APPENDIX

All spectra of isolated compounds from dichloromethane extract of *O. paniculata* twigs

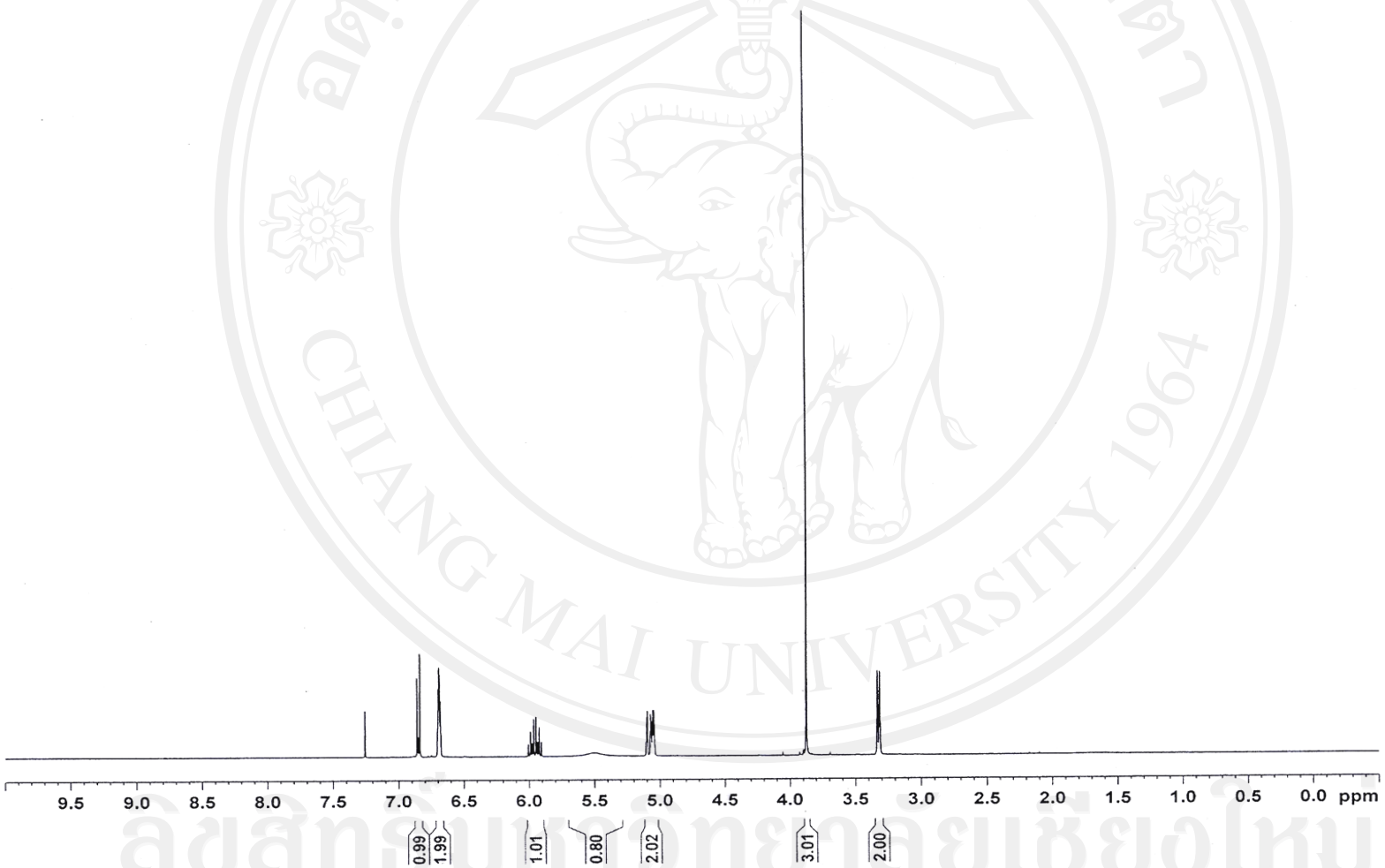

Figure 34 ^{1}H NMR spectrum of eugenol (**22**)

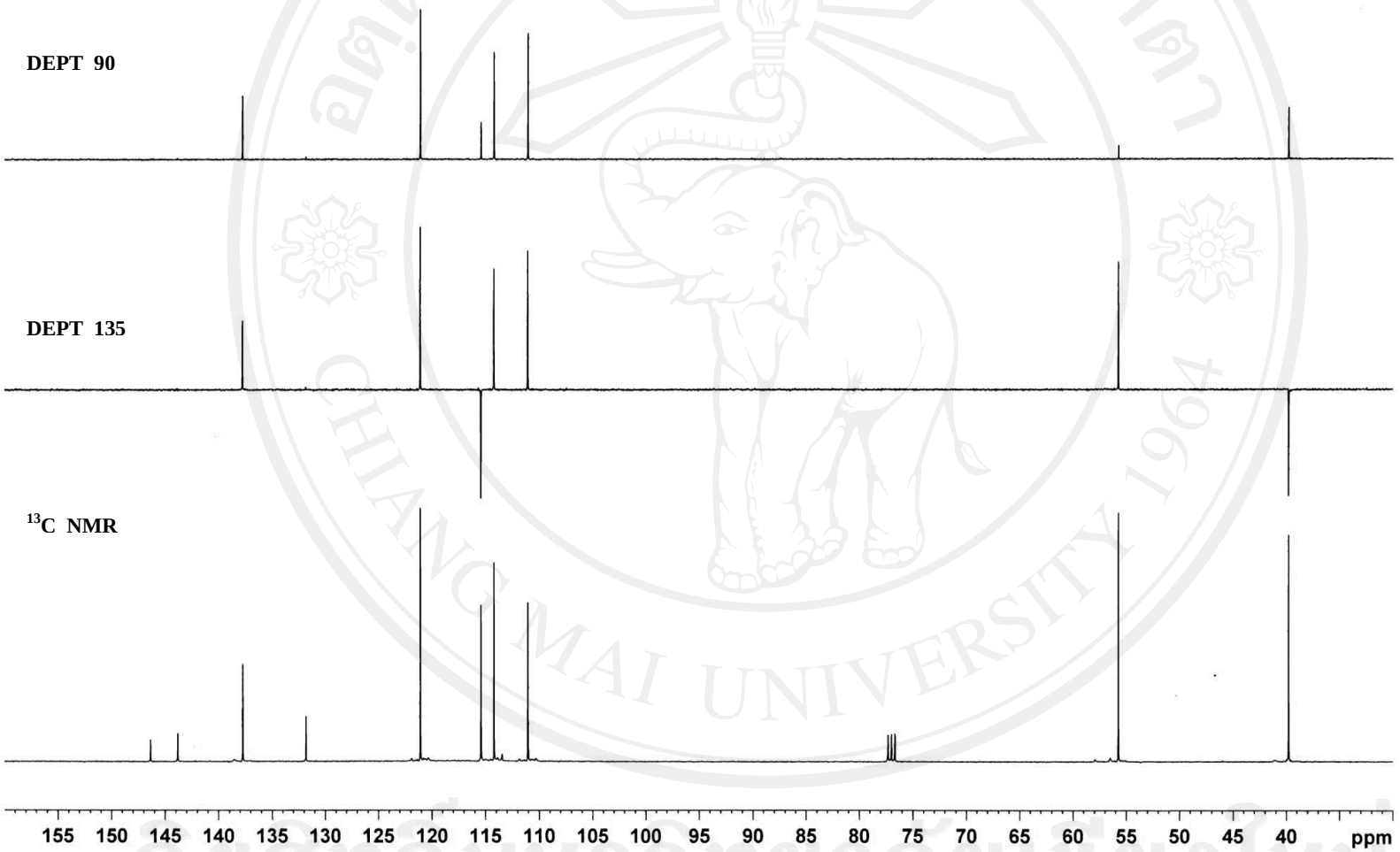

Figure 35 DEPT 90, DEPT 135 and ^{13}C NMR spectra of eugenol (**22**)

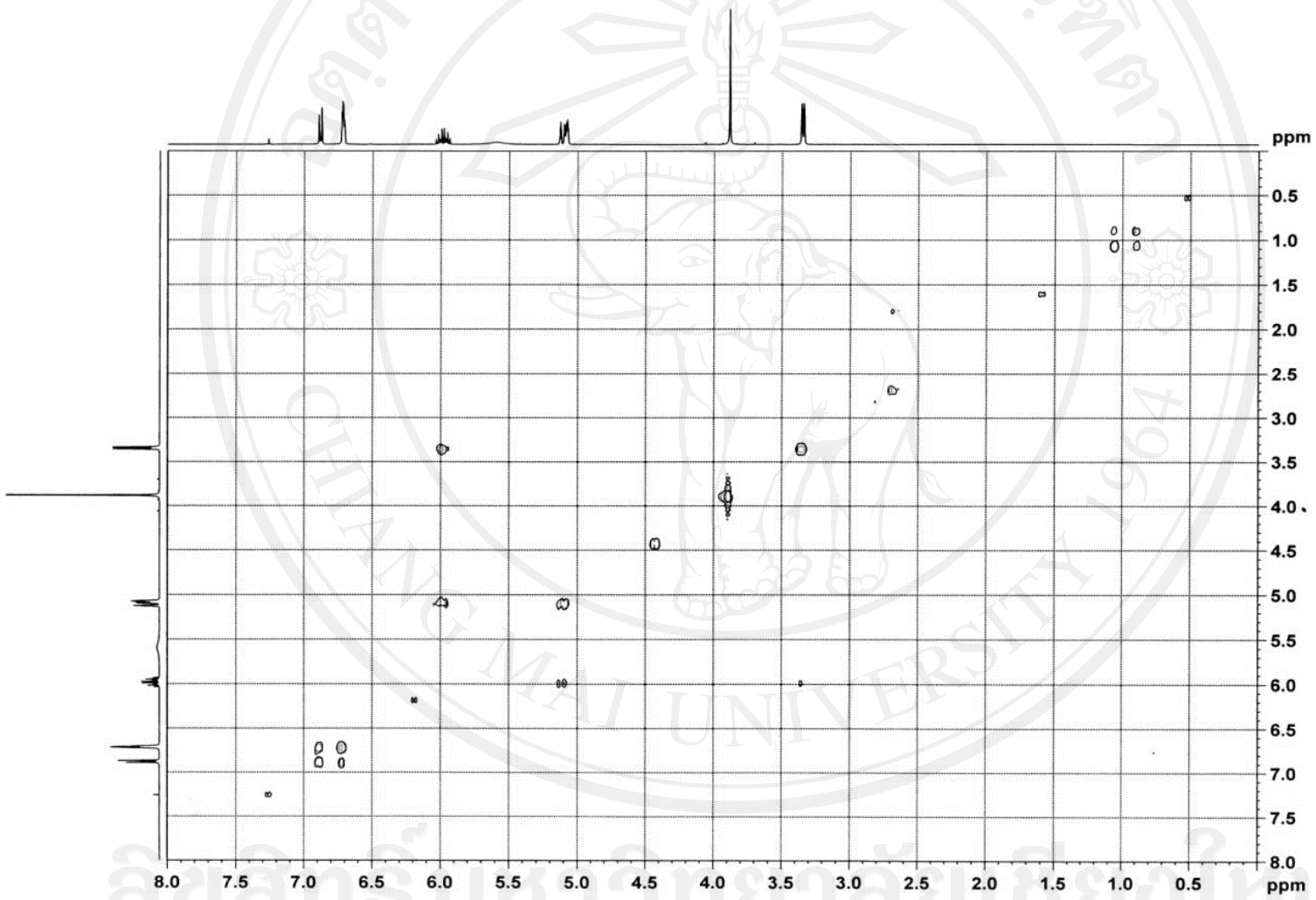

Figure 36 COSY spectrum of eugenol (**22**)

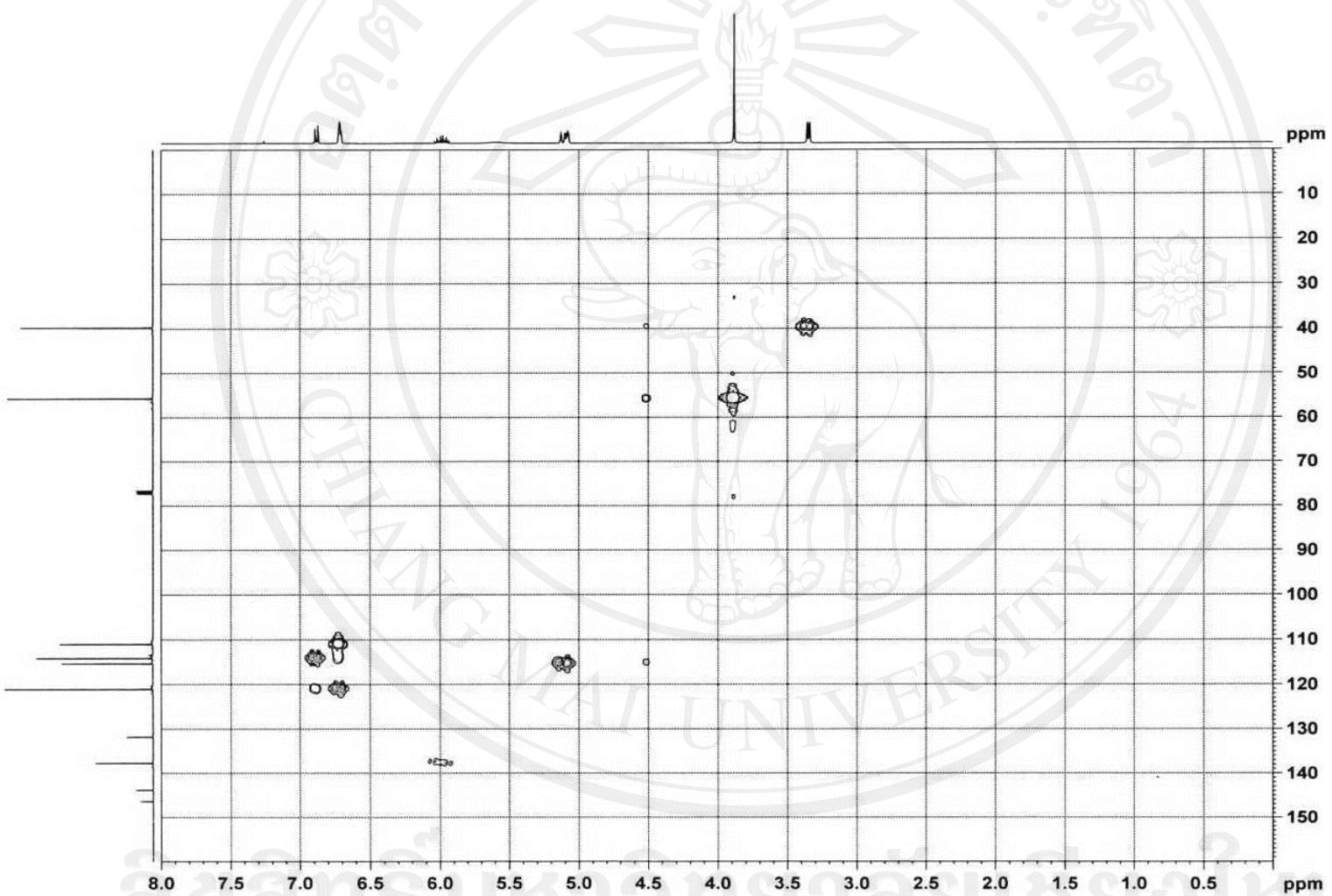

Figure 37 HMQC correlation of eugenol (**22**)

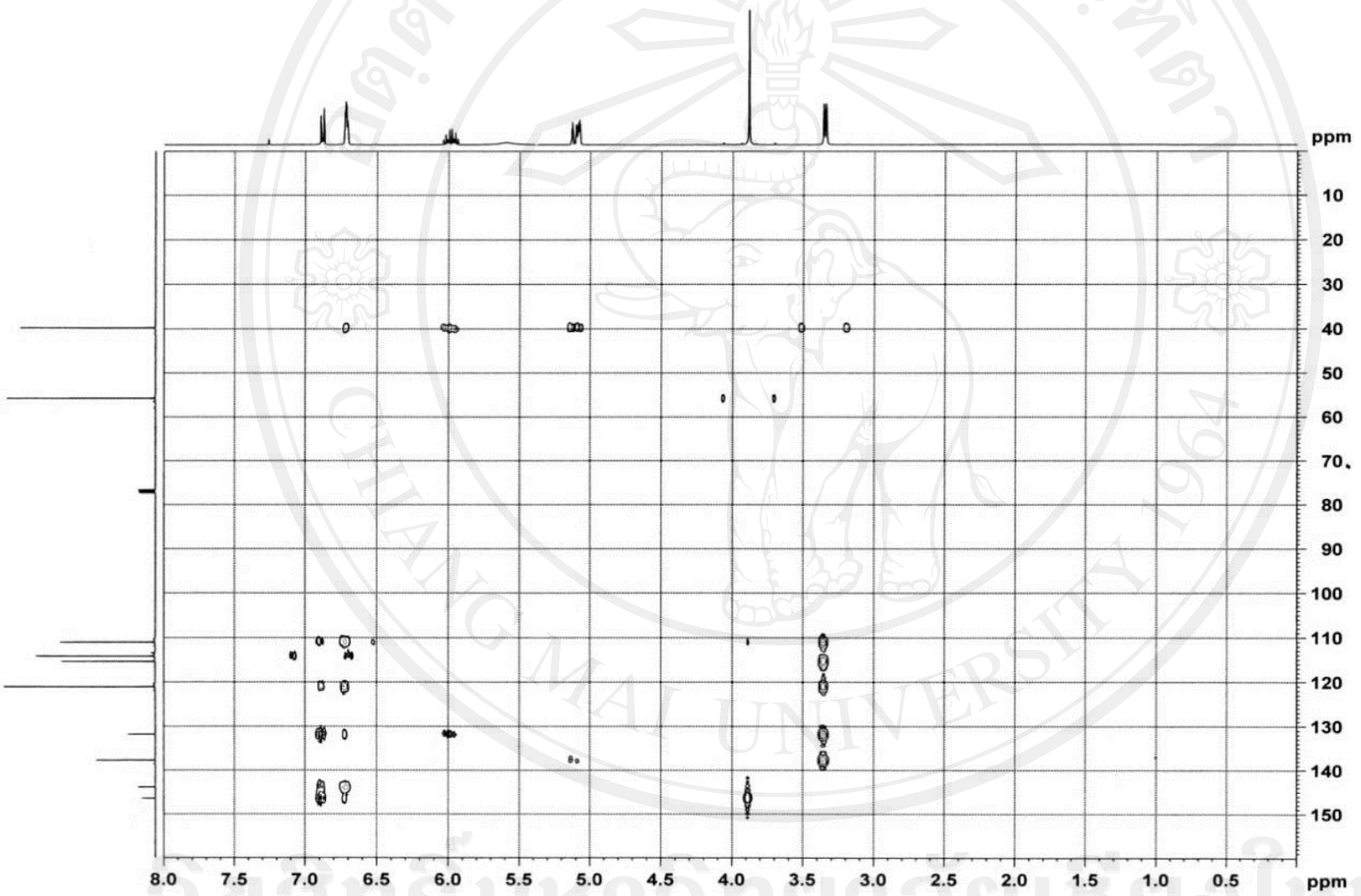

Figure 38 HMBC correlation of eugenol (**22**)

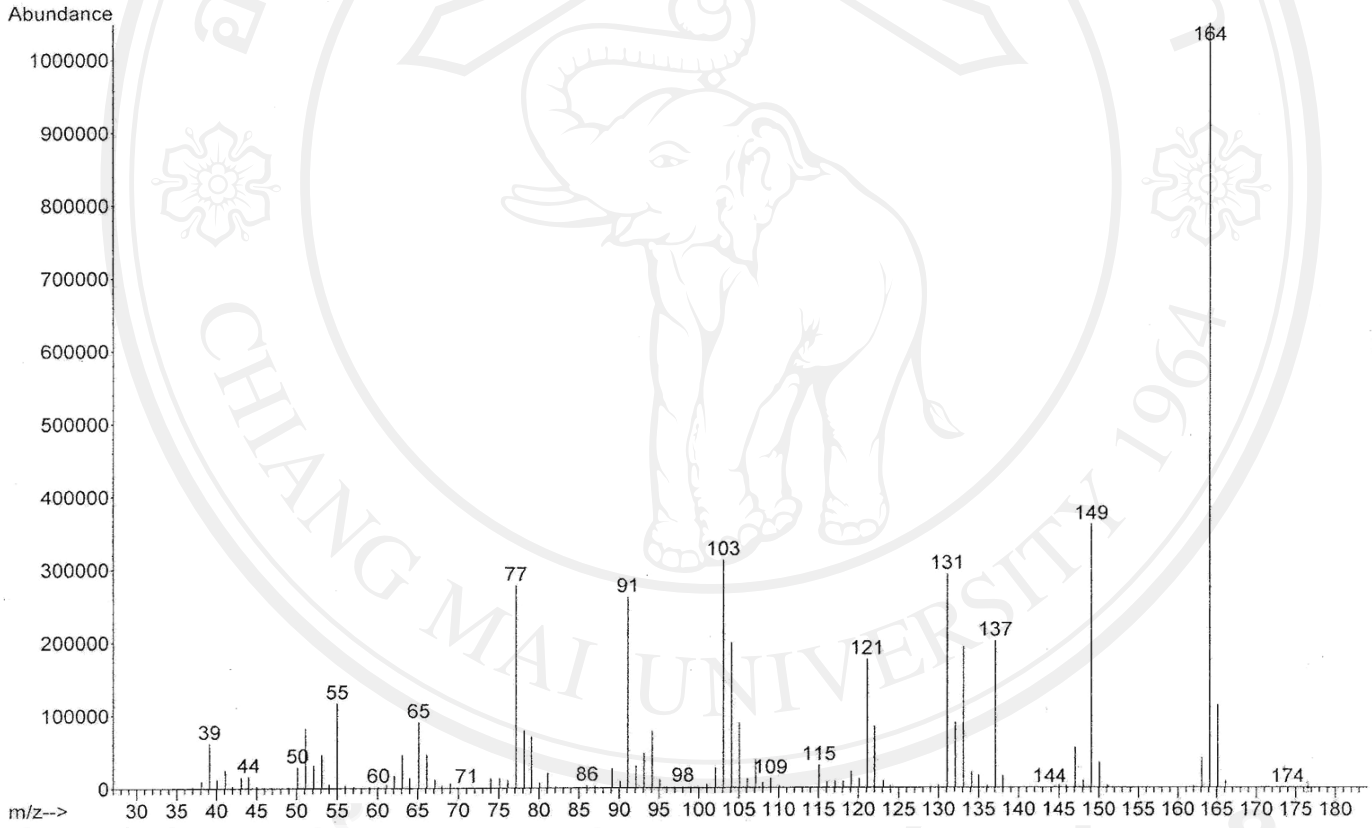

Figure 39 Mass spectrum of eugenol (**22**)

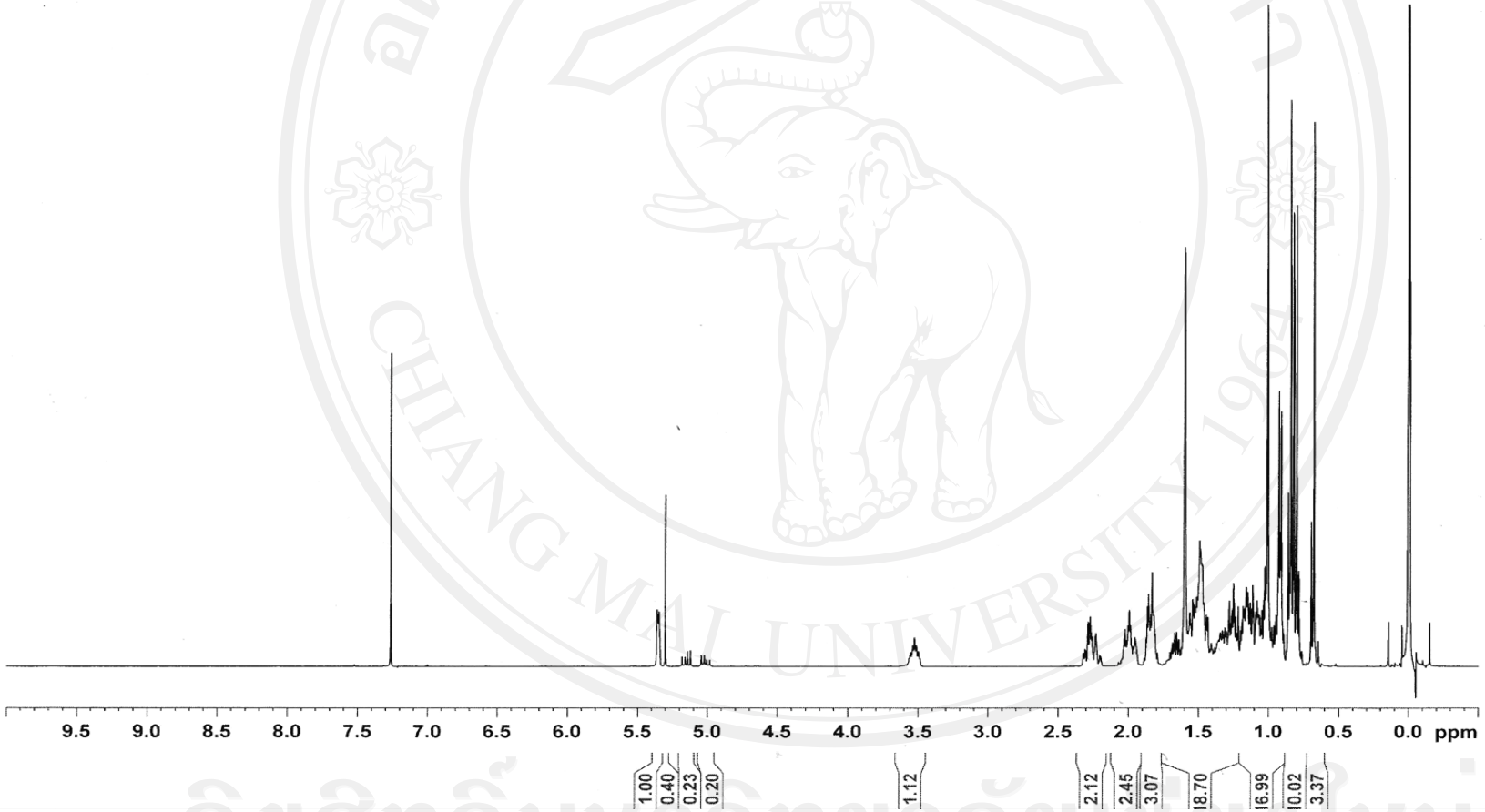

Figure 40 ^{1}H NMR spectrum of *β*-sitosterol (**23**) and stigmasterol (**24**)

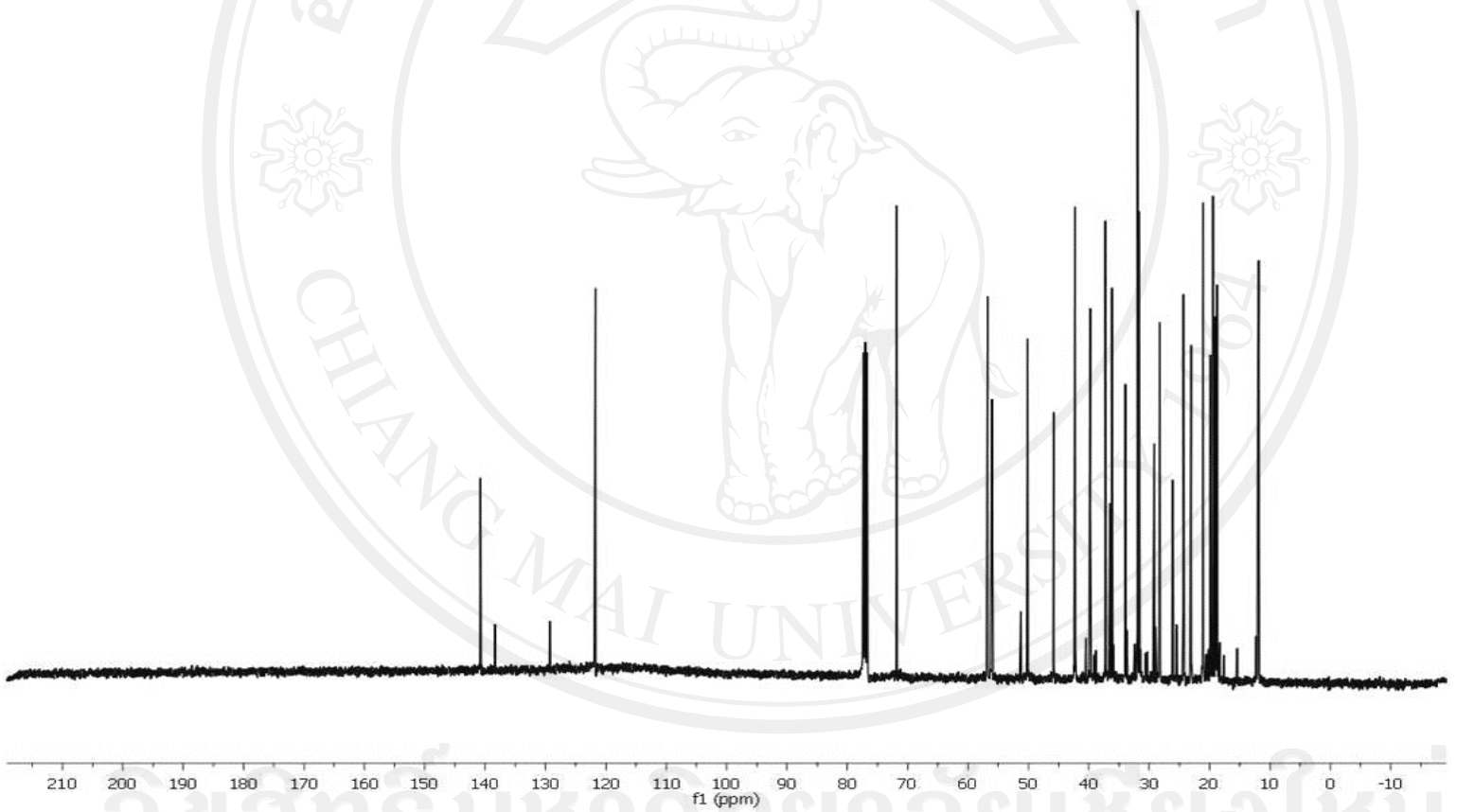

Figure 41 ^{13}C NMR of spectrum of β-sitosterol (**23**) and stigmasterol (**24**)

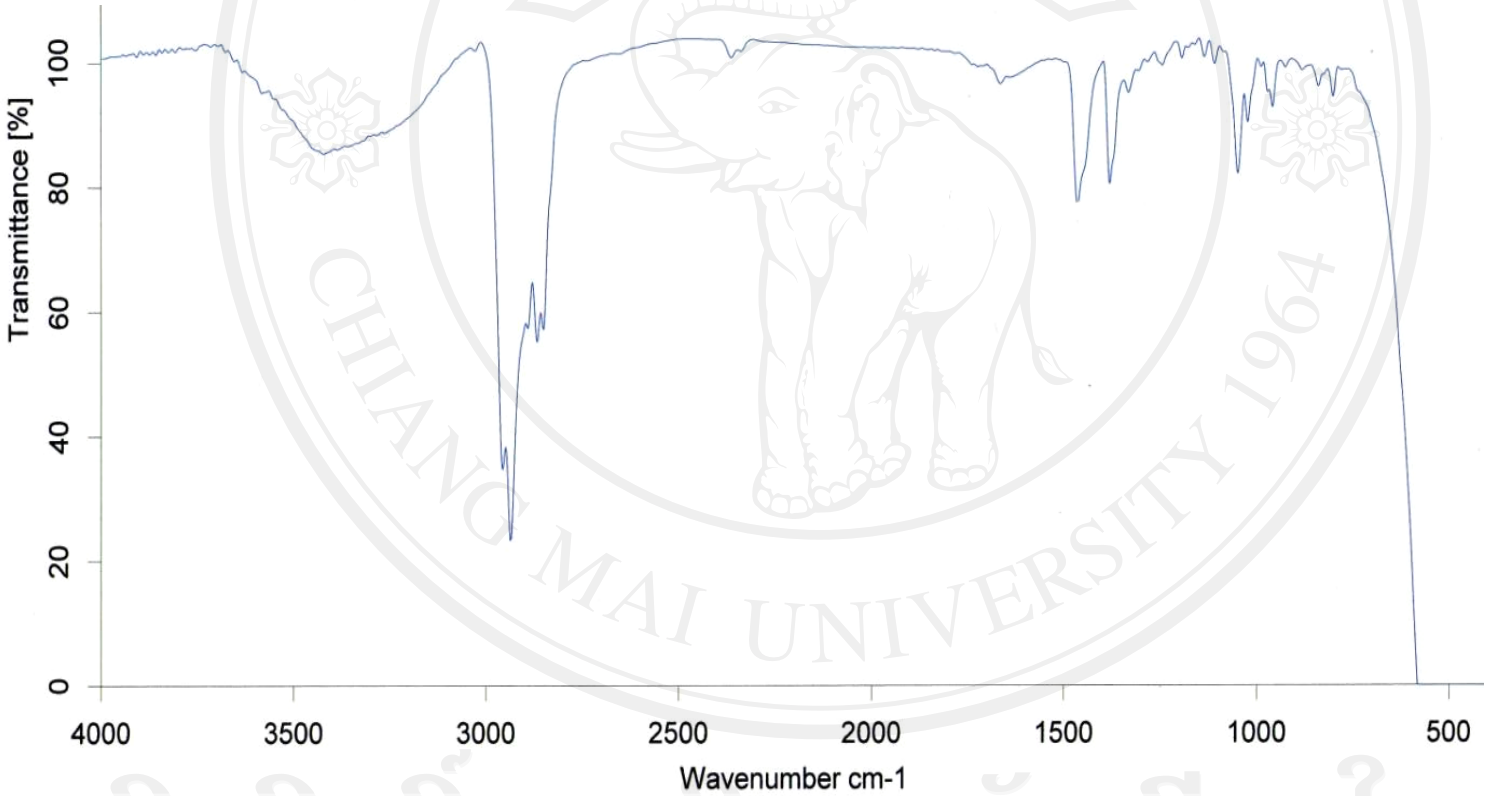

Figure 42 IR spectrum of spectrum of *β*-sitosterol (**23**) and stigmasterol (**24**)

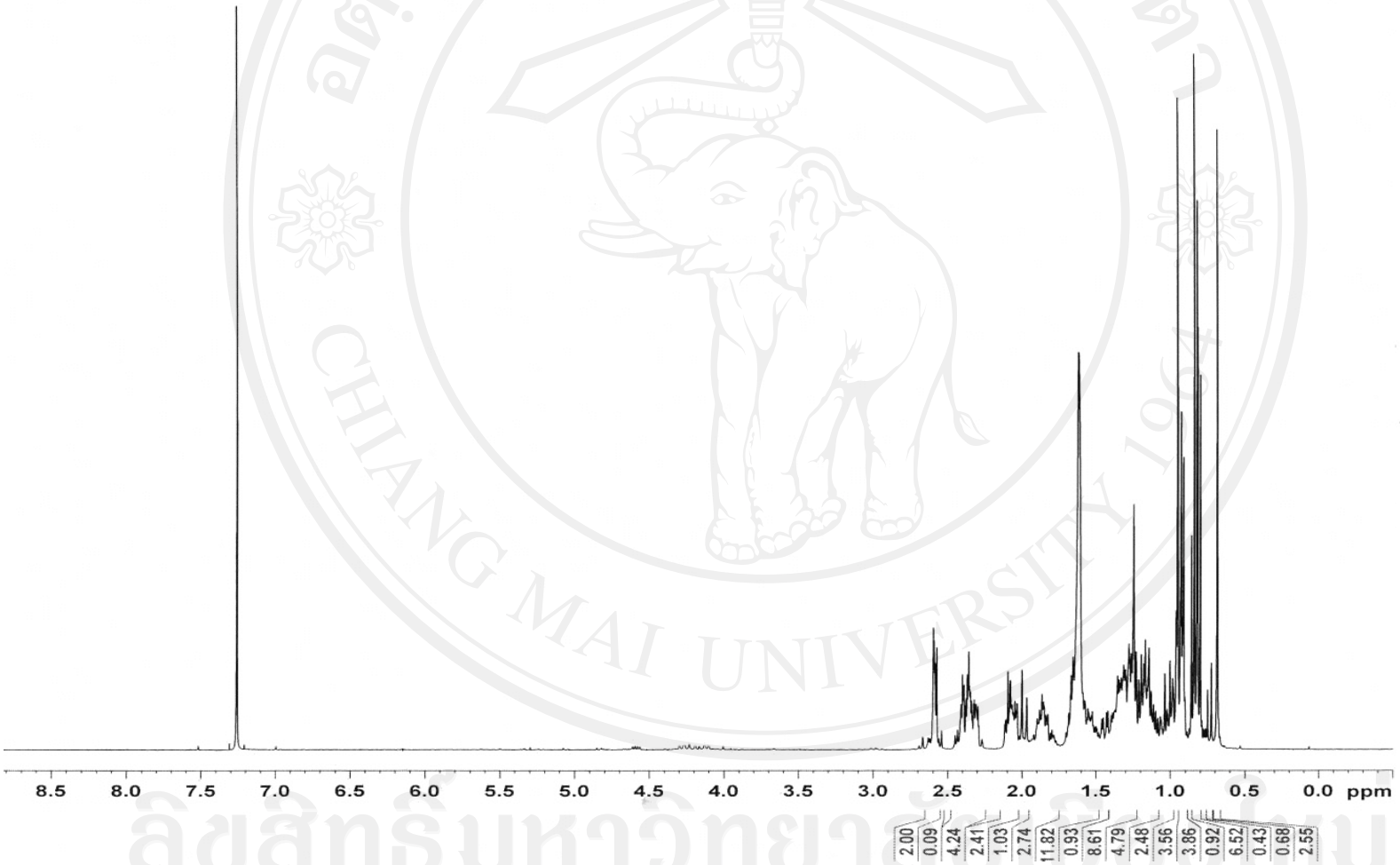

Figure 43 ^{1}H NMR spectrum of stigmastan-3,6-dione (**25**)

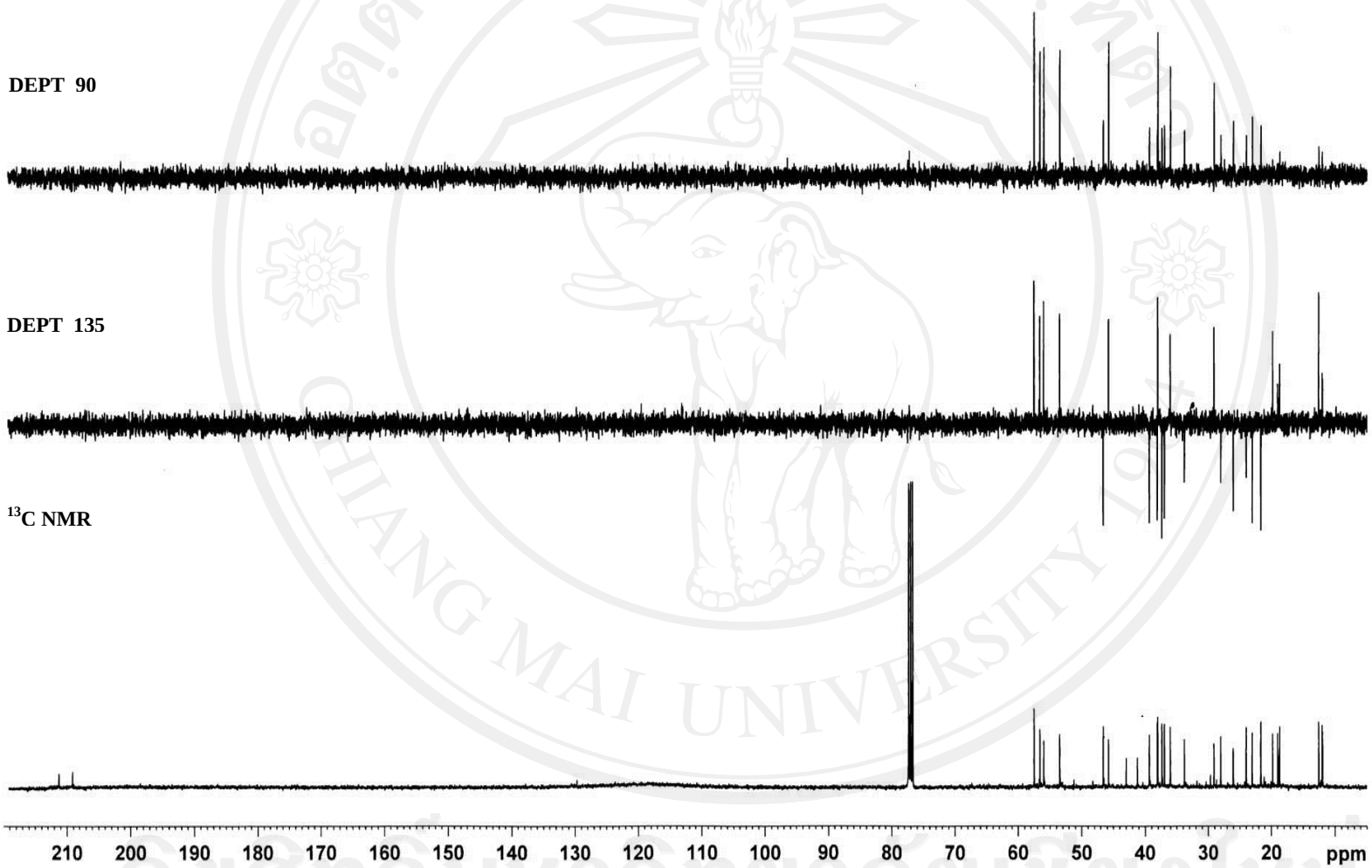

Figure 44 DEPT 90, DEPT 135 and ^{13}C NMR spectra of stigmastan-3,6-dione (**25**)

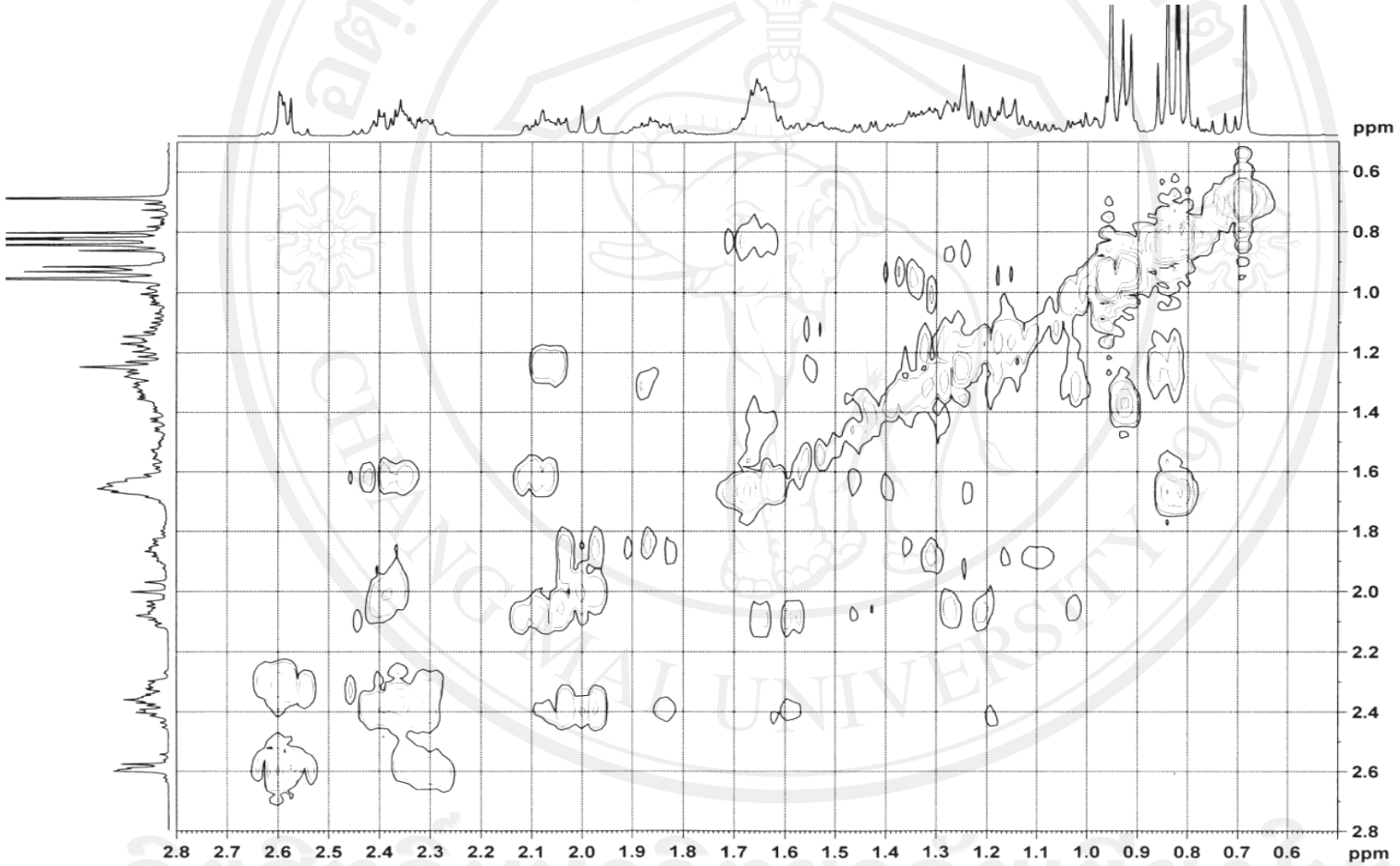

Figure 45 COSY spectrum of stigmastan-3,6-dione (**25**)

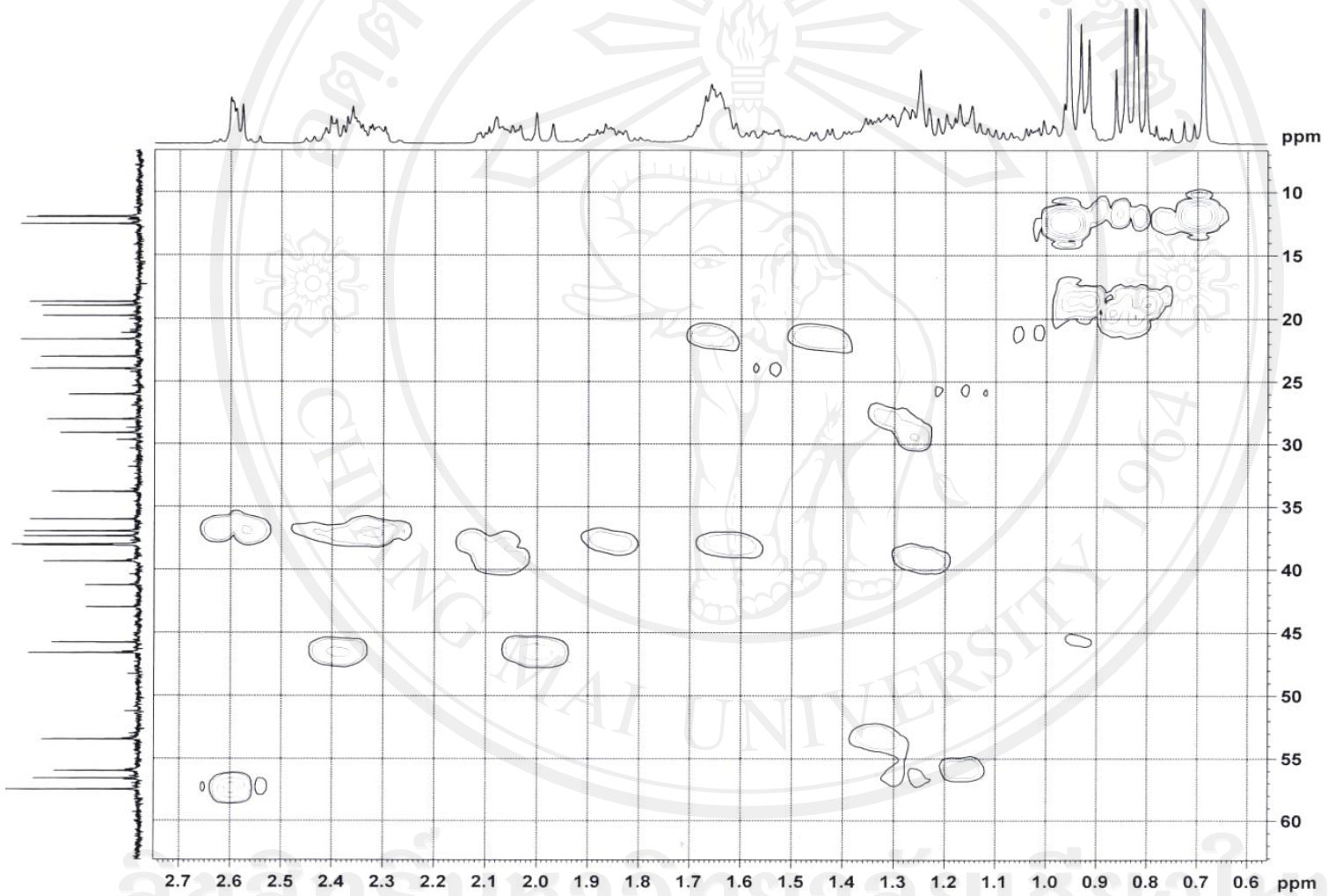

Figure 46 HMQC correlation of stigmastan-3,6-dione (**25**)

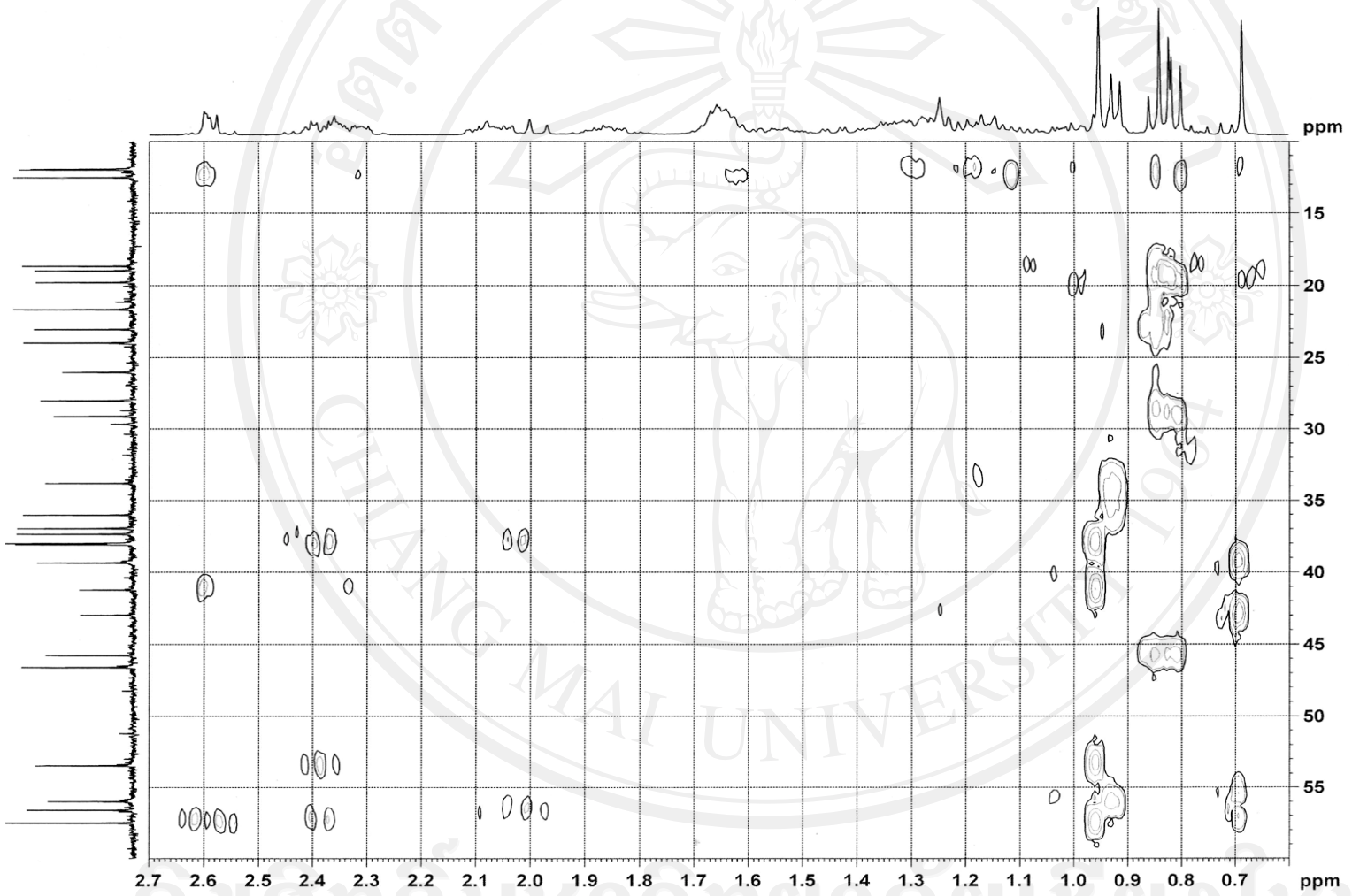

Figure 47 HMBC correlation of stigmastan-3,6-dione (**25)**

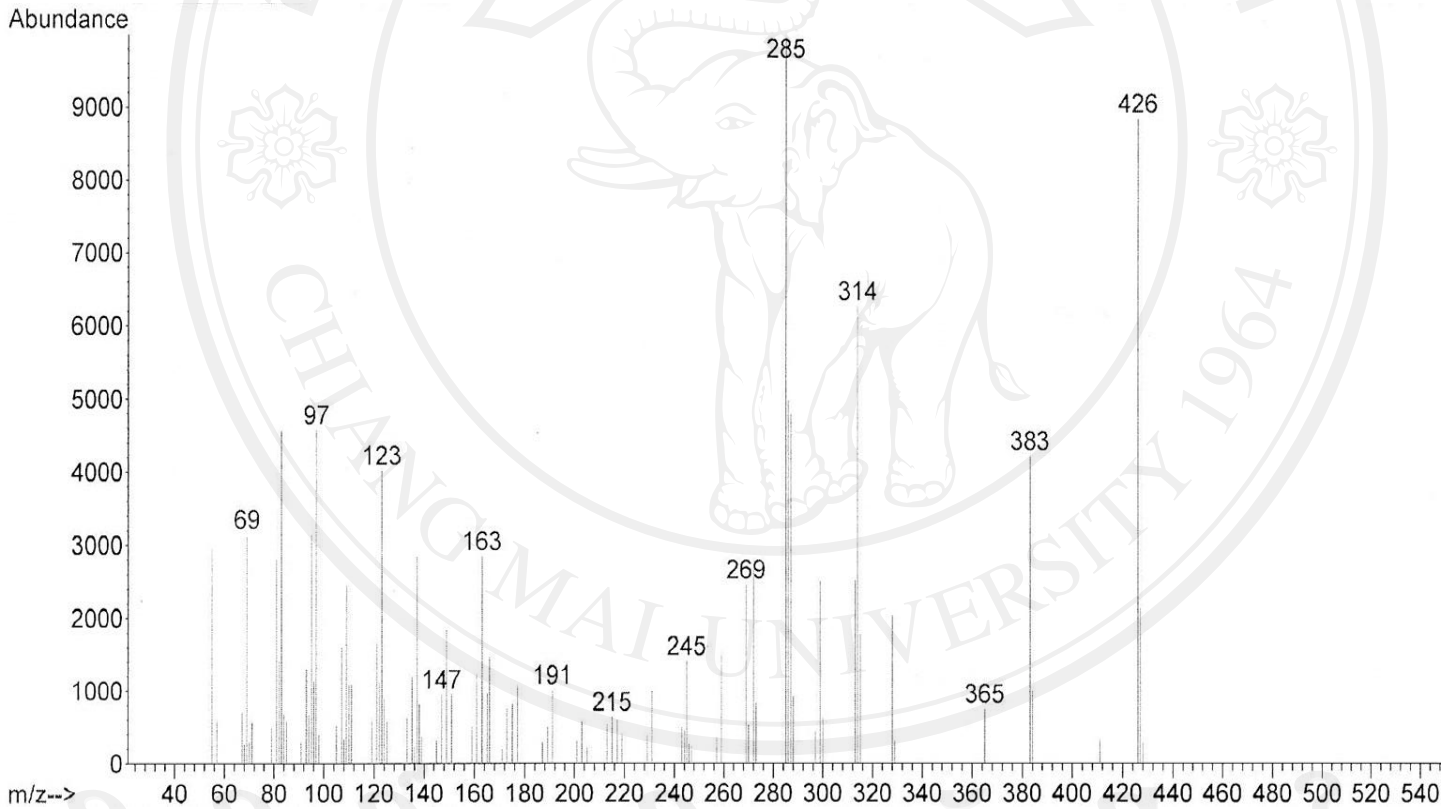

Figure 48 Mass spectrum of stigmastan-3,6-dione (**25)**

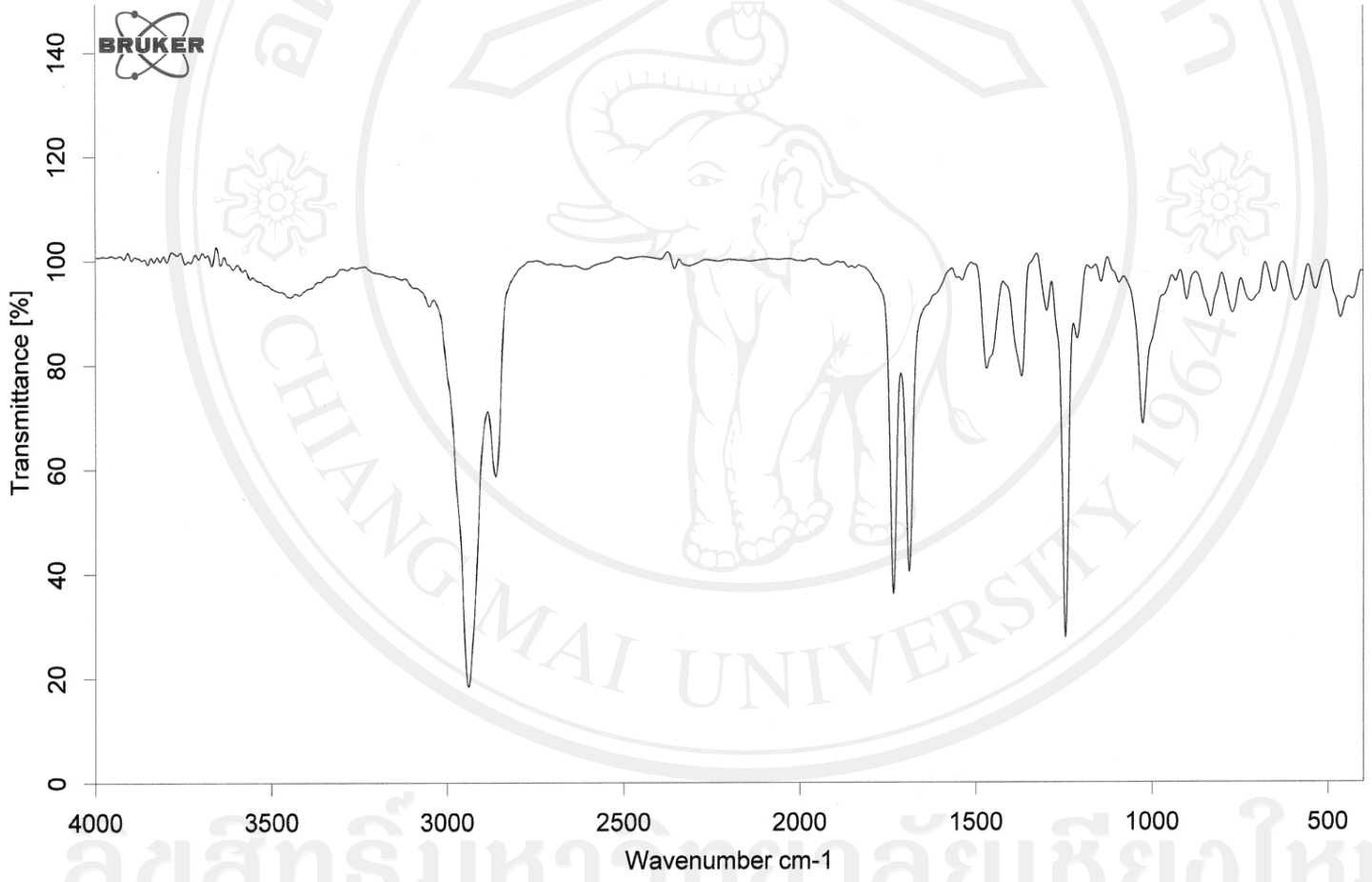

Figure 49 IR spectrum of stigmastan-3,6-dione (**25**)

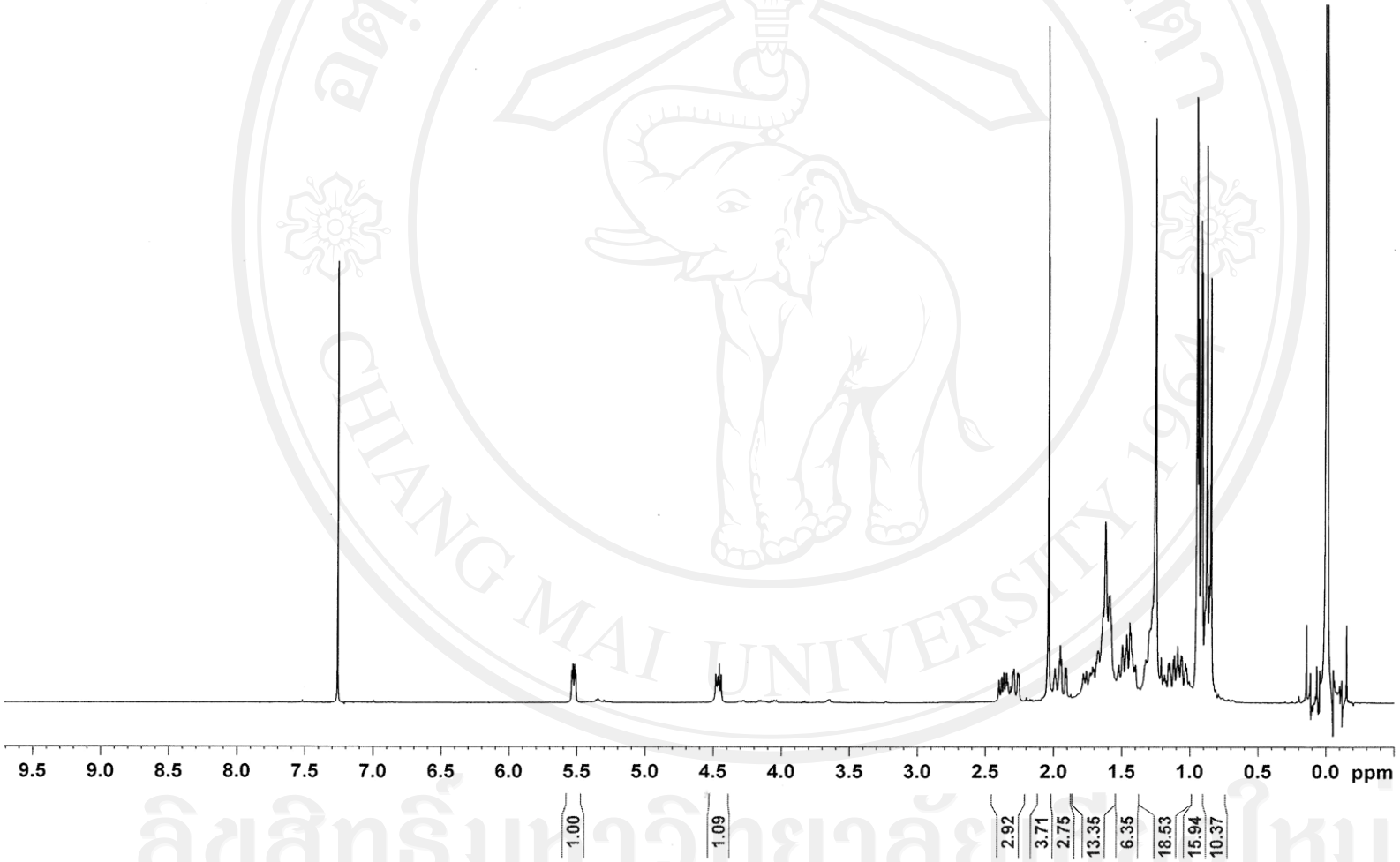

Figure 50 ^{1}H NMR spectrum of 3-acetyl aleuritolic acid (**26**)

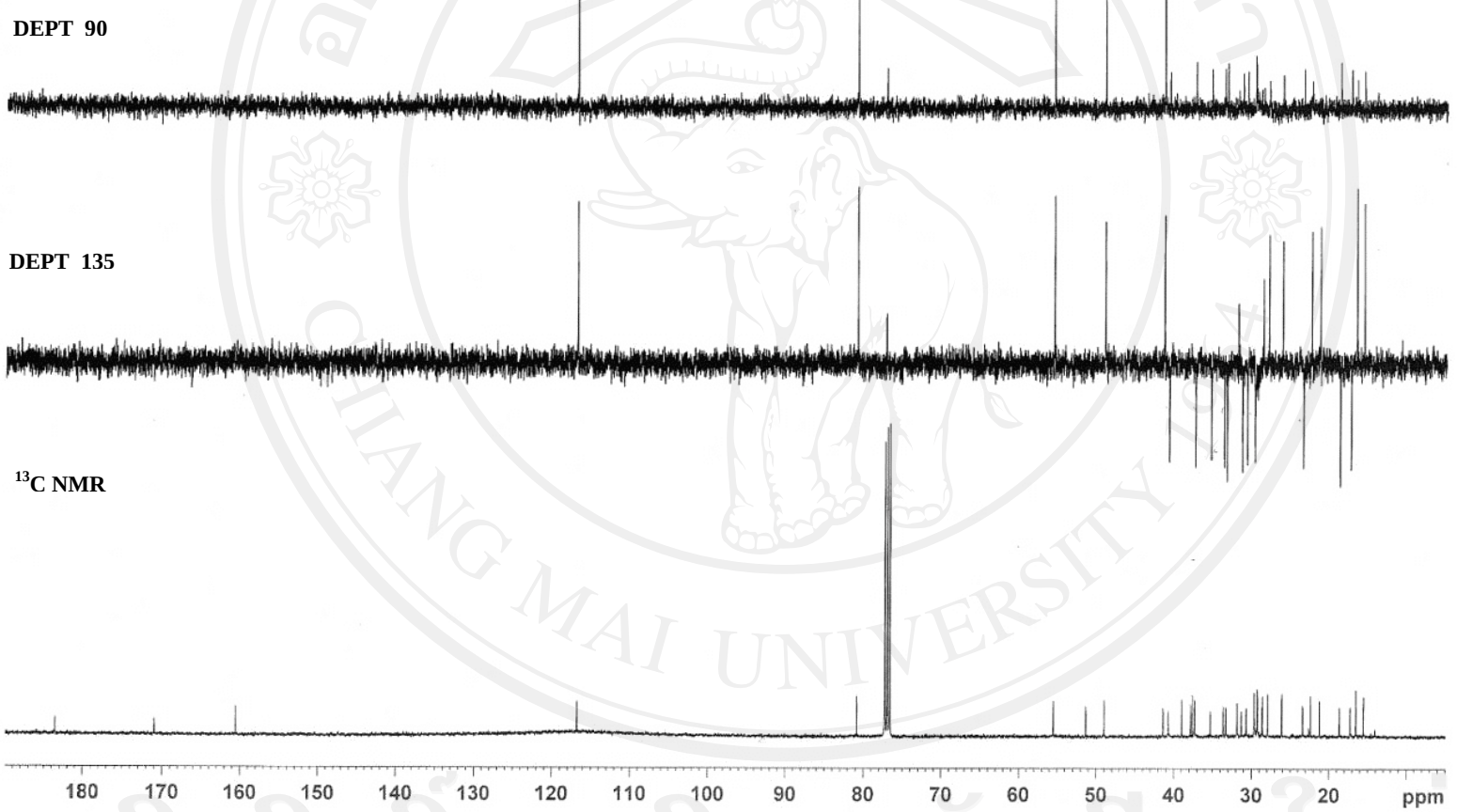

Figure 51 DEPT 90, DEPT 135,and ^{13}C NMR spectra of 3-acetyl aleuritolic acid (**26**)

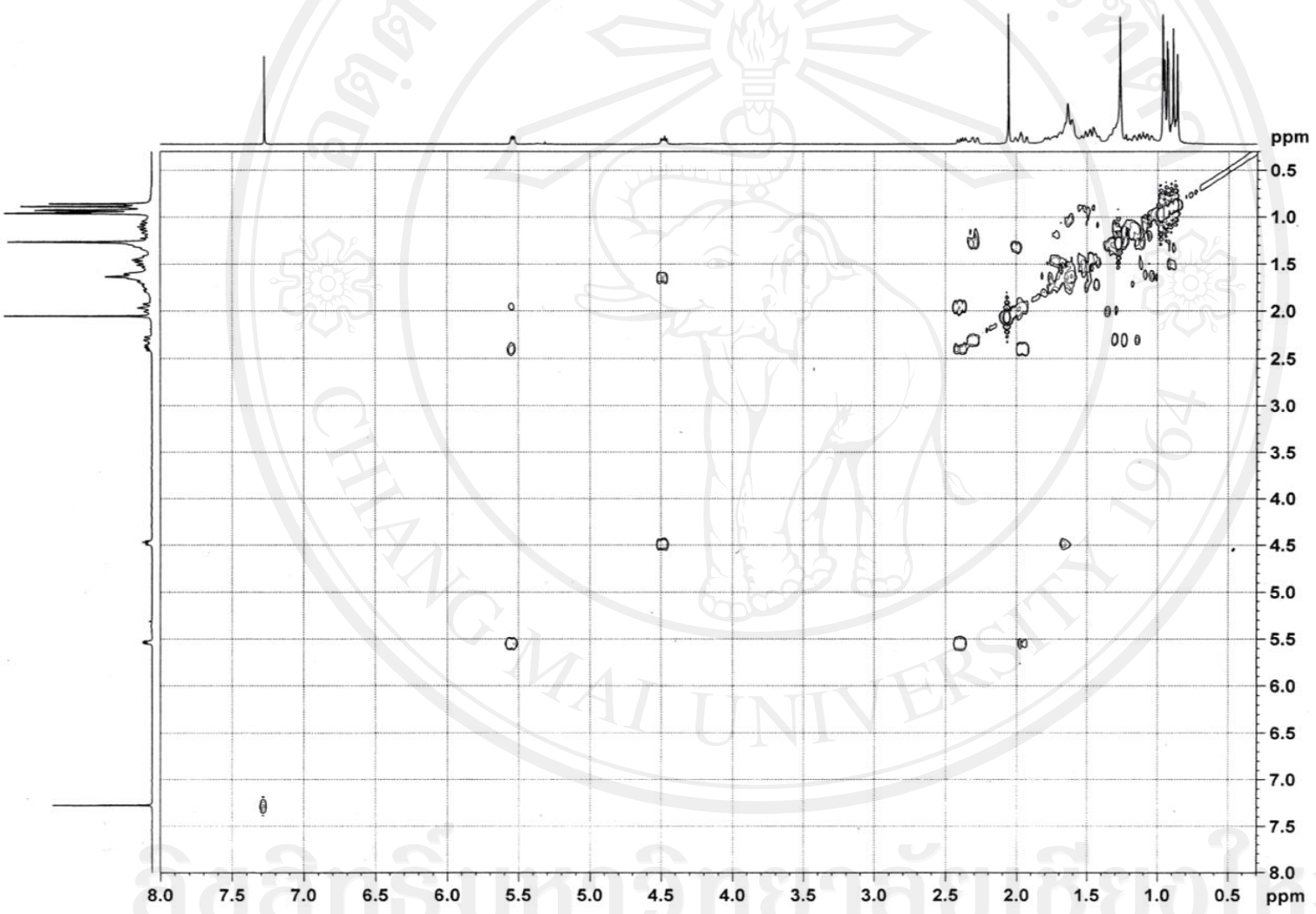

Figure 52 COSY spectrum of 3-acetyl aleuritolic acid (**26**)

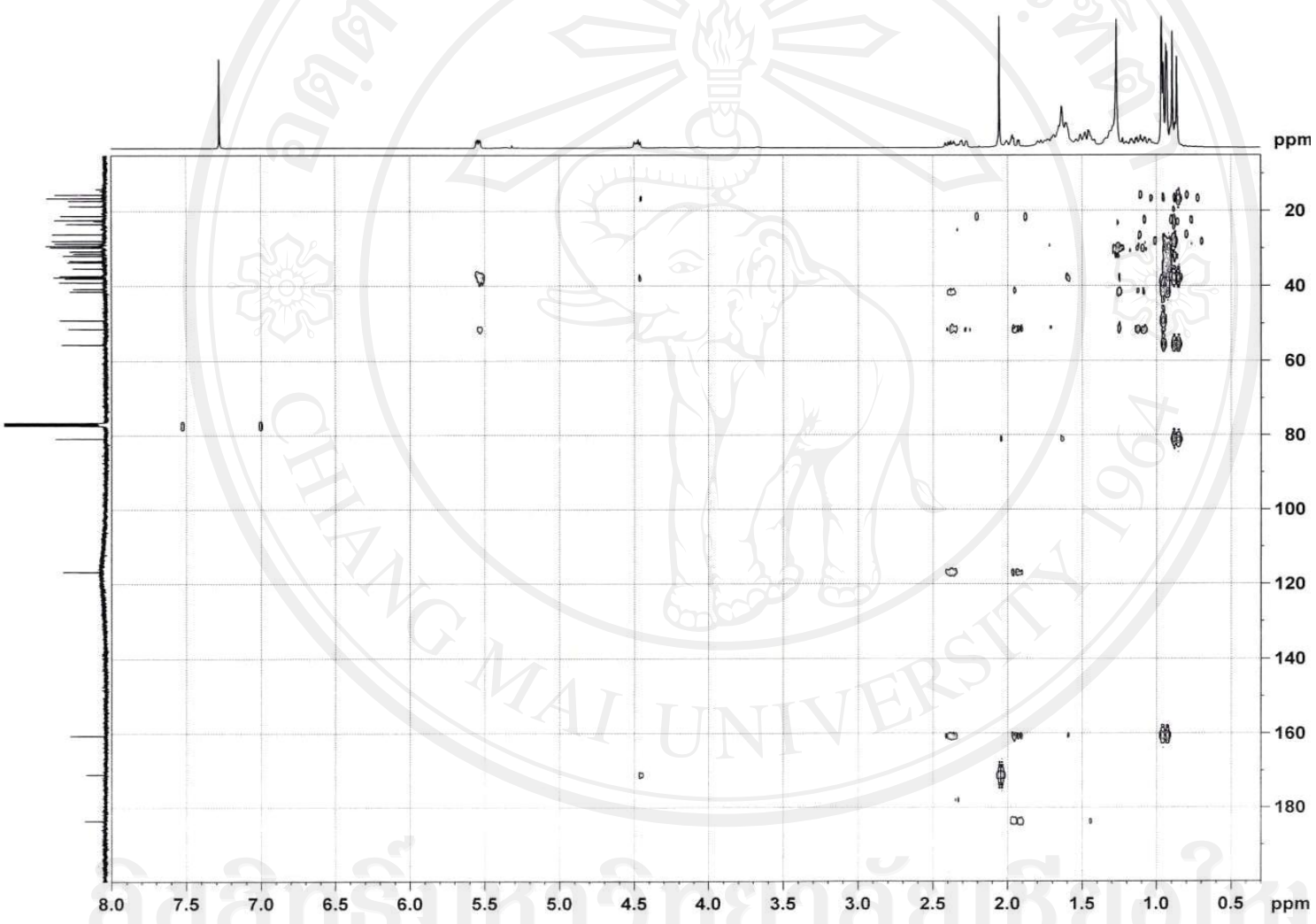

Figure 53 HMQC correlation of 3-acetyl aleuritolic acid (**26**

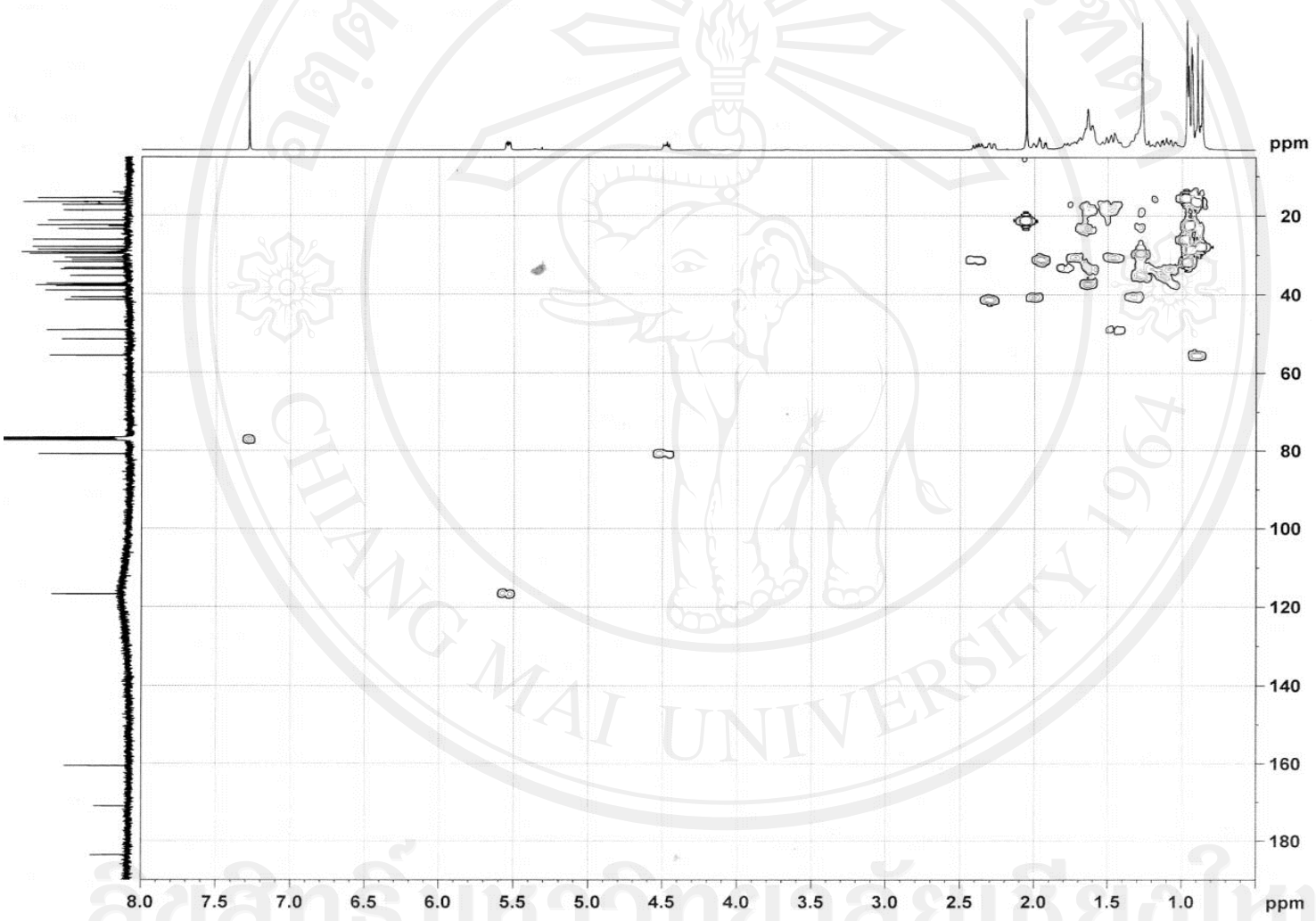

Figure 54 HMQC correlation of 3-acetyl aleuritolic acid (**26**)

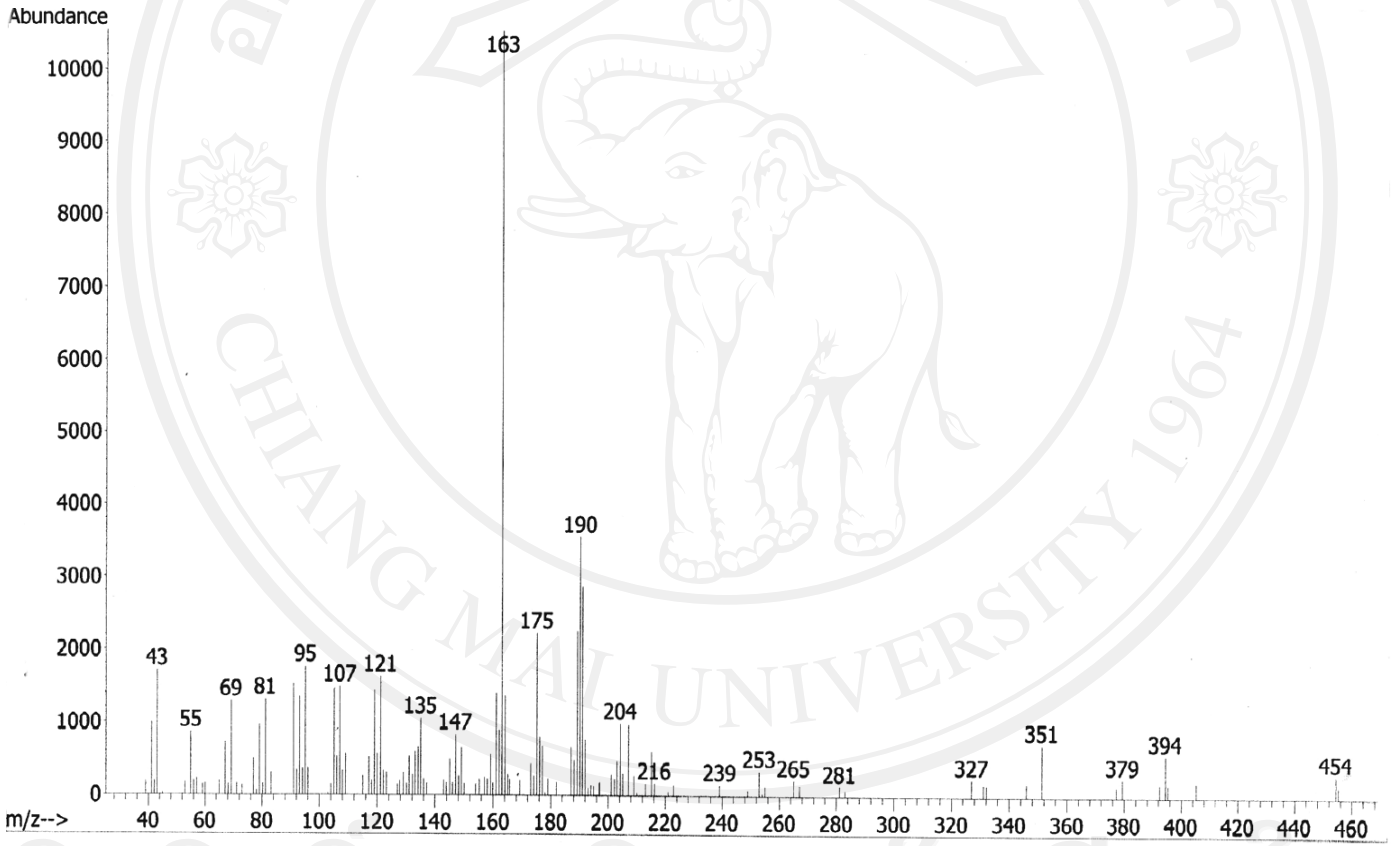

Figure 55 Mass spectrum of 3-acetyl aleuritolic acid (**26**)

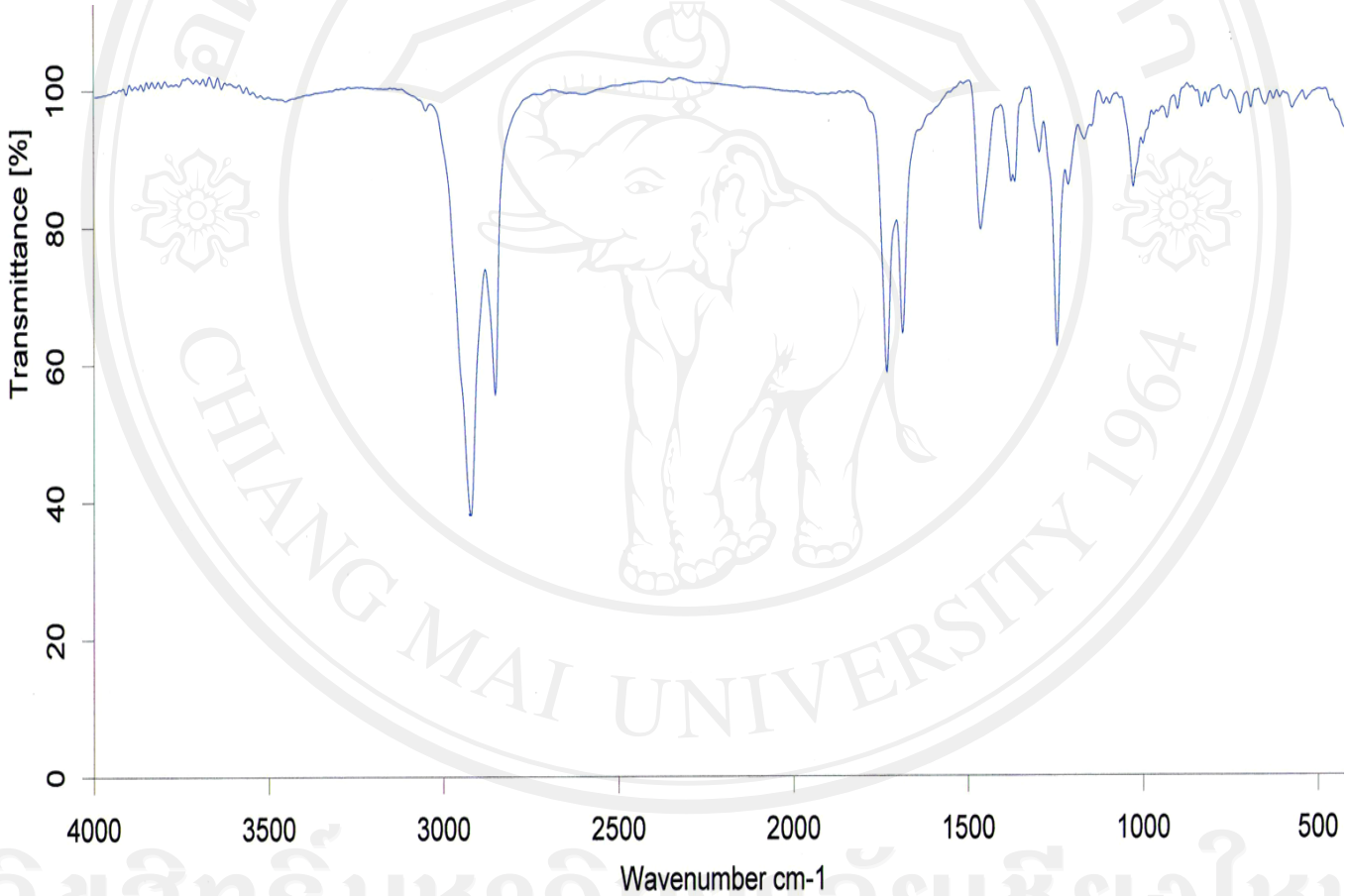

Figure 56 IR spectrum of 3-acetyl aleuritolic acid (**26**)

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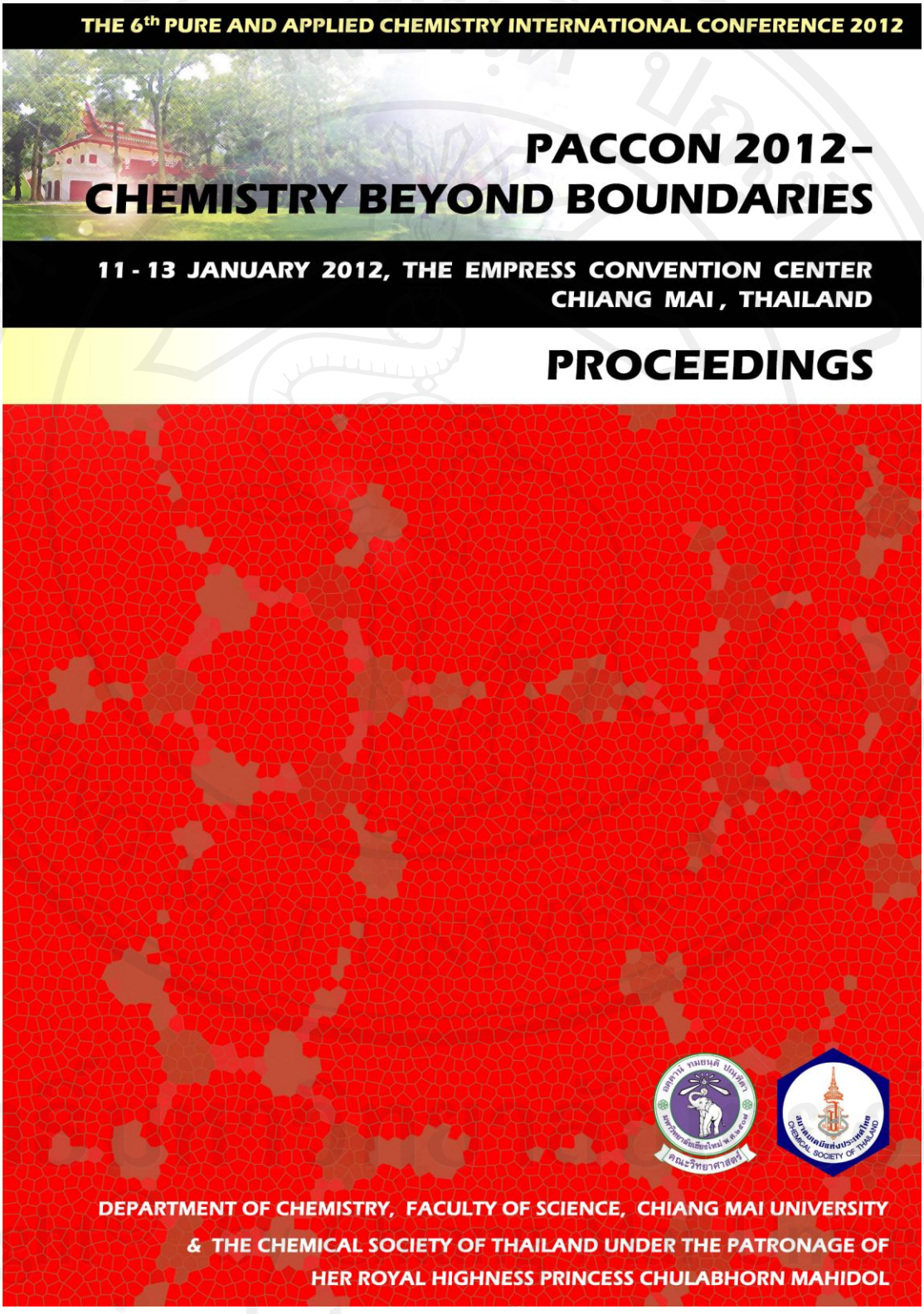
International Conference:

Neeranuth Intakaew, Puttinan Meepowpan*, and Siriwan Kettapan, "*Chemical Constituents of Dichloromethane Extract of Ostodes paniculata* Blume", The Sixth Pure and Applied Chemistry International Conference (PACCON VI), at Chiang Mai, Thailand (2012), pp. 1−3.



CONSTITUENTS OF THE DICHLOROMETHANE EXTRACT OF *OSTODES PANICULATA* BLUME

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Abstract

Ostodes paniculata Blume is highly inhibiting to the growth of P388 lymphocytic leukemia. The dichloromethane extract of the twigs of *O. paniculata* was investigated by chromatographic techniques. The chemical constituents of *O. paniculata* were found out the phytosterols, which were *β*-sitosterol (**4**) and stigmasterol (**3**) in ratio 78:22 and two known compounds as eugenol (**1**) and stigmastan-3,6-dione (**2**). These structures were elucidated by spectroscopic techniques on the basis of 1D and 2D NMR, MS and compared with the previous reports.

Introduction

Ostodes paniculata Blume is a plant belonging to Euphorbiaceae family. It is a large tree about 15 m. tall, dioecious; bark gray-brown; branches and leaves glabrous. It distributed throughout tropical (and parts of subtropical) Asia, including India, Myanmar, South of China, Indonesia and Thailand, ranging around North and North-East [1]. *O. paniculata*, commonly known as Makungdong, which is a rich source of activity compounds. Some of these compounds show interesting biological activities for instance antibacterial, antifungal, antihelminthic, antifertility, anti-inflammatory, antiispasmodic and inhibit the growth of P-388 lymphocytic leukemia system [2-4].

Since there has no report on phytochemical study, the present study aimed to investigates and isolates the chemical constituents from the twigs of *O. paniculata* and also chemotaxonomic studies on the genus.

Conclusions

These compounds (Fig. 1) as eugenol (**1**), stigmastane-3,6-dione (**2**), mixture of stigmasterol (**3**) and *β*-sitosterol (**4**) were isolated from the crude dichloromethane extract of the twigs of *Ostodes paniculata* Blume by chromatographic techniques.

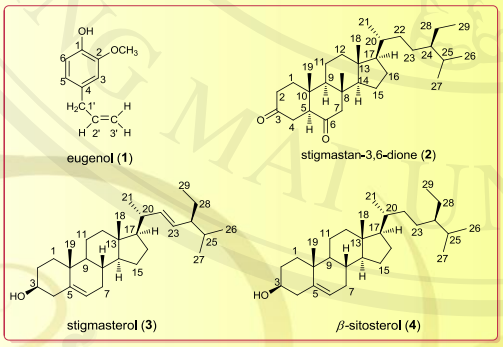

Fig. 1 Compounds **1-4** isolated from the twigs of *Ostodes paniculata* Blume

References

[1] D. Lucette, P. Gerard, R. Yvan, C. C. Dominique, *Tetrahedron* **55** (1999), pp. 7583–7588.
[2] A. C. H, C. E. L, C. V, G. C and C. W, *J. Braz. Chem. Soc.* **19** (2008), pp. 543-548.
[3] B. Li, G. G. Michael, *Fl China*. **11** (2008), pp. 27–272.
[4] B. S. D, G. K. A, J. Sudha, M. N. B and S. C. R, *Indian J. Exp. Biol*. **28** (1990), pp. 619–637.

Methods and Results

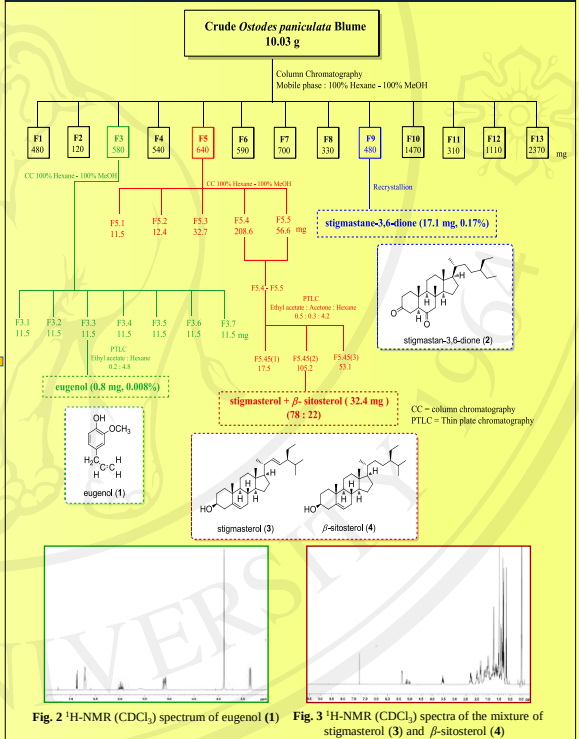

Fig. 2 ^{1}H-NMR ($CDCl_3$) spectrum of eugenol (**1**)

Fig. 3 ^{1}H-NMR ($CDCl_3$) spectra of the mixture of stigmasterol (**3**) and *β*-sitosterol (**4**)

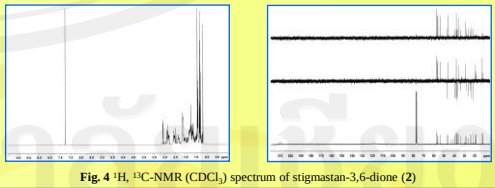

Fig. 4 ^{1}H, ^{13}C-NMR ($CDCl_3$) spectrum of stigmastan-3,6-dione (**2**)

Acknowledgements

Financial support from Science Achievement Scholarship of Thailand (SAST) is gratefully acknowledged. In addition, we also thank Department of Chemistry, Faculty of Science, Chiang Mai University for facilities supporting this research.

CHEMICAL CONSTITUENTS OF THE DICHLOROMETHANE EXTRACT OF OSTODES PANICULATA BLUME TWIGS

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Abstract: ***Ostodes paniculata*** **Blume is highly inhibiting to the growth of P388 lymphocytic leukemia. The dichloromethane extract of the twigs of *O. paniculata* were investigated by chromatographic techniques. The chemical constituents of *O. paniculata* were found it consist of two phytosterols, which were *β*-sitosterol (4) and stigmasterol (3) in the ratio 78:22 and two known compounds identified as eugenol (1) and stigmastan-3,6-dione (2). The structures were elucidated by spectroscopic techniques on the basis of 1D and 2D NMR, MS and compared with previous reports.**

1. Introduction

Ostodes paniculata Blume is a plant belonging to Euphorbiaceae family. It is a large tree about 15 m. tall, dioecious; bark gray-brown; branches and leaves glabrous. It distributed throughout tropical (and parts of subtropical) Asia, including India, Myanmar, South of China, Indonesia and Thailand, ranging around North and North-East [1]. *O. paniculata*, commonly known as Makungdong, is a rich source of active compounds. Two phorbol esters (Polycyclic diterpenes) which were 12-*O*-undecadienoylphorbol-13-acetate and ostodin showed interesting biological activities for instance antispasmodic activity on the guinea pig ileum *in vitro* [2], antileukemic activity ($ED_{50} \leq 4$ μg/ml) when tasted P-388 lymphocytic leukemia cell [3], antibacterial, antifungal, antihelminthic, antifertility, and anti-inflammatory [4-5].

Since there has been no report on phytochemical study, the present study aimed to investigate and isolate the chemical constituents from the twigs of this plant and also chemical taxonomic studies on the genus.

2. Materials and Methods

2.1 General Experimental Procedures

All NMR spectra, ^1H and ^{13}C NMR spectra, were recorded on Bruker DRX 400 MHzin $CDCl_3$which were given in ppm downfield from tetramethylsilane (TMS) were reported as δ-values in parts per million (ppm) relative to residue $CHCl_3$ as internal reference (^1H: δ 7.26, ^{13}C: δ 77.00) and coupling constants (*J* values) were reported in hertz (Hz). Peak multiplicities are indicated as follows: *s* (singlet), *d* (doublet), *t* (triplet), *brs* (broad singlets) and*m* (multiplet). Infrared spectra were taken with a FT-IR model TENSER 27 (Bruker) spectrometer and absorption frequencies were reported in reciprocal centimeters (cm^{-1}). Melting points of all compunds were measured with using an Electrothermal Melting Point apparatus (Sanyo, Model Gallenkamp). Flash column chromatography was performed employing Merck silica gel 60H. Preparative thin layerchromatography (PLC) plates were carried out using Merck silica gel 60 PF_{254}. Analytical thin layerchromatography was performed with Merck silica gel 60 F_{254} aluminumplates.

2.2 Plant Material

The twigs of *O. paniculata* were collected in the northern part of Thailand from Doi Suthep Pui National park in 2008. The plants were identified by Maxwell, J.F.A and the vocher specimen (NO. 06-651) has been deposited at the CMU Herbarium, Department of Biology, Faculty of Science, Chiang Mai University, Thailand.

2.3 Extraction and Isolation

The air-dried and chopped twigs of *O. paniculata* (2kg) were extracted with dichloromethane and methanol at room temperature (2,000 ml x 3days x 3times). The filtrate was evaporated under reduced pressure to get dichloromethane and methanol extracts (13.04, 22.54 g, respectively). The crude dichloromethane extract was separated by using column chromatography (CC) in polarity gradient system to give thirteen fractions (OP1-OP13). Fraction OP3 (0.580 g) was repeatedly separated by CC to afford **1** (0.80 mg). Fraction OP5 (0.640 g) was repeatedly separated by CC and recrystallized from the mixture of dichloromethane and hexane to afford **2** (17.1 mg). Fraction OP9 (0.480 g) was recrystallized from the mixture of dichloromethane and hexane to afford a mixture of **3** and **4** in the ratio of 78:22.

3. Results and Discussion

Chromatographic separation of dichloromethane extracts of the twigs of *Ostodes paniculata* yielded four known compounds. These compounds were identified as eugenol (**1**), stigmastan-3,6-dione (**2**) and mixture of stigmasterol (**3**) and *β*-sitosterol (**4**). Compound **1** was obtained as a pale yellow oilyliquid, and ^1H and ^{13}C NMR were shown in table 1. IR spectrum of this compound showed OH functional group at 3618 cm^{-1} and molecula rweight was estimated at *m/z* 165 (M+1) by Massspectrometry, which was agreeable with $C_{10}H_{12}O_2$. The structure of

compound **1** was determined from ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, DEPT135, DEPT90, HMQC and HMBC experiments. The ^{1}H NMR spectrum of compound **1** showed the single proton of hydroxyl group at 5.59 (1H, *brs*) with C-1(δ 143.8). The methoxy proton δ 3.88 (3H, *s*) with C-2 (δ 146.3). The aromatic proton at 6.88 (1H, *d*, *J* = 8.5 Hz), 6.70-6.72 (2H, *m*) could be assigned as 1,2,4-trisubstituted benzene.

In addition, the methylene proton of olefinic proton displayed chemical shift values at 5.07-5.10, 5.09-5.14 (2H, *m*, H-3') with C-3' (δ 115.4), from the HMBC correlation between H-3' (δ 5.07-5.14 and 5.07-5.09) with C-1' (δ 39.8), C-2' (δ 137.7). The methylene proton at 3.35 (2H, *d*, *J* = 6.7 Hz, H-1') was located at C-1' next to aromatic ring. The location of H-1' (δ 3.35) was suggested by HMBC with the saturated carbon δ 137.7 (C-2'), 115.4 (C-3'), 121.1 (C-3) 131.8 (C-4) and 114.2 (C-5). By comparison with literatures, compound **1** was thus identified as eugenol [6-7].

Table 1: ^{1}H and ^{13}C NMR data of compound **1** [5-6]

Position	δ_H	δ_C
1	-	143.8
2	-	146.3
3,6	6.70-6.72 (*m*, 2H)	121.1,111.0
4	-	131.8
5	6.88 (*d*,1H),*J* = 8.5Hz	114.2
1'	3.35 (*d*, 2H), *J* = 6.7 Hz	39.8
2'	5.93-6.04 (*m*, 1H)	137.7
3'	5.09-5.14 (*m*, 2H)	115.4
O-CH_3	3.88 (*s*, 3H)	55.7
O-H	5.59 (*brs*, 1H)	-

Compound **2** was obtained as a colorless amorphous solid, mp 192–194 °C and ^{1}H and ^{13}C NMR were shown in table 2. The molecular formula was assigned as $C_{29}H_{48}O_2$ based on the molecular ion peak at *m/z* 428. The IR spectrum exhibited 2(C=O) signals at 1716, 1708 cm^{-1} and C-O at 1239 cm^{-1}. In the ^{1}H NMR spectrum, the chemical shift at 2.00-2.59 (9H, *m*) are attributed to four methylene protons of H-1, H-2, H-4, H-7 and the methine proton H-5 that is connected to the ketone group. Chemical shifts at 1.00-2.00 (21H, *m*) are methylene and methyl protons that are not connected to the ketone group. Furthermore, six methyl signals display chemical shift at 0.69 (3H, *s*, Me-18), 0.95 (3H, *s*, Me-19), 0.92 (3H, *d*, *J* = 6.5 Hz, Me-21), 0.80 (3H, *d*, *J* = 6.8 Hz, Me-26), 0.84 (3H, *d*, *J* = 7.1 Hz, Me-27) and 0.86 (3H, *t*, *J* = 5.8 Hz, Me-29, subsequently). The ^{13}C NMR spectrum of compound **2** showed signals of four quaternary carbons, two of them at 211.2 (C-3), 209.1 (C-6), carbonyl carbon of ketone groups, the others are carbon which were substituted with methyl group at 41.2 (C-10), 43.0 (C-13). The structure was further confirmed by HMBC correlation between methylene proton at 2.11, 1.63 (H-1) with the carbon at 37.0 (C-2), 211.2 (C-3), 57.5 (C-5) and 12.5 (C-19) as well as another the methylene proton at 2.57, 2.33 (H-2) with the carbon at 37.3 (C-1), 211.2 (C-3) and 41.2 (C-10). In addition, the signal showed the methylene proton at 2.06 (H-4) with the carbon at 37.0 (C-2), 211.2 (C-3) and 57.5 (C-5) also the methine proton of δ 2.59 (H-5) with the carbon δ 211.2 (C-3), 38.1 (C-4), 209.1 (C-6), 41.2 (C-10) and 12.5 (C-19). From the spectroscopic data and comparison with the literatures, therefore compound **2** was identified as stigmastane-3,6-dione [7-8].

Table 2: ^{1}H NMR and ^{13}C NMR data of compound **2** [8-9]

Position	δ_H	δ_C
1	2.11, 1.63 (*m*, 2H)	37.3
2	2.57, 2.33 (*m*, 2H)	37.0
3	-	211.2
4	2.59, 2.06 (*m*, 2H)	38.1
5	2.59 (*m*, 1H)	57.5
6	-	209.1
7	2.38, 2.00 (*m*, 2H)	46.6
8	1.85 (*m*, 1H)	38.0
9	1.33 (*m*, 1H)	53.4
10	-	41.2
11	1.65, 1.42 (*m*, 2H)	21.6
12	2.06, 1.25 (*m*, 2H)	39.3
13	-	43.0
14	1.25 (*m*, 1H)	56.0
15	1.53 (*m*, 2H)	24.0
16	1.31 (*m*, 2H)	28.0
17	1.16 (*m*, 1H)	56.6
18	0.69 (*s*, 3H)	11.9
19	0.95 (*s*, 3H)	12.5
20	1.37 (*m*, 1H)	36.0
21	0.92 (*d*, 3H), *J* = 6.5 Hz	18.6
22	1.34, 1.02 (*m*, 2H)	33.8
23	1.16 (*m*, 2H)	26.0
24	0.92 (*m*, 1H)	45.7
25	1.25 (*m*, 1H)	29.1
26	0.80 (*d*, 3H), *J* = 6.8 Hz	19.0
27	0.84 (*d*, 3H), *J* = 7.1 Hz	19.8
28	1.28, 1.21 (*m*, 2H)	23.0
29	0.86 (*t*, 3H), *J* = 5.8 Hz	12.0

Mixture of compounds **3** and **4** were obtained as colorless solid. The EIMS spectrum of mixture compounds **3** and **4** showed a molecular ion peak at *m/z* 412 and 414 subsequently which corresponds to $C_{29}H_{48}O$ and $C_{29}H_{50}O$. The IR spectrum exhibited OH functional group at 3400 cm^{-1}, C=C at 1625 cm^{-1} and C-OH at 1360cm^{-1}. The ^{1}H NMR spectrum of mixture of compounds **3** and **4** showed signals of hydroxyl proton at 3.48-3.55 (1H, *m*), olefinic proton at 5.35 (1H, *d*, *J* = 5.2 Hz, H-6), two olefinic protons at 5.19, 5.06(1H, *dd*, *J* = 8.5, 8.6 Hz, H-22; 1H, *dd*, *J* = 8.6, 8.7 Hz, H-23, respectively) which were identified with the chemical shifts of H-22 and H-23 of stigmasterol [10]. These data including ^{1}H and ^{13}C NMR are also similar to the literatures of stigmasterol and β-sitosterol [11, 12]. The ratio of mixture compounds **3** and **4** could be determined by peak area integration of H-22, H-23 and H-6.

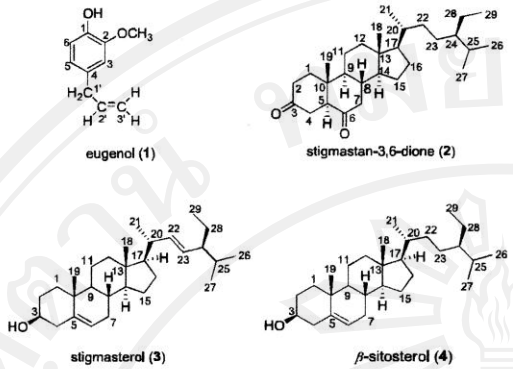

Figure 1.Structures of compounds **1-4** from *Ostodes paniculata* Blume twigs.

4. Conclusion

Eugenol (**1**), stigmastane-3,6-dione (**2**) and a mixture of stigmasterol (**3**) and *β*-sitosterol (**4**) have been isolated from the crude dichloromethane extract of the twigs of *Ostodes paniculata* Blume by chromatographic techniques.

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