CHAPTER 6

INSTRUMENTAL ANALYSIS AND STRUCTURAL ELUCIDATION OF ISOLATED COMPOUNDS

6.1 Introduction

Identification of compounds usually involves a combination of different techniques including nuclear magnetic resonance (NMR), mass spectrometry (MS), ultraviolet (UV) and infrared (IR) spectrometry. Other ways of confirming the identification of the compounds include calculation of the R_f values in different solvent systems and determination of melting point. In this study NMR and MS techniques were used as tools for identifying the structure of compounds. Retardation factor (R_f) values were calculated in most cases using CEF solvent system as the mobile phase. UV spectrometry was not required as structure elucidation was completed in all cases without the use of this technique.

6.2 Nuclear magnetic resonance spectroscopy [NMR]

For structural elucidation purposes, compounds isolated were subjected to instrumental analysis. The Nuclear Magnetic Resonance (NMR) [both ^{1}H and ^{13}C] spectra was determined with the assistance of the regional analytical center at Rand Afrikaans University, Johannesburg, and also at the Department of Chemistry and Biochemistry, Medical University of Southern Africa using Varian Unity Inova 300 and 600 MHz NMR system. Some analyses were at the Hans-Knoll institute, Jena, Germany using a DPX-300, "DNMR, DPX-500 (Bruker, Germany).

6.2.1 NMR sample preparation

Isolated compounds were dried, weighed (5-10 mg) and dissolved in a maximum of 2 ml of deuterated solvents [Merck] used for NMR. Pyridine and chloroform were used as solvents of choice, because the compounds under investigation were soluble in them. The solutions [free of insoluble impurities] were then pipetted into NMR tubes [commonly of 5 mm diameter to a depth of 2-3 cm] using a clean Pasteur pipette and taken to either of the above universities for proton (^{1}H), carbon 13 (^{13}C), distortionless enhancement through polarization transfer (DEPT), correlated spectroscopy (COSY), heteronuclear multiple quantum

coherence (HMQC), heteronuclear multiple bond connectivity (HMBC) and mass spectrometer (MS) analysis. For NMR analysis, the tubes were lowered into a probe placed between the poles of a magnet. The probe had a transmitter and receiver coils connected to it. The magnet was then adjusted to give the highest level of homogeneity and the tube was spun. The spectrum was then taken using the instrument controls.

6.2.2 One-dimensional spectroscopy

6.2.2.1 1H-NMR

Proton magnetic resonance (PMR/^{1}H NMR) was widely employed. The spectrum appears predominantly in the range 0–10 ppm downfield from the reference signal of trimethylsilane. Proton NMR gives a measure of the absorptions of the different proton signals from a compound. The integral of the signal is proportional to the number of protons it represents, and the nature of the hydrogen is established by the chemical shift. The absorption of a signal is generally proportional to the number of protons coming into resonance frequency of the signal with the result that the area under the absorption peak is proportional to the number of protons being detected. A nucleus in a region of high electron density experiences a chemical field proportionately weaker than those in a region of low electron density, and a higher field has to be applied to bring it into resonance. Such nuclei are said to be shielded by the electrons. A high electron density shields a nucleus and causes resonance to occur at relatively high field [with low delta value]. Likewise, a low electron density causes resonance to occur at relatively low field [i.e. with high chemical shift value] and the nucleus is said to be deshielded (Friebolin, 1998).

6.2.2.2 ^{13}C-NMR

^{13}C-NMR was used to determine the precise frequency at which each carbon comes into resonance and is determined not only by the applied field $\beta_{0,}$ but also by minute differences in the magnetic environment experienced by each nucleus. These minute differences are caused largely by the variation in electrons in the neighborhood of each nucleus, with the result that each chemically distinct carbon atom in a structure, when it happens to be a ^{13}C, will come into resonance at a slightly different frequency from all the others. Each upward line in a ^{13}C spectrum corresponds to one carbon atom.

6.2.2.3 DEPT

Distortionless enhancement through polarization transfer [DEPT] is a technique that allows a separate spectrum to be obtained for the ^{13}C of CH_3, CH_2, and CH. So called because the impulse sequence used forces part of the higher sensitivity associated with proton detection on to ^{13}C, a process that enhances the ^{13}C signal intensity by polarization transfer from ^{1}H to ^{13}C.

6.2.3 Two-dimensional spectroscopy

Two-dimensional (2-D) spectroscopy is a more recent innovation. The spectrum contains signals dispersed according to two characteristic frequencies rather than one so that the numbers of distinct signals that can be resolved are more than in a normal 1D spectrum. In 'resolved' 2-D experiments, chemical shifts and hetero or homonuclear spin couplings are separated into two dimensions. The 'correlation' experiments meanwhile differ from resolved experiments in that they contain a mixing period during which coherence is transferred or evolves in the spin system. The result is a 2-D spectrum exhibiting connectivities, i.e. cross peaks between signals from coupled spins. The most common method of presenting this data is by contour plot as it is able to cope with crowded spectra and also allows easier determination of the frequency co-ordinates of peaks (Friebolin, 1998).

6.2.3.1 Homonuclear correlations

6.2.3.1.1 ^{1}H- ^{1}H Correlation Spectroscopy (COSY)

Correlated Spectroscopy (COSY) is a two-dimensional experiment that indicates all the spin-spin coupled protons in one spectrum. In a COSY spectrum, two essentially identical chemical shift axes are plotted orthogonally (although the resolution on each chemical shift axes is normally different). All peaks that are mutually spin-spin coupled are shown by cross peaks, which are symmetrically placed about the diagonal. By introducing extra delays in the pulse sequence, COSY spectra can be obtained to emphasize long-range coupling. In such long range COSY or delayed COSY spectra, this may be used to uncover coupling of the order of 1Hz and thereby giving connectivities over 4 or 5 bonds.

6.2.3.2 Heteronuclear correlations

Heteronuclear correlation spectroscopy is analogous to COSY. However, a different experimental regime is required since two types of nuclei with different Larmor frequencies are involved (Neri and Tringali 2001). It differs from COSY only in that the second pulse is applied simultaneously to both nuclei. It can be used to work out one-bond correlations or longer distance correlations and thus aids in building up a picture.

6.2.3.2.1 Heteronuclear Multiple Quantum Coherence (HMQC) (^{1}H-^{13}C COSY)

This ^{1}H-^{13}C COSY spectrum allows the use of the carbon spectrum to unambiguously assign often severely overlapping proton signals. It has the advantage that even in complicated molecules there is little overlapping of the correlation peaks, which combine the large chemical shifts of ^{13}C NMR and those of ^{1}H NMR spectroscopy.

6.2.3.2.2 Heteronuclear Multiple Bond Connectivity (HMBC) (Long range ^{13}C-^{1}H COSY)

In this sequence, a time delay of about 50 ms in the pulse sequence is used to determine two and three bond coupling. It is crucial in constructing extended pieces of the molecular skeleton that can be joined together to complete structure elucidation. Since many ^{1}H-^{13}C (two-bond) and ^{1}H-C-C-^{13}C (three-bond) coupling constants are rather similar in value and lie in the range of 2-20 Hz, then ^{13}C chemical shifts are now correlated with the chemical shift of those protons separated from them by two and three bonds.

6.2.3.2.3 NOESY spectroscopy

The nuclear Overhauser effect (n.O.e) results from through-space interactions of magnetic nuclei. It is defined as a change in the integrated NMR absorption intensity of a nuclear spin when the NMR absorption of another spin is saturated. It allows moieties that are spatially proximal (but may be too far to couple through bonds) to be located and it makes a major contribution to the determination of stereochemistry (Neri and Tringali 2001).

6.3 Mass spectrometry

6.3.1 Sample preparation

Approximately 1 mg of each isolated compounds was dried, put in 2 ml vial and sent to either of the analytical centres mentioned in Section 6.2 for mass spectrometry analysis.

6.3.2 Mass spectrometry (MS)

Specific identification of molecules is more certain with the use of mass spectrometer (MS). In electron impact mass spectrometry (EIMS) the effluent which contains the separated and vaporized compounds, passes into the ion chamber of the mass spectrometer, which is under a high vacuum. A beam of electrons accelerated from a filament, which ionizes and fragments them, bombards the molecules. Initially, one electron is removed from each molecule to form a positively charged molecular ion (M^+). Breakage of bonds relative to bond strength occurs rapidly in the molecular ion to generate fragment ions. The various ions are accelerated into the analyzer portion of the mass spectrometer where they are sorted according to their mass to charge ratios (*m/z* values) that are equivalent to the molecular weights of the fragments. The ion signal is amplified by an electron multiplier and the mass spectrum is plotted from low to high mass. The *m/z* values are plotted against relative abundance of the ions. The most abundant ion (base peak) in the spectrum is assigned as 100%

6.3.2.1 Electron Impact Spray MS (EIS-MS)

For all the compounds isolated from the Hans Knöll Institute (HKI) in Jena Germany, ESIMS, triple quadruple mass spectrometer Quattro (VG Biotech, England); EIMS, 70 eV direct inlet, high resolution with perfluorokerosine as a standard, MAT 95 XL (Finnigan, Germany) was used for the analysis. The EIS-MS is an MS technique performed to give a more precise molecular ion that could be of great assistance for molecular formula determination.

6.4 IR

For IR analysis, IFS55 spectrometer (Bruker, Germany) was used for the analysis.

6.5 Results and Discussions

6.5.1 Identification of compounds

Most of the compounds had the basic triterpenoids skeleton (**Fig 6-1**). The result of compounds 1-5 are as discussed in the following sections.

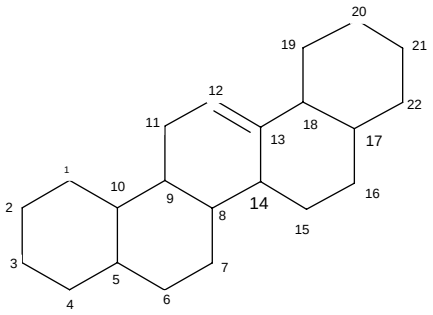


Figure 6-1: Skeletal unit of olean-12-ene type of pentacyclic triterpenoid isolated from *C. imberbe*

Compound **1** crystallized in most of the fractions and was isolated in large quantity. It has been previously isolated from the same species by Katerere *et al*., 2002 and Rogers and Subramony, 1988, and appeared to be the major constituent in the leaves of *C. imberbe*. Compound **2**, **3** and **4** have been isolated from different plant species. Compound **5**, a glycoside, was isolated and reported here for the first time.

From previous work done; pentacyclic triterpenes have been isolated from *C. imberbe* and related species of Combretaceae (Roger, 1989b; Rogers and Subramony, 1988 and Rogers and Verotta, 1996). This was of great assistance in the preliminary analysis of NMR data, which indicated that the isoprenoids were triterpenoid compounds in nature. IR analysis indicated the presence of olefinic (ca 1652.84cm^{-1}), hydroxyl (ca.3440 cm^{-1}), carboxylic acid OH (*ca*.2923.37 cm^{-1}), methyl (*ca*.1456 cm^{-1}) and carboxylic acid C=O (*ca*.17717 cm^{-1}) in most of the compounds.

6.5.1.1 Compound 1

Compound **1** crystallized as a white solid from the first silica gel 60 column after elution with 100% ethyl acetate. NMR spectra were suggestive of an oleane-12- skeleton that has previously been isolated from this plant with a carboxylic function, a trisubstituted double bond, carbon bearing OH bonds and an AB system.

This pattern was observed in all other compounds isolated in this study, suggesting a similarity in the chemical structure in the main skeletal unit (**Fig. 6-1**) modified by the various constituents.

MS analysis of Compound **1** (**Fig. 6-3**) gave a molecular formula of $C_{30}H_{48}O_4$ (m/z 472) and characteristic peak at m/z 248 $(M\text{-}C_{14}H_{24}O_2)^+$, m/z 454 $(M\text{-}H_20)^+$, m/z 187 $M\text{-}C_{16}H_{27}O_4)^+$, m/z 130 $(C_{10}H_{10})^+$, and m/z 93 $(C_7H_9)^+$ in an EI mass spectrum due to a retro-Diels-Alder fragmentation of an oleanene skeleton with a free carboxylic function either in ring D or E. There was an initial loss of water, then a Wagner-Meerwein rearrangement with subsequent lost of another water (**Fig. 6-2**). The rearranged fragment then undergoes retro-Diels fragmentation. This structural type was further supported by the indication of seven degree of unsaturation and the ^1H NMR spectrum. It contained resonance for seven skeletal methyl groups and a broad triplet at δ_H 5.21 for olefenic proton (H-12), carboxylic acid functionality (δ_c 182.0) and two hydroxyl groups (δ_H 3.6, δ_c, 72.38 and δ_H 3.71, δ_c 73.35). The assignment presented resulted from a combination of ^1H-^1H COSY, HMQC and HMBC experiments. The free carboxylic acid group was assigned to C-30, since it showed an HMBC correlation to H_3-29 and the nature of the fragmentation pattern suggested the presence of the two hydroxyl groups in ring A. Using this information along with the ^1H-^1H COSY, HMQC and HMBC data the partial structure of Compound **1** which was suggestive of a 1,3-dihydroxy-12-oleanen-29-oic acid, corresponding to 1α, 3β-dihydroxyoleanen-12-29-oic previously isolated from the same species by Rogers and Subramony 1988 after perfect comparison of the experimental data.

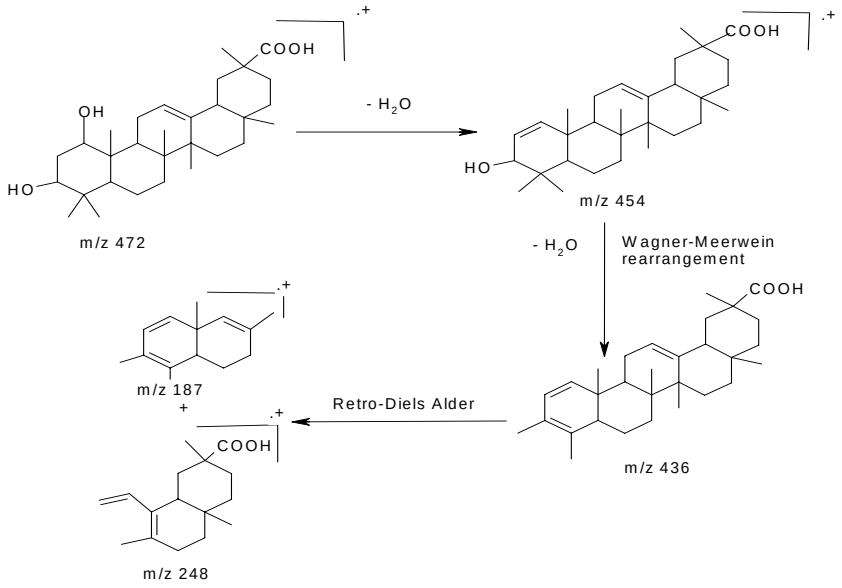


Figure 6-2: Fragmentation pattern of compound 1

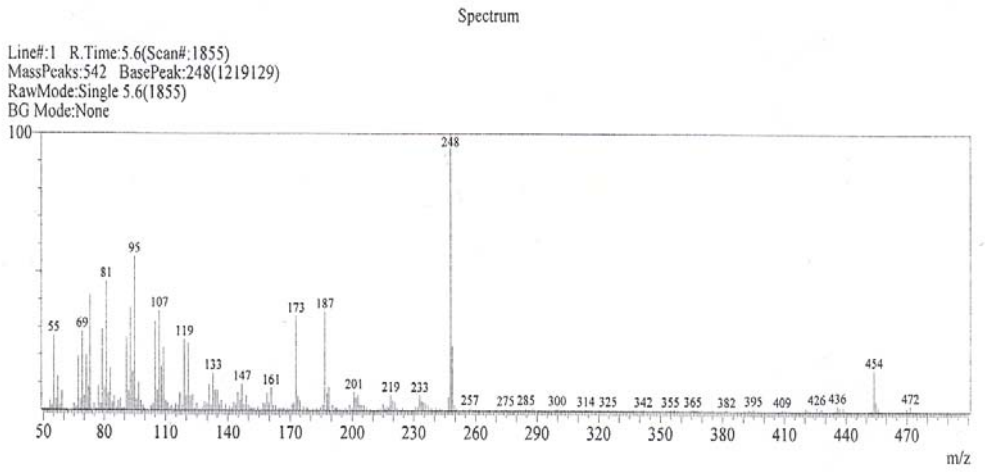


Figure 6-3: EI-MS spectrum of compound 1

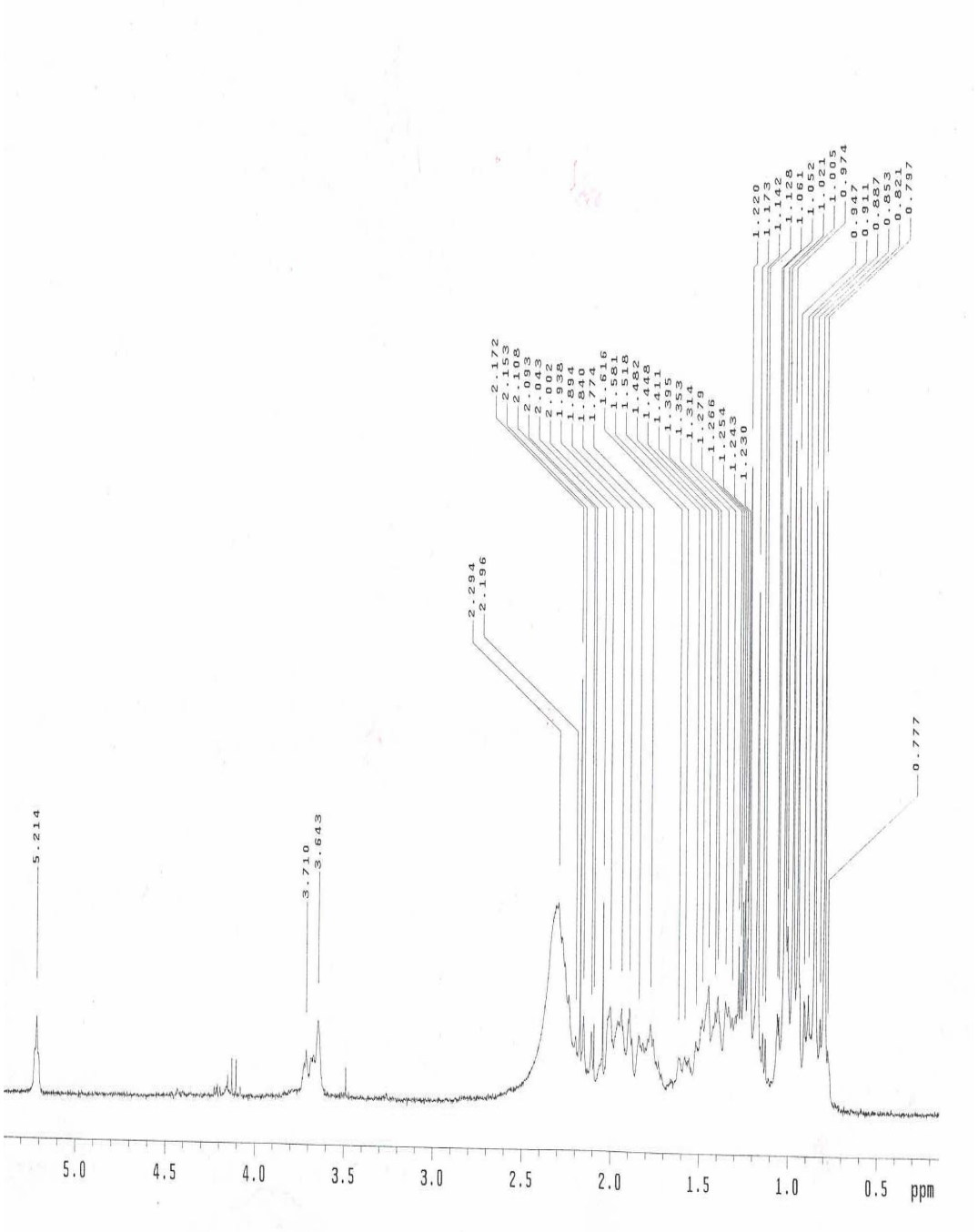


Figure 6-4: H NMR spectrum of Compound 1

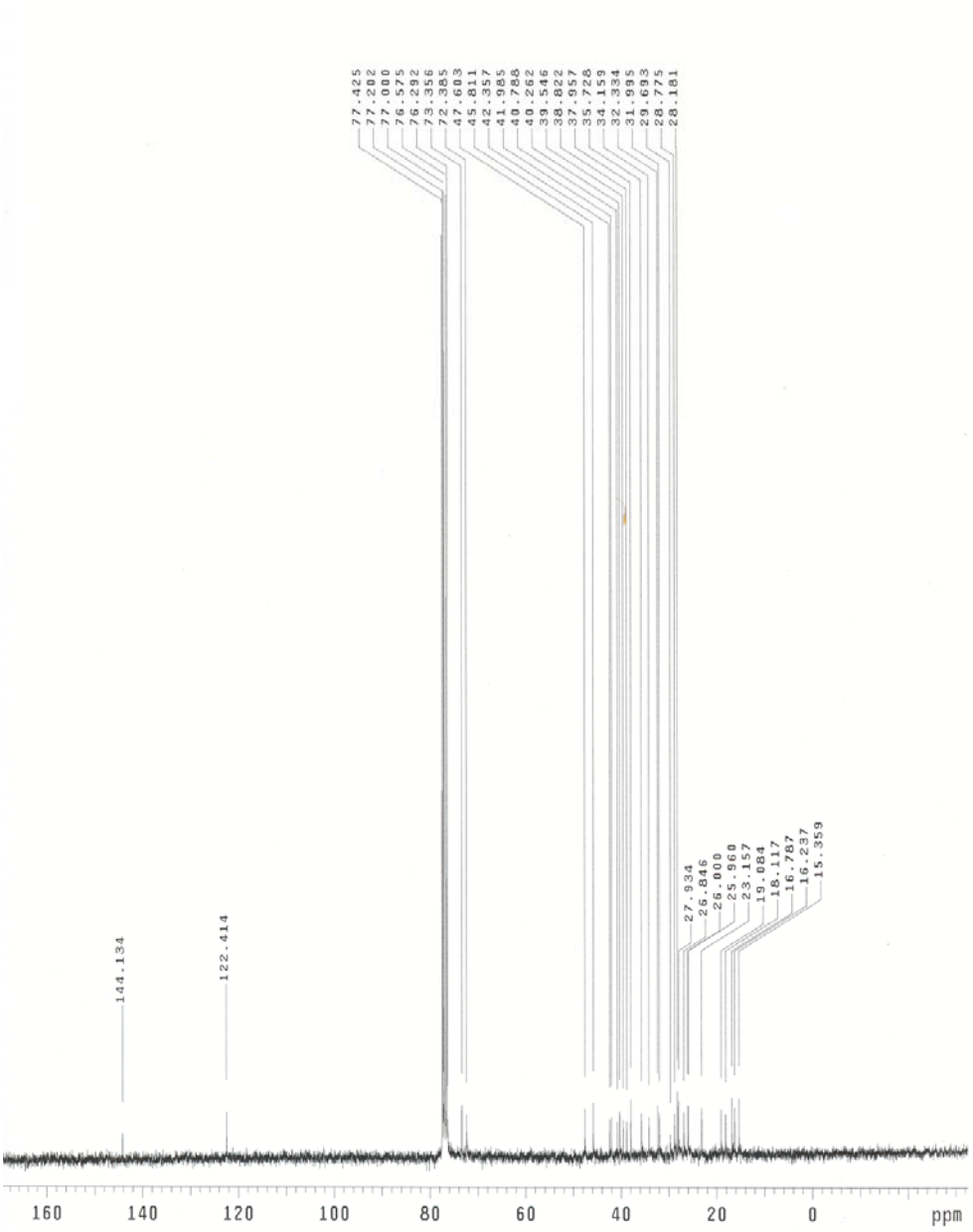


Figure 6-5: ^{13}C NMR spectrum of Compound 1

6.5.1.2 Compound 2

The ^{13}C (**Table 6-1**), ^{1}H and mass spectral data of Compound **2, 3** and **4** showed a strong resemblance to those of Compound **1**. The only difference between these three molecules could be observed in Ring A and E. Compound **2** and **3** had a similar molecular formula $C_{30}H_{48}O_3$ (m|z 456) by mass spectrometry, revealing the presence of seven degrees of unsaturations within the molecule. The ^{13}C NMR spectrum of compounds 1-4 is shown on Table 5-6.The differences in their structures were observed in Ring E where Compound **2** the ^{13}C (**Fig. 6-6**) had resonance at δ_c 182.87 at C-30 indicating the acid functionality and Compound **3** which had ^{13}C resonance at δ_c 210 to indicated a carbonyl functionality at C-24 and two hydroxyl groups (δ_c 79.05, δ 68.15) at C-30 and C-3 as observed in the HMBC correlations. The ^{13}C NMR resonances at δ_c 182.6, 143.5, 122.5, 79.0, HMBC correlations together with their literature comparison suggest Compound 2 to be another oleanene and identified as 3-hydroxyl-12-olean-30-oic (Mukherjee, *et al*., 1994).

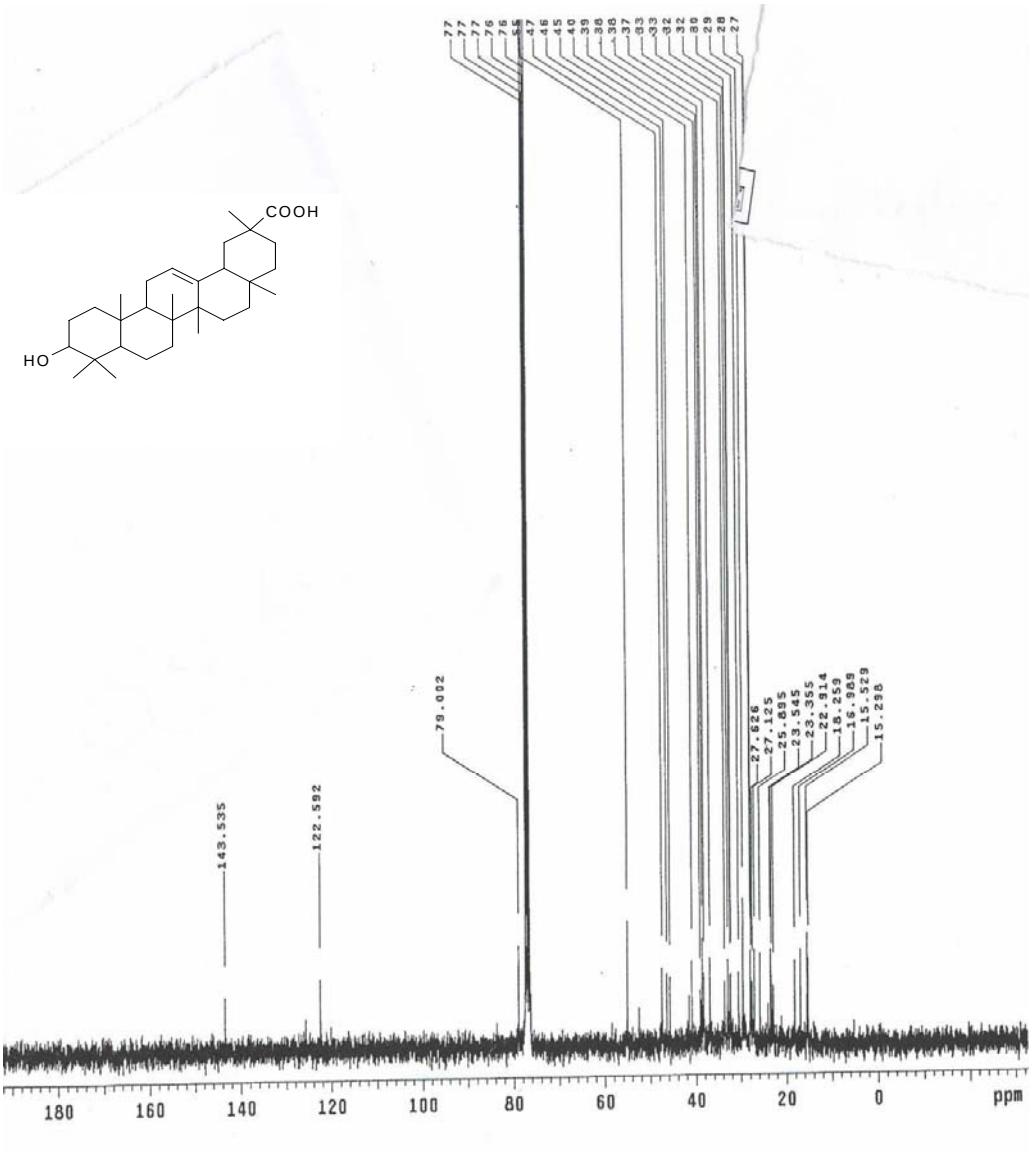


Figure 6-6: ^{13}C NMR spectrum of Compound 2

6.5.1.3 Compound 3

Compound **3** was isolated as a white powder. EI-MS mass analysis indicated the presence of a molecular ion at m/z 457, and characteristic peaks at 439 (M-H_20), 190 and 247 (**Fig. 6-8**), which corresponds to the

molecular formula $C_{30}H_{48}O_3$. The ^{13}C NMR resonances at δ_c144.8, δ_c122.6, δ_c 79.0, δ_c 68.1, HMMC correlations together with literature structure comparison (De Sousa *et al.,* 1990) suggested Compound **3** to be 3, 30-dihydroxyl-12-oleanen-22-one. The proton spectra of Compound **3** (**Fig. 6-9**) determine at the Rand Afrikaans University is different from those determined in MEDUNSA and HKI Germany.

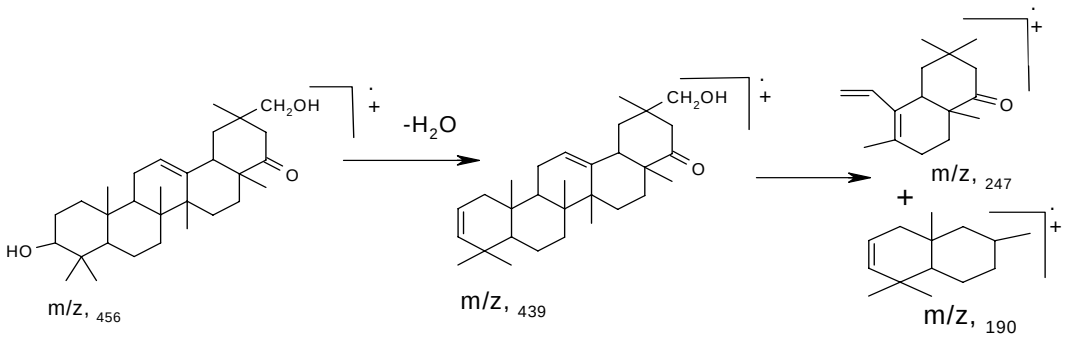


Figure 6-7: Fragmentation pattern of compound 3

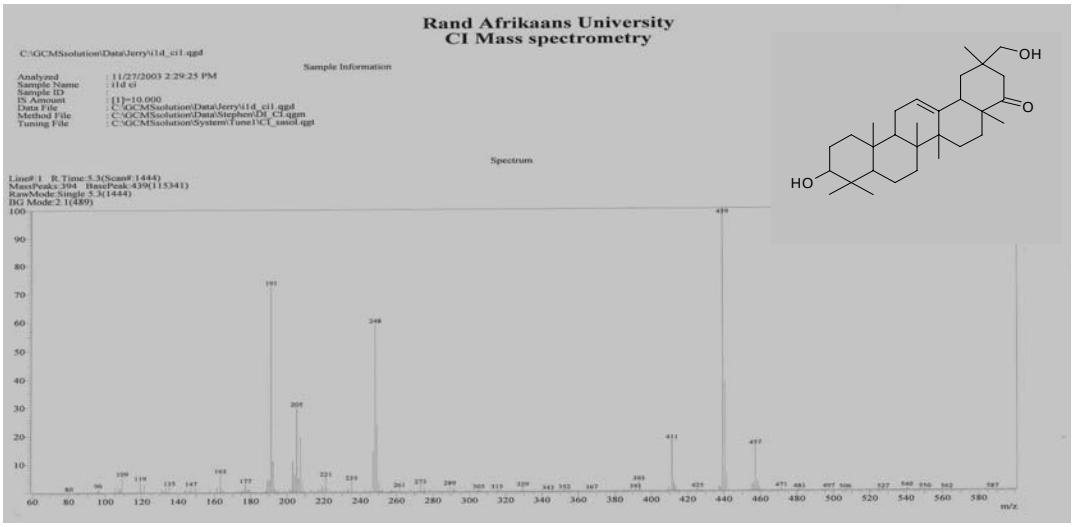


Figure 6-8: Electron impact mass spectrum of Compound 3 indicating the m/e of each fragment

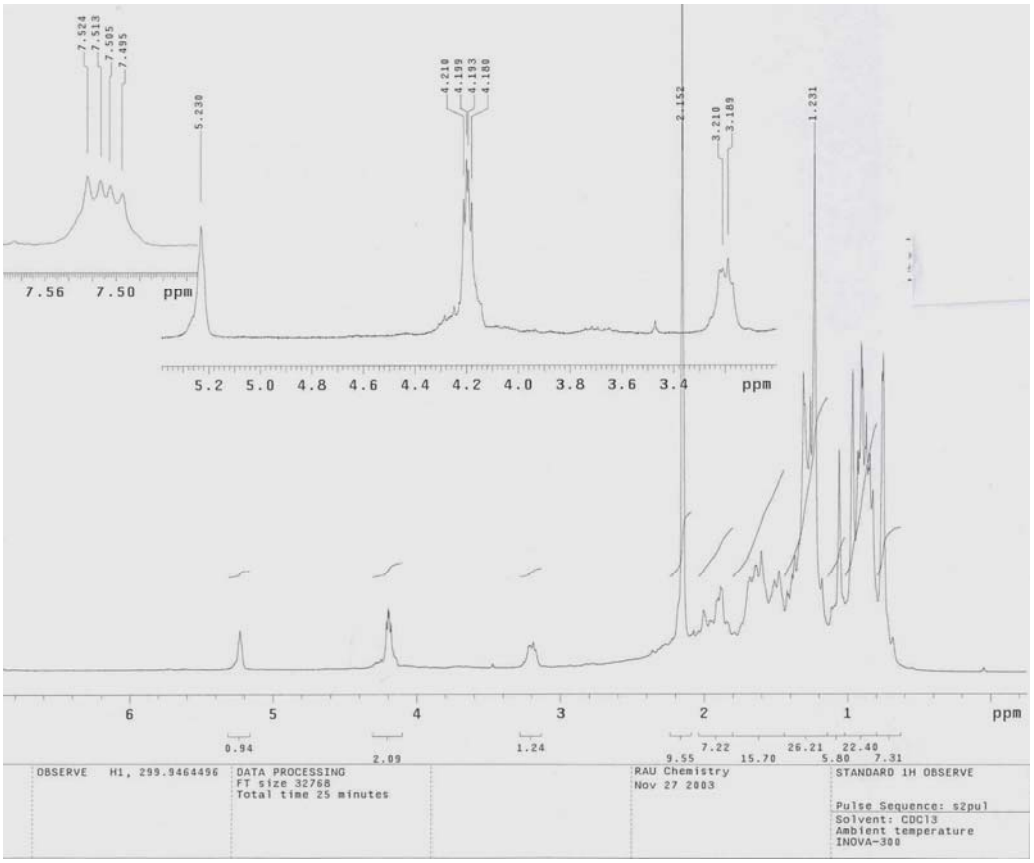


Figure 6-9: ^{1}H NMR spectrum of Compound 3

6.5.1.4 Compound 4

Compound 4 was isolated as white crystals and had $[M+Na]^{+}$ ion peak at m/z 511 in the positive ion ESI-MS suggesting a molecular formula of $C_{30}H_{48}O_5$. Comparing the ^{13}C of Compound **4** with Compound **1**, there was a very strong resemblance. The only difference was observed in the number of hydroxyl groups present in each compound. Compound **4** showed the presence of three hydroxyl groups (**Fig. 6-10**) at δ_c 66.85, δ_H 4.23 δc 73.71, δ_H 3.77 δc 74.60, δ_H 3.57 along with signal at δ_c 182.69 indicating a carboxylic acid group and at δ 149.19, 126.70 indicating the presence of the olefenic carbon C-12 and C-13. DEPT analysis (**Fig. 6-11**) also showed that Compound **4** had seven methyl (CH_3-) groups, eight methylene groups (-CH_2-) and seven methine groups (-CH-). The ^{13}C and ^{1}HNMR, DEPT as well as literature comparison suggest Compound **4** to be another oleanene, (1, 3, 24-trihydroxyl-12-olean-29-oic acid) that has been reported by Duan and Takaihi (2001) previously.

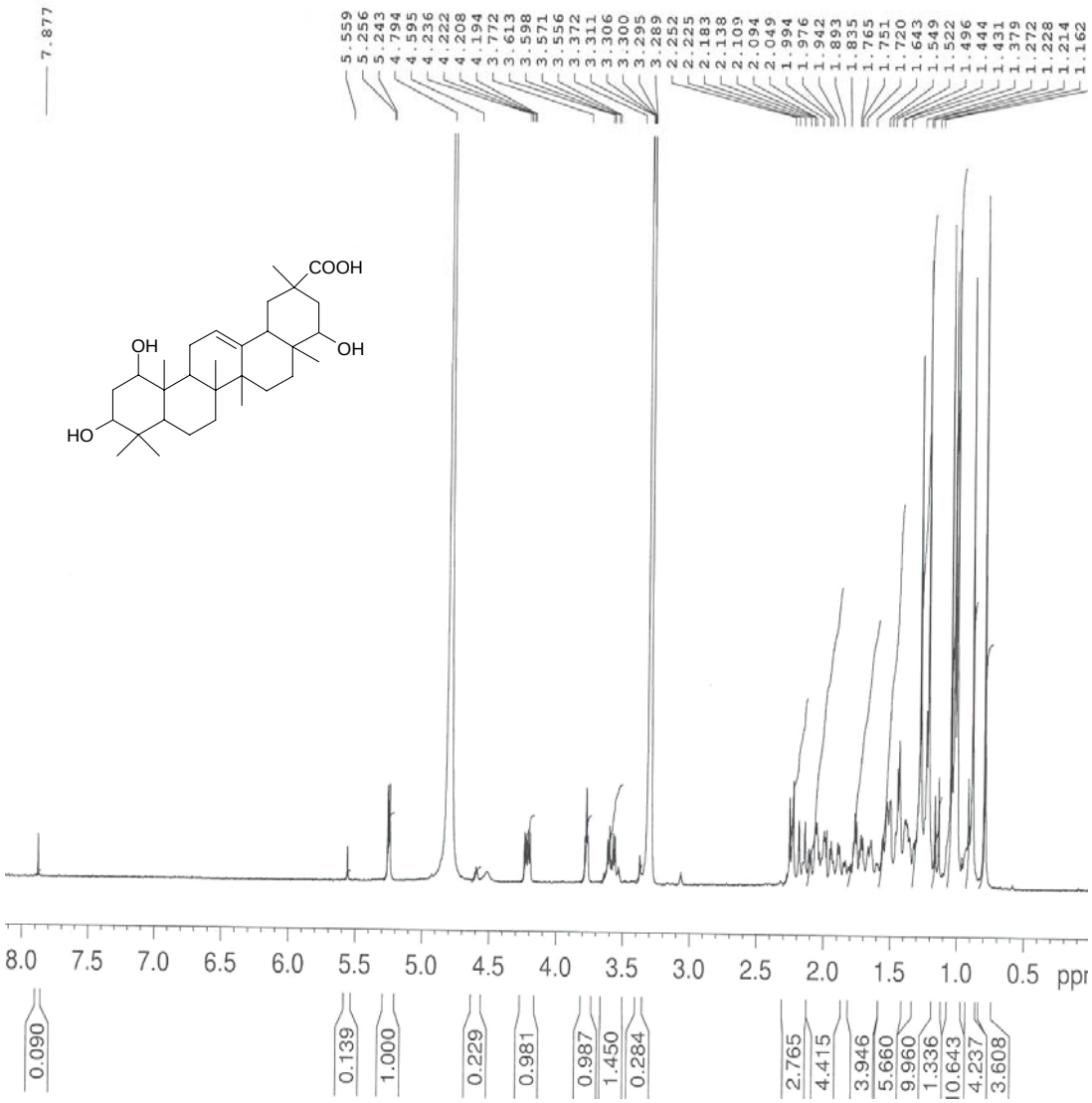


Figure 6-10: ^{1}H NMR spectrum of Compound 4

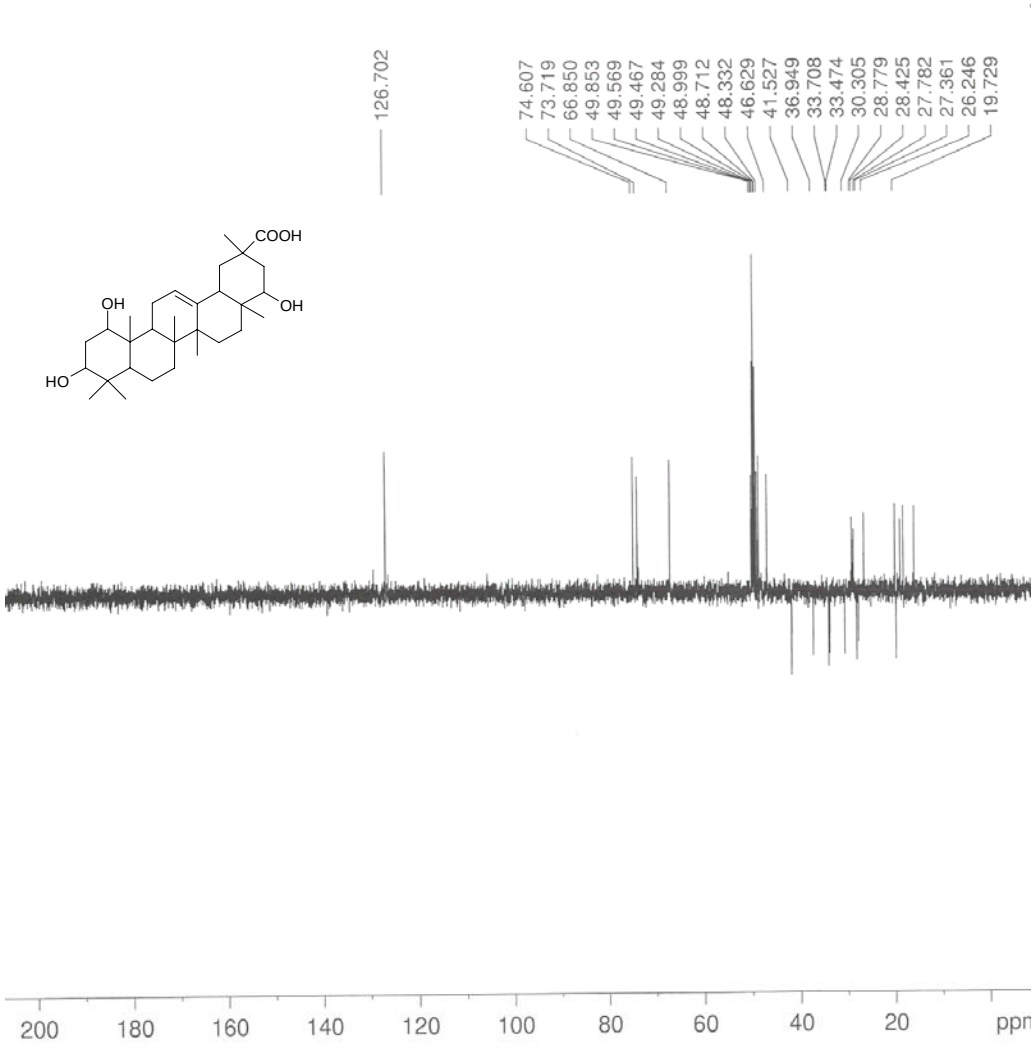


Figure 6-11: DEPT NMR spectrum of Compound 4 differentiating CH_3-, CH_2-, and CH- carbons

6.5.1.5 Compound 5

Compound **5** was isolated as white crystals and has a molecular formula of $C_{40}H_{62}O_{11}$ as determined by ^{1}H, ^{13}C NMR, DEPT (**Fig. 6-13**) data and a $[M+Na]^+$ ion peak at *m/z* 718.0 in the positive ESIMS. The IR spectrum indicated similar structural features as in compounds **1-4** (presence of olefinic (ca 1652.84cm^{-1}), hydroxyl (ca.3440 cm^{-1}), carboxylic acid OH (*ca*.2923.37 cm^{-1}), methyl (*ca*.1456 cm^{-1}) and carboxylic acid

C=O (*ca.*17717 cm^{-1}). ^1H NMR signals **(Fig. 6-12**) at δ_H 5.25 were indicating a vinylic ($R_2C=CHR$) moiety and those at δ_H 3.6 and 3.7 hydroxylated methines in the aglycone. The signals at δ_H 4.70 and δ_c 98.62 indicate an anomeric CH group (acetal) of an oxygen-linked sugar moiety. The ^{13}C NMR data of 5 (**Table 6-2**) were almost identical to those of 1α, 3β, 23-trihydroxy-12-oleanen-29-oic acid (Rogers and Subranomy, 1988) except for an additional sugar moiety and the missing hydroxyl group at C-3 that had been replaced with the ether linked sugar moiety, as indicated by δ_c 67.0. The location of the new sugar moiety was confirmed by HMBC correlations between H-3 and the anomeric carbon and between the anomeric proton and C-3. Two acetylated methines were indicated at δ_c 75.4, δ_H 4.90 (C-4') and δ_c 73.9, δ_H 5.11 (C-2') through correlations to two acetyl groups. All ^1H and ^{13}C NMR data were assigned through HMQC, HMBC (Table 6-3) and COSY experiments.

According to the coupling constants and correlation peaks in NOESY spectrum, 5 showed the same relative stereochemistry in the triterpenoid skeleton as 1α, 3β, 23-trihydroxy-12-oleanen-29-oic acid and the anomeric proton located at β position in sugar moiety. The relative configuration of the chiral centres at C-1, C-3, C-4, C-8, C-15, C-20 and C-10 was determined by detailed NOESY analysis. Correlations between H-1 (δ_H 3.55) and H-3 (δ_H 4.19), H_3-25 (δ_H 1.00) and H_3-26 (δ_H 1.05) and H_3-28 (δ_H 0.93) and H_3-23 (δ_H 0.7) indicated these protons to be oriented on the same side of the ring system. On the other hand, NOESY correlations between H-9 (δ_H 2.52) and H-27 (δ_H 1.30) and none of the above protons indicated these two protons to be located on the other ring plane. Thus, compound **5** was elucidated as 1α, 23β-dihydroxy-12-oleanen-29-oic-acid-3β-O-α-2,4-diacetylrhamnopyranoside. This compound is reported here for the first time.

Table 6-1. ^{13}C NMR data of compounds 1-4 in CH_3OH.

Carbon	1	2	3	4
C-1	73.3	39.2	39.0	74.6
C-2	35.8	27.1	27.2	36.9
C-3	72.3	79.0	68.1	73.7
C-4	40.2	39.2	39.0	40.2
C-5	47.6	55.1	55.2	48.4
C-6	18.1	18.2	18.2	18.6
C-7	31.9	32.3	32.4	33.1
C-8	39.5	40.9	40.0	41.5
C-9	38.8	52.5	53.5	36.9
C-10	42.3	41.5	42.0	42.8
C-11	23.1	23.3	23.6	26.4
C-12	122.4	122.5	122.6	126.7
C-13	144.1	143.5	144.8	149.1
C-14	41.9	45.8	46.5	43.2
C-15	26.6	27.6	28.3	27.8
C-16	27.4	27.9	28.1	28.4
C-17	32.8	32.5	33.2	33.4
C-18	46.7	46.4	46.7	46.6
C-19	41.6	39.2	42.2	42.8
C-20	42.8	46.4	42.5	43.2
C-21	29.8	30.6	31.1	30.3
C-22	36.8	37.0	205.0	66.8
C-23	28.1	28.0	28.1	36.9
C-24	18.1	16.9	17.5	17.9
C-25	16.2	15.5	16.6	19.5
C-26	16.7	15.2	17.4	17.9
C-27	26.0	25.8	26.2	26.2
C-28	28.7	29.6	28.7	28.7
C-29	19.0	22.9	21.4	19.7
C-30	181.4	181.3	79.0	182.6

All measured in *d*- CH_3OH relative to TMS, - No carban at that position.

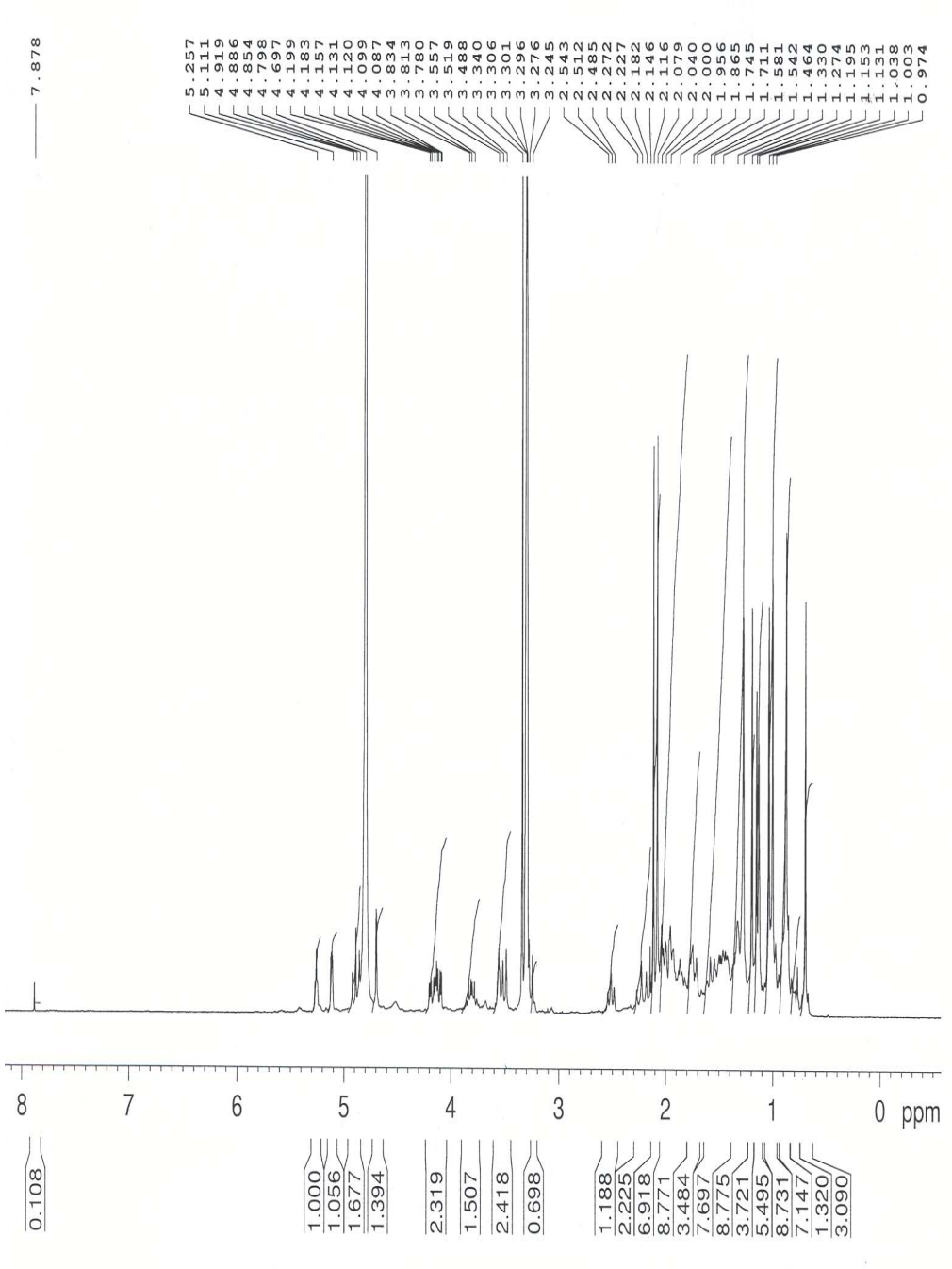


Figure 6-12: ^{1}H NMR spectrum of compound 5

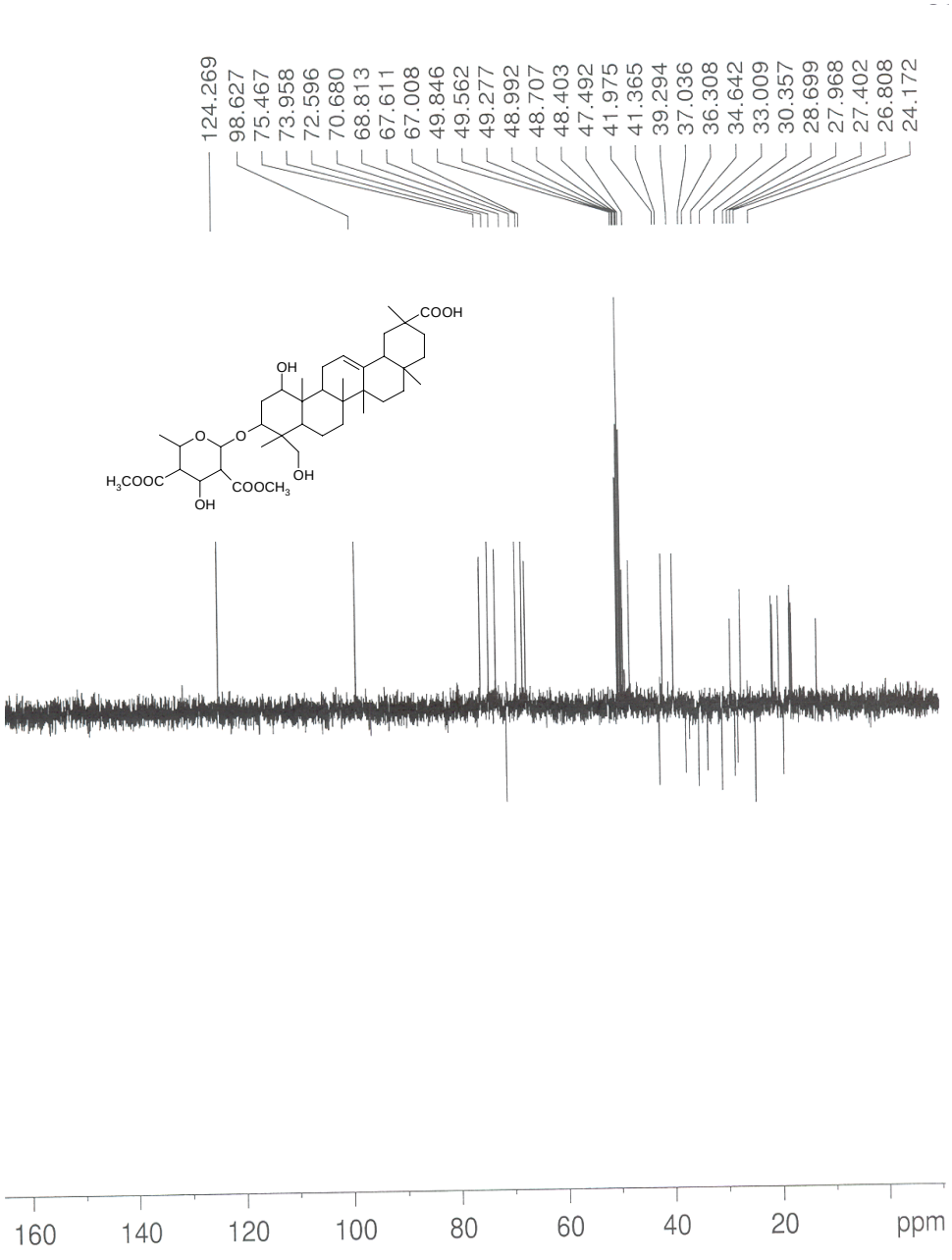


Figure 6-13: DEPT spectrum of Compound 5 indicating the presence of CH_3-, CH_2- and CH- carbons in the compound

Table 6-2: ^{13}C (75.4 MHz) and ^{1}H (300 MHz) NMR data of 5 in CD_3OD.

Position		^{13}C (δ_C)	^{1}H (δ_H, mult., *J* in Hz)
C-1	CH	72.6	3.55 *t* br
C-2	CH_2	34.6	1.74 *m*; 2.0, *m*
C-3	CH	67.0	4.17 *dd* (9.8, 4.5)
C-4	C	43.2	-
C-5	CH	41.3	1.75 *m*
C-6	CH_2	18.9	1.45 *m*, 1.54 *m*
C-7	CH_2	33.0	1.30 *m*, 1.60 *m*
C-8	C	40.7	-
C-9	CH	39.2	2.52 *dd* (9.3, 9.3)
C-10	C	41.8	-
C-11	CH_2	24.1	1.95 *m*
C-12	CH	124.3	5.25 *s*
C-13	C	145.3	-
C-14	C	43.5	-
C-15	CH_2	27.4	1.86 *m*
C-16	CH_2	27.9	2.05 *m*
C-17	C	33.5	-
C-18	CH	47.5	2.05 *m*
C-19	CH_2	41.9	1.30 *m*, 2.23 *m*
C-20	C	43.6	-
C-21	CH_2	30.4	1.34 *m*, 1.86 *m*
C-22	CH_2	37.0	1.32 *m*, 1.49 *m*
C-23	CH_3	12.6	0.70 *s*
C-24	CH_2	70.6	3.26 *d* (9.3), 3.50 *d* (9.3)
C-25	CH_3	17.3	1.00 *s*
C-26	CH_3	17.7	1.05 *s*
C-27	CH_3	26.8	1.27 s
C-28	CH_3	28.6	0.88 s
C-29	CH_3	19.7	1.20 *s*
C-30	C	182.8	-
C-1`	CH	98.6	4.60 *s* br
C-2`	CH	73.9	5.11 *m*
C-3`	CH	68.8	4.11 *dd* (3.5, 9.8)
C-4`	CH	75.4	4.90, *dd* (9.8, 9.8)

C-5`	CH	67.6	3.81 *m*
C-6`	CH_3	17.6	1.14 *d* (6.4)
C-1"	C=O	172.2	-
C-2"	CH_3	21.0	2.12 *s*
C-3"	C=O	172.2	-
C-4"	CH_3	20.8	2.08 *s*

All measured in *d*- CH_3OH relative to TMS, No carbon at that position (-)

Table 6-3. HMBC correlations for methyl groups in compound 5.

Position	δ_H	δc	^2J	^3J
23	0.7	12.6	43.2 (C-4)	67.0 (C-3), 41.3 (5), 70.6 (24)
25	1.0	17.3	41.8 (C-10),	72.6 (C-1), 41.3 (C-5), 39.2 (C-9)
26	1.05	17.7	40.7 (C-8)	39.2 (C-9), 33.0 (C-7), 43.5 (C18)
27	1.30	26.8	43.5 (C-14)	40.7 (C-8), 27.4 (C15), 145.3 (C-13)
28	0.90	28.6	33.5 (C-17)	47.5 (C-18), 27.9 (C-16), 37.0 (C-22)
29	1.20	19.7	43.6 (C-20)	182.8 (C-30), 30.4 (C-21), 41.9 (C-19)
Rh´-6	1.5	17.6	67.6 Rh´-5)	75.4 (Rh´-4)

6.5.1.6 Compounds 6

Compound **6** (150 mg) that was isolated as white crystals with a molecular ion peak at 694.8 $[M+NH_4]^+$ in the ESIMS. The corresponding molecular weight of 676 g/mol suggested a molecular formula of $C_{38}H_{60}O_{10}$ containing nine degrees of unsaturation. IR analysis indicated the presence of olefinic (1653 cm^{-1}), hydroxyl (3440 cm^{-1}), methyl (1456 cm^{-1}) and carboxylic acid (hydroxyl at 2923 cm^{-1} and 1772 cm^{1}) moieties. The triterpenoid skeleton was easily deduced from ^1H NMR data (**Fig. 6-13**) by the appearance of vinylic (R_2C=CHR, δ_H 5.24) and hydroxylated methine (RCHOH, δ_H 3.55) .s (**Table 6-5**). There was also a crowded signal indicating the presence of $–CH_3$, $-CH_2$, and –CH protons (δ_H 0.5-δ_H 2.0). The ^{13}C NMR spectrum (**Fig. 6-15**) confirmed these findings, additionally it showed the typical carboxyl at δ_c 183.1 of the $–CO_2H$ constituent. Altogether, eight methyl groups, ten methylenes, eleven methines and nine quaternary carbons were revealed by DEPT data. The ^1H **(Fig. 6-14**) and ^{13}C NMR data of compound **6** were similar to those of 7 except for differences in signals of rings A and E relating to different hydroxylation patterns. In 5, the methyl group at C-23 carries an ether linked substitution as indicated by the new methylene group at δ_C 70.3/δ_H 3.25 dd, 3.50 dd. In ring E, C-22 is a regular methylene and not oxgenated like in compound **7** (δ_c 31.1).

According to HMBC **(Fig. 6-16)** the carboxylic acid carbonyl is located at C-20 and one of the hydroxyl groups was located at C-1 (δ_c 72.6) as earlier stated by HMQC. Assisted by the ^{1}H-^{1}H COSY, the spin system of the complete rhamnose-skeleton (Rh') was traced. Using HMQC and ^{13}C spectra, the proton appearing as a singlet at δ_H 4.69 was assigned as anomeric proton of the sugar unit. The proton at δ_H 4.93 (Rh'-4) was assigned to be located next to an acetyl substituent of the sugar moiety. C-23 was unequivocally assigned as site of glycosylation by a HMBC correlation between H-Rh-1 (δ_H 4.69) and the oxymethylene C-23. This position has been shown as a preferred site of glycosylation in similar compounds from the related *C. imberbe* (Roger and Subramony, 1988). The relative configurations of the chiral centres at C-1, C-3, C-4, C-8, C-10, C-14, C-17, and C-20 were determined by NOESY analysis: correlations between H-1 (δ_H 3.55) and H-27 (δ_H 1.26), H-24 (δ_H 0.70) and H-25 (δ_H 1.00), H-18 (δ_H 2.01) and H-28 (δ_H 0.87), H-18 (δ_H 2.01) and H-30 (δ_H 1.19), H-Rh'-2 (δ_H 3.90) and H-Rh'-4 (δ_H 4.93), H-Rh'-1 (δ_H 3.55) and H-Rh'-4 (δ_H 4.93) indicated that these protons were oriented in the same direction. On the other hand, the NOESY correlations between H-23 (δ_H 3.50) and H-27 (δ_H 1.26), H-Rh'-6 (δ_H 1.13) and H-Rh'-3 (δ_H 4.14) indicated these to orient in the opposite direction. On the basis of the above analysis, compound **6** was thus elucidated as 1α,3β-dihydroxy-12-oleanen-29-oic-acid-23β-O-α-4-acetylrhamnopyranoside. This compound has not been reported previously. A similar compound has been isolated from *C. imberbe* by Katerere *et al*., 2003 with the acetyl rhamnosides attched at position 4 of the triterpene aglycone.

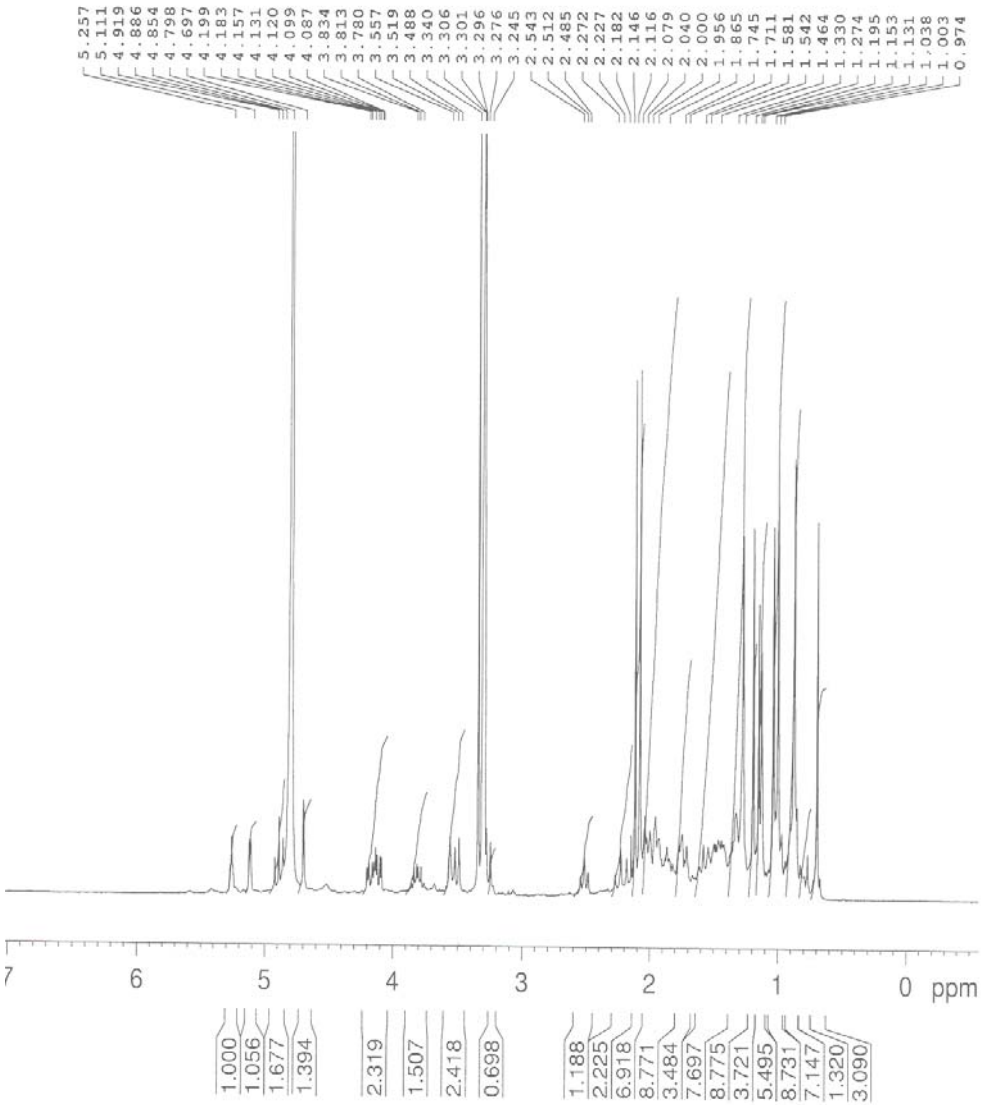


Figure 6-14: ^{1}H NMR spectrum of compound 6

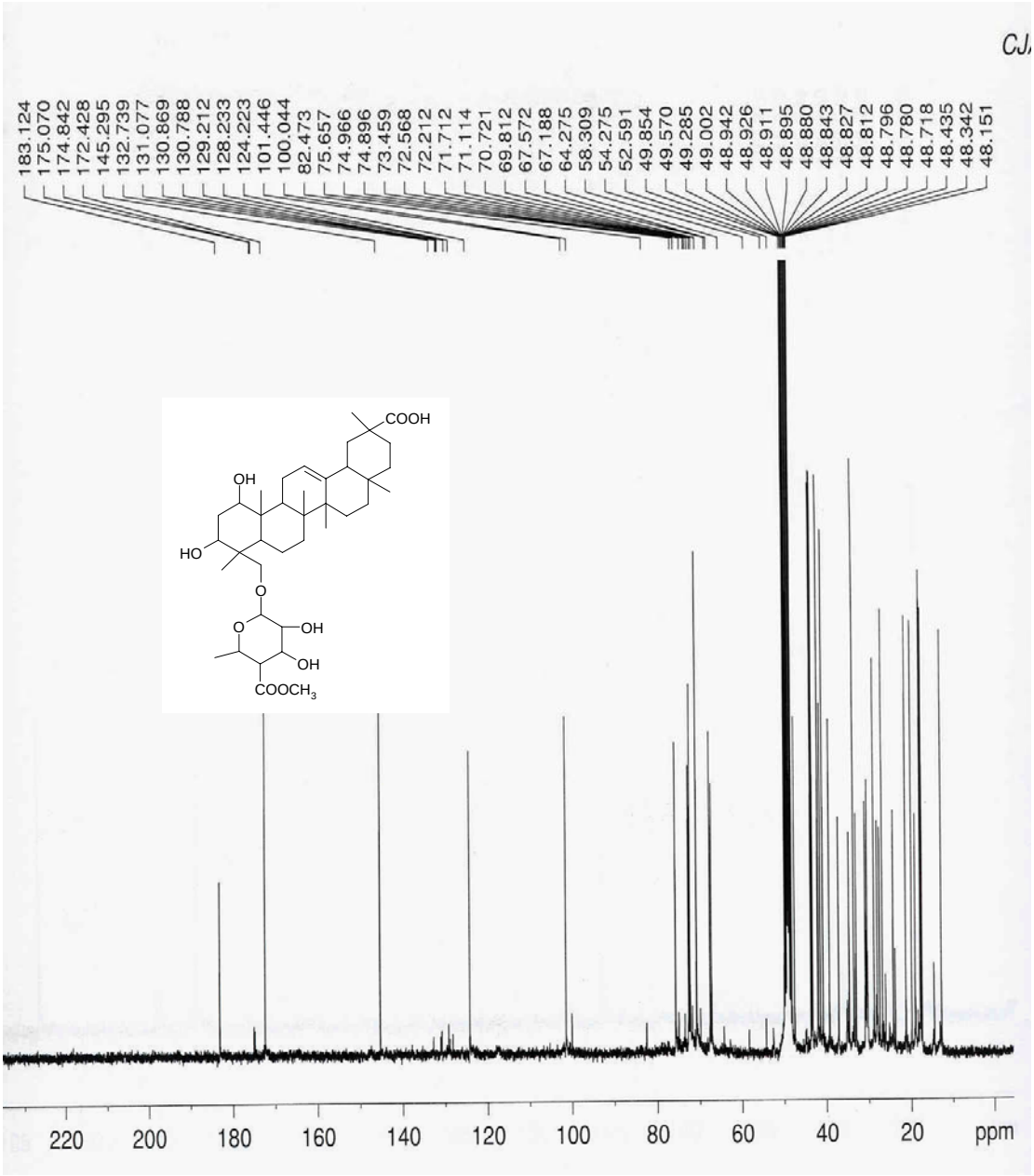


Figure 6-15: ^{13}C NMR spectrum of Compound 6

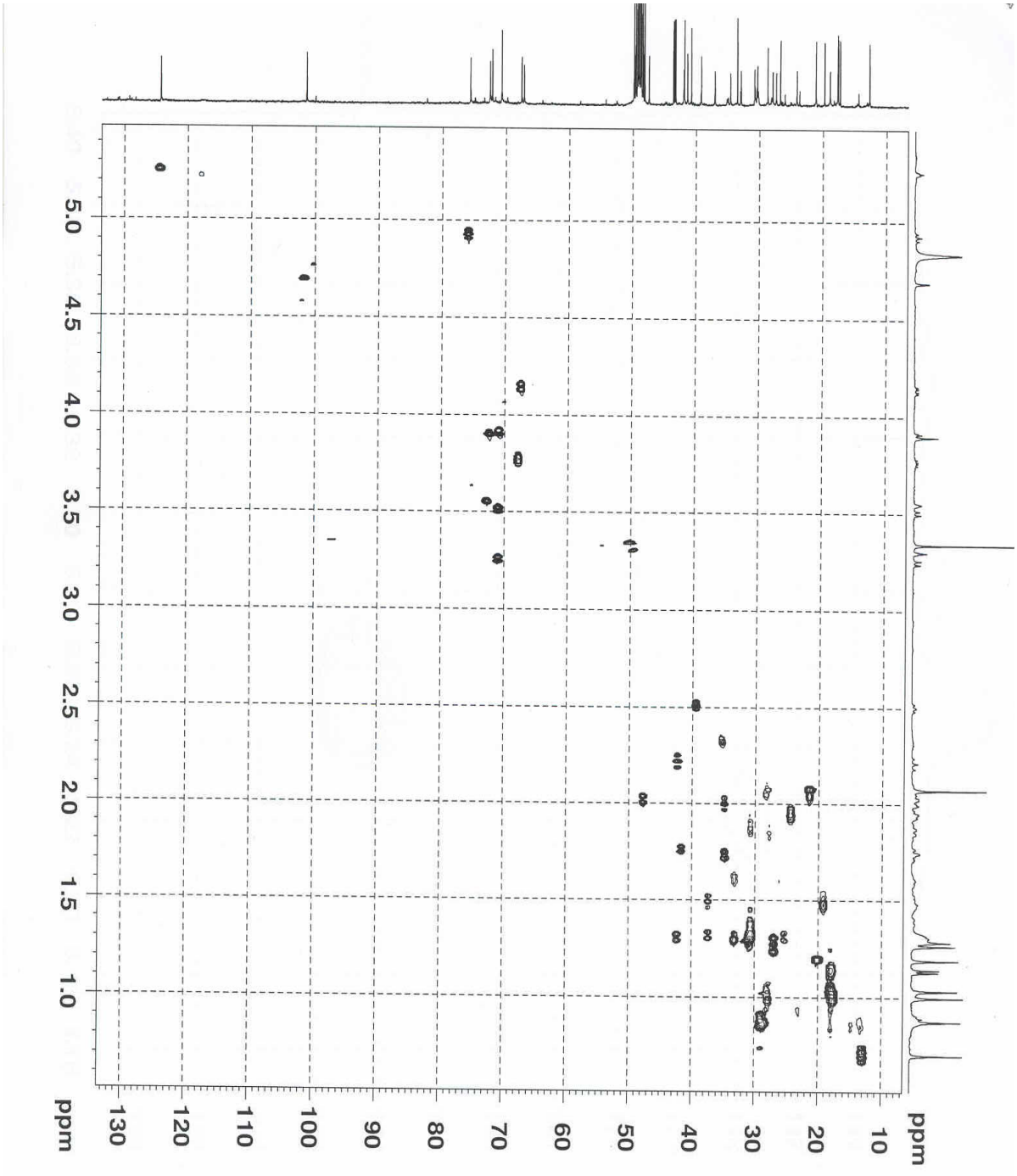


Figure 6-16: HMBC spectrum of Compound 6.

6.5.1.7 Compound 7

MS analysis of Compound **7** indicated a molecular ion peak ($M+Na^+$) of m/z 495.8 and suggested a molecular formula of $C_{30}H_{48}O_4$ according to ESI-MS, indicating seven degrees of unsaturation. This structural type was further supported by the ^1H NMR spectrum **(Fig. 6-17**), which contained resonance for seven skeletal methyl groups, ten methylene groups, five methine groups, eight quaternary carbons (from DEPT, **Fig. 6-18** and **Fig. 6-19**) and a broad triplet at δ_H 5.25 for a olefenic proton (H-12), a carboxylic acid functionality (δ_c 182.90) and two-hydroxyl groups (δ_H 3.6, $\delta_{c,}$ 74.01 and δ_H 3.75, δc 72.97). The final attribution of the ^1H, ^{13}C (**Table 6-4**), DEPT, and EI-MS of Compound **7** were in complete agreement with literature data of 1α, 22β dihydroxy-12-oleanen-oic that has previously been isolated (Nakano K, 1997). Compound **7** was therefore elucidated as 1α, 22β-dihydroxyl-12-oleanen-30-oic acid.

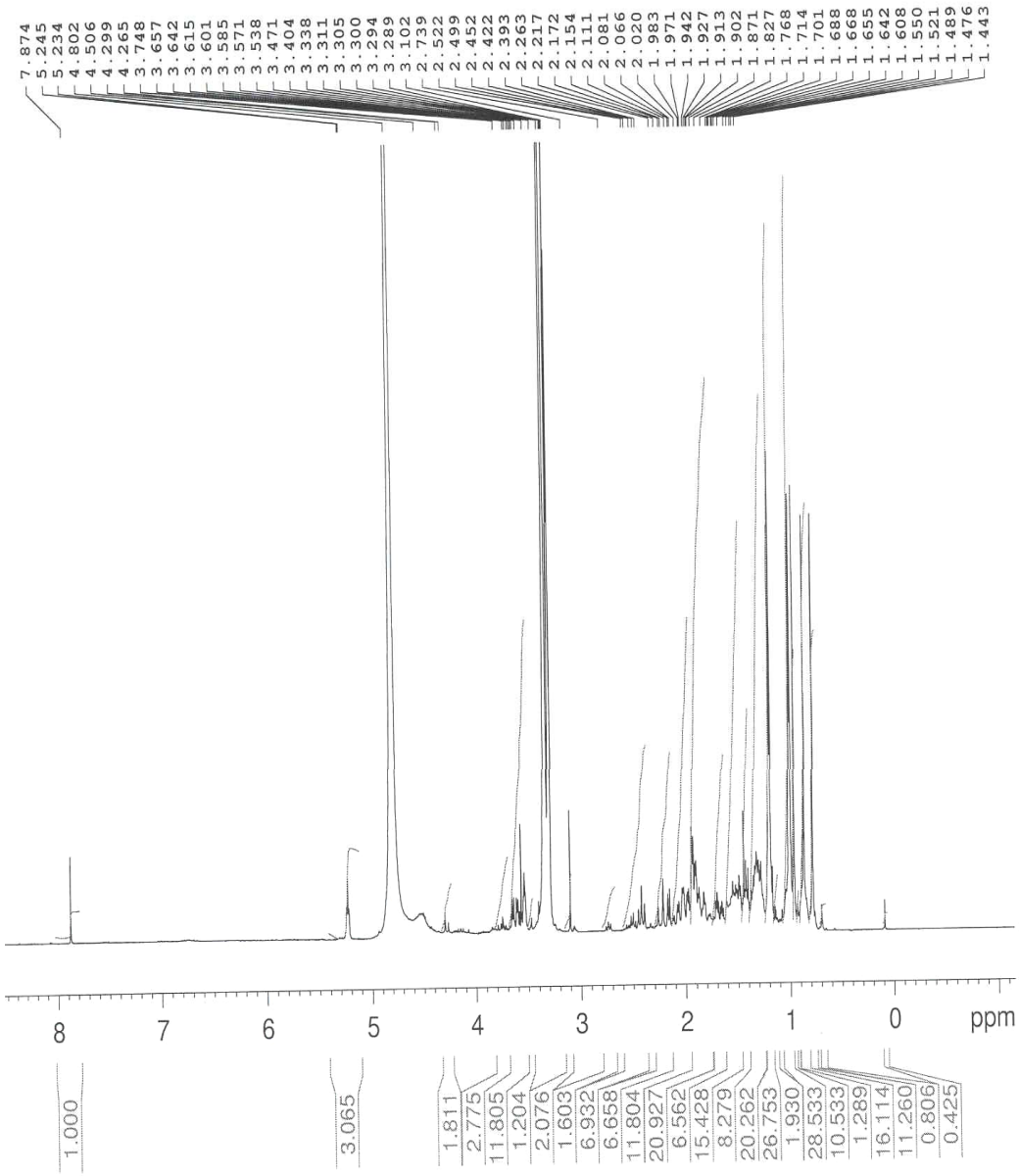


Figure 6-17: ^{1}H NMR of compound 7

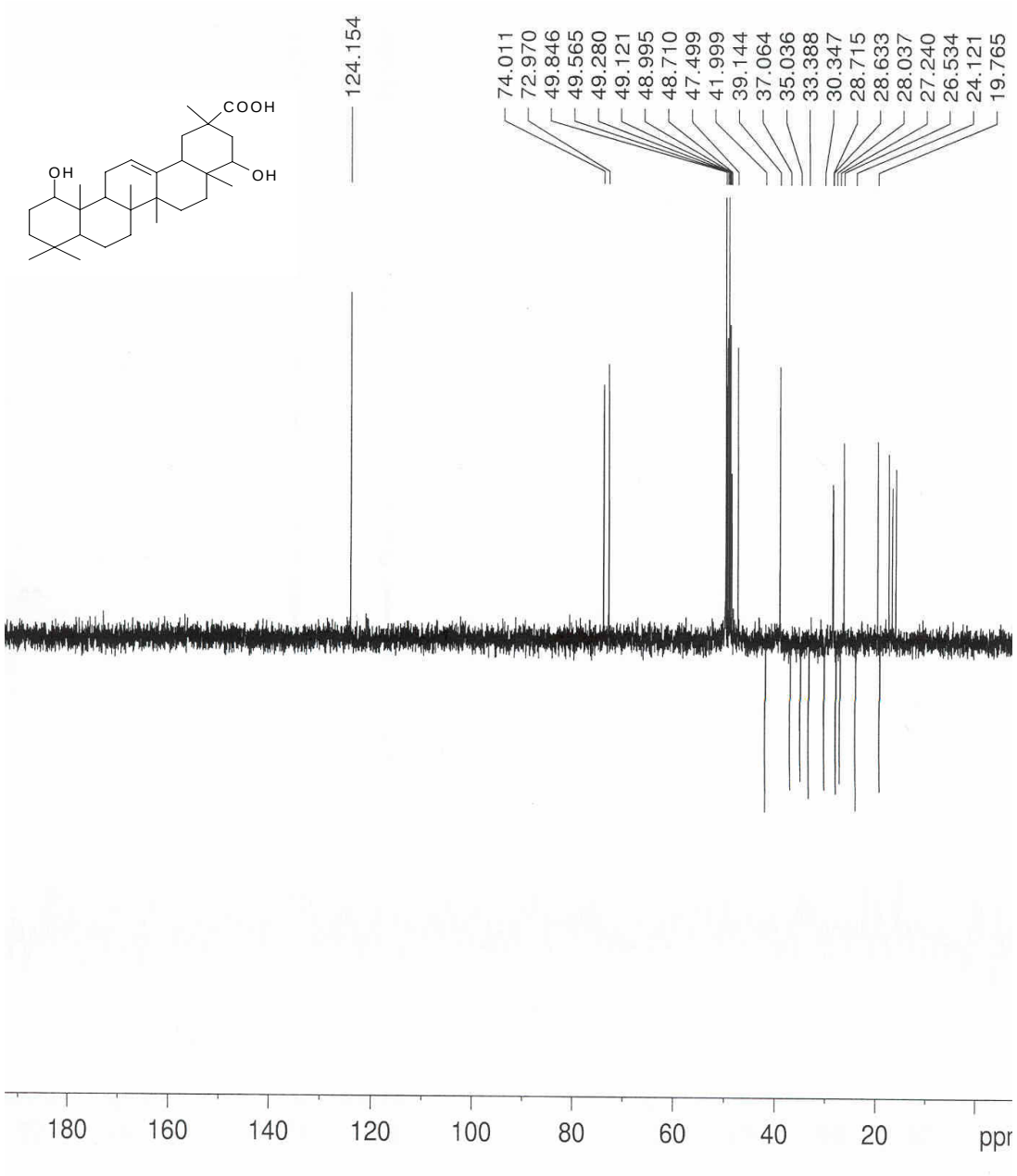


Figure 6-18: DEPT spectrum of Compound 7

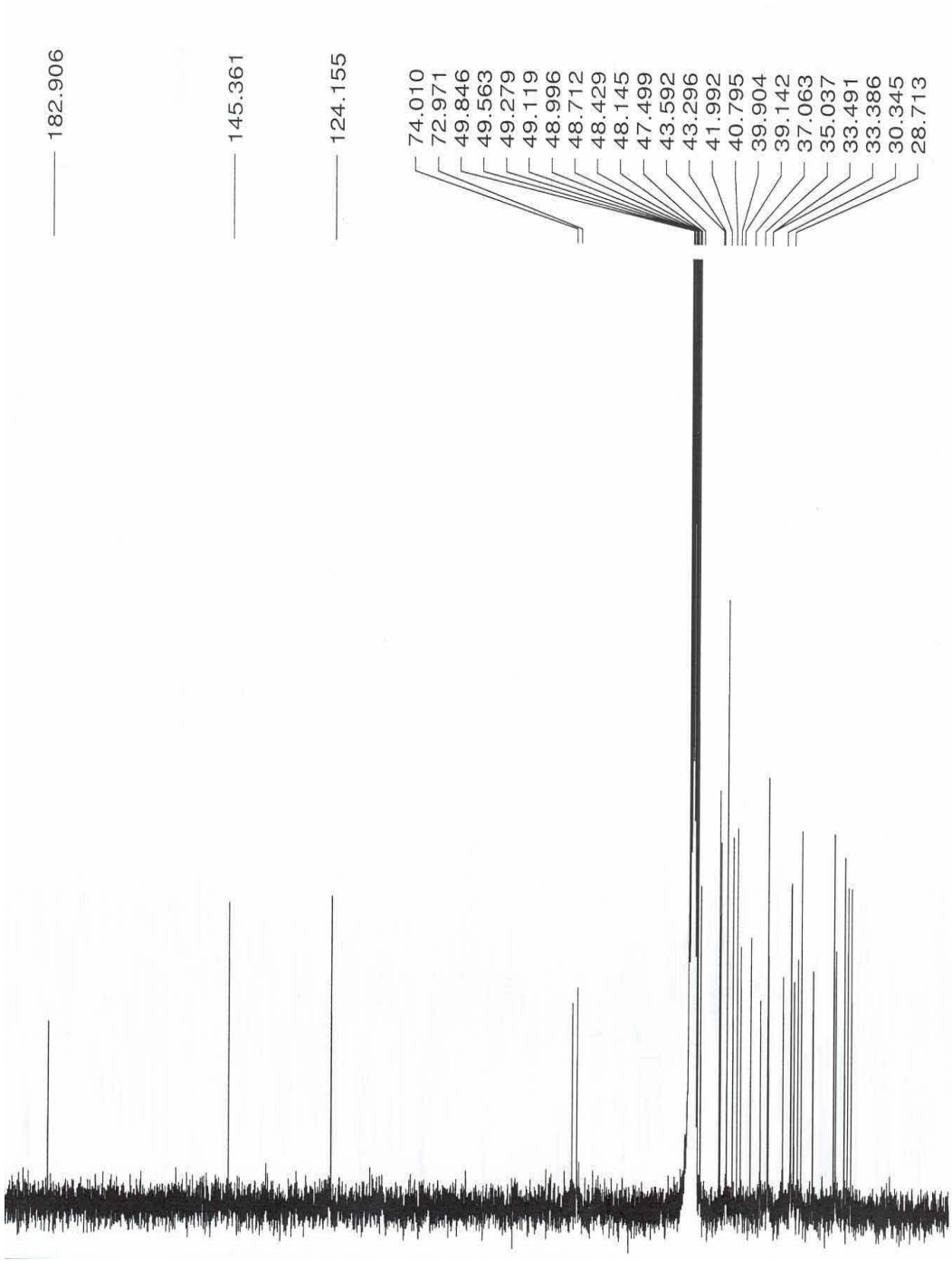


Figure 6-19: ^{13}C NMR of Compound 7

6.5.1.8 Compound 8

Compound **8** was isolated as a cream powder with a molecular ion peak [M+NH_4^+] at 591.0 based on ESI-MS suggesting a molecular formula of $C_{35}H_{36}O_6$ and indicating eight degrees of unsaturation. Its ^{1}H NMR spectrum (**Fig. 6-20**) revealed the presence of four hydroxyl groups at δ_H 4.15, 4 δ_H.45, δ_H 4.65, and δ_H 4.85, olefenic protons at δ_H 5.2, and δ_H 5.3; and an ester linkage proton at 4.22. Five methyls groups, eleven methylenes groups, sixteen methine groups, and three quaternary carbons were revealed by DEPT (**Fig. 6-21**) The ^{13} C NMR indicated the presence of an ester linkage carbon at δ_c 100.8; four hydroxyl groups at δ_c 61.22, δ_c 70.24, δ_c73.57 and δ_c 76.78; five signals at δ_c 110.86, δ_c121.29, δ_c129.66, δ_c 136.90 indicating olefenic carbons. The structure of Compound **8** was established as 24-ethylcholesta-7, 22,25-trien-3-ol-O-β-D-glucopyranoside based on above data was in perfect agreement with literature of the same compound that has been isolated previously from *Clerodendron inerme* (Atta.ur-Rahman *et al*., 1997).

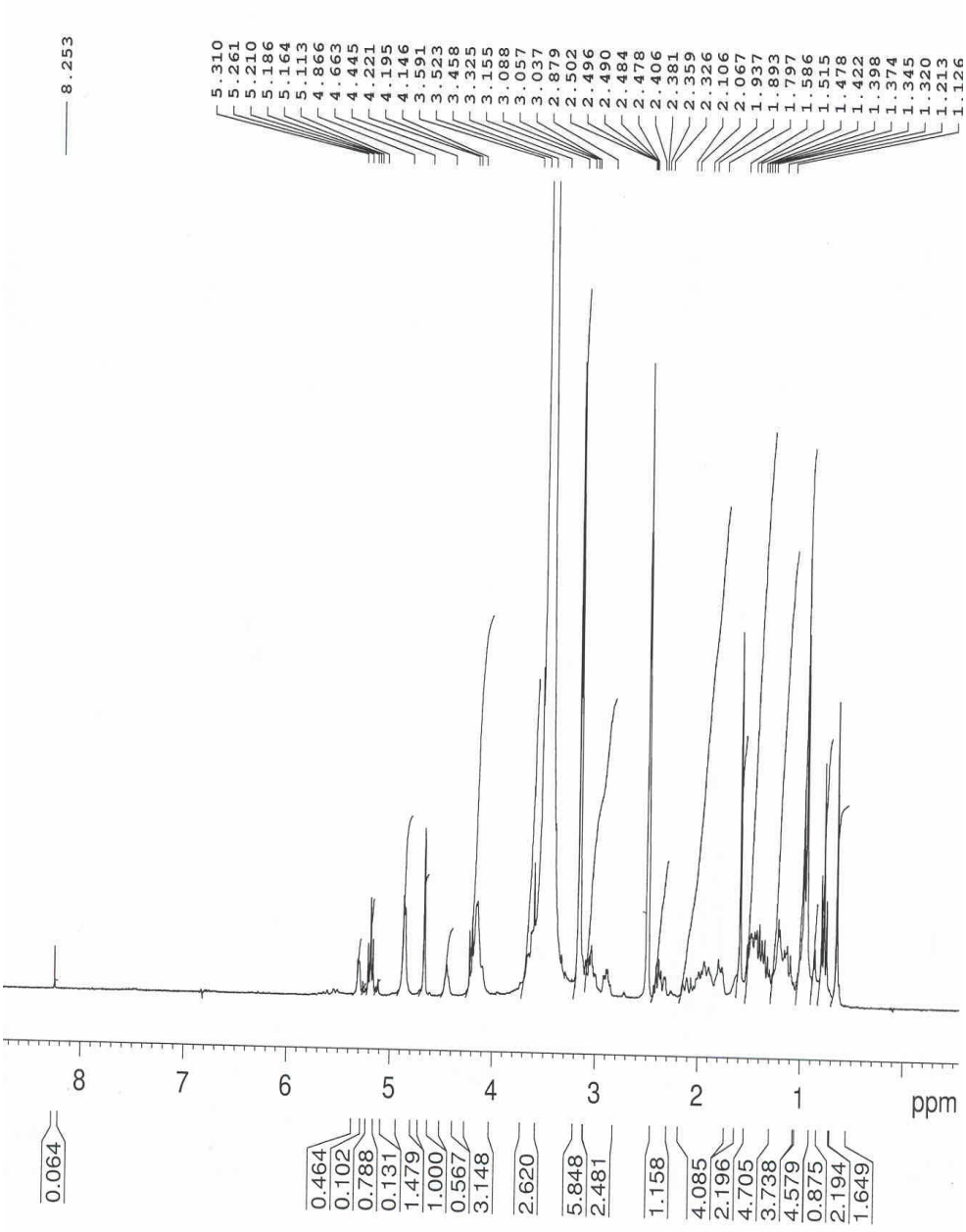


Figure 6-20: ^{1}H-NMR of Compound 8

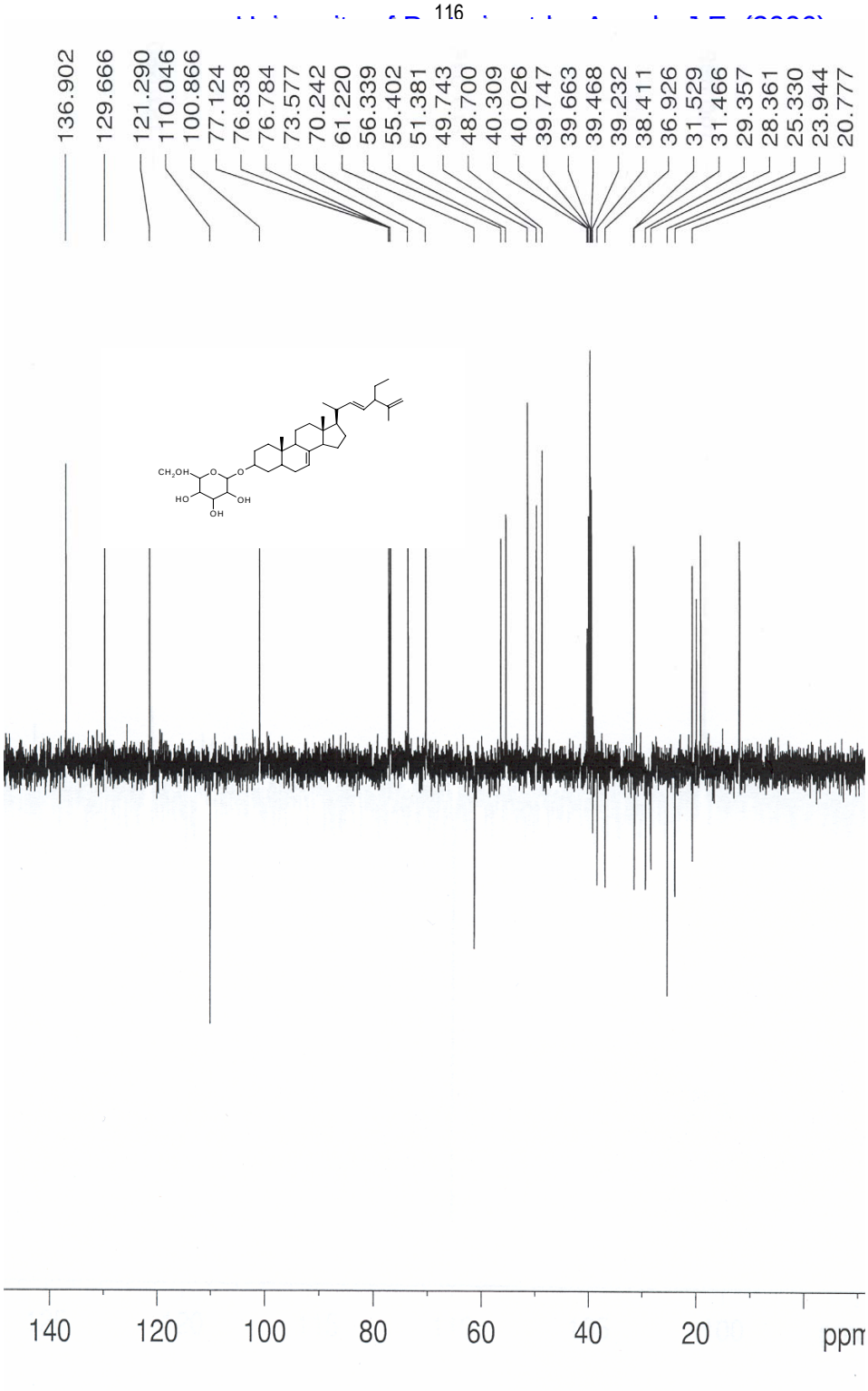


Figure 6-21: DEPT spectrum of Compound 8.

Table 6-4: ^{13}C (75.4 MHz) and ^{1}H (300 MHz) NMR data of 7 and 8 in CD_3OD.

Carbon numbers	7	8
1	72.9	36,9
2	33.4	31.4
3	35	77.0
4	43.2	38.4
5	45.2	140.5
6	19.3	121.2
7	33.3	29.3
8	40.7	31.5
9	39.9	49.7
10	43.5	36.3
11	24.1	21.1
12	124.1	38.4
13	145.3	41.8
14	43.2	56.3
15	30.3	23.9
16	27.2	28.4
17	28	55.3
18	39.1	11.9
19	41.9	19.1
20	43.6	40.4
21	30.3	19.9
22	74	136.8
23	16.1	129.6
24	16.8	51.3
25	19.7	147.7
26	17.5	110.0
27	26.5	20.7
28	28	25.3
29	19.3	12.0
30	182.9	-
Rh'-1		100.0
Rh'-2		76.7
Rh'-3		76.7
Rh'-4		70.1
Rh'-5		76.8
Rh'-6		61.1

Table 6-5: ^{1}H (300 MHz) and ^{13}C (75.4 MHz) NMR data of 6 (CD_3OD)

Position	Carbon type	^{13}C (δ_C)	^{1}H (δ_H)[a]
1	CH	72.6	3.55 brs
2	CH_2	34.6	1.74 m, 1.93 m
3	CH	67.2	4.14 dd 13.2, 4.5
4	C	43.3	
5	CH	41.4	1.76 m
6	CH_2	18.9	1.45 m
7	CH_2	33.0	1.59 m; 1.28 m
8	C	40.8	
9	CH	39.3	2.50 dd 8.2, 8.2
10	C	42.0	
11	CH_2	24.1	1.93-1.98 m
12	CH	124.3	5.24 s
13	C	145.3	
14	C	43.5	
15	CH_2	27.4	1.86 dd 4,2, 8.0
16	CH_2	28.0	2.07 m
17	C	33.6	
18	CH	47.5	2.01 brd
19	CH_2	41.9	1.30 m; 2.21 dd 13.4, 13.4
20	C	43.6	
21	CH_2	30.4	1.87 m
22	CH_2	37.1	1.47 brt, 1.30 dd
23	CH_2	70.7	3.50 d, 9.6; 3.25 d, 9.6
24	CH_3	12.8	0.70 s
25	CH_3	17.4	1.00 s
26	CH_3	17.8	1.03 s
27	CH_3	26.8	1.26 s
28	CH_3	28.8	0.87 s
29	C	183.1	
30	CH_3	19.8	1.19 s
Rh'-1[b]	CH	101.5	4.69 brs
Rh'-2	CH	72.2	3.90 brs
Rh'-3	CH	70.7	3.91 dd cov

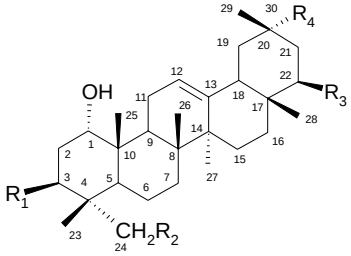
Rh'-4	CH	75.7	4.93 brt, 9.6
Rh'-5	CH	67.6	3.76 m
Rh'-6	CH_3	17.7	1.13 d, 10.0
Ac-1[c]	C	172.5	
Ac-2	CH_3	21.2	2.07 s

[a] multiplicity, *J* im Hz; [b] Rhamnopyranoside (Rh'); [c] acetyl (Ac)

It is important to mention that in related studies, triterpends have been isolated in closely related genera Terminalia. In some of these studies, a new A-*seco*-triterpene was isolated from the stem bark of *Terminalia glaucescens* Planchon and other known triterpenes, friedelin, β-sitosterol, stigmasterol, lupeol, betulinic acid, β-amyrin and long chain fatty acids were also isolated (Atta-ur-Rahman *et al*., 2002). Also, a new triterpene, 2α-hydroxymicromeric acid, and two known compounds, maslinic acid and 2α-hydroxyursolic acid have been isolated from *Terminalia chebula leaves (*Chandan Singh, 1990). This may show an indication ofa chemotaxonomic correlationship between generals in Combretaceae.

6.6 Summary

A total of eight structures **(Fig. 6-22 and Fig. 6-23**) were elucidated with the aid of the NMR (1H, 13C, HMBC, COSY, and HMQC), MS and IR data that were available. All compounds had an olean-12-ene skeleton, except Compound **8**.

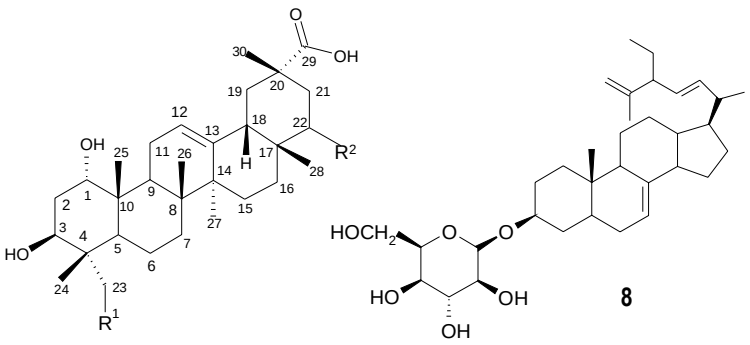


Compounds	R_1	R_2	R_3	R_4
1	OH	H	H	COOH
2	H	H	H	COOH
3	H	H	=O	CH_2OH
4	H	H	OH	COOH
5	2,4-Di-Ac-O-Rh	OH	H	H

Figure 6-22: Compounds 1-5 isolated from *C. imberbe.*

Compounds **1-5** were isolated from *C. imberbe*. Compound **1** was a known compound and elucidated as 1α, 3β-dihydroxy-12-oleanen-29-oic. Compound **2** very similar to compound **1** was elucidated as 3-hydroxyl-12-oleanene-29-oic. Compound **3**, isolated as a white powder had molecular formula similar to Compound **2** but appeared to have a different structure. It was elucidated as 3, 30-dihydroxyl-12-oleanen-22-one, see page 100. Compound **4** was isolated as white crystals and its structure elucidated as 1, 3, 24-trihydroxyl-12-olean-29-oic acid, see page 103.

Compound **5** and **6** were new compounds with optical activities of α_D^{26}: 36.6.Cc μg/ml CH_3OH and α_D^{26}: 31.39°.Cc ug/ml. CH_3OH respectively. ^1H NMR, ^{13}NMR, ^1H COSY, HMBC, HMQC and NEOSY data were used to elucidate their structures as 1,3, 23-trihydroxy-12-oleanen-29-oic acid-3β-O-α-L-2, 4 diacetylrhamnopyranoside and 1, 5-dihydroxyl-12-oleanen-29-oic acid 3β-O-α-L-4-acetylrhamnopyranoside respectively. Compounds **6-8 (Fig. 6-23)** were isolated from *C. padoides*. Compound **7** was found to be similar to Compound **6** but differ in the positions of their hydroxyl group. It was therefore elucidated as a 1, 22-dihydroxyl-12-oleanen-30-oic acid, see page 110.



Compounds	R^1	R^2
6	4-Ac-O-Rh	H
7	H	OH

Figure 6-23: Compounds 6-8 isolated from *C. padoides.*

Compound **8** was a steroid glycoside and was much different in structure the other compounds isolated. Its structure was established as 24-ethylcholesta-7, 22, 25-trien-3-ol-O-β-D-glucopyranoside on the bases of ^1H NMR, ^{13}C NMR, DEPT ESI-MS and literature comparison (Atta.Ur-Rahman *et al.,* 1997).