CHAPTER 4

RESULTS AND DISCUSSION

4.1 Biological activities screening of dichloromethane extract of *V. parishii* leaves

The results of bioactivity tests of dichloromethane extract of plant from BIOTEC, NSTDA, were shown in Table 1.

Table 1 Bioactivities screening of dichloromethane extract of *V. parishii*

Testing type	IC_{50} or MIC (μg/ml)
K1 Strain*	N/A
H37Ra strain**	N/A
Cytotoxicity**	N/A

N/A=Inactive

*K1 Strain = Anti-malaria (*Plasmodium falciparum*)

% inhibition	Anti-malarial activity
< 50%	Inactive

** H37Ra strain = Anti-*Mycobacterium tuberculosis* (Anti-TB)

% inhibition	Anti-TB activity
< 90%	Inactive
≥ 90%	Active (MIC included)

*** Cytotoxicity = Cytotoxicity against Vero cells (African green monkey kidney)

% inhibition	Activity
> 50%	Non-cytotoxic
≤ 50%	Cytotoxic (IC_{50} included)

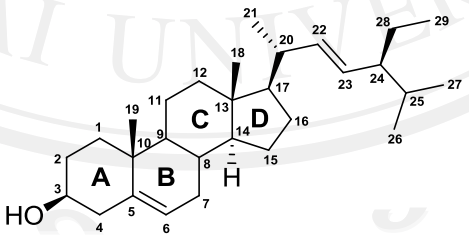
The bioactivity evidences of dichloromethane extract of *V. parishii* leaves were inactive both anti-plasmodial and cytotoxicity activities. However, this extract was studied chemical components to prove the chemical taxonomy of this plant.

4.2 Structure elucidation of isolated compounds

From the investigation of phytochemical constituents from the dichloromethane extract of *V. parishii* Hook f. leaves, air dried leaves of this plant were extracted with

dichloromethane (3 days x 3 times). The extract was isolated chemical constituents by chromatography and recrystallization to give nine compounds, consisting of stigmasterol (**58**), *α*-amyrin acetate (**9**), lupeol acetate (**59**), lupeol palmitate (**60**), lupenone (**61**), lupeol (**41**) *α*-amyrin (**13**), hentriacontane (**62**), and palmitic acid (**63**), were found in this extract. The structures of all compounds were characterized on the basis of spectrum and physical evidence. These were referred to the data from spectroscopic techniques such as ^{1}H NMR and ^{13}C NMR, COSY, DEPT, HMQC, HMBC, mass spectrometry, and IR spectroscopy.

Stigmasterol (**58**); (3*β*,22*E*)-stigmasta-5,22-dien-3-ol for a systematic name, a common phytochemical compound had been isolated from fraction VPD-F07 and VPD-F08 of the dichloromethane extract of *V. parishii* leaves. This compound was a colorless crystalline solid and had melting points between 167.3 – 168.5 °C compared with mp 163.0–164.0 °C from the previous study.[36] Moreover, the IR spectrum indicated two significant signals that contained stretching of hydroxyl at 3457 and stretching of carbon of double bond at 1638 cm^{-1}. The ^{1}H NMR spectrum of this compound showed some obvious peaks. For instance, H-3 proton of hydroxyl group had a signal at 3.51 ppm which was a multiplet, H-6 proton of the methine group of endocyclic double bond in ring B displayed peak at 5.34 ppm which was a multiplet signal, and H-22 and H-23 protons of double bond outside ring D showed two doublet of doublets peaks at 5.14 (*J* = 15.2, 8.5 Hz) and 5.01 (*J* = 15.2, 8.6) ppm, respectively.



stigmasterol (**58**)

Figure 29 Labeling number of each carbon in structure of stigmasterol (**58**)

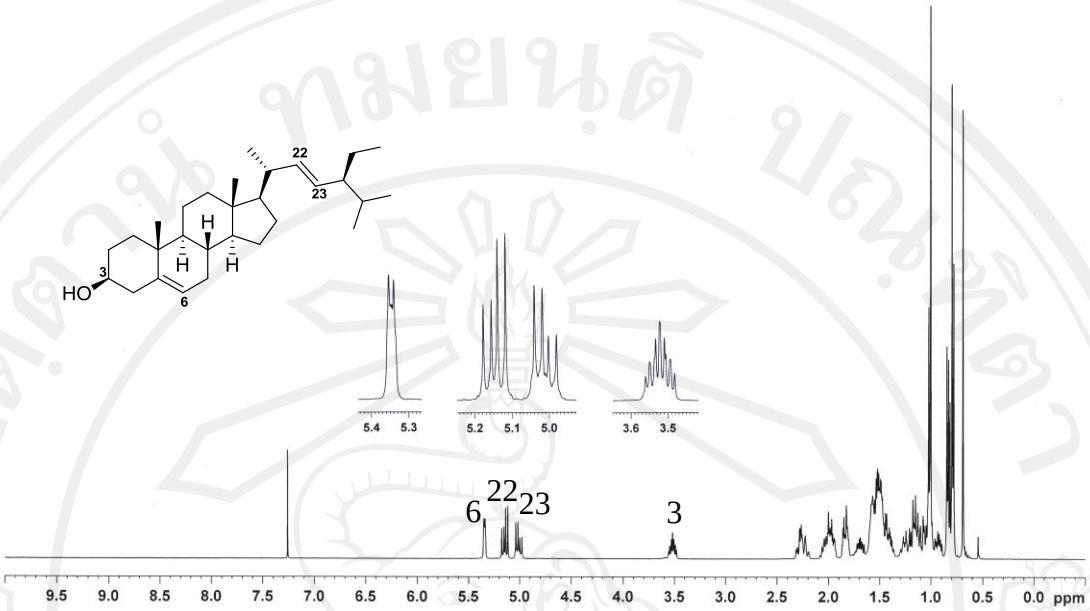


Figure 30 ^{1}H NMR spectrum of stigmasterol (**58**)

The ^{13}C NMR spectrum of this compound displayed several characteristic signals, C-3 carbon that had hydroxyl group had a signal at 71.8 ppm, C-5 and C-6 carbons of endocyclic double bond showed peaks at 140.7 and 121.7 ppm, and C-23 and C-24 carbons of double bond outside ring D displayed signals at 138.3 and 129.3 ppm, respectively. All chemical shifts of ^{1}H and ^{13}C NMR of stigmasterol, which were confirmed data with previous reports [37], as shown in Table 2.

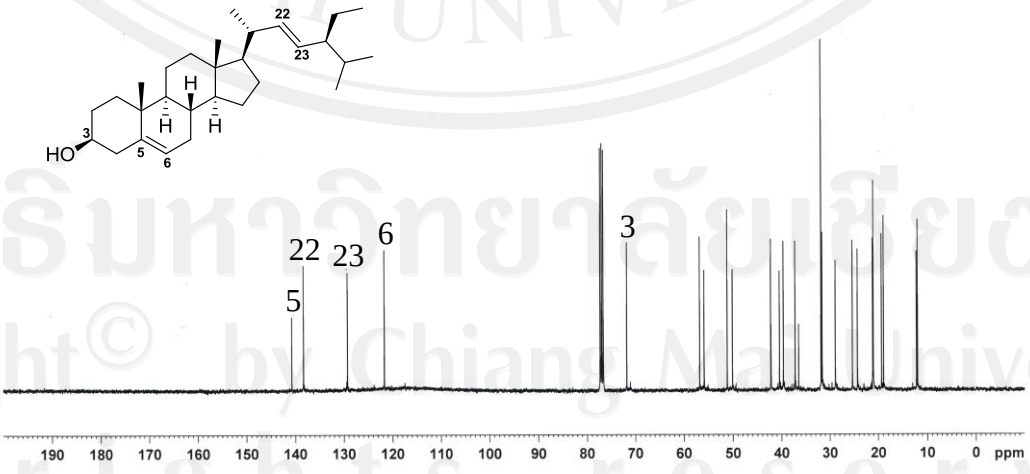


Figure 31 ^{13}C NMR spectrum of stigmasterol (**58**)

Table 2 ^{1}H NMR and ^{13}C NMR spectrum of stigmasterol (**58**) ($CDCl_3$) at 400 MHz (for ^{1}H NMR) and 100 MHz (for ^{13}C NMR) (*J* in Hz)

Carbon position	Chemical shift (δ; ppm)	
	^{1}H NMR data	^{13}C NMR data
1	1.85 [*m*, 1H]	31.3
	1.08 [*m*, 1H]	
2	1.83 [*m*, 1H]	31.6
	1.51 [*m*, 1H]	
3	3.51 [*m*, 1H]	71.8
4	2.30 [*m*, 1H]	42.3
	2.23 [*m*, 1H]	
5	-	140.7
6	5.34 [*m*, 1H]	121.7
7	1.97 [*m*, 1H]	31.6
	1.50 [*m*, 1H]	
8	1.46 [*m*, 1H]	31.9
9	0.94 [*m*, 1H]	50.2
10	-	36.5
11	1.46 [*m*, 2H]	21.1
12	2.00 [*m*, 1H]	39.7
	1.18 [*m*, 1H]	
13	-	42.2
14	1.01 [*m*, 1H]	56.9
15	1.56 [*m*, 1H]	24.4
16	1.72 [*m*, 1H]	28.9
	1.27 [*m*, 1H]	
17	1.15 [*m*, 1H]	55.9
18	0.69 [*s*, 3H]	12.0
19	1.10 [*s*, 3H]	19.4
20	2.06 [*m*, 1H]	40.2

Table 2 ^{1}H NMR and ^{13}C NMR spectrum of stigmasterol (**58**) ($CDCl_3$) at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR) (*J* in Hz) (cont.)

Carbon position	Chemical shift (δ; ppm)	
	^{1}H NMR data	^{13}C NMR data
21	1.02 [*m*, 3H]	21.2
22	5.14 [*dd*, 1H];	138.3
	J = 15.2, 8.5 Hz	
23	5.01 [*dd*, 1H];	129.3
	J = 15.2, 8.6 Hz	
24	1.53 [*m*, 1H]	51.2
25	1.55 [*m*, 1H]	31.9
26	0.84 [*s*, 3H]	21.2
27	0.79 [*s*, 3H]	19.0
28	1.43 [*m*, 1H]	25.4
	1.18 [*m*, 1H]	
29	0.81 [*s*, 3H]	12.2

Moreover, this compound was measured the exact mass from EI-MS technique that received the molecular ion peak at *m/z* 412 [M^+]. The other key fragment ions that were compared with a few references [38, 39] showed signals at *m/z* 394, 231, and 213, respectively. Explaining mechanism of fragmentation of them is shown in Figure 32.

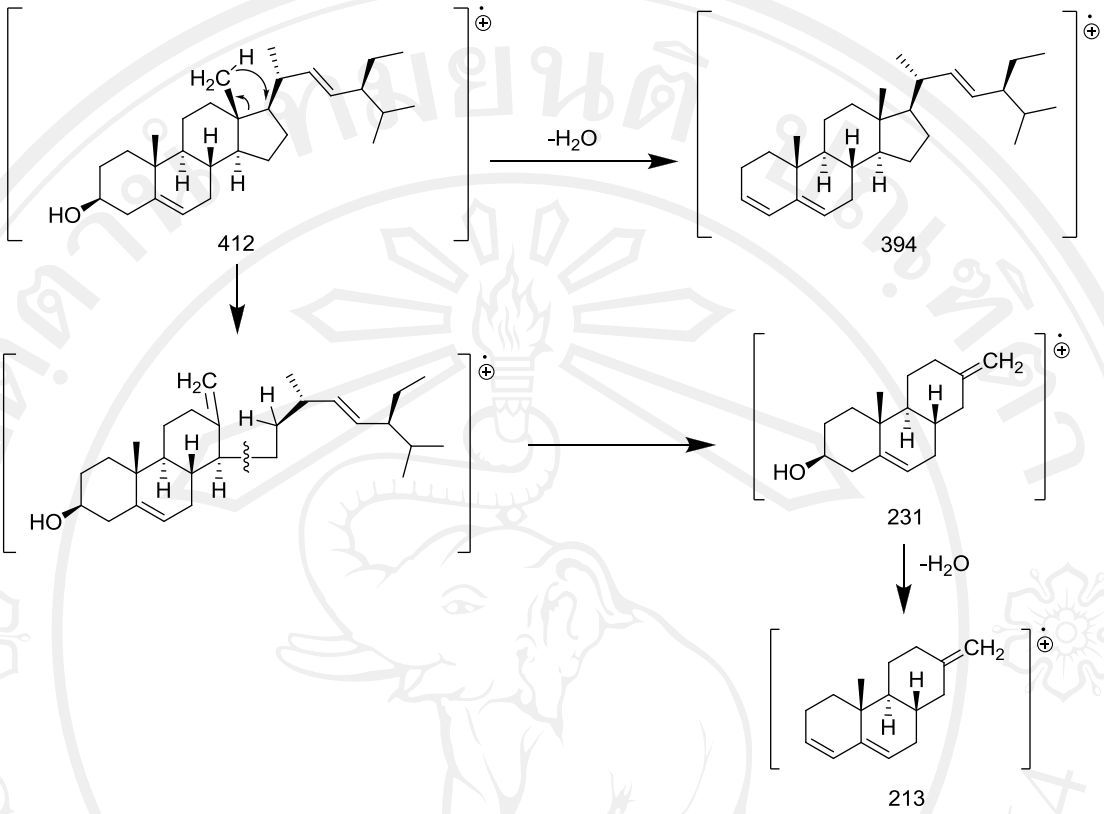
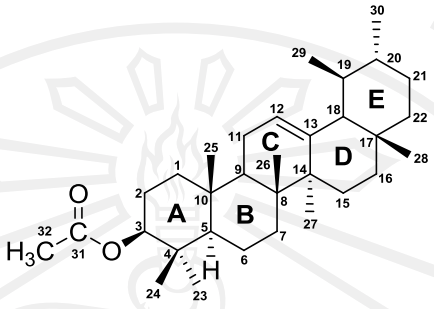


Figure 32 Mechanism of key fragment ions of stigmasterol (**58**) from EI-MS

α-Amyrin acetate (**9**); (3*β*)-urs-12-en-3-yl acetate for a systematic name, a triterpene, which was a white solid, was found in fraction VPD-03 of the dichloromethane extract of *V. parishii* leaves. This compound was measured its melting point that was obtained and recorded the output between 219.0 – 222.0 °C that conform to the previous study which was showed the melting point between 220.0 – 222.0 °C.[3] Besides, the IR spectrum of compound showed three vibrational signals, consisting of the stretching of carbonyl at 1735, the stretching of carbon of double bond at 1592, and the stretching of ether bond in acetate group at 1245 cm^{-1}. From measuring optical rotation of compound, the value was observed and recorded as $[\alpha]^{26.6}_{D}$ +85.3°, whereas the reference was reported as $[\alpha]^{25}_{D}$ +76.1°.[40] The ^{1}H NMR spectrum of this compound indicated several characteristic signals, for instance, the H-3 that bonded with carbon 3 showed multiplet signal at 4.50 ppm, H-12 which was a proton of double bond in ring C also displayed a triplet peak with the coupling

of constant (*J*) 3.6 Hz, at 5.12 ppm. In additon, three protons of methyl group of acetate group at position 32 showed a sharp singlet peak at 2.04 ppm.



α-amyrin acetate (**9**)

Figure 33 Labeling number of each carbon in structure of α-amyrin acetate (**9**)

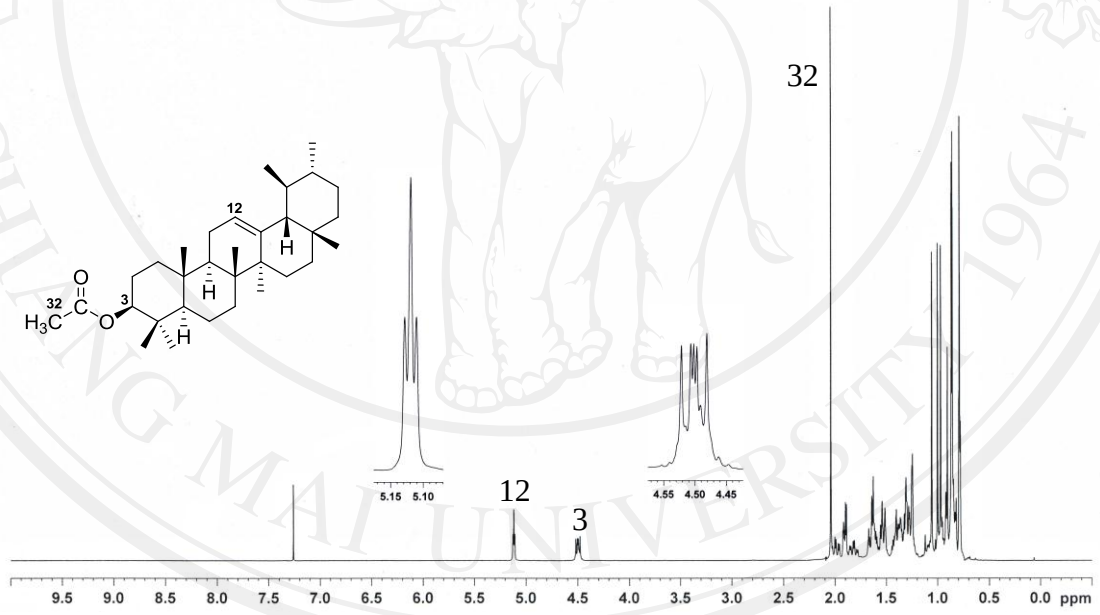


Figure 34 ^{1}H NMR spectrum of α-amyrin acetate (**9**)

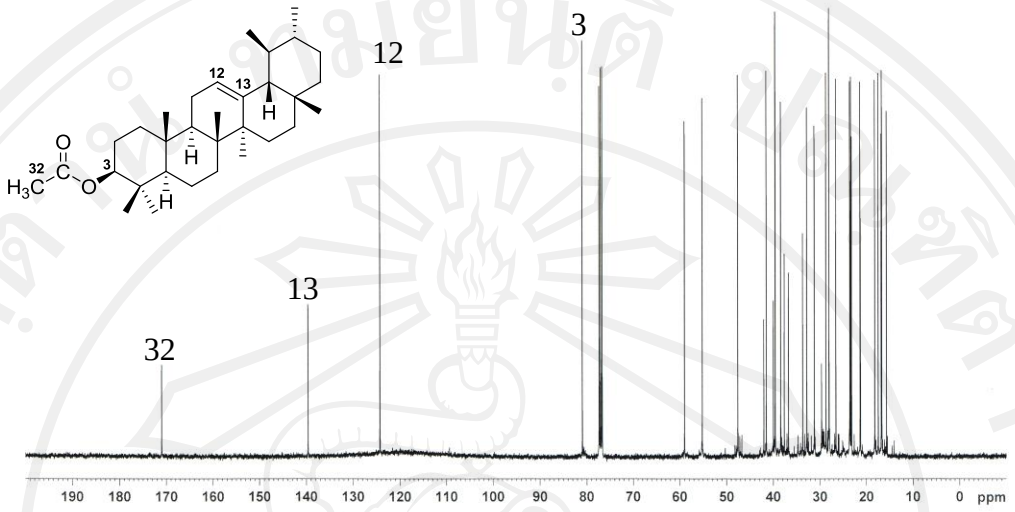


Figure 35 ^{13}C NMR spectrum of α-amyrin acetate (**9**)

Similarly, ^{13}C NMR spectrum of compound also displayed some significant peaks such as C-3 carbon which adjoined with acetate group at 80.9 ppm, C-12 and C-13 of double bond in ring C at 124.3 and 139.6 ppm, respectively, and C-32 of methyl group of acetate group at 171.0 ppm. All results of ^{1}H NMR and ^{13}C NMR of α-amyrin acetate (**9**) from the experiment, which were confirmed with the references [40, 41], were shown in Table 3 and 4.

Table 3 ^{1}H NMR spectrum of α-amyrin acetate (**9**) ($CDCl_3$) at 400 MHz

Carbon position	Chemical shift (δ; ppm)
3	4.50 [*m*, 1H]
12	5.12 [*t*, 1H];
	J = 3.6 Hz
23	0.86 [*s*, 3H]
24	0.87 [*s*, 3H]
25	0.97 [*s*, 3H]
26	1.00 [*s*, 3H]
27	1.06 [*s*, 3H]
28	0.79 [*s*, 3H]

Table 3 ^{1}H NMR spectrum of α-amyrin acetate (**9**) ($CDCl_3$) at 400 MHz (cont.)

Carbon position	Chemical shift (δ; ppm)
29	0.79 [*s*, 3H]
30	0.91 [*d*, 3H]; J = 4.5 Hz
32	2.04 [*s*, 3H]

Table 4 ^{13}C NMR spectrum of α-amyrin acetate (**9**) ($CDCl_3$) at 100 MHz

Carbon position	Chemical shift (δ; ppm)
1	38.4
2	23.6
3	80.9
4	37.6
5	55.2
6	18.2
7	31.2
8	39.6
9	47.6
10	36.8
11	23.4
12	124.3
13	139.6
14	40.0
15	28.1
16	26.6
17	33.7
18	47.6
19	41.5
20	33.7
21	33.8

Table 4 ^{13}C NMR spectrum of α-amyrin acetate (**9**) ($CDCl_3$) at 100 MHz (cont.)

Carbon position	Chemical shift (δ; ppm)
22	38.4
23	28.0
24	16.7
25	15.7
26	16.8
27	28.0
28	28.0
29	28.7
30	23.2
31	171.0
32	21.4

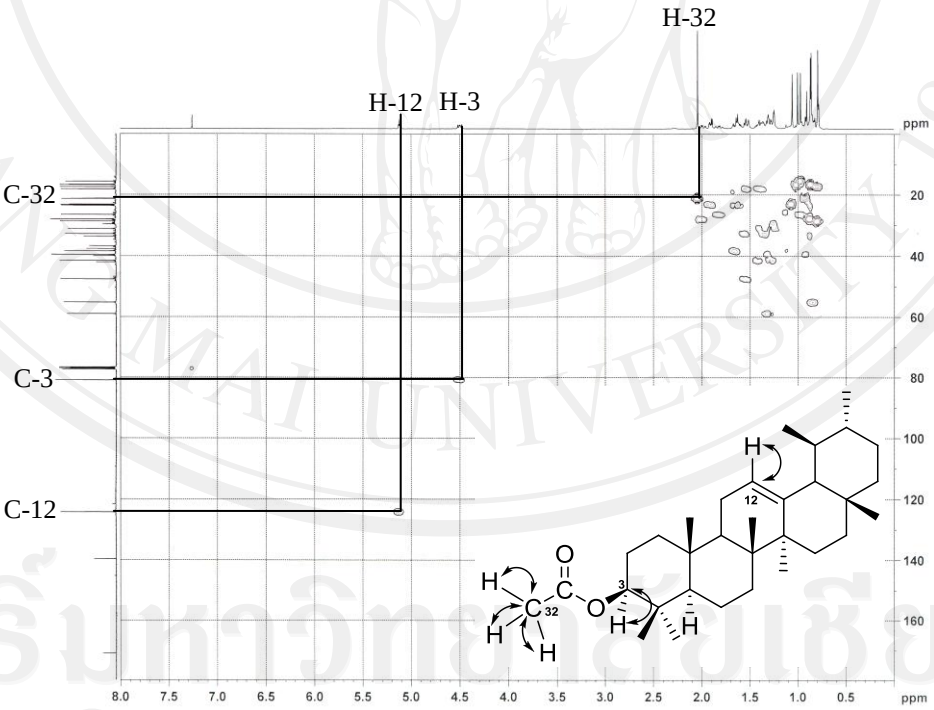


Figure 36 HMQC data of α-amyrin acetate (**9**)

Moreover, this compound was approved the structure by HMQC data that indicates the correlation between each proton and carbon especially important signals. For instance, H-3 proton at 5.12 ppm was coupled with C-3 at 80.9 ppm, H-12 proton at 5.12 ppm accorded with C-12 at 124.3 ppm, and H-32 protons, three protons of methyl group of acetate group, at 2.04 ppm correlated with C-32 at 21.4 ppm, respectively.

The environment of each proton on functional groups were confirmed by HMBC data, for instance, proton 3 on acetate carbon that coupled with carbon 2, 4, and 31 (carbonyl carbon), proton 12 (proton of endocyclic double bond) which had relationship with carbon 11 and 13, and methyl protons in acetate group (the protons on carbon 32) with carbon 31 (carbonyl carbon).

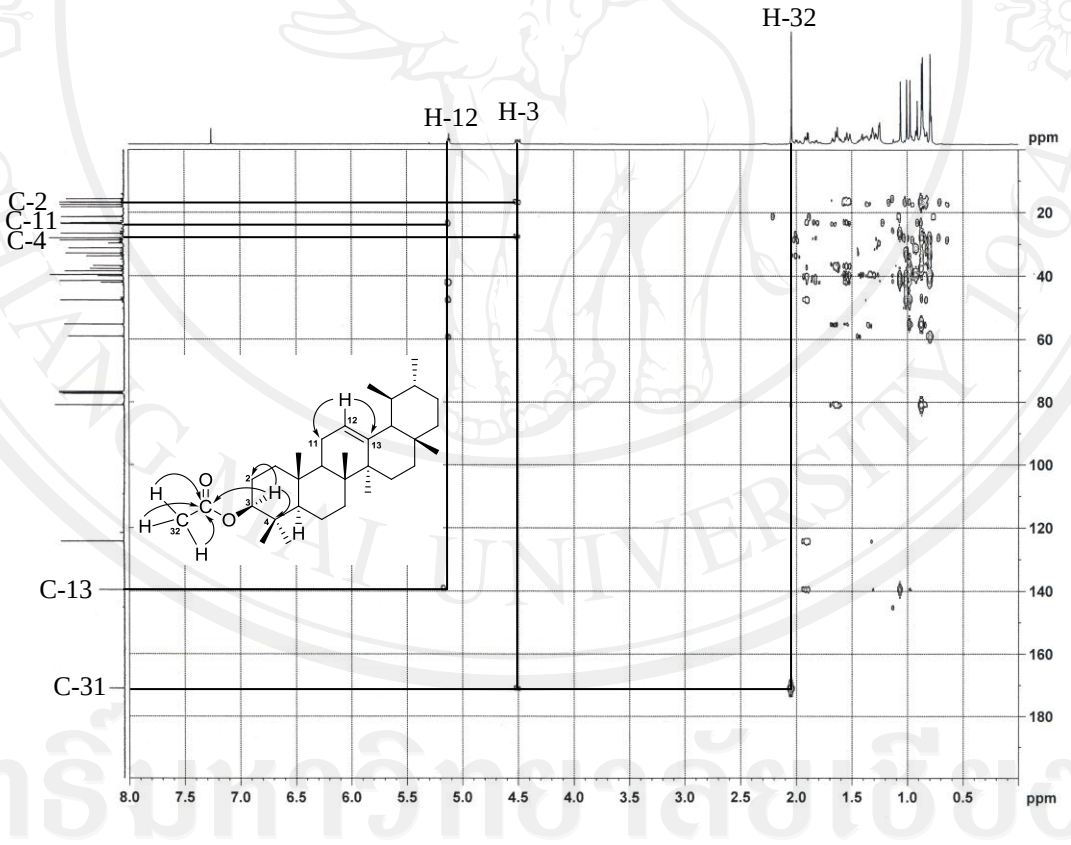


Figure 37 HMBC data of α-amyrin acetate (**9**)

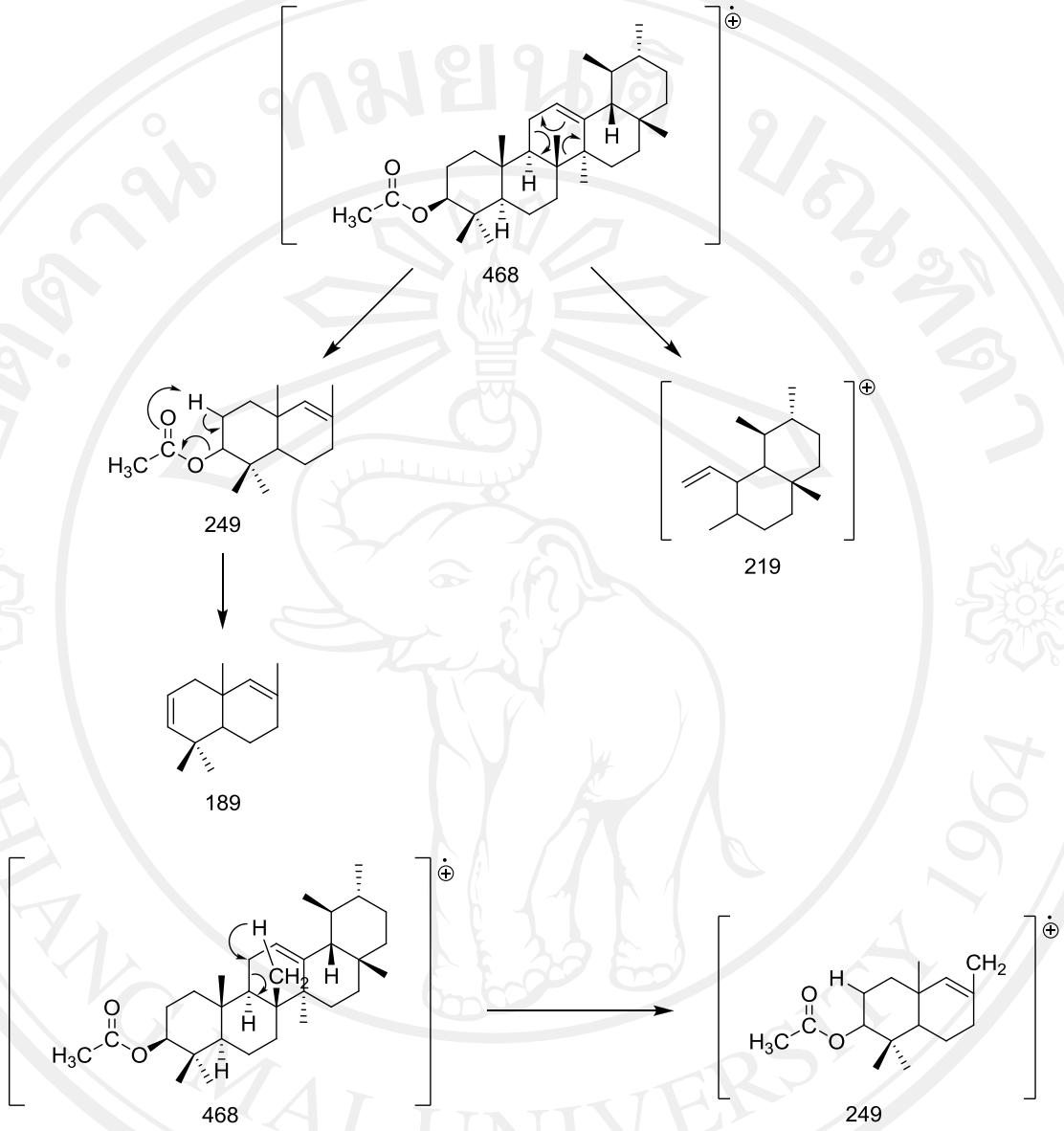
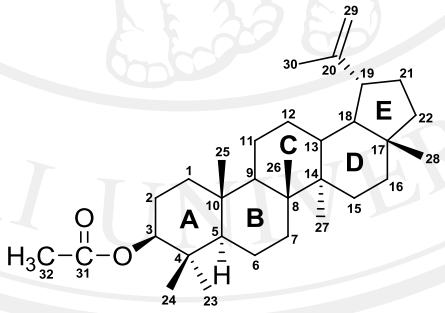


Figure 38 Mechanism of key fragment ions of *α*-amyrin acetate (**9**) from EI-MS

From the EI-MS technique, there was obtained its molecular ion peak at *m/z* 468 [M^+]. The other fragments which were the key fragments were observed and recorded at *m/z* 249, 219 and 189, respectively. Fragmentation of important fragments of *α*-amyrin acetate (**9**) was indicated their probability in Figure 38 compared with the previous report.[42]

Lupeol acetate (**59**); which was called lup-20(29)-en-3-yl acetate in the systematic name, was obtained a white solid. This isolated compound, a triterpene,

was separated from fraction VPD-03 of the dichloromethane extract of *V. parishii* leaves same as *α*-amyrin acetate (**9**). Melting point was found to be in the range of 210.0 – 212.0 °C that was confirmed with the previous report which was shown the melting point between 216.0 – 217.0 °C.[43] The IR spectrum of compound displayed three important signals containing the stretching of carbonyl at 1734, the stretching of carbon of double bond at 1643, and the stretching of ether bond in acetate group at 1245 cm^{-1}. In addition, This compound was measured the optical rotation value and recorded the result as $[\alpha]^{29.3}_{D}$ +51.4°, compared with $[\alpha]^{20}_{D}$ +47.3° from the literature.[44] The ^{1}H NMR spectrum of the compound displayed especial peaks, for example, H-3 proton adhered on the C-3 carbon showed doublet of doublets signal at 4.47 ppm, which had coupling constant (*J*) 10.4, 5.4 Hz. The H-19 proton that adjoined on C-19, which bonded with tertiary carbon of terminal double bond, was also another important peak that indicated triplet signal (*J* = 11.1, 5.8 Hz) at 2.37 ppm. In addition, two protons at position-29, which were methylene protons, showed two signals at 4.56 ppm that was doublet of doublets (*J* = 2.4, 1.3 Hz) and 4.68 ppm which was doublet (*J* = 2.3 Hz), respectively, and H-32 was identified as three protons of methyl group of acetate group displayed at 2.04 ppm.



lupeol acetate (**59**)

Figure 39 Labeling number of each carbon in structure of lupeol acetate (**59**)

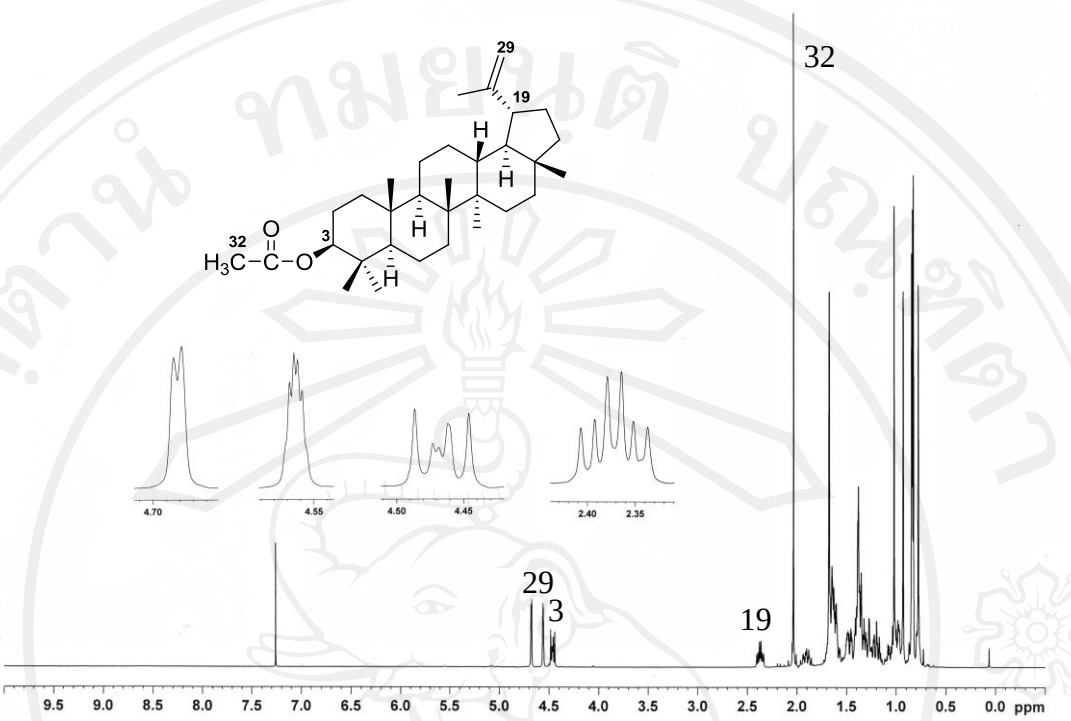


Figure 40 ^{1}H NMR spectrum of lupeol acetate (**59**)

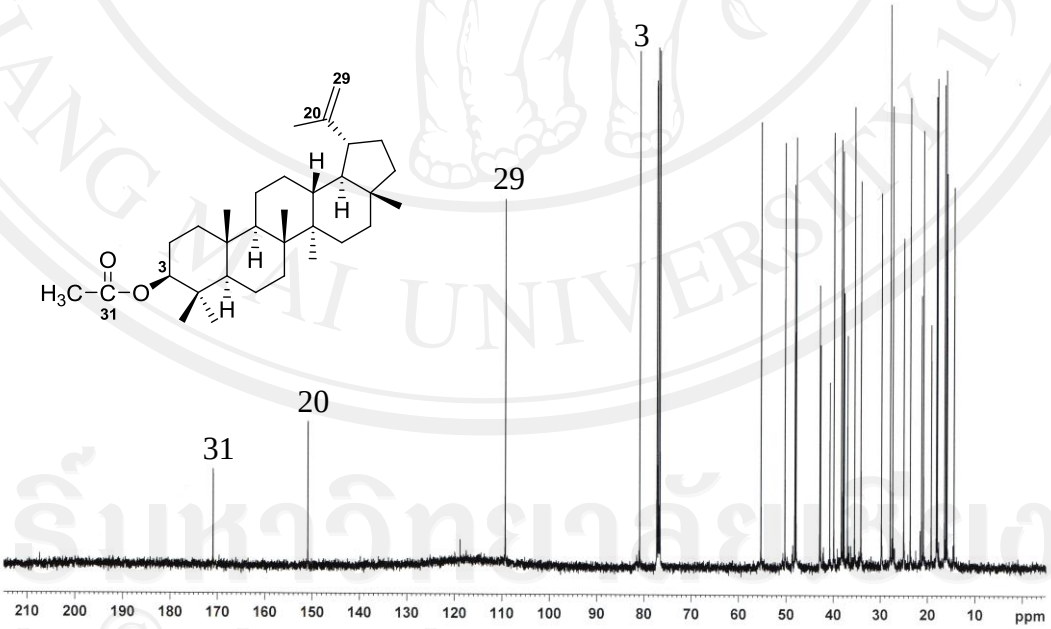


Figure 41 ^{13}C NMR spectrum of lupeol acetate (**59**)

Their ^{13}C NMR spectrum also showed especial signals, for instance, C-3 carbon that adjoined acetate carbon indicated peak at 80.9 ppm, C-20 carbon was identified as tertiary carbon of double bond outside ring E showed peak at 150.9 ppm, C-29 carbon, which was methylene carbon of terminal double bond, displayed signal at 109.3 ppm, and C-31 carbon, carbonyl carbon of acetate group, showed obvious peak at 171.0 ppm.

The correlation between ^{1}H and ^{13}C NMR spectrum were confirmed by HMQC. From this data, they presented the results, for instance, H-3 proton was coupled with C-3 carbon, H-19 which accorded with C-19, two protons (H-29) of methylene group correlated with C-29 carbon, The methyl protons (H-32 protons) of acetate group were linked with C-32 carbon. Therefore, we could deduce all protons, which were identified, were bonded with all correlated carbon. All spectrum data of lupeol acetate (**59**), which were showed in Table 5 and 6, were identical with previous report. [45]

Table 5 ^{1}H NMR spectrum of lupeol acetate (**59**) ($CDCl_3$) at 400 MHz

Carbon position	Chemical shift (δ; ppm)
3	4.47 [*m*, 1H]
5	0.79 [*m*, 1H]
6	0.84 [*m*, 2H]
12	1.65 [*m*, 2H]
19	2.37 [*dt*, 1H];
	J = 11.1, 5.8 Hz
21	1.38 [*m*, 1H]
	1.86-1.96 [*m*, 1H]
22	1.63 [*m*, 2H]
23	0.84 [*s*, 3H]
24	0.84 [*s*, 3H]
25	0.84 [*s*, 3H]
26	1.02 [*s*, 3H]

Table 5 ^{1}H NMR spectrum of lupeol acetate (**59**) ($CDCl_3$) at 400 MHz (cont.)

Carbon position	Chemical shift (δ; ppm)
27	0.93 [*s*, 3H]
28	0.83 [s, 3H]
29	4.56 [*dd*, 1H];
	J = 2.4, 1.3 Hz
	4.68 [*d*, 1H];
	J = 2.3 Hz
30	1.68 [*s*, 3H]
32	2.04 [*s*, 3H]

Table 6 ^{13}C NMR spectrum of lupeol acetate (**59**) ($CDCl_3$) at 100 MHz

Carbon position	Chemical shift (δ; ppm)
1	38.3
2	23.7
3	80.9
4	37.7
5	55.3
6	18.1
7	34.2
8	40.8
9	50.3
10	37.0
11	20.9
12	25.5
13	38.0
14	42.8
15	27.4
16	35.5
17	42.8

Table 6 ^{13}C NMR spectrum of lupeol acetate (**59**) ($CDCl_3$) at 100 MHz (cont.)

Carbon position	Chemical shift (δ; ppm)
18	48.0
19	48.2
20	150.9
21	29.8
22	39.9
23	27.9
24	15.9
25	16.1
26	16.4
27	14.5
28	17.9
29	109.3
30	19.2
31	171.0
32	21.6

The HMBC data indicated two or three bonds coupling of proton with carbon. For example, proton 3 on acetate carbon had relationship with carbon 2, 4 and 31 (carbonyl carbon), proton 19 coupled with carbon 18, 20 and 21, proton 29 (two proton of methylene double bond) which was outside ring E had correlation with carbon 20, and methyl protons (H-32) in acetate group were related by carbonyl carbon (C-31).

In EI-MS experiment, this compound was observed and recorded its molecular ion peak at *m/z* 468 [M^+]. The other fragments that were the key fragments were noticed and recorded at *m/z* 408, 249 and 189, respectively. Fragmentation of important fragments of lupeol acetate (**59**) was indicated their mechanism in Figure 44 compared with previous study.[42]

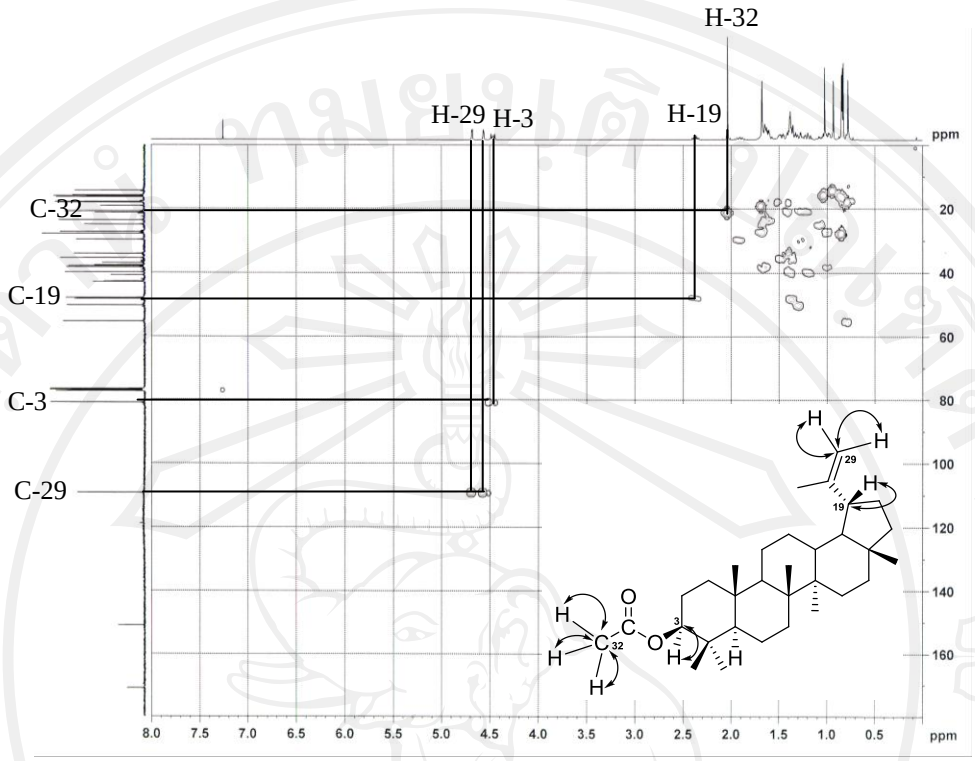


Figure 42 HMQC data of lupeol acetate (**59**)

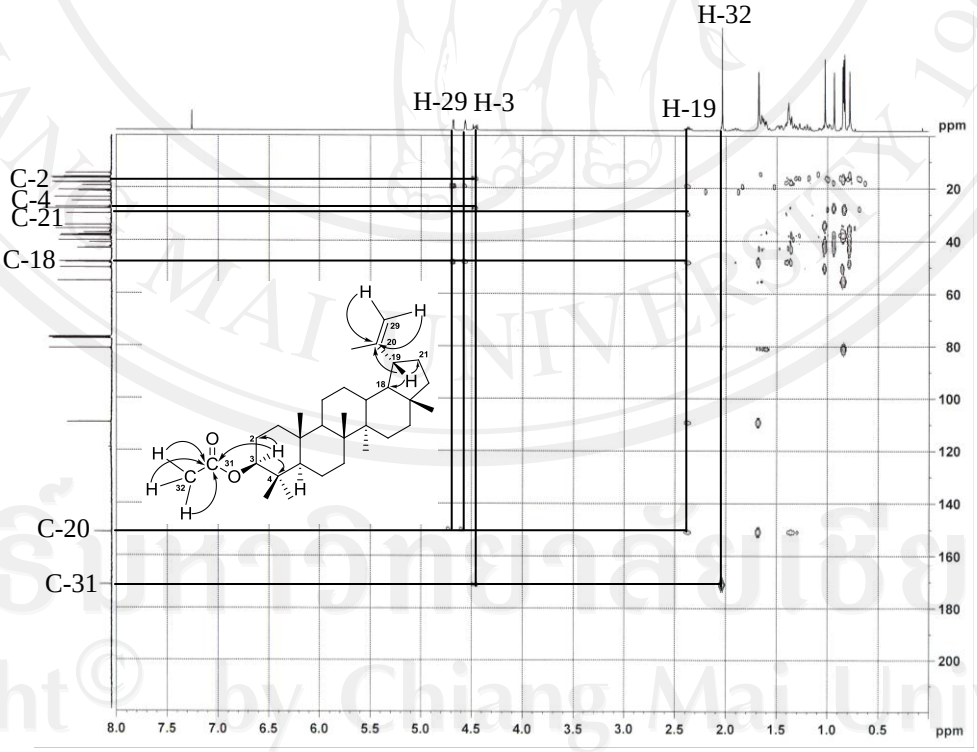


Figure 43 HMBC data of lupeol acetate (**59**)

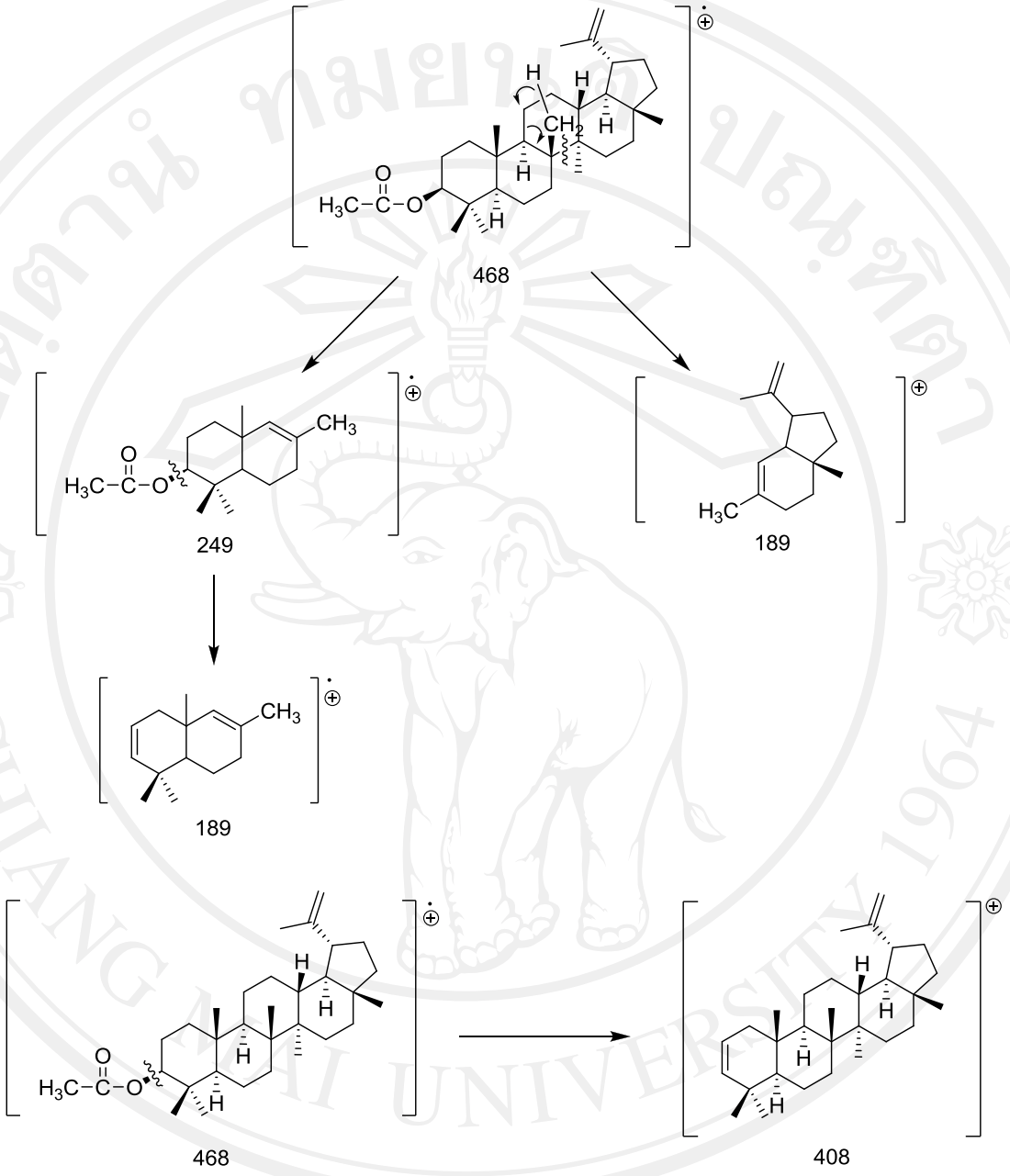
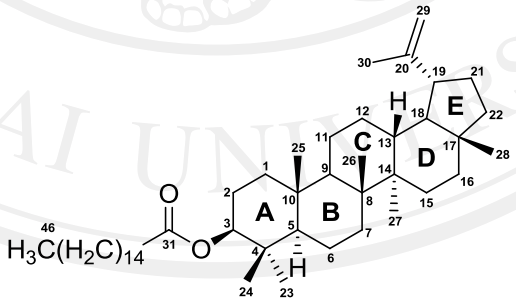


Figure 44 Mechanism of key fragment ions of lupeol acetate (**59**) from EI-MS

Lupeol palmitate (**60**); a triterpene that was called as (3*β*)-lup-20(29)-en-3-yl palmitate in the systematic name, was isolated from fraction VPD-03 of the dichloromethane extract same *α*-amyrin acetate (**9**) and lupeol acetate (**59**). The compound was measured and recorded its melting point between 79.0 – 80.5 °C that conform to the result in previous report shown between 80.0 – 81.0 °C.[46] Measuring of IR spectrum of this compound indicated three significant signals that

consisted stretching of carbonyl at 1728, stretching of carbon of double bond at 1639, and stretching of ether bond in carboxylate group at 1178 cm^{-1}. Furthermore, the compound was measured the optical rotation value to give result as $[\alpha]^{29.1}_{D}$ +35.0°, which the value was reported as $[\alpha]^{20}_{D}$ +34.0° in previous study.[47] The ^{1}H NMR spectrum of this compound from isolation process indicated characteristic signals like compound **59**. For example, H-3 proton, which adjoined C-3 carbon, showed doublet of doublet peak at 4.47 ppm (J = 10.6, 5.6 Hz), H-19 proton adhered on C-19 carbon, which associated with tertiary carbon of terminal double bond outside ring E, displayed doublet of triplet signal (J = 11.0, 5.8 Hz) at 2.37 ppm, and H-29 protons that had two protons were identified as methylene protons of terminal double bond outside ring E showed peaks at 4.57 ppm, multiplet signal, and 4.68 ppm, broad doublet signal (J = 2.1 Hz), respectively.

To confirm the number of proton in main core that bonded with C-3, D_2O was added to lupeol palmitate solution for changing H_2O form in $CDCl_3$ at 1.56 ppm to D_2O form at 4.76 ppm. Integration of peak area of main core structure before added D_2O (89.85) was compared with after added D_2O (76.79), which were shown in Figure 48 and 49, which the number of proton in main core structure should be the value as 73.



lupeol palmitate (**60**)

Figure 45 Labeling number of each carbon in structure of lupeol palmitate (**60**)

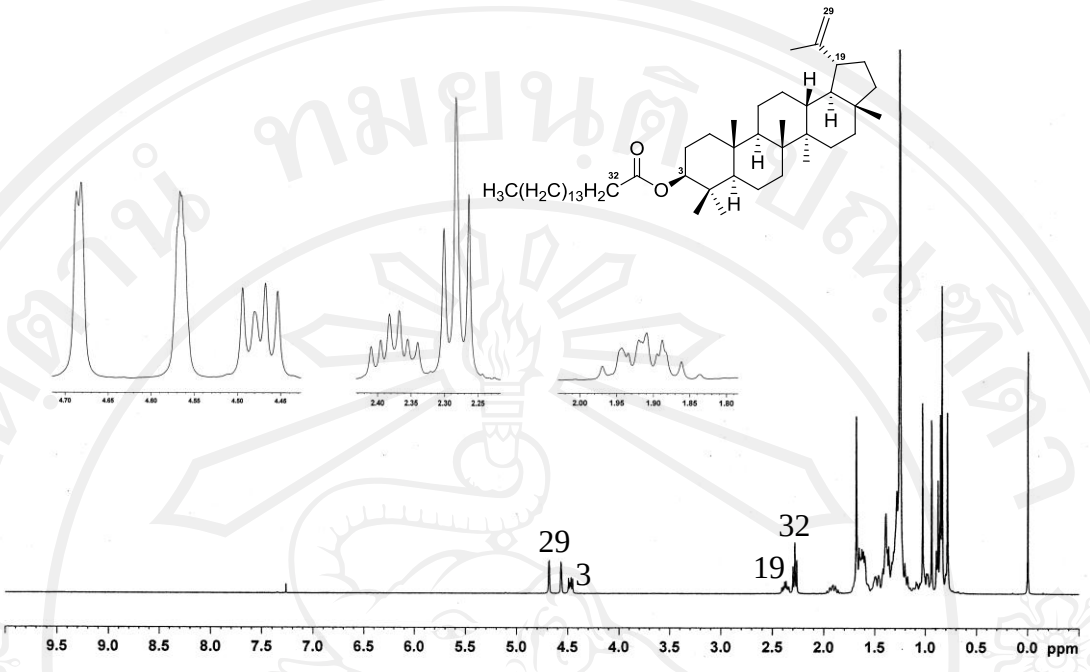


Figure 46 ^{1}H NMR spectrum of lupeol palmitate (**60**)

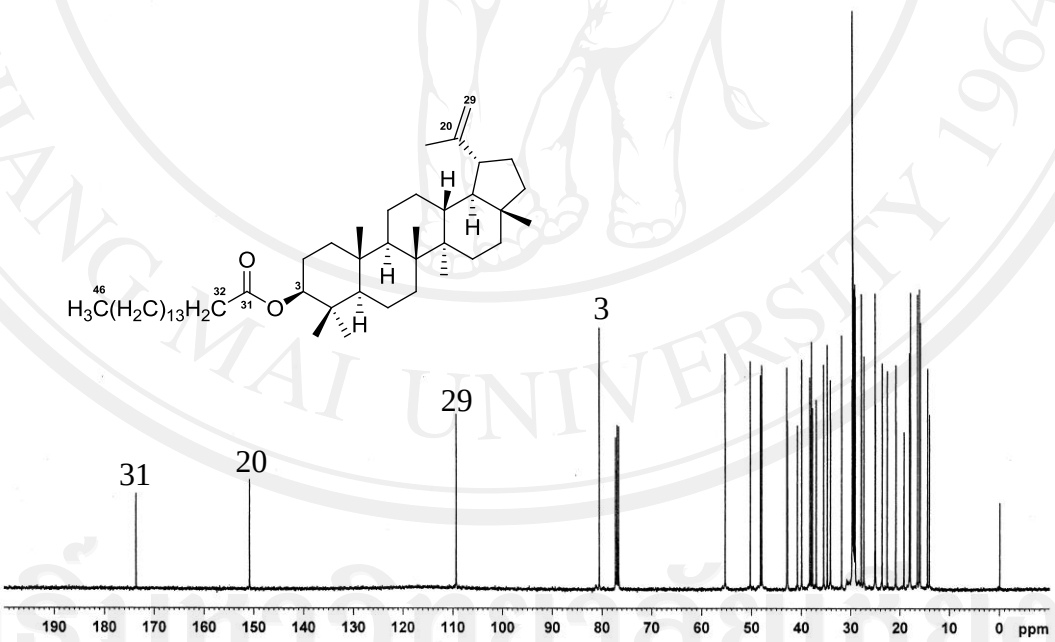


Figure 47 ^{13}C NMR spectrum of lupeol palmitate (**60**)

The ^{13}C NMR spectrum of this compound performed significant signals, for example, C-3 carbon that bonded with carboxylate group showed peak at 80.5 ppm,

C-20 carbon, which was tertiary carbon of double bond outside ring E, indicated peak at 150.9 ppm, C-29 carbon that was methylene group of double bond outside ring E displayed peak at 109.4 ppm, C-31 carbon identified as carbonyl carbon of carboxylate group showed evident signal at 173.6 ppm, and C-34 to 43 carbons that were side chain of alkyl branch of carboxylate group indicated between 29.2–29.7 ppm. All spectrum of the separated compound, compared with previous report [47, 48], were shown in Table 7 and 8.

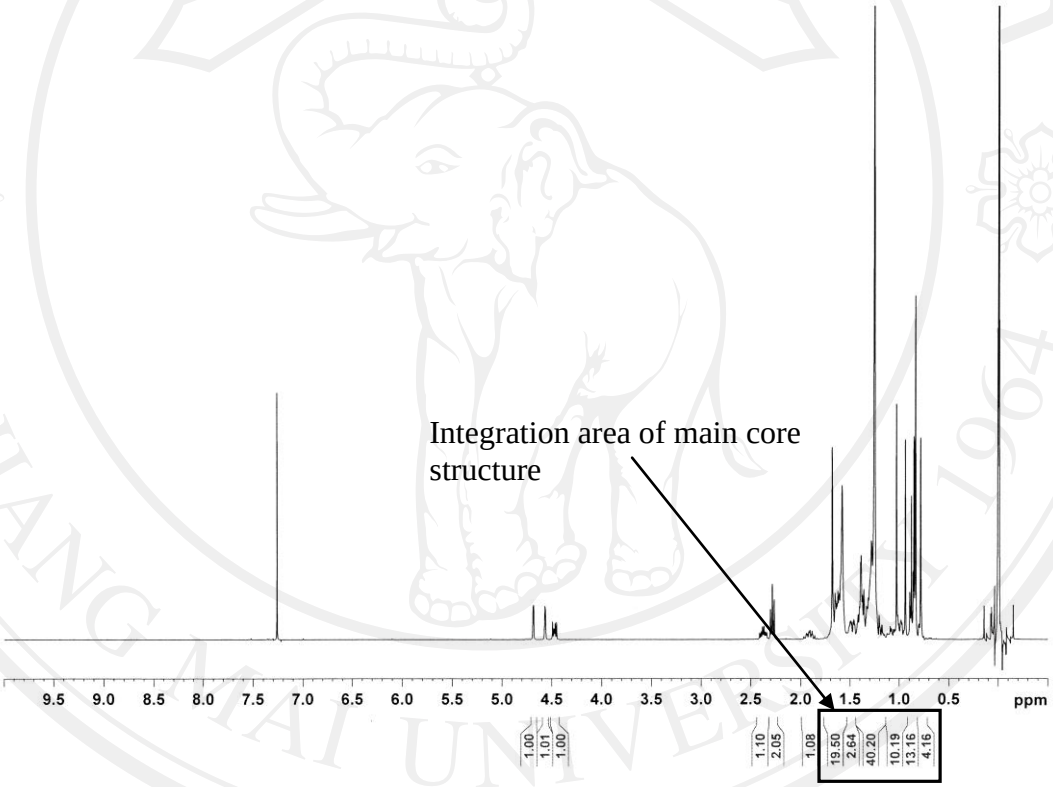


Figure 48 ^{1}H NMR spectrum of lupeol palmitate (**60**) before added D_2O

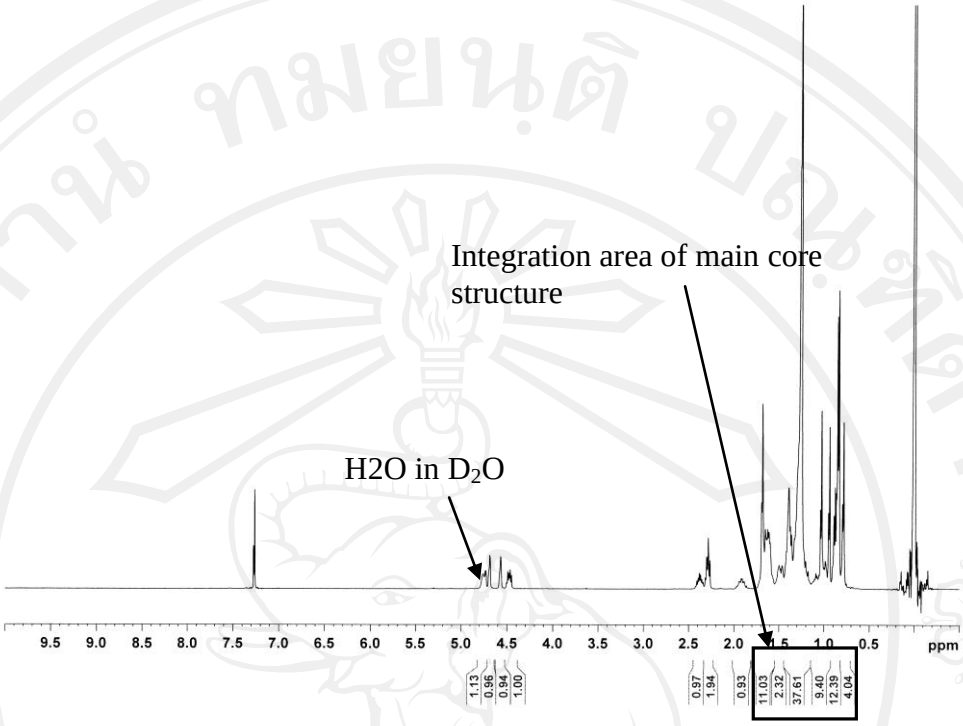


Figure 49 ^{1}H NMR spectrum of lupeol palmitate (**60**) after added D_2O

Table 7 ^{1}H NMR spectrum of lupeol palmitate (**60**) ($CDCl_3$) at 400 MHz

Carbon position	Chemical shift (δ; ppm)
3	4.47 [*m*, 1H]
5	0.79 [*m*, 1H]
6	0.85 [*m*, 2H]
12	1.65 [*m*, 2H]
19	2.37 [*dt*, 1H]; *J* = 11.0, 5.8 Hz
21	1.60 [*m*, 2H]
22	1.61 [*m*, 2H]
23	0.88 [*s*, 3H]
24	0.79 [*s*, 3H]
25	0.85 [*s*, 3H]
26	1.03 [*s*, 3H]
27	0.94 [*s*, 3H]

Table 7 ^{1}H NMR spectrum of lupeol palmitate (**60**) ($CDCl_3$) at 400 MHz (cont.)

Carbon position	Chemical shift (δ; ppm)
28	0.84 [*s*, 3H]
29	4.57 [*m*, 1H]
	4.68 [br *d*, 1H];
	J = 2.1 Hz
30	1.68 [*s*, 3H]
32	2.28 [*t*, 2H];
	J = 7.4 Hz
46	1.20 [*s* ,3H]

Table 8 ^{13}C NMR spectrum of lupeol palmitate (**60**) ($CDCl_3$) at 100 MHz

Carbon position	Chemical shift (δ; ppm)
1	37.8
2	23.7
3	80.5
4	38.0
5	50.3
6	18.0
7	31.9
8	38.4
9	40.8
10	37.0
11	20.9
12	25.2
13	35.6
14	42.8
15	27.4
16	34.8

Table 8 ^{1}3C NMR spectrum of lupeol palmitate (**60**) ($CDCl_3$) at 100 MHz (cont.)

Carbon position	Chemical shift (δ; ppm)
17	42.9
18	48.2
19	47.9
20	150.9
21	38.0
22	39.9
23	27.9
24	15.9
25	16.5
26	16.1
27	14.5
28	18.2
29	109.4
30	19.3
31	173.6
32	34.2
33	25.0
34-43	29.2 – 29.7
44	29.7
45	22.7
46	14.1

To confirm the evidence, HMQC was used to explain correlation between ^{1}H and ^{13}C NMR spectrum of this compound. In the result, it displayed the correlation such as H-3 proton was coupled by C-3 carbon, H-19 proton associated with C-19 carbon, two methylene protons (H-29) showed correlation with C-29 that was methylene carbon of terminal double bond.

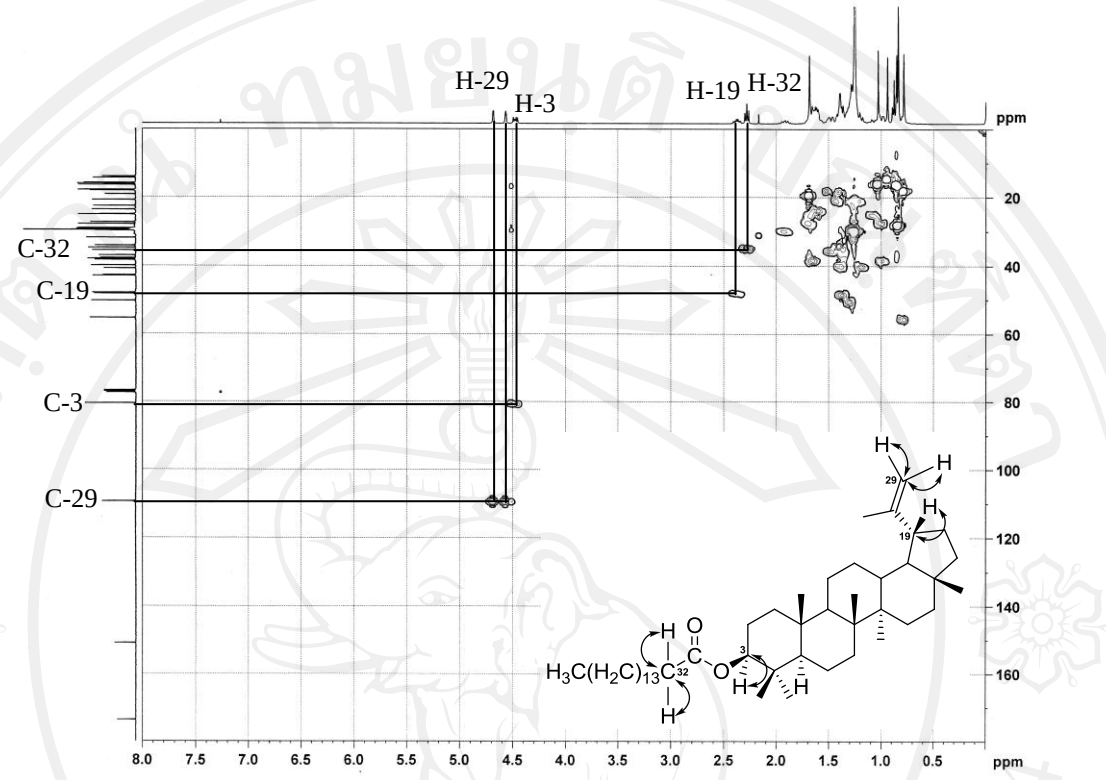


Figure 50 HMQC data of lupeol palmitate (**60**)

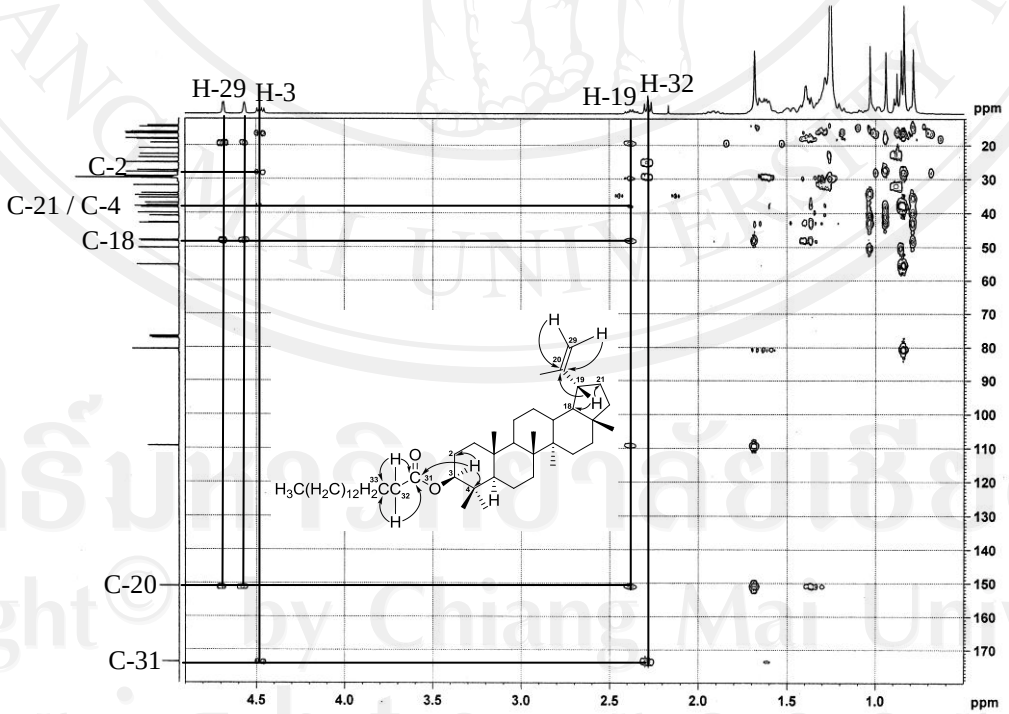


Figure 51 HMBC data of lupeol palmitate (**60**)

The HMBC experiment displayed two or three bonds coupling between proton and carbon such as proton 3 on carbon that linked with palmitate chain (C-3) with carbon 2, 4, and 31 (carbonyl carbon), proton 19 with carbon 18, 20, and 21, two protons outside ring E (H-29) with carbon 20, and two protons (H-32) on carbon 32 that bonded with carbonyl group with carbonyl carbon (C-31).

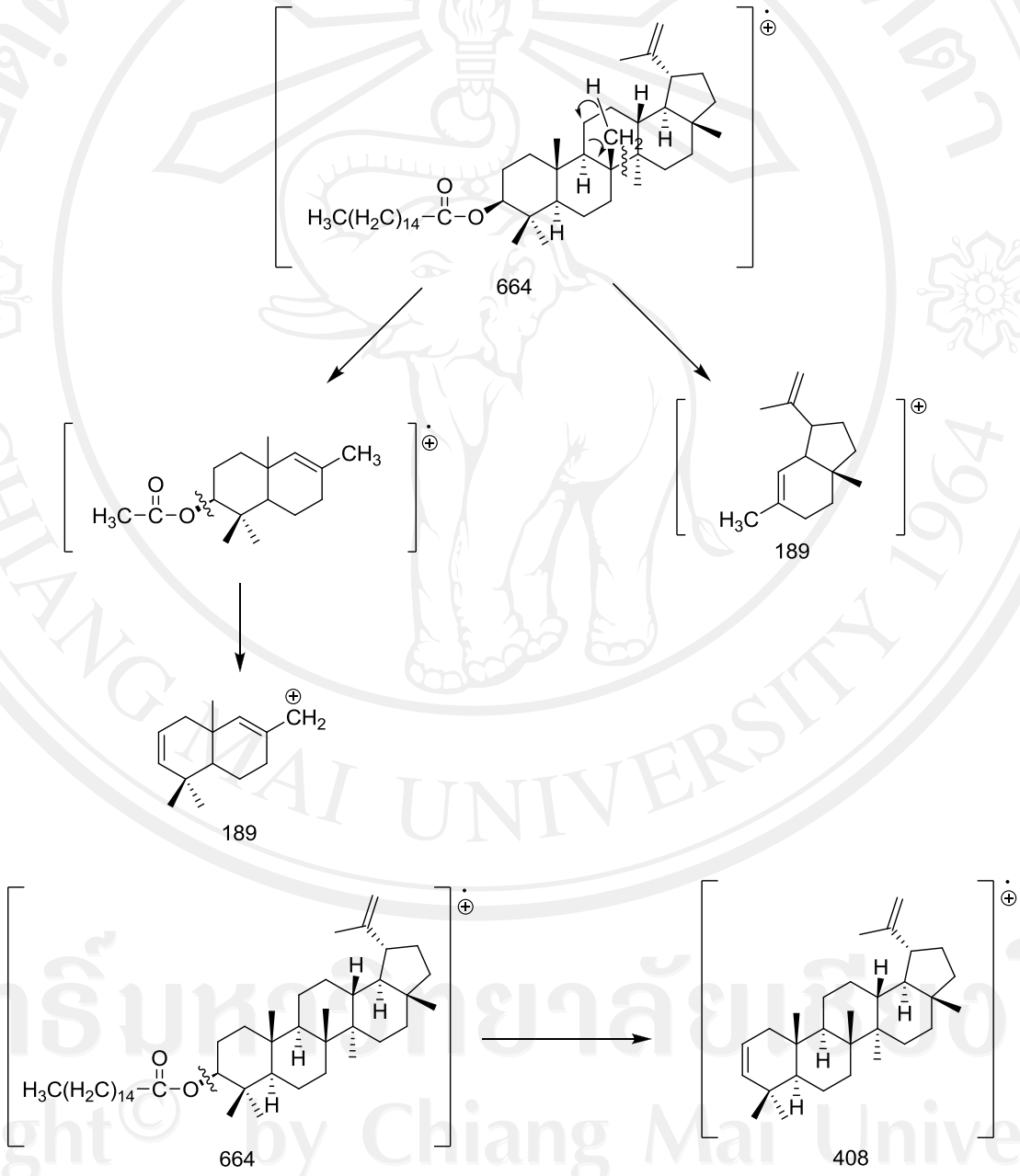
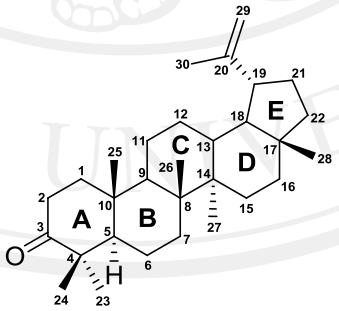


Figure 52 Mechanism of key fragment ions of lupeol palmitate (**60**) from EI-MS

From EI-MS method, the molecular ion peak [M^+] of this compound was not found in the experiment. However, the other key fragments were observed from mass spectrum at *m/z* 408 and 189. The mechanism of fragmentary process, like compound **59**, was shown in Figure 52.

Lupenone (**61**); which is called lup-20(29)-en-3-one in the systematic name, it was a white solid that was found in fraction VPD-F04 of dichloromethane extract of *V. parishii* leaves. This compound was measured its melting point and recorded the values between 166.0 – 168.0 °C, compared with previous study that reported as 168.0 – 170.0 °C.[49] Likewise, the IR spectrum of isolated compound showed a few obvious signals which consisted stretching of carbonyl at 1706, and stretching of carbon of double bond at 1639 cm^{-1}. The compound was also measured the optical rotation technique to give the value as $[\alpha]^{29}_D$ +20.1°, which was compared with $[\alpha]^{27}_D$ +20.7° in previous report.[50] From the spectroscopic techniques, the ^1H NMR spectrum of this compound indicated some important peaks. For instance, two protons at C-2 position displayed two obvious multiplet peaks at 2.40 and 2.47 ppm respectively, and the protons on terminal methylene carbon (H-29) showed two significant peaks at 4.57, which was a doublet of doublet peak with coupling constant (*J*) 2.4 and 1.3 Hz, and 4.69 ppm, a doublet peak with coupling constant (*J*) 2.4 Hz.



lupenone (**61**)

Figure 53 Labeling number of each carbon in structure of lupenone (**61**)

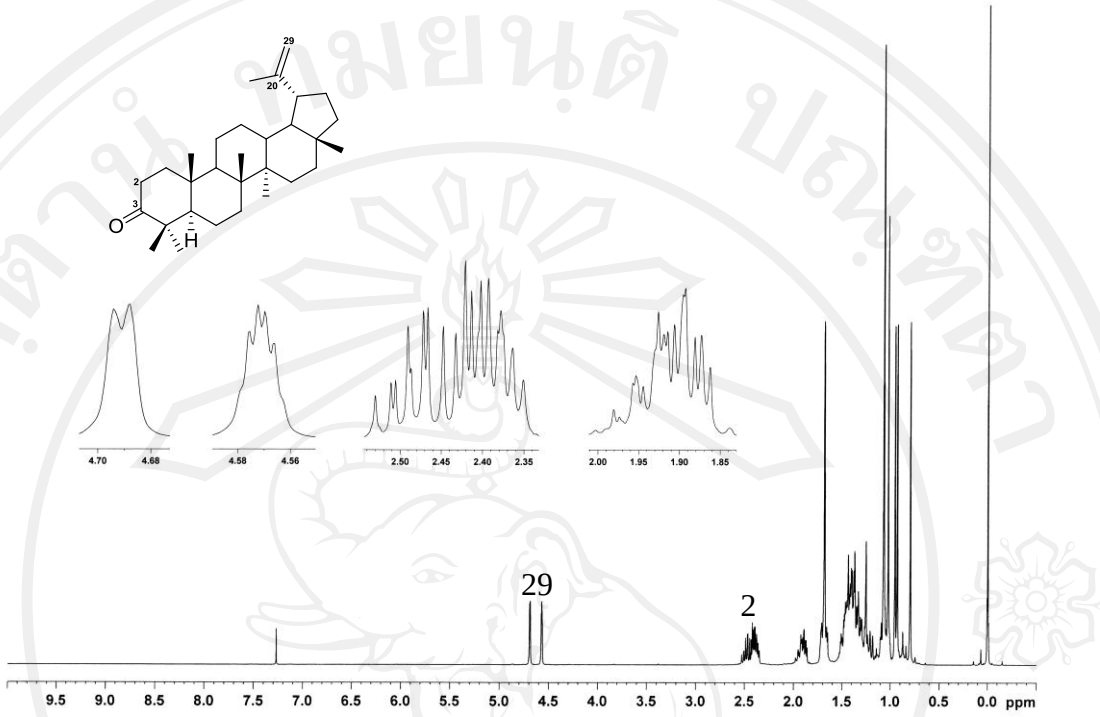


Figure 54 ^{1}H NMR spectrum of lupenone (**61**)

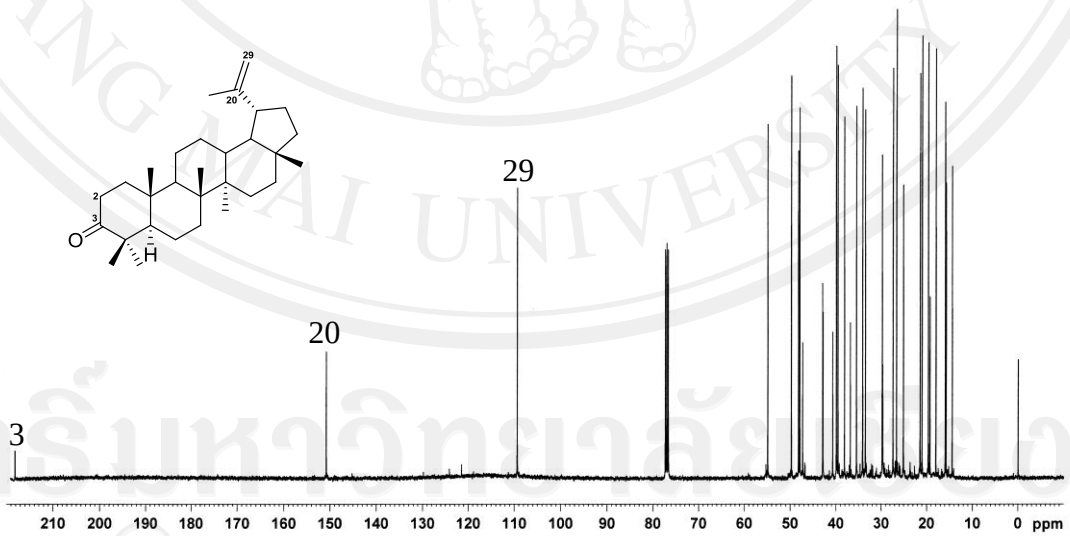


Figure 55 ^{13}C NMR spectrum of lupenone (**61**)

In the same way, ^{13}C NMR spectrum of compound indicated obvious signals. For example, carbon 3 which had carbonyl group showed peak at 218.2 ppm, and carbon 20 and 29 of terminal double bond outside ring E displayed two clear peaks at 150.8 and 109.4 ppm, respectively. All spectrum of lupenone, ^{1}H and ^{13}C NMR, were confirmed with previous report.[51] For all data from the experiment, they were shown in Table 9 and 10.

Table 9 ^{1}H NMR spectrum of lupenone (**61**) ($CDCl_3$) at 400 MHz

Carbon position	Chemical shift (δ; ppm)
2	2.40 [*m*, 1H]
	2.47 [*m*, 1H]
19	2.38 [*m*, 1H]
23	1.07 [*s*, H]
24	1.02 [*s*, H]
25	0.93 [*s*, H]
26	1.07 [*s*, H]
27	0.96 [*s*, H]
28	0.80 [*s*, H]
29	4.57 [*dd*, 1H];
	J = 2.4, 1.3 Hz
	4.69 [*d*, 1H];
	J = 2.4 Hz
30	1.68 [*s*, H]

Table 10 ^{13}C NMR spectrum of lupenone (**61**) ($CDCl_3$) at 100 MHz

Carbon position	Chemical shift (δ; ppm)
1	39.6
2	34.1
3	218.2
4	47.3

Table 10 ^{13}C NMR spectrum of lupenone (**61**) ($CDCl_3$) at 100 MHz (cont.)

Carbon position	Chemical shift (δ; ppm)
5	54.9
6	19.6
7	33.5
8	40.7
9	49.8
10	36.8
11	21.4
12	25.1
13	38.1
14	43.0
15	27.4
16	35.3
17	42.9
18	48.2
19	47.9
20	150.8
21	29.8
22	39.9
23	26.6
24	21.0
25	16.0
26	15.8
27	14.4
28	18.0
29	109.4
30	19.3

To make sure the result, HMQC data which confirmed the correlation between proton and carbon were also used to identify this compound. From this method, some important signals of NMR spectrum of compound were considered, for instance, two protons at C-2 position at 2.40 and 2.47 ppm had relationship with carbon 2 at 34.1 ppm, and two protons of double bond outside ring E at 4.57 and 4.69 ppm correlated with carbon 29 at 109.4 ppm, respectively. To explain clearly, these correlations were shown in Figure 56.

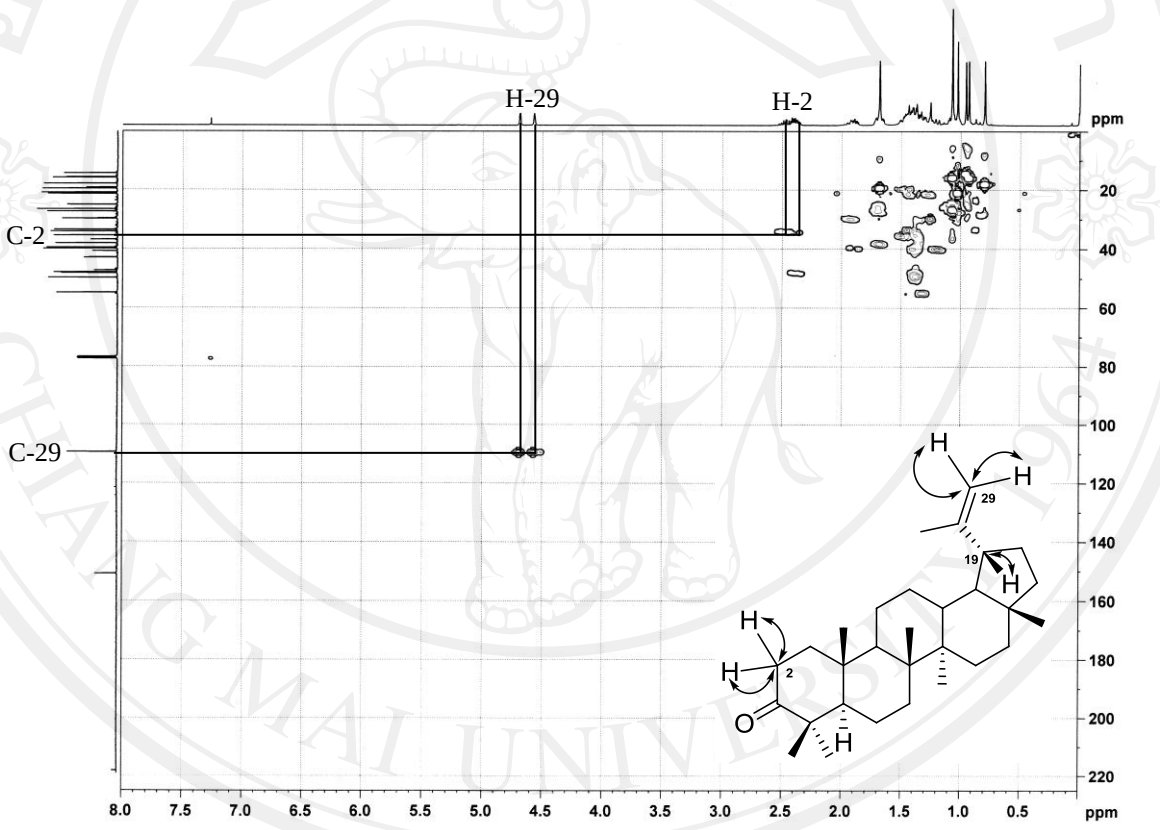


Figure 56 HMQC data of lupenone (**61**)

The two or three bonds coupling between proton and carbon of this triterpene were explained by HMBC data. For example, two protons on carbon 2 (H-2) had relationship with carbon 1 and carbon 3 (carbonyl carbon in ring A), and two protons of terminal methylene of double bond (H-29) correlated with carbon 20.

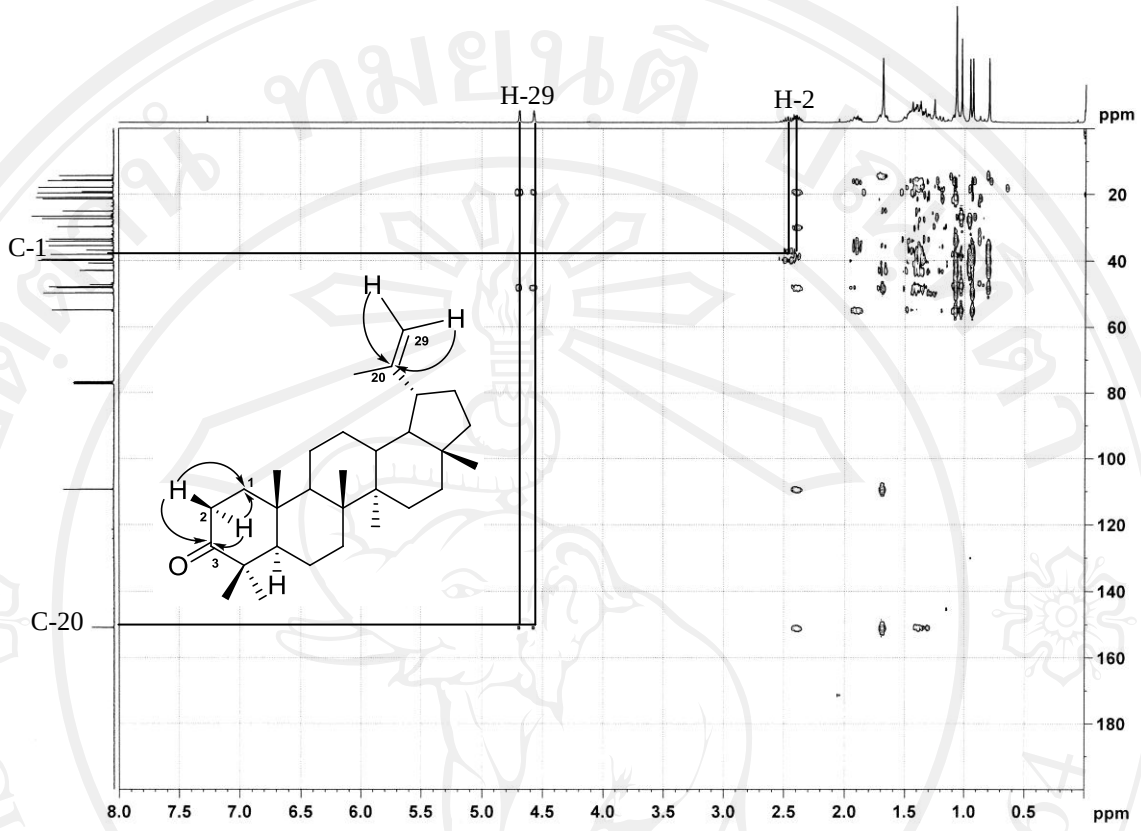


Figure 57 HMBC data of lupenone (**61**)

From EI-MS method, the molecular ion peak [M^+] of this triterpene was found as *m/z* 424. Moreover, the other key fragments were observed from mass spectrum at *m/z* 409, 381, 205 and 189. The probability of fragmentation of lupenone was similar with other triterpenes that can describe same previous report [52], which was shown in Figure 58.

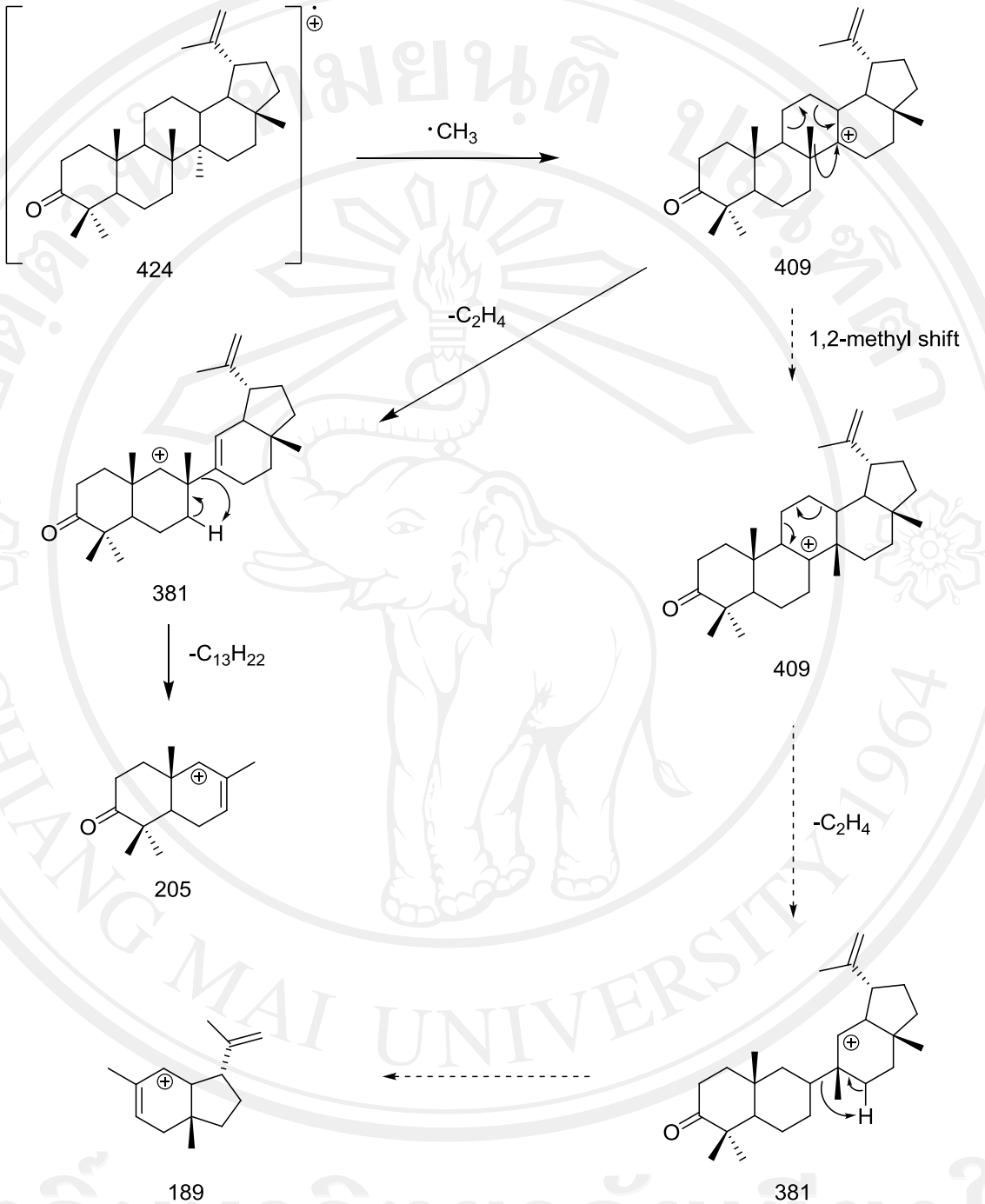
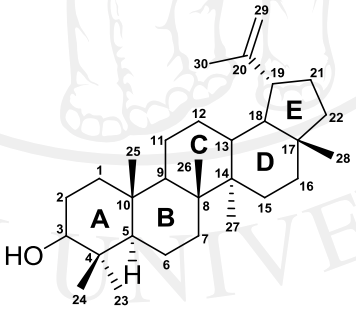


Figure 58 Mechanism of key fragment ions of lupenone (**61**) from EI-MS

Lupeol (**41**); which was called (3*β*,13*ζ*)-lup-20(29)-en-3-ol in the systematic name, a white solid compound, was isolated from sub-fraction VPD-F05-I of fraction VPD-F05 of dichloromethane extract of *V. parishii* leaves. Measuring of its melting point gave the value between 212.0 – 213.0 °C (the value was reported as 213.0 °C in the literature, [53]). The IR spectrum of this compound indicated two significant signals, consisting of stretching of hydroxyl at 3390, and stretching of carbon of double bond at 1593 cm^{-1}. Moreover, the optical rotation value of compound was measured and recorded as $[\alpha]^{28.9}_{D}$ +29.2°, compared with the previous literature that was reported as $[\alpha]^{25}_{D}$ +25.7°.[53] The ^{1}H NMR spectrum of this triterpene displayed some special peaks, for example, a proton on C-3 position, near hydroxyl group, showed triplet signal at 3.19 ppm with coupling constant (*J*) 5.7 Hz, a proton on C-19 position bonded with tertiary carbon of double bond outside ring E indicated doublet of triplet peak at 2.38 ppm with coupling constant (*J*) 11.1, 5.8 Hz, and two protons of terminal double bond, the protons on carbon 29, displayed two obvious signals at 4.56, a doublet of doublets peak (*J* = 2.3, 1.4 Hz) and 4.68 ppm, a doublet peak (*J* = 2.3 Hz).



lupeol (**41**)

Figure 59 Labeling number of each carbon in structure of lupeol (**41**)

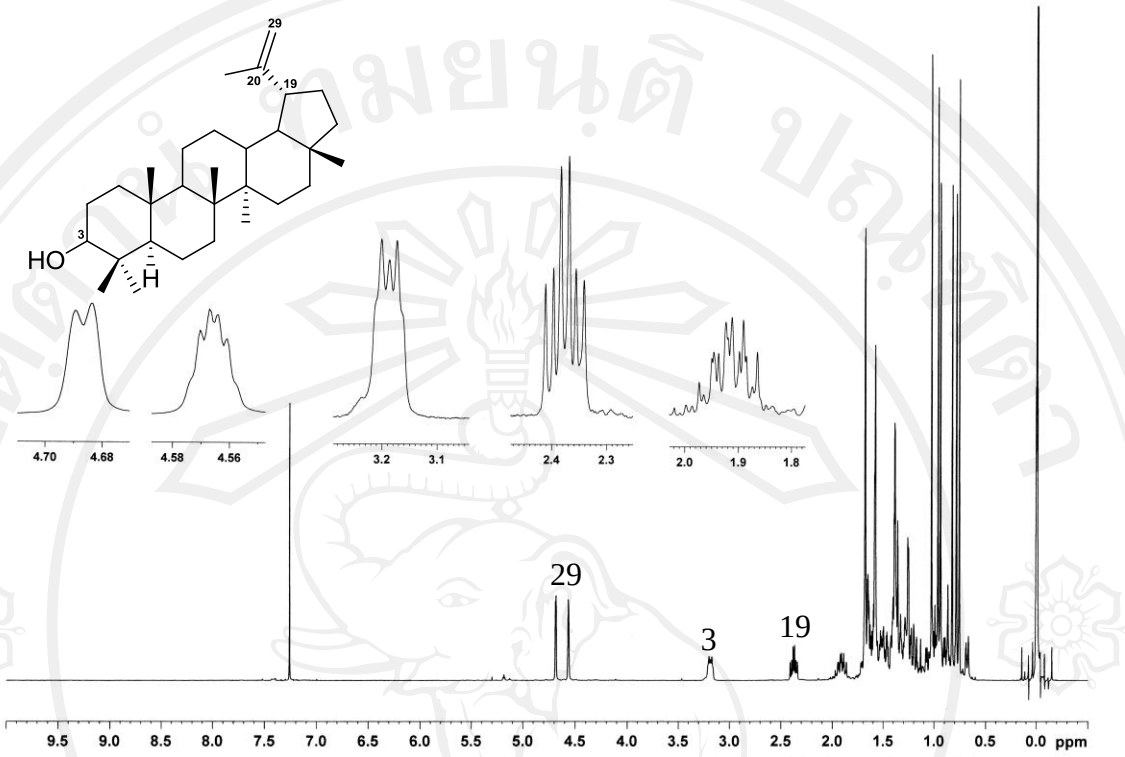


Figure 60 ^{1}H NMR spectrum of lupeol (**41**)

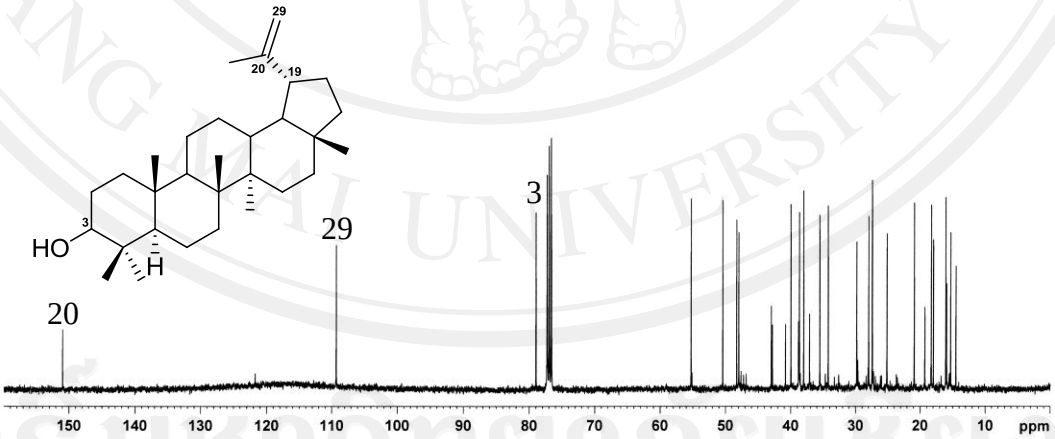


Figure 61 ^{13}C NMR spectrum of lupeol (**41**)

Furthermore, ^{13}C NMR spectrum of this compound also showed some important signals such as hydroxyl carbon (C-3) at 79.0 ppm, carbon 20 and 29 of terminal double bond outside ring E at 151.0 and 109.3 ppm respectively. All spectrum from

the experiment, which were shown in Table 11 and 12, were compared with previous literature.[53, 54]

Table 11 ^{1}H NMR spectrum of lupeol (**41**) ($CDCl_3$) at 400 MHz

Carbon position	Chemical shift (δ; ppm)
3	3.19 [*t*, 1H];
	J = 5.7 Hz
19	2.38 [*dt*, 1H];
	J = 11.1, 5.8 Hz
23	0.76 [*s*, H]
24	0.79 [*s*, H]
25	0.83 [*s*, H]
26	0.94 [*s*, H]
27	0.97 [*s*, H]
28	1.03 [*s*, H]
29	4.56 [*dd*, 1H];
	J = 2.3, 1.4 Hz
	4.69 [*d*, 1H];
	J = 2.3 Hz
30	1.69 [*s*, H]

Table 12 ^{13}C NMR spectrum of lupeol (**41**) ($CDCl_3$) at 100 MHz

Carbon position	Chemical shift (δ; ppm)
1	38.7
2	27.4
3	79.0
4	38.9
5	55.3
6	18.3
7	34.3

Table 12 ^{13}C NMR spectrum of lupeol (**41**) ($CDCl_3$) at 100 MHz (cont.)

Carbon position	Chemical shift (δ; ppm)
8	40.8
9	50.4
10	37.2
11	20.9
12	25.2
13	38.1
14	42.8
15	27.4
16	35.6
17	43.0
18	48.0
19	48.3
20	151.0
21	29.8
22	40.0
23	28.0
24	15.4
25	16.1
26	16.0
27	14.5
28	18.0
29	109.3
30	19.3

To confirm the correlation between ^{1}H and ^{13}C spectrum, HMQC data of the triterpene was considered in this method. For instance, the proton on C-3 position at 3.19 ppm was correlated with hydroxyl carbon (C-3) at 79.0 ppm, the proton on C-19 position at 2.38 ppm had relationship with carbon 19 that adjoined with tertiary carbon of terminal double bond at 48.3 ppm, and two protons on C-29 position at 4.56 and 4.69 ppm were coupled with carbon 29 of double bond outside ring E.

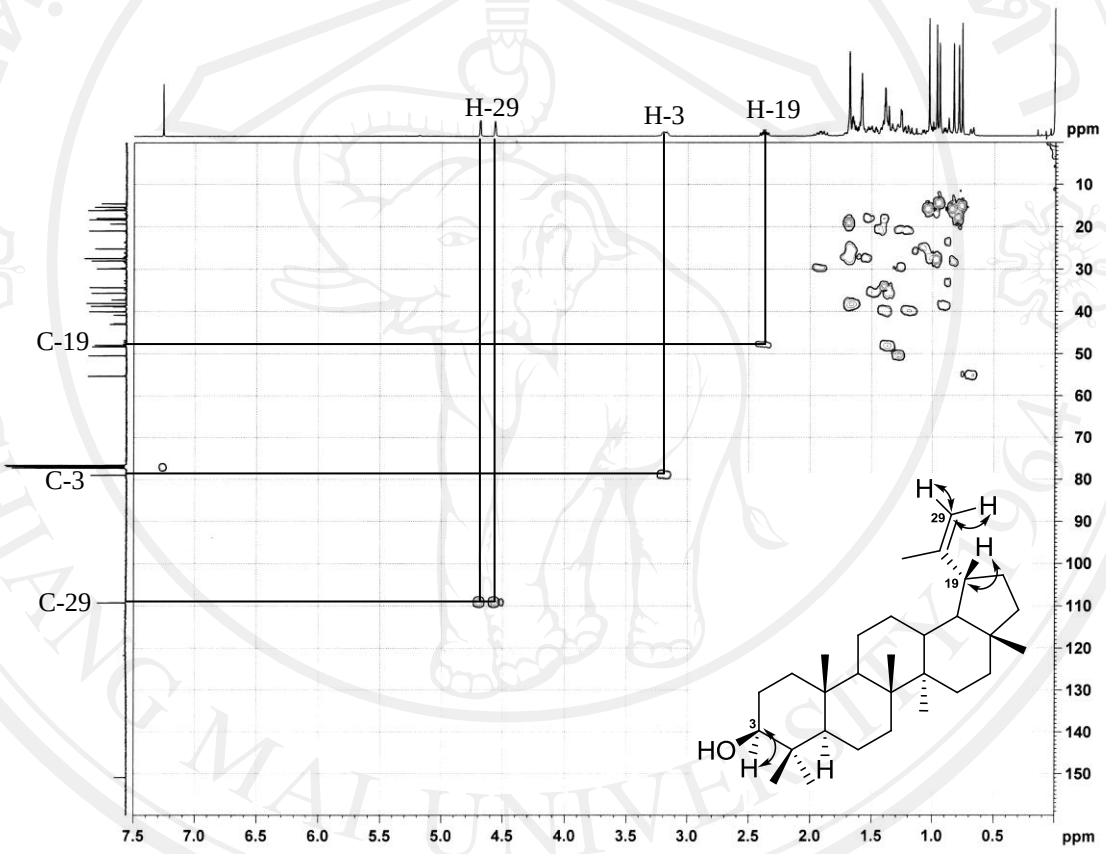


Figure 62 HMQC data of lupeol (**41**)

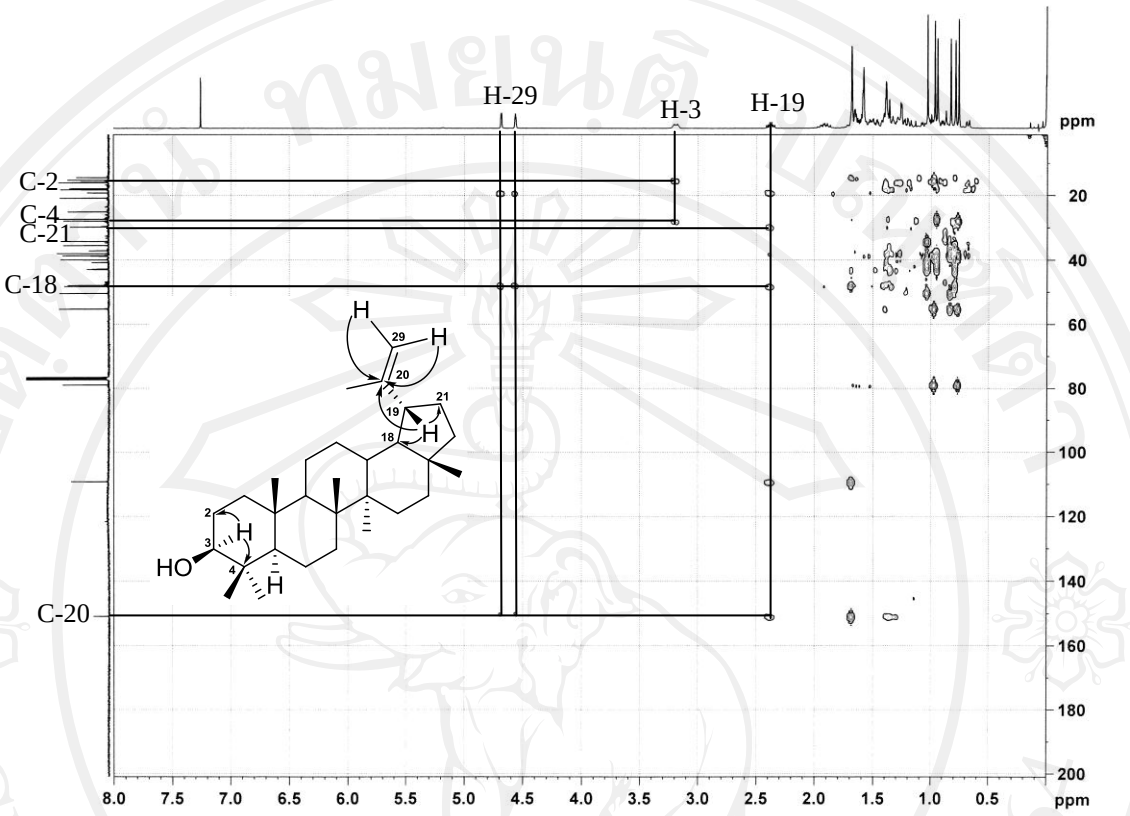


Figure 63 HMBC data of lupeol (**41**)

The HMBC data of this compound displayed relationship of two or three bonds coupling between proton and carbon such as proton 3 on hydroxyl carbon with carbon 2 and 4, proton 19 on carbon that bonded with terminal double bond with carbon 18, 20, and 21, and terminal methylene protons (H-29) with carbon 20.

From EI-MS result, the molecular ion peak [M^+] of this compound was found as *m/z* 426. Moreover, the other key fragments were observed from mass spectrum at *m/z* 424, 409, 381, 205 and 189. The probability of fragmentation of lupeol was similar with compound **61** which was explained the mechanism in previous literature [52], which was shown in Figure 64.

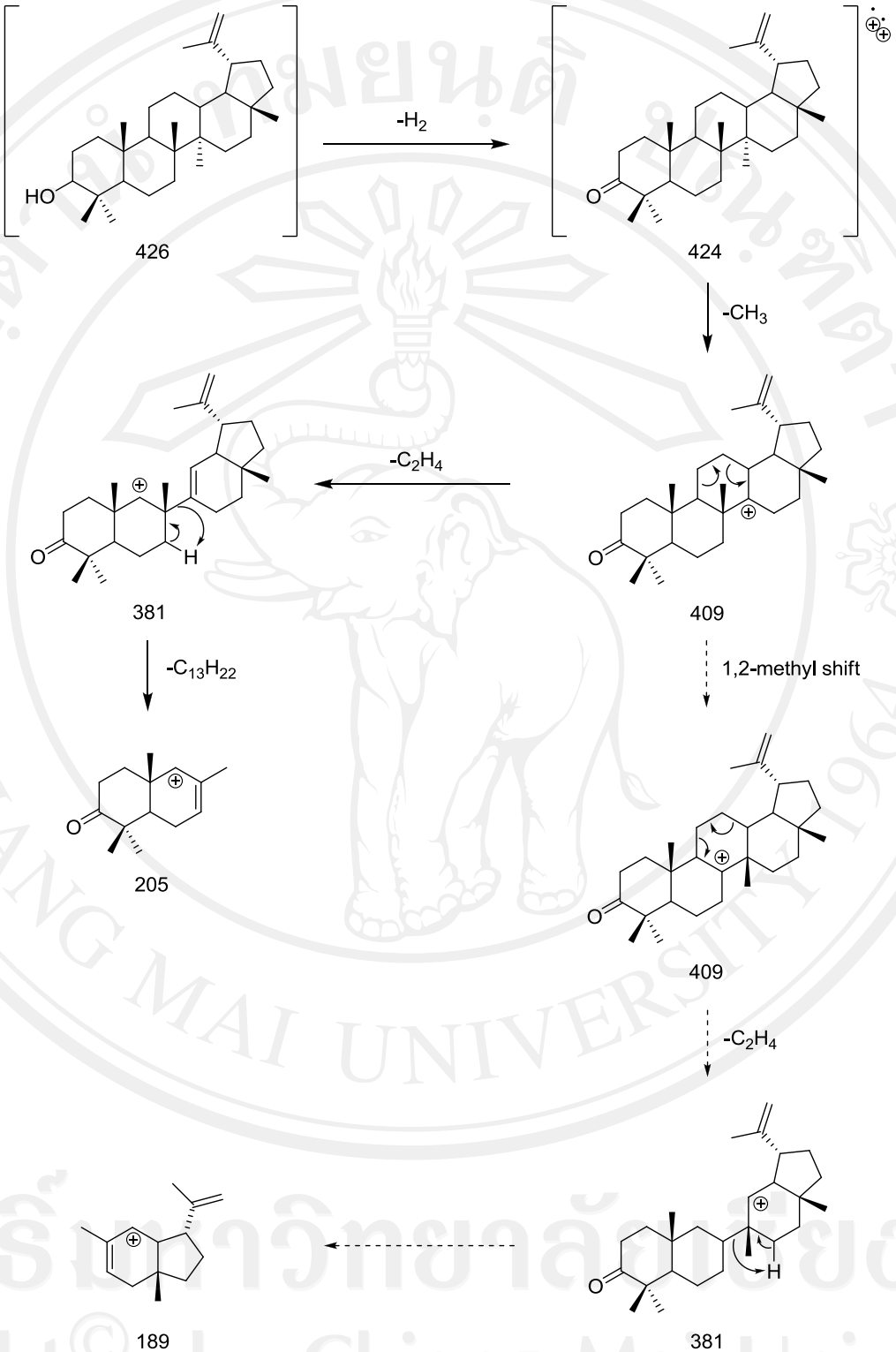


Figure 64 Mechanism of key fragment ions of lupeol (**41**) from EI-MS

α-Amyrin (**13**); called 12-ursen-3-*β*-ol in the systematic name, a white solid triterpene, was found in sub-fraction VPD-F05-H of fraction VPD-F05 of dichloromethane of *V. parishii* leaves. This compound was measured its melting point and recorded the value between 183.0 –185.0 °C, compared with mp 184.0 – 185.0 °C in previous study.[55] From the IR spectrum, it showed two significant signals, containing of stretching of hydroxyl group at 3440, and stretching of carbon of double bond at 1639 cm^{-1}. Besides, measuring of the optical rotation of this compound gave the value as $[\alpha]^{29.2}_{D}$ +58.8° (the previous literature reported as $[\alpha]^{28}_{D}$ +66.4°, [50]). The ^{1}H NMR spectrum of this triterpene displayed some obvious signals, for example, the proton on C-3 position showed a quartet peak at 3.23 ppm with coupling constant (*J*) 5.2 Hz, and the proton on carbon 12 of endocyclic double bond in ring C indicated a triplet signal at 5.13 ppm with coupling constant (*J*) 2.6 Hz, respectively.

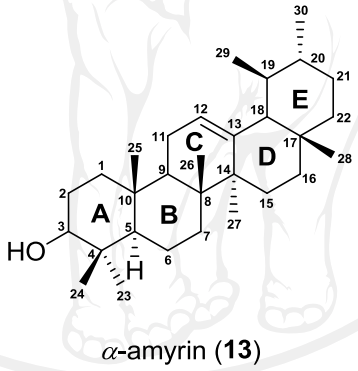


Figure 65 Labeling number of each carbon in structure of *α*-amyrin (**13**)

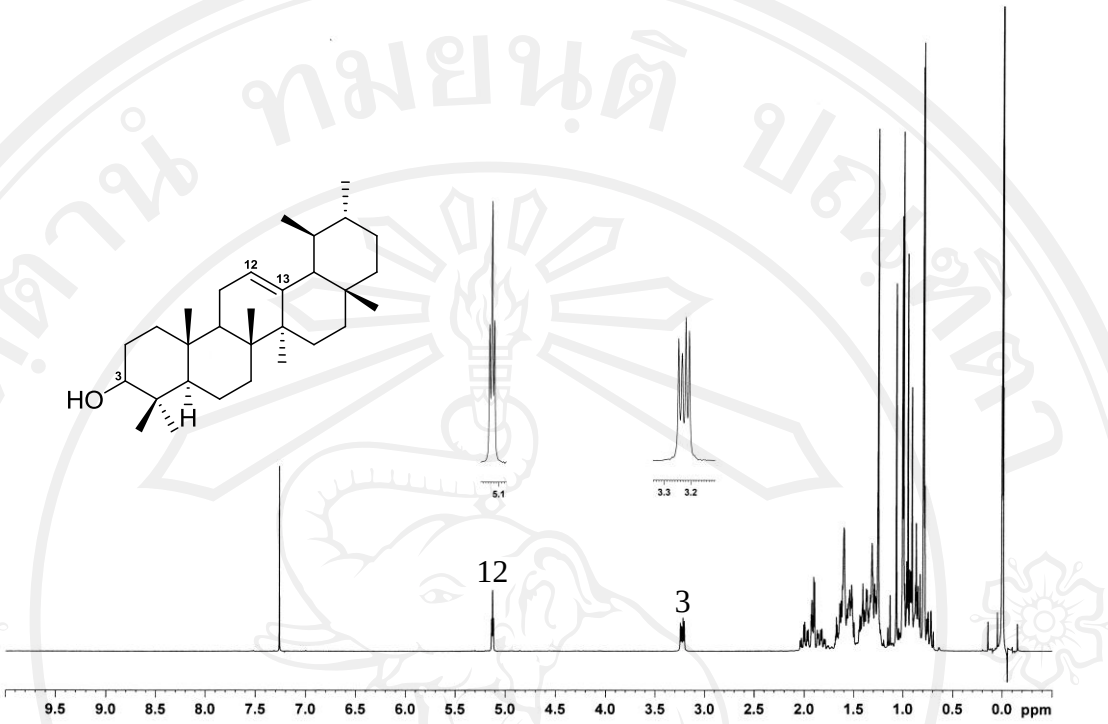


Figure 66 ^{1}H NMR spectrum of α-amyrin (**13**)

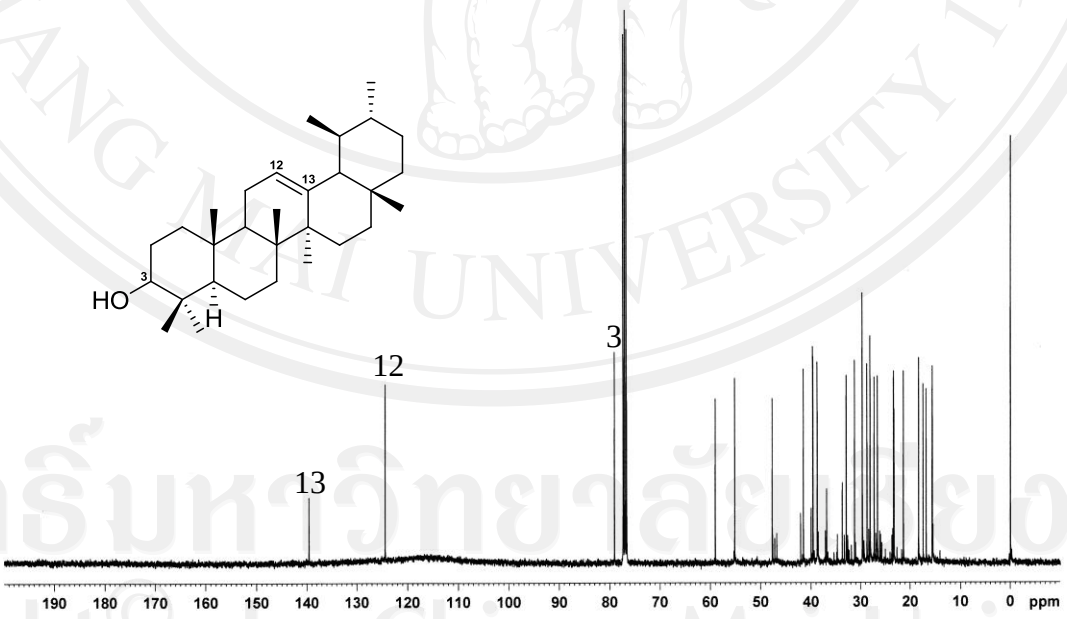


Figure 67 ^{13}C NMR spectrum of α-amyrin (**13**)

Likewise, the ^{13}C NMR spectrum of compound **13** also indicated some important peaks. For instance, carbon 3 that bonded with hydroxyl group showed signal at 79.1 ppm, and endocyclic double bond carbons, C-12 and C-13, displayed two peaks at 124.4 and 139.6 ppm, respectively. All spectrum of this compound from the experiment, presented in Table 13 and 14, were compared with other reports.[41, 55]

Table 13 ^{1}H NMR spectrum of α-amyrin (**13**) ($CDCl_3$) at 400 MHz

Carbon position	Chemical shift (δ; ppm)
3	3.23 [*q*, 1H];
	J = 5.2 Hz
12	5.13 [*t*, 1H];
	J = 3.6 Hz
23	1.00 [*s*, H]
24	0.79 [*s*, H]
25	0.95 [*s*, H]
26	1.01 [*s*, H]
27	1.07 [*s*, H]
28	0.80 [*s*, H]
29	0.78 [*s*, H]
30	0.91 [*s*, H]

Table 14 ^{13}C NMR spectrum of α-amyrin (**13**) ($CDCl_3$) at 100 MHz

Carbon position	Chemical shift (δ; ppm)
1	38.8
2	27.3
3	79.1
4	38.8
5	55.2
6	18.3

Table 14 ^{13}C NMR spectrum of α-amyrin (**13**) ($CDCl_3$) at 100 MHz (cont.)

Carbon position	Chemical shift (δ; ppm)
7	32.9
8	39.6
9	47.7
10	36.9
11	23.4
12	124.4
13	139.6
14	42.1
15	29.7
16	26.6
17	33.7
18	59.1
19	39.7
20	39.6
21	31.2
22	41.5
23	28.7
24	15.6
25	15.7
26	16.8
27	23.5
28	28.1
29	17.5
30	21.4

To confirm the result, the correlation between ^{1}H and ^{13}C NMR spectrum were considered from HMQC data, consisting of the proton on C-3 position at 3.23 ppm was related with hydroxyl carbon (C-3) at 79.1 ppm, and the proton of endocyclic double bond (H-12) at 5.13 ppm were correlated with endocyclic double bond in ring C at 124.4 ppm.

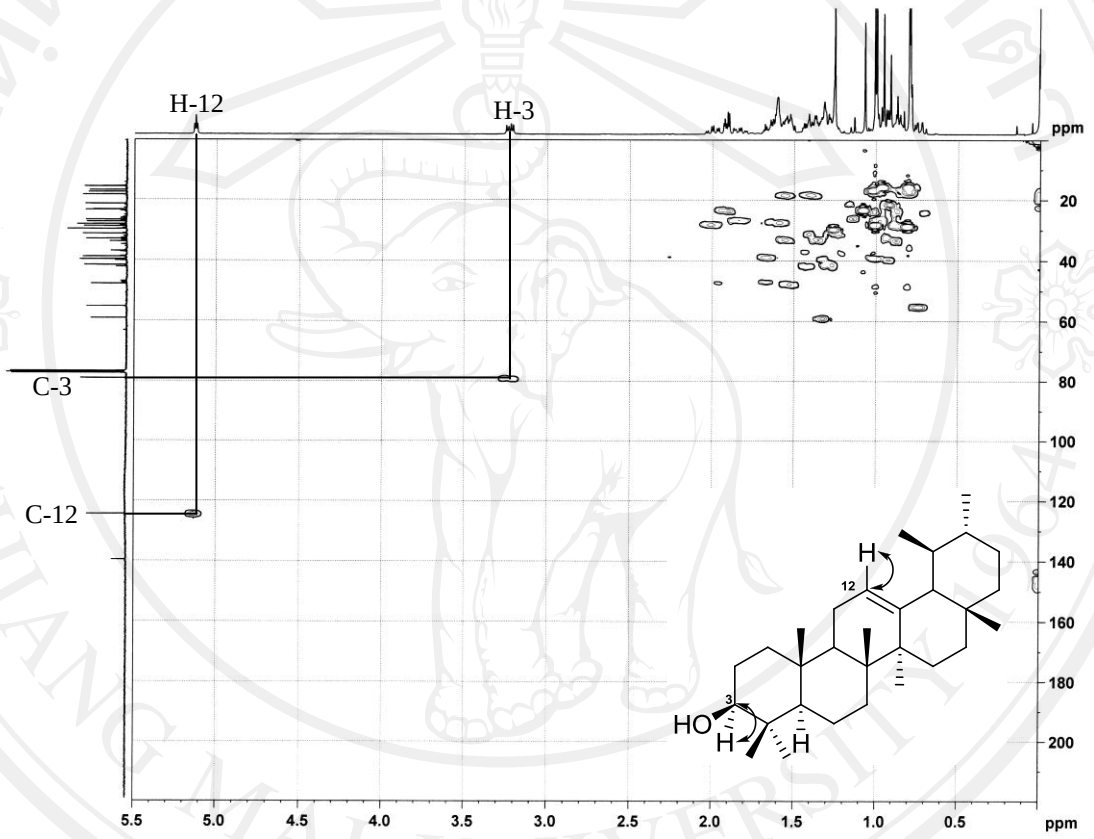


Figure 68 HMQC data of *α*-amyrin (**13**)

The coupling between two or three bonds of proton and carbon was descripted by HMBC data. For instance, proton 3 on hydroxyl carbon (C-3) related with carbon 2 and 4, and proton 12 of endocyclic double bond in ring C indicated relationship with carbon 11 and 13.

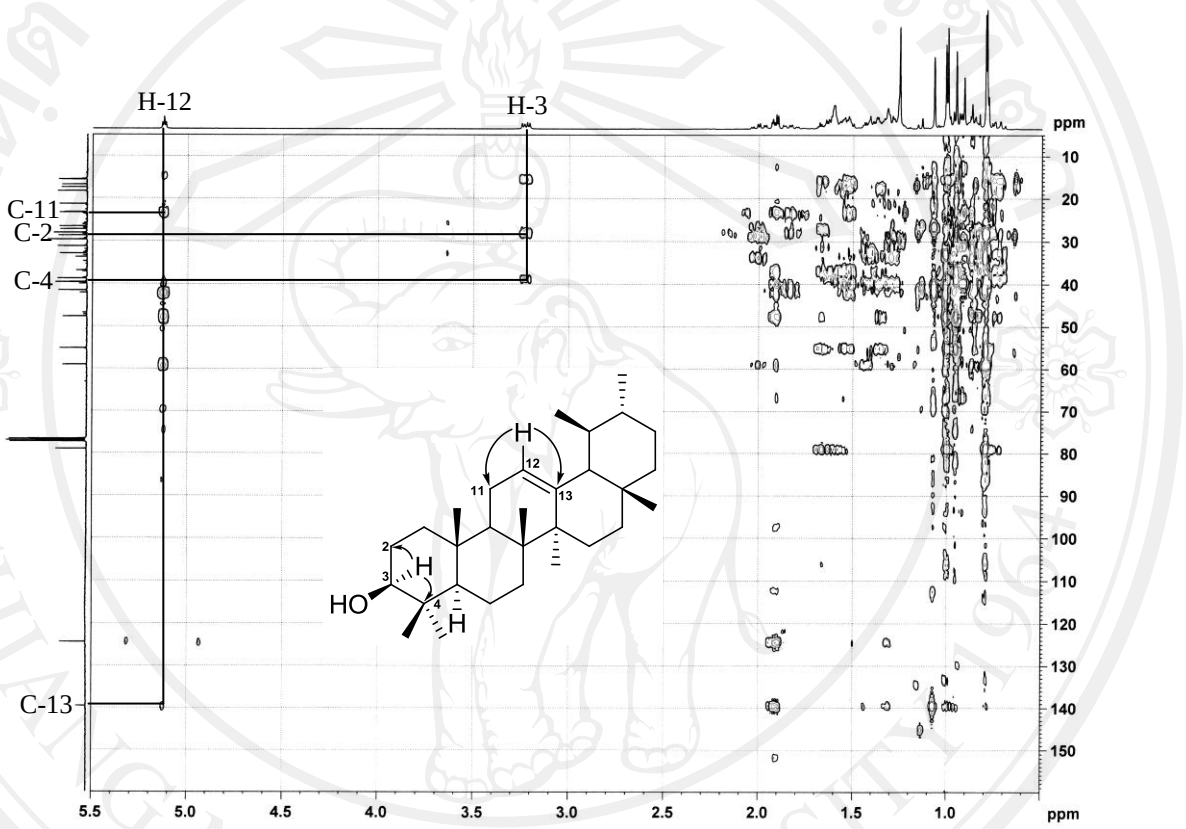


Figure 69 HMBC data of α-amyrin (**13**)

From EI-MS data, the molecular ion peak [M^+] of compound **13** was found as *m/z* 426. Furthermore, the other key fragments were observed from mass spectrum at *m/z* 219, 207, and 189. The mechanism of fragmentation of this triterpene was explained base on previous literature [38, 39], which was shown in Figure 70.

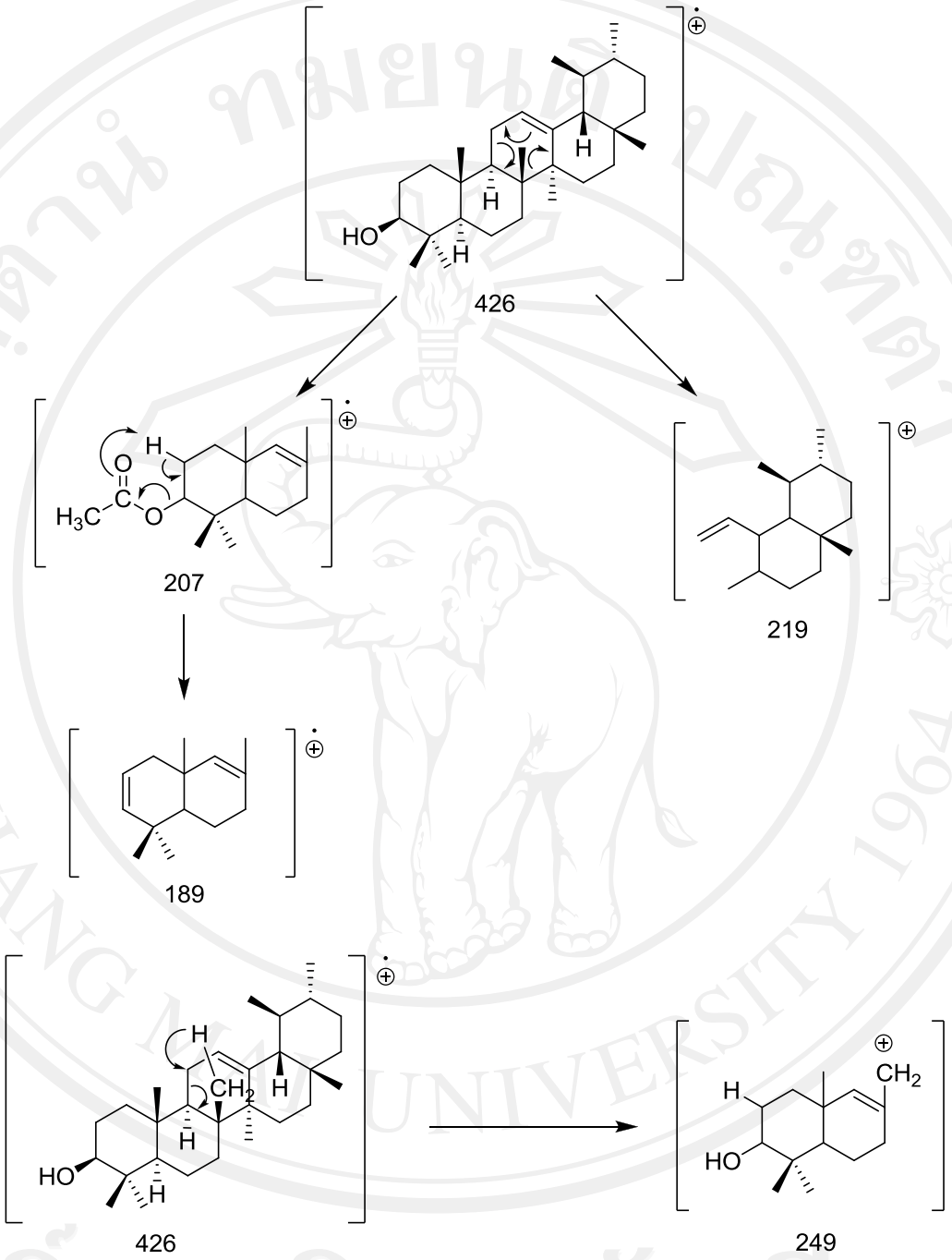
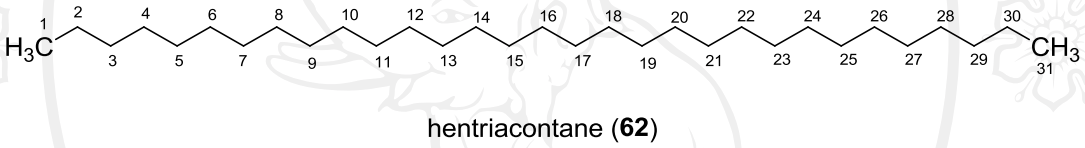


Figure 70 Mechanism of key fragment ions of *α*-amyrin (**13**) from EI-MS

Hentriacontane (**62**); a phytochemical hydrocarbon that were found in many plants [56-60], was recrystallized by 100% dichloromethane from fraction VPD-F01 of dichloromethane extract of *V. parishii* leaves in white solid form. This compound was measured its melting point which was observed and recorded between 68.8 – 69.5 °C, compared with mp 68.0 – 68.5 °C that were reported in previous literatures [61, 62]. Moreover, the IR spectrum of this hydrocarbon showed common signals of alkane, stretching of CH_3 and CH_2 at 2985, 2924, and 2851 cm^{-1}. The ^1H NMR of compound indicated two general peaks of hydrocarbon chain, containing of methyl protons of C-1 and C-31 position at 0.90 ppm, triplet signal with coupling constant (*J*) 6.7 Hz, and methylene protons, H-2 – H-30, which was multiplet peak at 1.28 ppm.



hentriacontane (**62**)

Figure 71 Labeling number of each carbon in structure of hentriacontane (**62**)

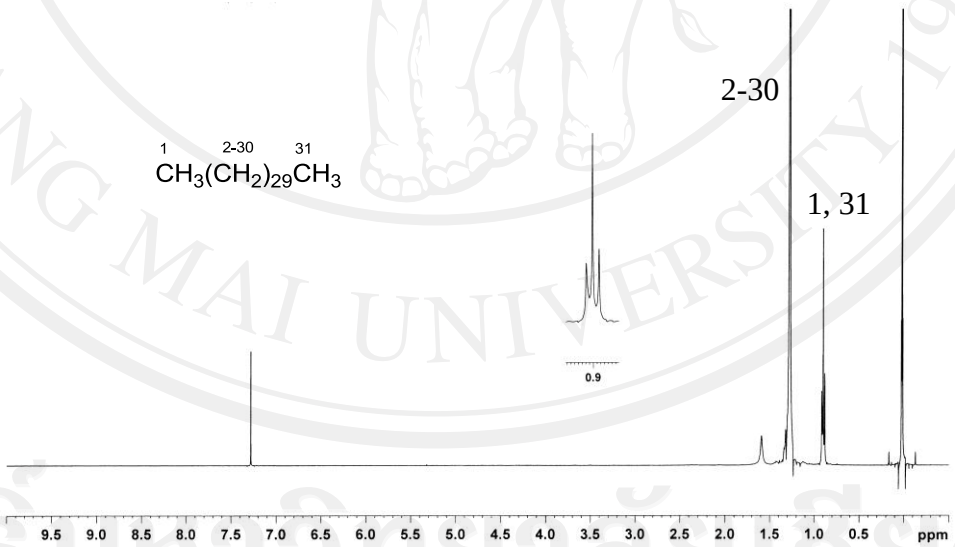


Figure 72 ^1H NMR spectrum of hentriacontane (**62**)

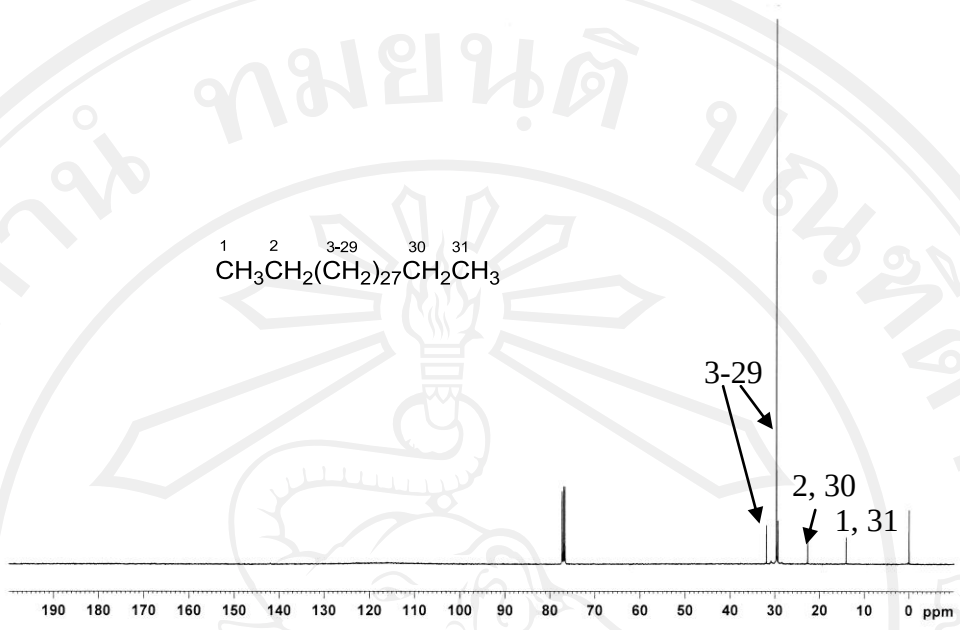
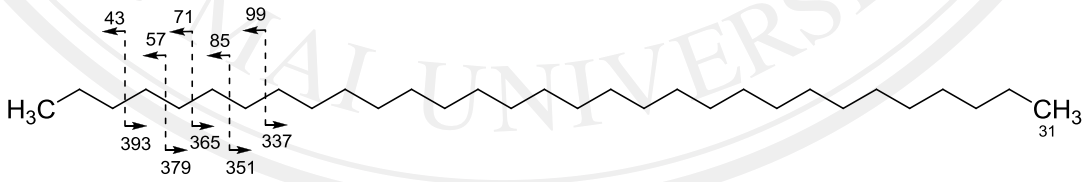


Figure 73 ^{13}C NMR spectrum of hentriacontane (**62**)

In the same way, the ^{13}C NMR spectrum of this hydrocarbon indicated common signals of saturated chain, consisting of methyl carbons, C-1 and C-31, showed peak at 14.1 ppm, methylene carbon that bonded with methyl group, C-2 and C-30, displayed peak at 22.7 ppm, and methylene carbons, C-3 – C-29, in saturated chain at 29.4, 29.7, and 31.9 ppm.

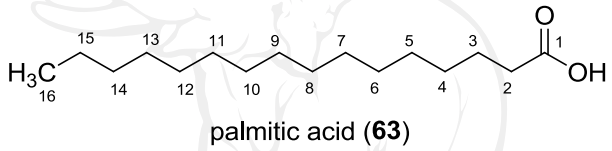


436

Figure 74 Mechanism of key fragment ions of hentriacontane (**62**) from EI-MS

From EI-MS data, the molecular ion peak [M^+] of the compound was found as *m/z* 436. Moreover, the other key fragments were observed from mass spectrum at *m/z* 393, 379, 365, 351, 337, 99, 85, 71, 57 and 43. The fragmentation of hentriacontane was explained the mechanism in previous literatures [38, 39], which was shown in Figure 74.

Palmitic acid (**63**); or hexadecanoic acid is a common fatty acid found in many species [63, 64] which was recrystallized from fraction VPD-F07 by hexane and dichloromethane with ratio 1:1. This compound was measured its melting point which was observed and recorded between 61.0 – 62.0 °C (the literature reported as 62.9 °C [65]). Besides, the IR spectrum of this hydrocarbon showed special signals of stretching of hydroxyl group at 3445 cm^{-1}, and stretching of carbonyl group at 1638 cm^{-1}. The ^{1}H NMR spectrum of this fatty acid showed common signals, containing of a triplet peak (J = 6.5 Hz) of methyl protons, H-16, at 0.88 ppm, a singlet signal of methylene protons in side chain, H-4 – H-15, at 1.25 ppm, and H-2 (a triplet peak with J = 7.4 Hz) and H-3 (a quentet peak with J = 7.1 Hz) as methylene protons near carboxylic group at 2.34 and 1.62 ppm, respectively.



palmitic acid (**63**)

Figure 75 Labeling number of each carbon in structure of palmitic acid (**63**)

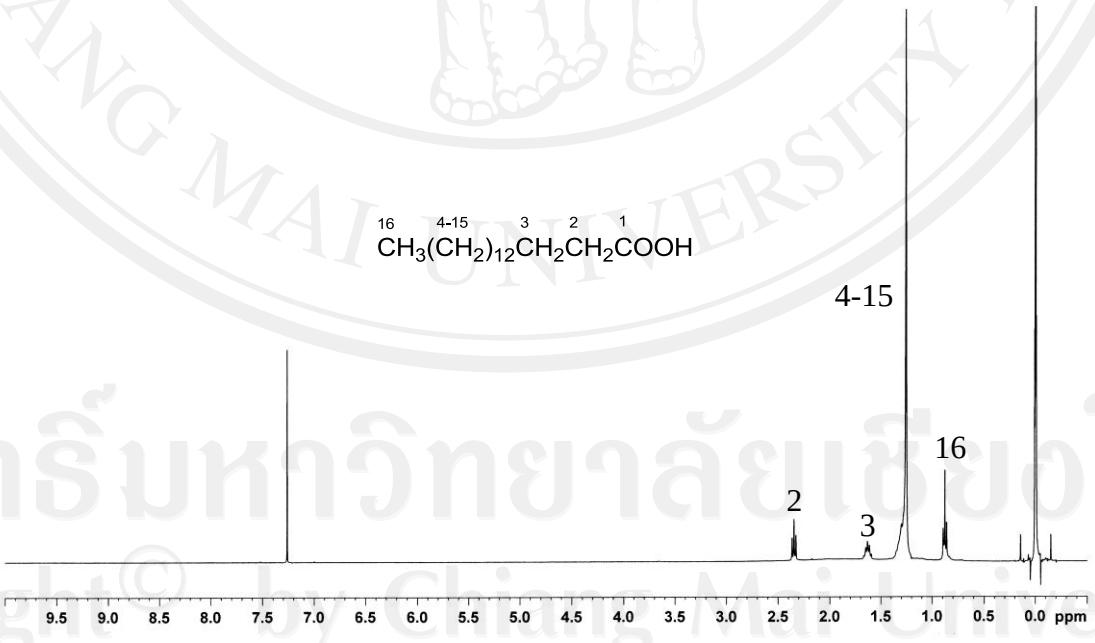


Figure 76 ^{1}H NMR spectrum of palmitic acid (**63**)

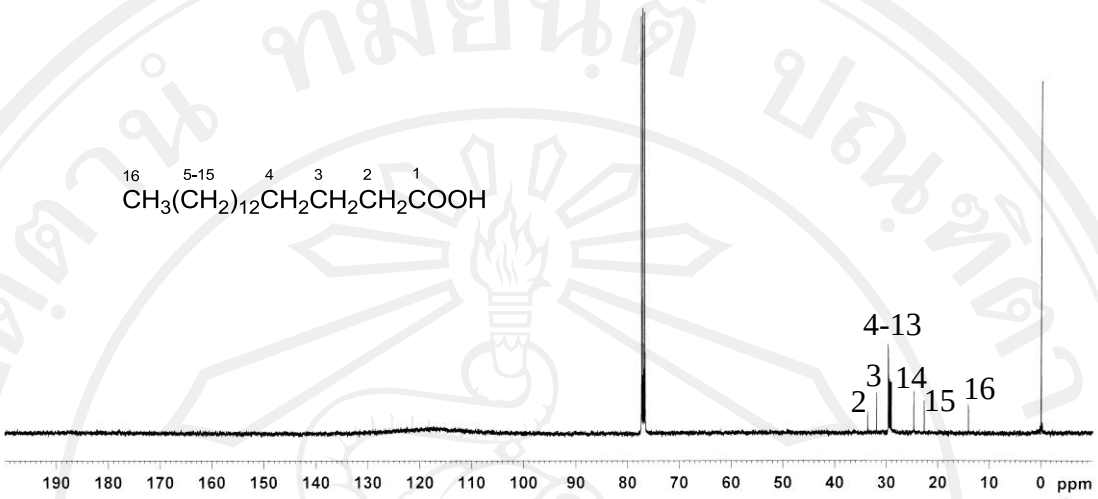


Figure 77 ^{13}C NMR spectrum of palmitic acid (**63**)

Likewise, the ^{13}C NMR spectrum of this compound displayed common signals of saturated chain, consisting of methyl carbons, C-16, showed peak at 14.1 ppm, methylene carbon that bonded with methyl group, C-15, displayed peak at 22.7 ppm, methylene carbons, C-2 – C-14, in side chain between 24.7 – 33.7 ppm, respectively.

To make sure the number of proton in side chain, D_2O was added to palmitic acid solution for changing H_2O form in $CDCl_3$ at 1.56 ppm to D_2O form at 4.76 ppm. And then, integration of peak area of hydrocarbon chain before added D_2O (48.95) was compared with after added D_2O (33.72), indicated in Figure 78 and 79, which the number of proton in main core structure should be the value as 31.

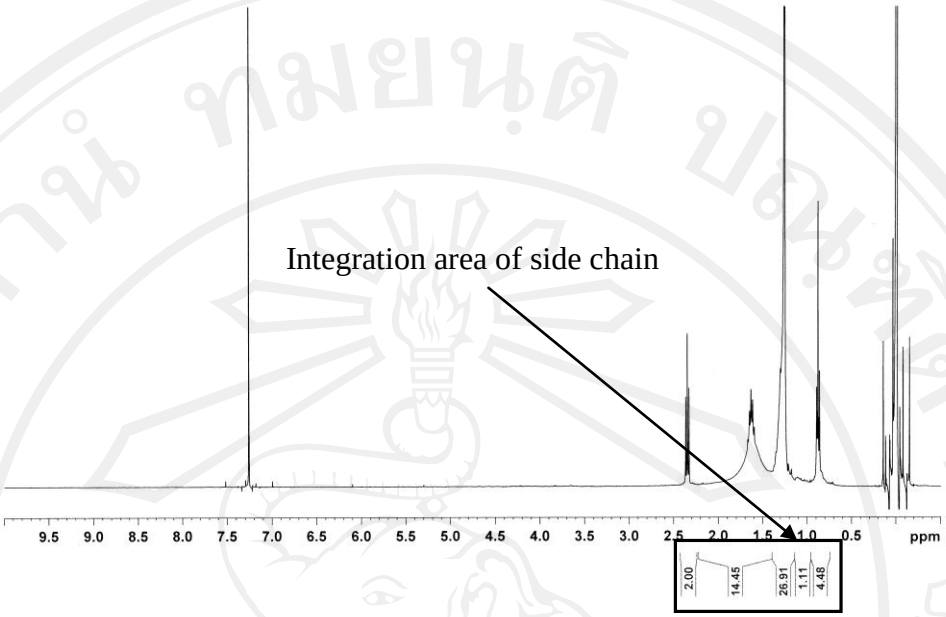


Figure 78 ^{1}H NMR spectrum of palmitic acid (**63**) before added D_2O

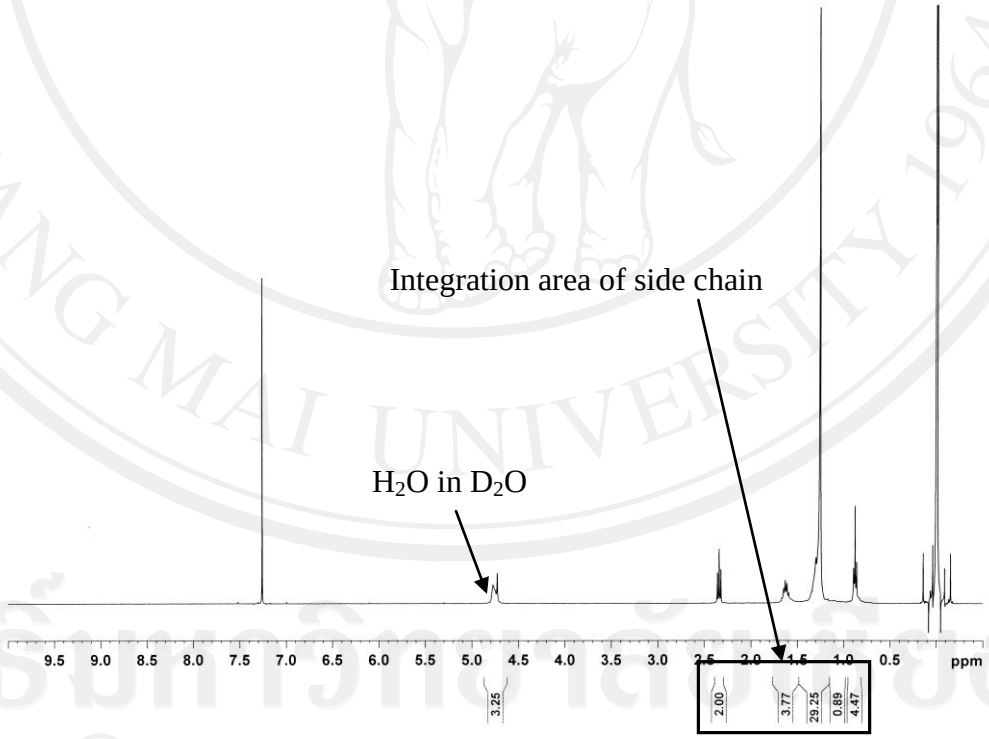


Figure 79 ^{1}H NMR spectrum of palmitic acid (**63**) after added D_2O

4.3 Biological activities of isolated compounds from previous literatures

The nine compounds, stigmasterol (**58**), α-amyrin acetate (**9**), lupeol acetate (**59**), lupeol palmitate (**60**), lupenone (**61**), lupeol (**41**) α-amyrin (**13**), hentriacontane (**62**), and palmitic acid (**63**), separated from *V. parishii* leaves were well known, which were discovered and reported from other species. Furthermore, five triterpenes that were found in *V. parishii* were reported their bioassay in previous literatures, shown in Table 15.

Table 15 Bioassay of five triterpenes from previous literatures

Compound	Experimental Model	Activity	Reference
lupeol (**41**)	Antiplasmodial activities		
	Plasmodium falciparum (BHz26/86)[a]	45% GI[b] *in vitro* at 25 μg/mL	[69]
	P. falciparum (FCR-3)[c]	IC_{50}[d] *in vitro* 41 μg/mL	[53]
	P. falciparum (K1)[e]	IC_{50}[d] *in vitro* 5.0 μg/mL	[70]
	Anti-inflammatory and anti-arthritic activities		
	TPA1[f]	18 (0.5 mg/ear)	[71]
	TPA2[g]	36.2% (0.5 mg/ear)	[72]
	TPA3[h]	IC_{50} 0.48 mg/ear	[73]
lupeol acetate (**59**)	Anti-inflammatory and anti-arthritic activities		
	Croton oil[i]	72% (0.42 μM/ear)	[74]
lupeol palmitate (**60**)	Anti-inflammatory and anti-arthritic activities		
	Croton oil[i]	54% (0.42 μM/ear)	[74]
lupenone (**61**)	Antiplasmodial activities		
	P. falciparum (K1)[e]	I[j] *in vitro* 20 μg/mL	[75]

Table 15 Bioassay of five triterpenes from previous literatures (cont.)

Compound	Experimental Model	Activity	Reference
α-amyrin acetate (**9**)	Anti-inflammatory activities		
	TPA-induced inflammation in mice	IC_{50}[d] 0.61 μmol/ear	[76]

[a]BHz26/86 = chloroquin-resistant strain

[b]GI = growth inhibition

[c]FCR-3 = chloroquin-resistant strain

[d]IC_{50} = half inhibitory concentration

[e]K1 = multidrug-resistant strain

[f]TPA1 = TPA-ear oedema 2h pre-treated with test compound

[g]TPA2 = TPA-ear oedema with simultaneous administration of test compound

[h]TPA3 = without specification

[i]Croton oil = Croton oil-ear edema

[j]I = inactive

4.4 Biosynthesis of isolated triterpenes in *V. parishii*

The six triterpenes, *α*-amyrin acetate (**9**), lupeol acetate (**59**), lupeol palmitate (**60**), lupenone (**61**), lupeol (**41**) *α*-amyrin (**13**), which were isolated from *V. parishii*, were well known compounds which had biosynthesis from Mevalonate pathway.[77] The biosynthetic mechanism of triterpenes is shown in Figure 80.

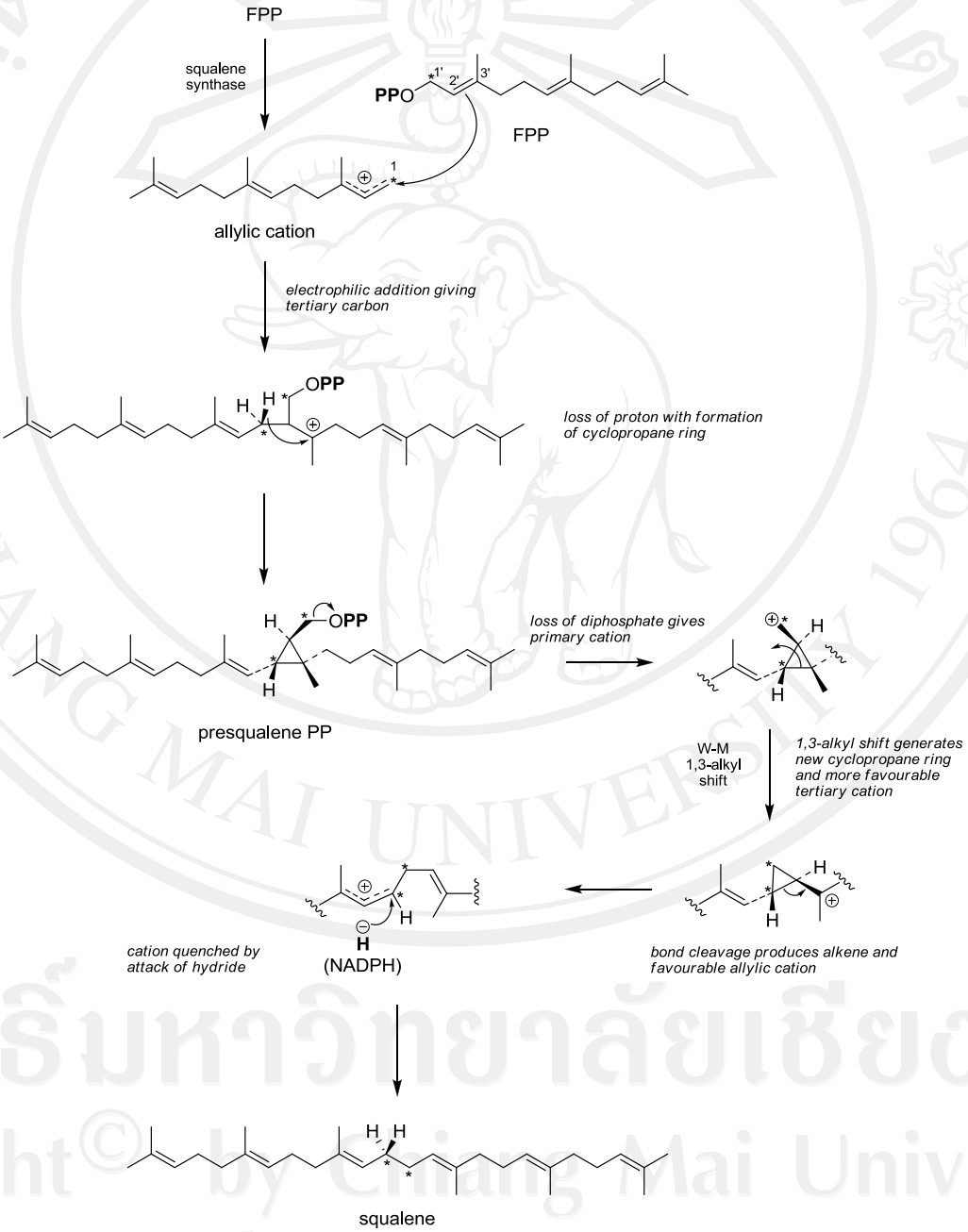


Figure 80 Biosynthesis of triterpenes *via* Mevalonate pathway

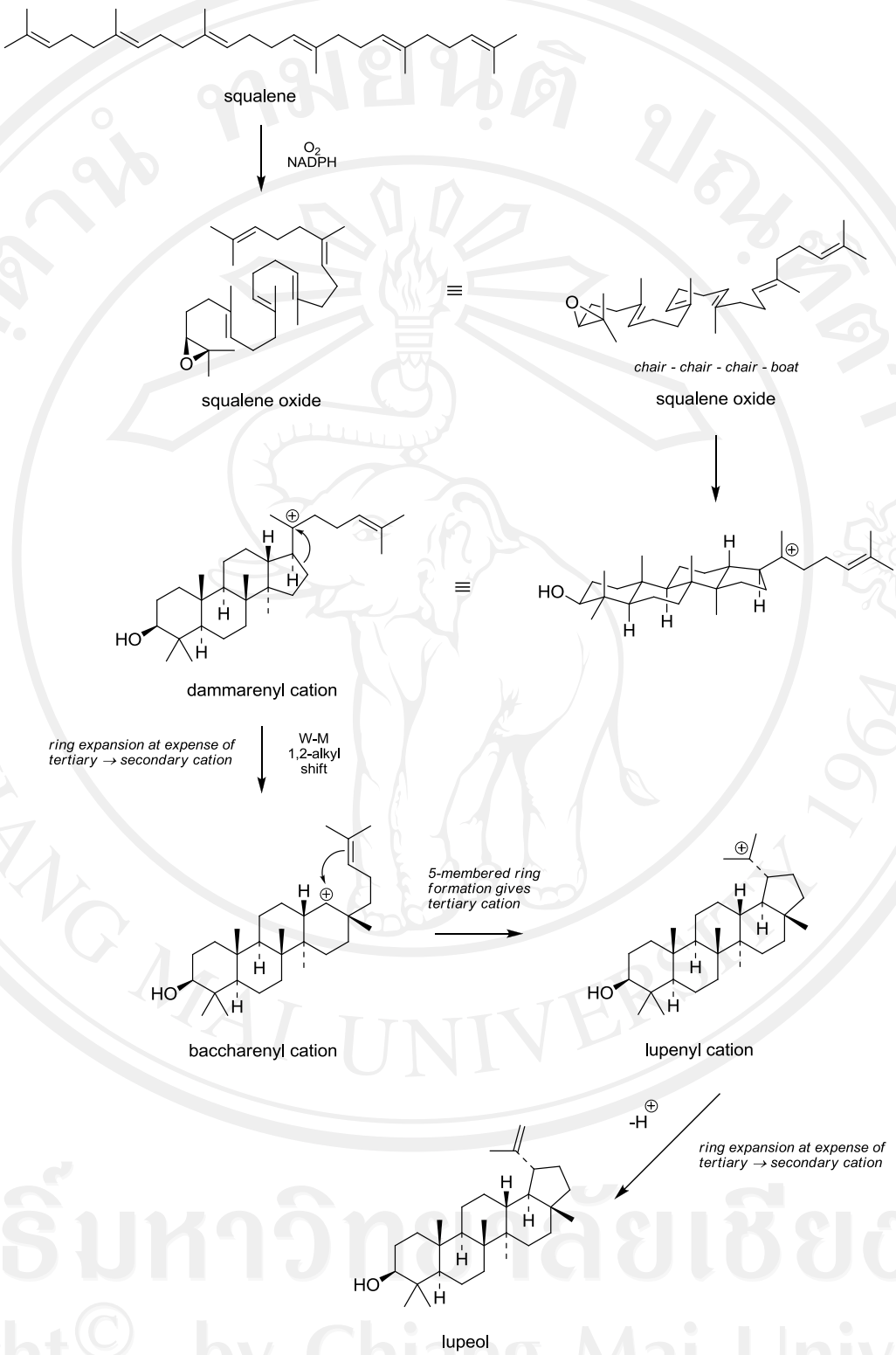


Figure 80 Biosynthesis of triterpenes *via* Mevalonate pathway (cont.)

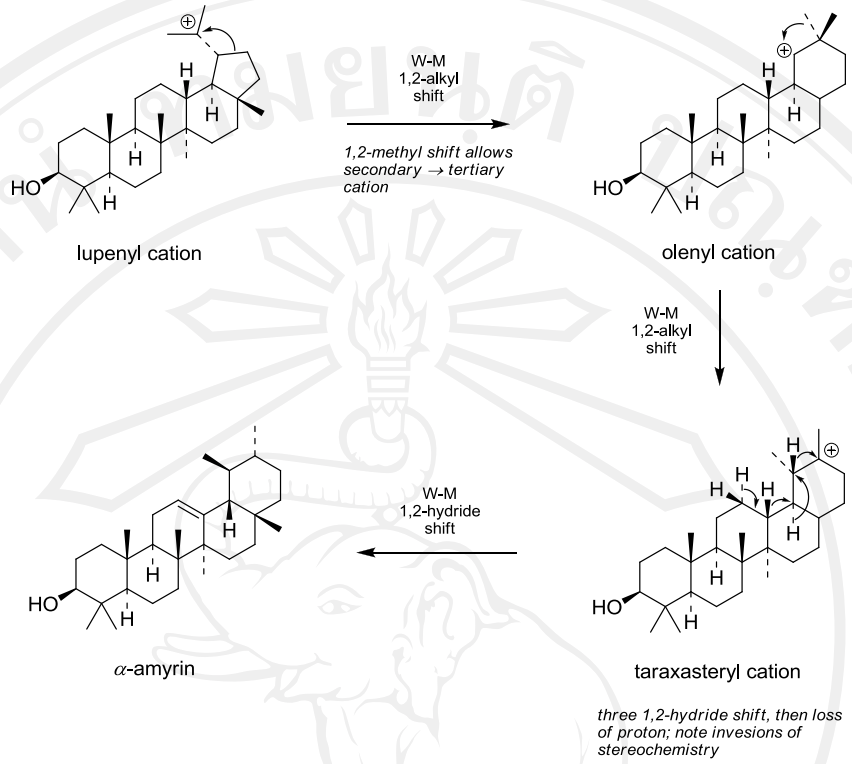


Figure 80 Biosynthesis of triterpenes *via* Mevalonate pathway (cont.)