oily mixture was analysed by GC and GC-MS spectrometry and the fragmentation pattern of the compounds were matched against those reported in the reference libraries [14]. The total ion chromatogram of these methyl esters of fatty acids is shown in Figure 1.

Methyl hexadecanoate **1**. MS m/z 270 (M^+, 3%), 239 (M^+ – OMe, 2%, $C_{16}H_{31}O_1^+$), 227, 199, 185, 143, 129, 87 (50%, $CH_2CH_2CO_2CH_3^+$), 74 (base peak, 100%, $C_3H_6O_2^+$), 55.

(Z,Z) methyl 9,12-octadecadienoate **2**. MS m/z 294 (M^+, 2%), 263 (M^+ – OMe, 2%, $C_{18}H_{31}O_1^+$), 150, 123, 109, 95, 81, 67 (base peak, 100%), 55.

(Z) methyl 9-octadecenoate **3**. MS m/z 296 (M^+, 2%), 264 (M^+ – OMe, 2%, $C_{18}H_{33}O_1^+$), 222, 123, 110, 96, 83, 74, 55(base peak, 100%).

Methyl octadecanoate **4**. MS m/z 298 (M^+, 2%), 267 (M^+ – OMe, 2%, $C_{18}H_{35}O_1^+$), 255, 199, 143, 129, 97, 87 (50%, $CH_2CH_2CO_2CH_3^+$), 74 (base peak, 100%, $C_3H_6O_2^+$), 55.

Methyl eicosanoate **5**. MS m/z 326 (M^+, 2%), 295 (M^+ – OMe, 2%, $C_{20}H_{39}O_1^+$), 283, 241, 227, 199, 185, 171, 143, 129, 97, 87 (50%, $CH_2CH_2CO_2CH_3^+$), 74 (base peak, 100%, $C_3H_6O_2^+$), 55.

Methyl docosanoate **6**. MS m/z 354 (M^+, 2%), 323 (M^+ – OMe, 2%, $C_{22}H_{43}O_1^+$), 311, 269, 255, 199, 143, 129, 111, 97, 87 (50%, $CH_2CH_2CO_2CH_3^+$), 74 (base peak, 100%, $C_3H_6O_2^+$), 55.

Methyl tetracosanoate **7**. MS m/z 382 (M^+, 3%), 351 (M^+ – OMe, 2%, $C_{24}H_{47}O_1^+$, 339), 199, 185, 157, 143, 129, 97, 87 (50%, $CH_2CH_2CO_2CH_3^+$), 74 (base peak, 100%, $C_3H_6O_2^+$), 55.

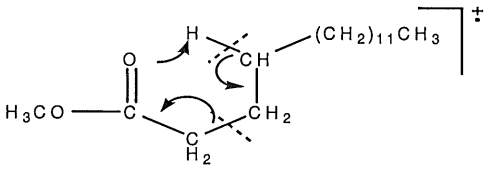
β–sitosterol **8**. White crystalline solid, m.p. 119°C. ^{1}H NMR ($CDCl_3$, 399.65 MHz): δ0.66–1.03 (CH_3, s), 1.04–2.26 (CH_2, CH, m), 3.50 (H–3, m), 5.35 (H–6, d broad). ^{13}C NMR ($CDCl_3$, 399.65 MHz): δ71.9 (C–O, C–3), 140.8 (C olefinic, C–5), 121.8 (C olefinic, C–6), 36.6 (C quaternary, C–10), 42.4 (C quaternary, C–13).

3β–acetoxyurs-12-en-28-oic acid **9**. White crystals, m.p. 258–260°C. MS: m/z 498 (M^+, 10.0), 453 (2.0), 248 (100.0), 203 (45.0), 133 (30.0), 69 (12.0), 43 (27.0). IR: KBr (cm^{-1}) 3276.47 (O–H stretching), 2938.02 (C–H), 1716.34 (C=O). ^{1}H NMR ($CDCl_3$, 499.7 MHz): δ5.23 (1H, t, J = 3.5 Hz, H–12), 4.49 (1H, dd, J = 10.5, 7.0 Hz, H–3), 2.19 (2H, d, J = 12 Hz, H–18), 2.04 (3H, s, MeC=O), 1.71 (2H, m, CH_2), 1.65 (2H, m, CH_2), 1.63 (2H, CH_2), 1.52 (H, CH), 1.32 (H, m, CH), 1.09 (2H, –CH_2), 1.08 (2H, s, CH_2), 1.06 (3H, Me–27), 0.95 (3H, Me–23), 0.95 (3H, Me–25), 0.83 (1H, CH), 0.84 (3H, s, Me–24), 0.85 (3H, Me–29), 0.86 (3H, Me–30), 0.77 (3H, Me–26). ^{13}C NMR ($CDCl_3$, 499.7

MHz): δ80.91 (C–O, C–3), 38.81 (C quaternary, C–4), 39.47 (C quaternary, C–8), 36.89 (C quaternary, C–10), 125.75 (C=C, C–12), 137.95 (C=C, C–13), 41.92 (C quaternary, C–14), 47.90 (C quaternary, C–17), 52.60 (C–18), 182.52 (COOH, C–28), 171.02 (C=O, C–31). The carbons and protons of 3β–acetoxyurs-12-en-28-oic acid **9** were assigned from the DEPT, HSQC and HMBC experiments and by comparison with published data [15].

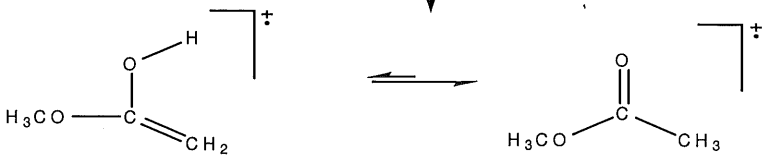
RESULTS AND DISCUSSION

Elution with 2% ethyl acetate-hexane gave an oily mixture which contained methyl ester of fatty acids hexadecanoic, (*Z,Z*)-9,12-octadecadienoic, (*Z*)-9-octadecenoic, octadecanoic, eicosanoic, docosanoic and tetracosanoic identified through GC–MS spectrometry. The EIMS of all the methyl ester fatty acids gave a M^+ – OMe peak that corresponded to the $R–C{\equiv}O^+$ fragment ion. The ion $R–C{\equiv}O^+$ gives an easily recognizable peak for esters and for methyl esters occurring at M^+ – OMe [16]. The EIMS of methyl hexadecanoate, methyl octadecanoate, methyl eicosanoate, methyl docosanoate and methyl tetracosanoate showed very intense base peaks at m/z 74 which can be assigned to the fragment ion $C_3H_6O_2^+$. A methyl ester of an aliphatic acid unbranched at α carbon gives a strong peak at m/z 74, which is the base peak in straight-chain methyl esters from C_6 to C_{26} due to the McLafferty rearrangement [16]. This moiety can be deduced by the location of the peak resulting from the following cleavage:



m/z = 270, molecular ion peak

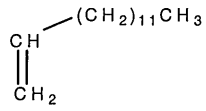
For methyl hexadecanoate

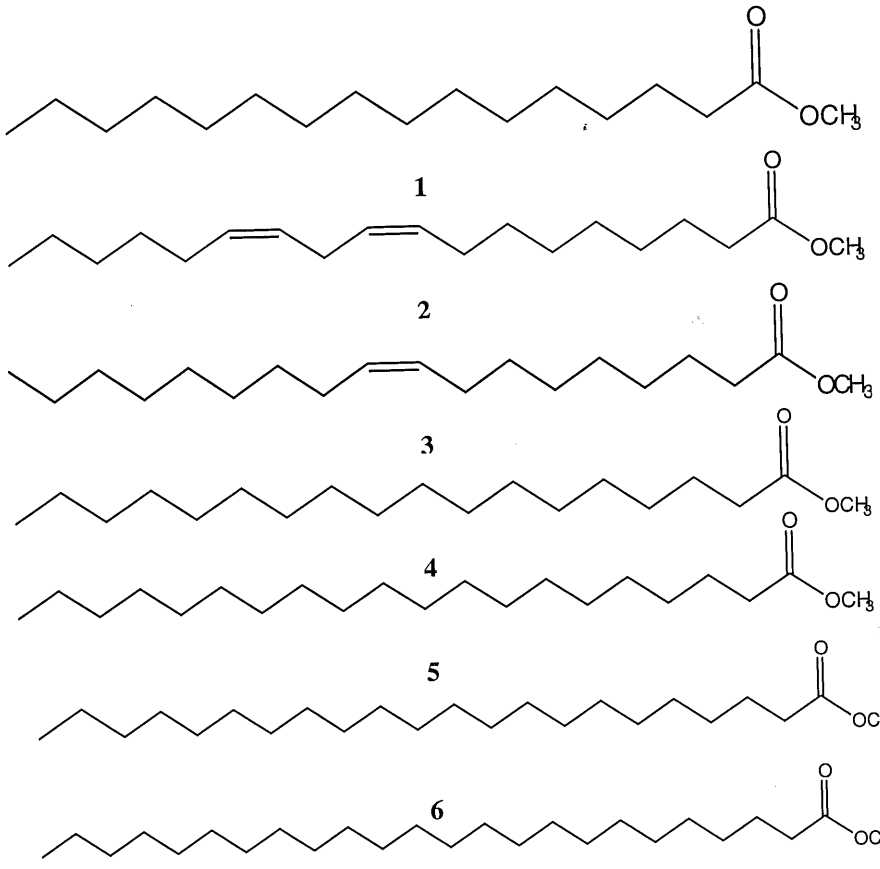


m/z = 74, ($C_3H_6O_2^+$) base peak

For methyl hexadecanoate

+





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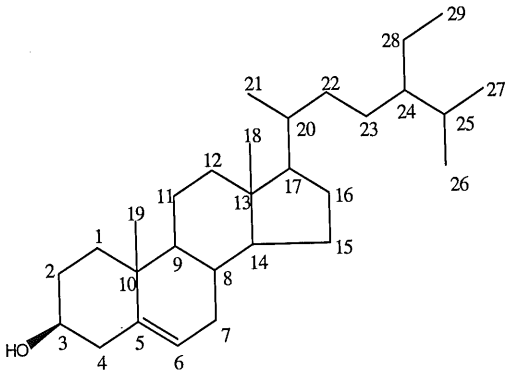

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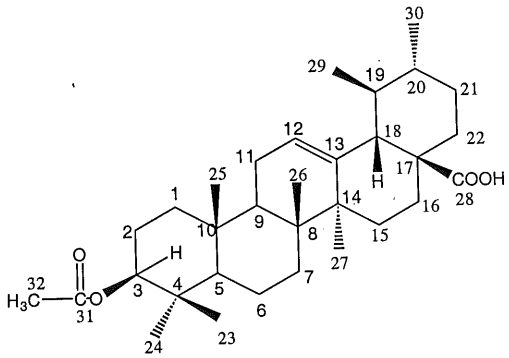

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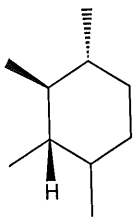

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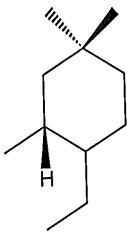

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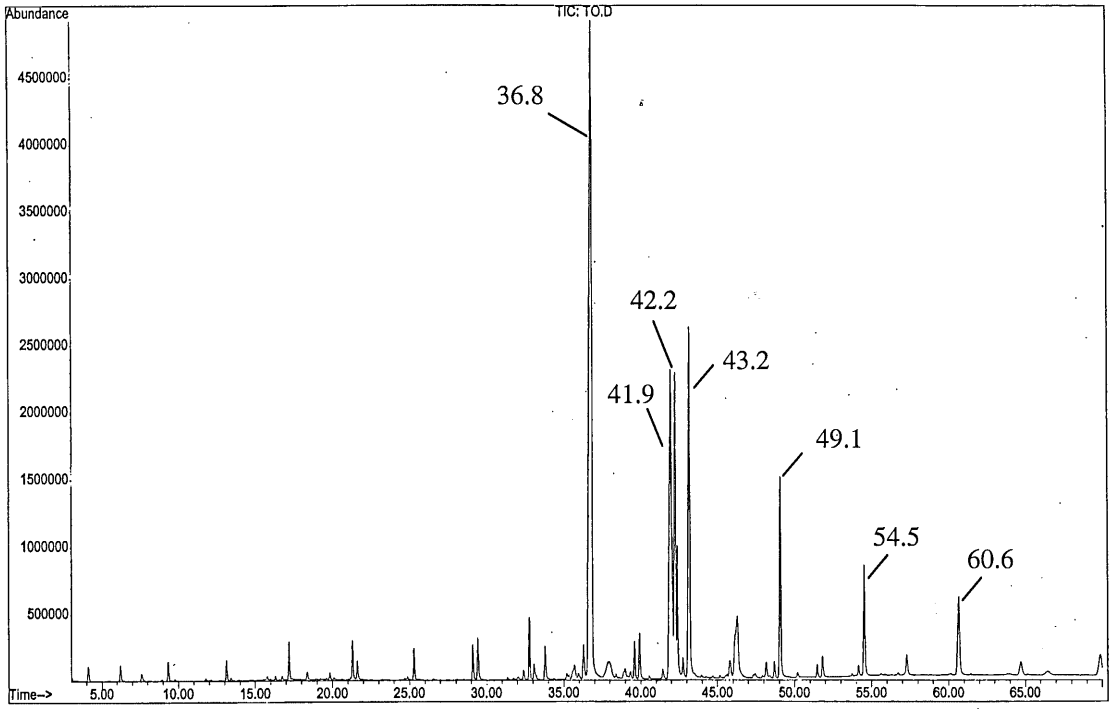

Figure 1. The total ion chromatogram of the oily mixture containing fatty acid methyl esters

Table 1. Methyl esters fatty acids and their retention times

Methyl esters fatty acids	Molecular Weight	Retention time, minute	Area Percentage, %
Methyl hexadecanoate **1**	270.26	36.85	33.05
(Z, Z) Methyl 9,12-octadecadienoate **2**	294.26	41.99	13.71
(Z) Methyl 9-octadecenoate **3**	296.27	42.29	8.64
Methyl octadecanoate **4**	298.29	43.21	10.71
Methyl eicosanoate **5**	326.32	49.11	5.24
Methyl docosanoate **6**	354.35	54.55	2.78
Methyl tetracosanoate **7**	382.38	60.66	2.97

The EIMS of each of the methyl ester fatty acids also showed a peak at m/z 87, which was consistent with the $CH_2CH_2CO_2CH_3^+$ due to the loss of alkyl group $CH_3(CH_2)_n$ depending on the length of alkyl chain of the fatty acid [16]. Thus,

the content of the oily mixture consists of methyl esters of fatty acids. Of the seven methyl ester fatty acids, methyl hexadecanoate **1**, methyl octadecanoate **4**, methyl eicosanoate **5**, methyl docosanoate **6** and methyl tetracosanoate **7** were found to be saturated long chain methyl ester fatty acids.

β–sitosterol **8** was obtained as white crystalline solid with melting point 119°C. The ^{1}H NMR spectrum showed six singlet peaks at δ0.76, 0.86, 0.90, 0.92, 1.00 and 1.06 corresponding to six methyl groups of β–sitosterol. Carbon C–O, carbon olefinic and carbon quaternary locations can be determined by comparing the obtained data to the ^{13}C NMR spectrum data that was reported [17]. Chemical shifts on δ140.8 and δ121.8 showed the presence of C olefinic C–5 and C–6 [17]. The peak at δ71.9 was assigned to the oxymethine proton C–3 [17]. The signals at δ36.6 and 42.4 were consistent with the position of the quaternary carbon, C–10 and C–13, respectively [17].

3β–acetoxyurs-12-en-28-oic acid **9** was obtained as white crystals with melting point 258–260°C. The IR spectrum showed bands at 3400 cm^{-1} for hydroxyl group of COOH and carbonyl absorption at 1716 cm^{-1}. The EIMS gave a molecular ion peak at m/z 498 consistent with the molecular formula $C_{32}H_{50}O_4$ (Figure 2). The ^{1}H NMR spectrum showed signals for one olefinic proton at δ5.23 (t, J = 3.5 Hz) assigned to H–12, and for one oxymethine proton at δ4.49 (dd, J = 10.5, 7.0 Hz). There were also strong resonances due to the methyl groups in the region of δ0.77–1.06. The singlet peak at δ2.04 was consistent with the acetoxyl group (CH_3COO^-) at C–3. The ^{13}C NMR spectrum included the signals for oxygen bearing carbon (δ80.9) and trisubstituted double bond at δ125.8 and 137.00. The EIMS further showed abundant ions due to retro Diels-Alder cleavage at m/z 248 ($C_{16}H_{24}O_2$, 100%) and m/z 192. Other peaks corresponding to m/z 203 and 133 were consistent with initial loss of COOH from the fragment ion at m/z 248 followed by cleavage of C18–C19 and C21–C22 bonds, respectively (Figure 2). All these observations suggested that compound **2** was a pentacyclic triterpene of the α-amyrin class (ursane) with a Δ^{12} double bond [18, 19] and one acetoxy group at C–3. The presence of H–18 as a doublet at δ2.19 (J = 11.5 Hz) again suggested an ursane skeleton **10** rather than an oleanane **11**. The acetoxyl group was assigned to C–3, on biogenetic grounds, in β and equatorial configuration based on larger coupling constant of H–3α at δ4.49 [20]. The proton NMR data were consistent with a derivative of ursolic acid. The R_f data was also comparable to a synthetic sample obtained through acetylation of ursolic acid. The assignment of various carbon atoms in the ^{13}C NMR spectrum were made through comparison with published spectra [15] and confirmed through NMR experiments.

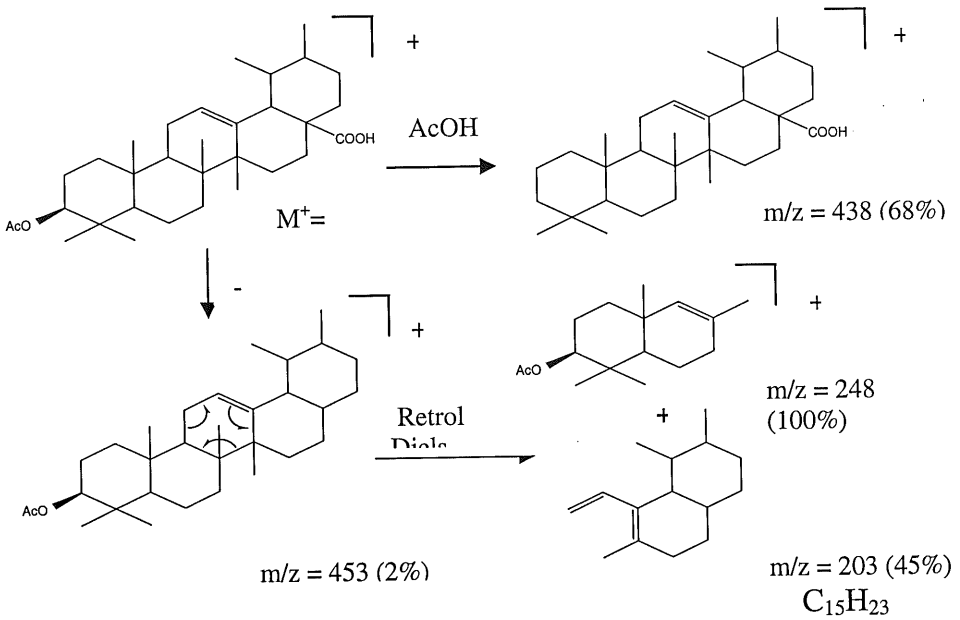

Figure 2. The ion fragmentations of 3β-acetoxyurs-12-en-28-oic acid

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