APPENDIX

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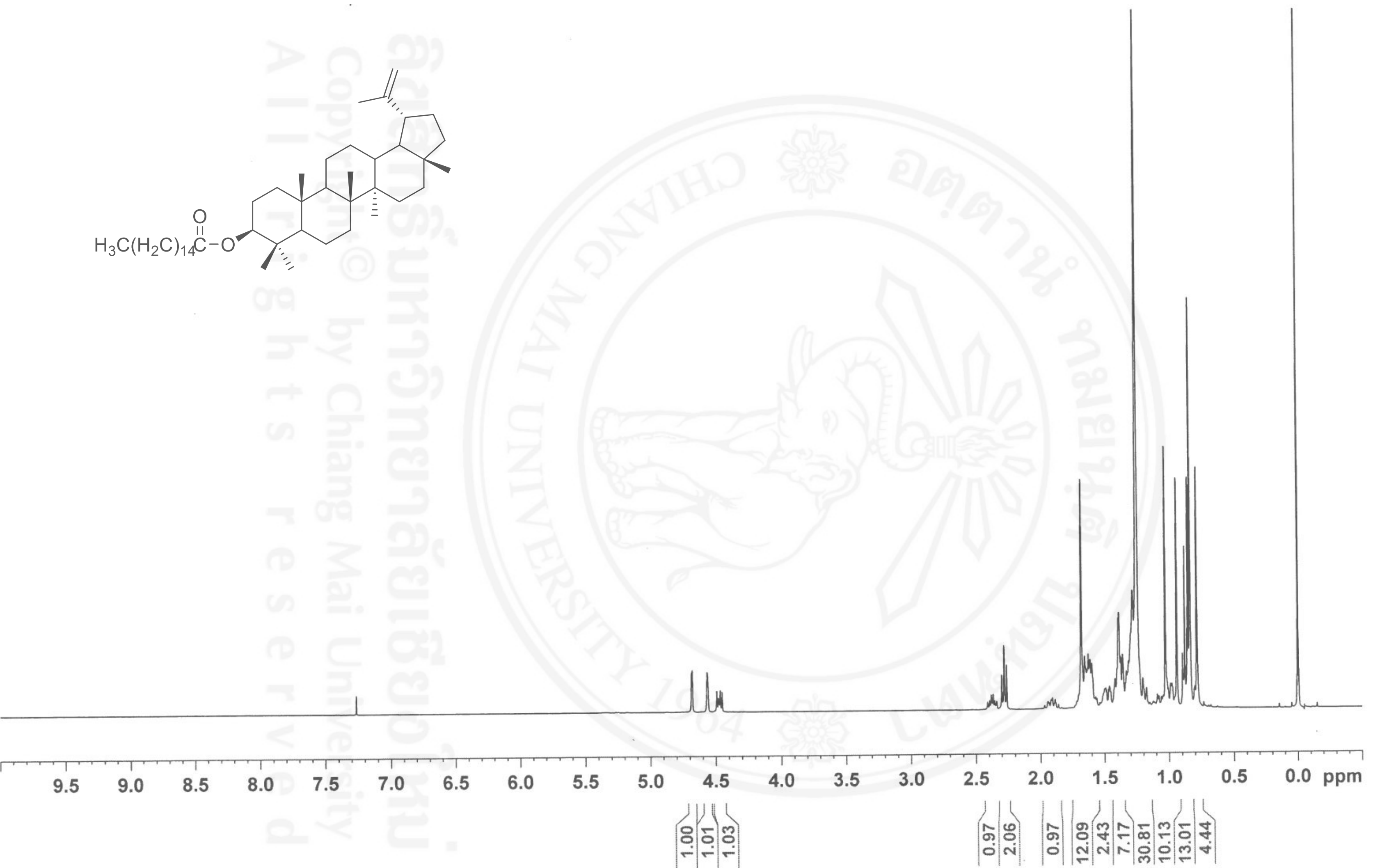

Figure 1 ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum of lupeol palmitate (**134**)

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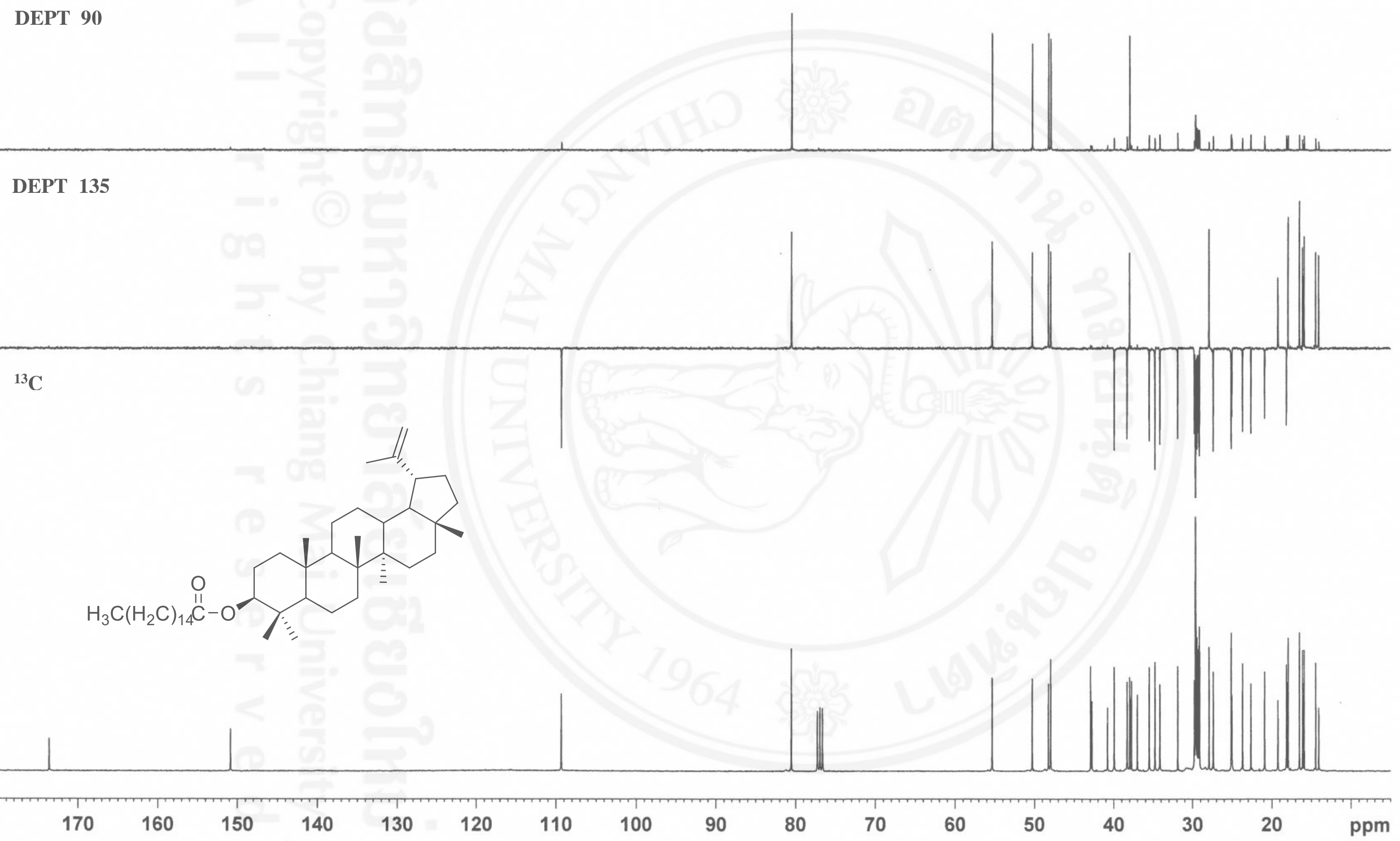

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Figure 2 ^{13}C NMR (100 MHz, in $CDCl_3$) and DEPT spectra of lupeol palmitate (**134**)

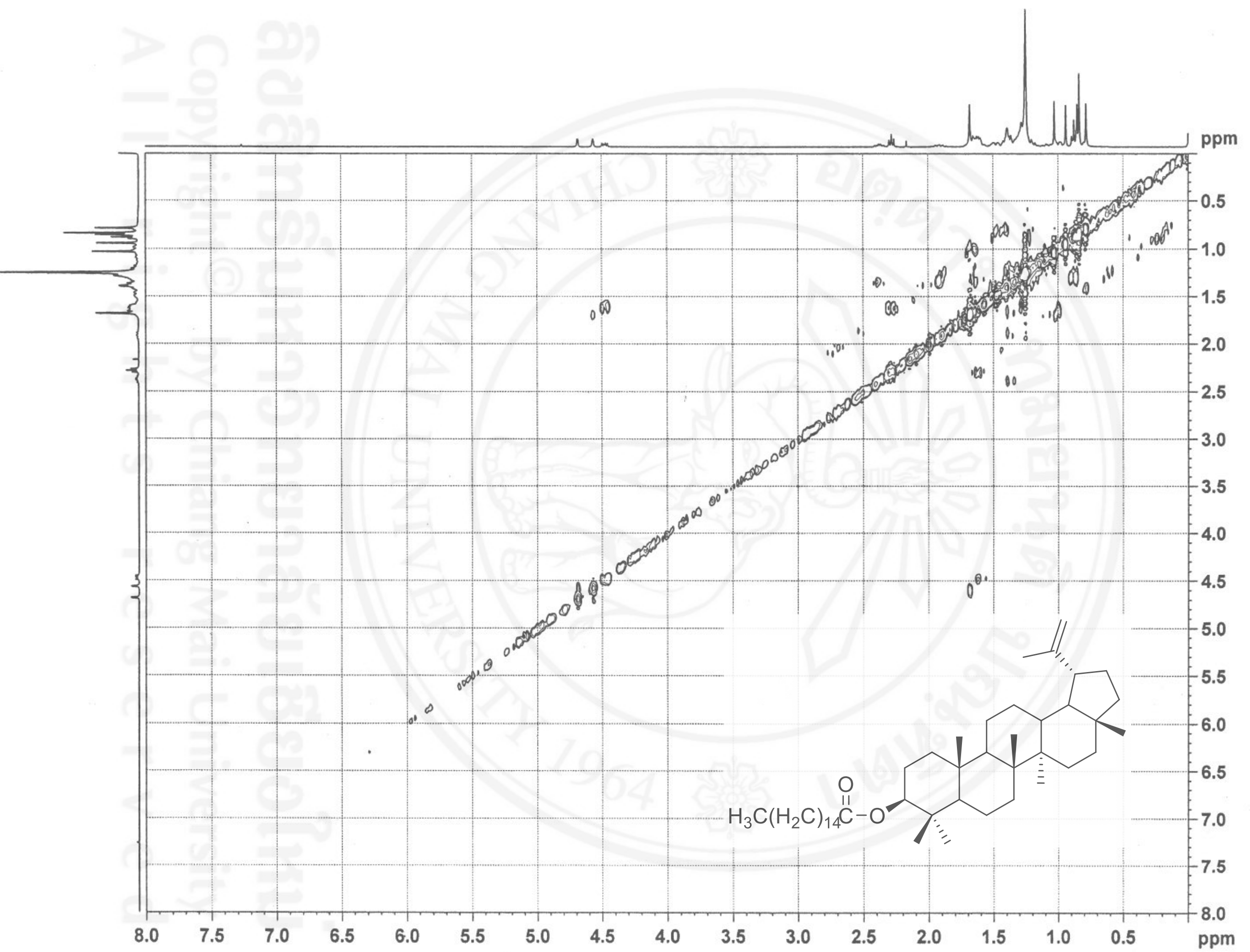

Figure 3 ^{1}H-^{1}H COSY (in $CDCl_3$) spectrum of lupeol palmitate (**134**)

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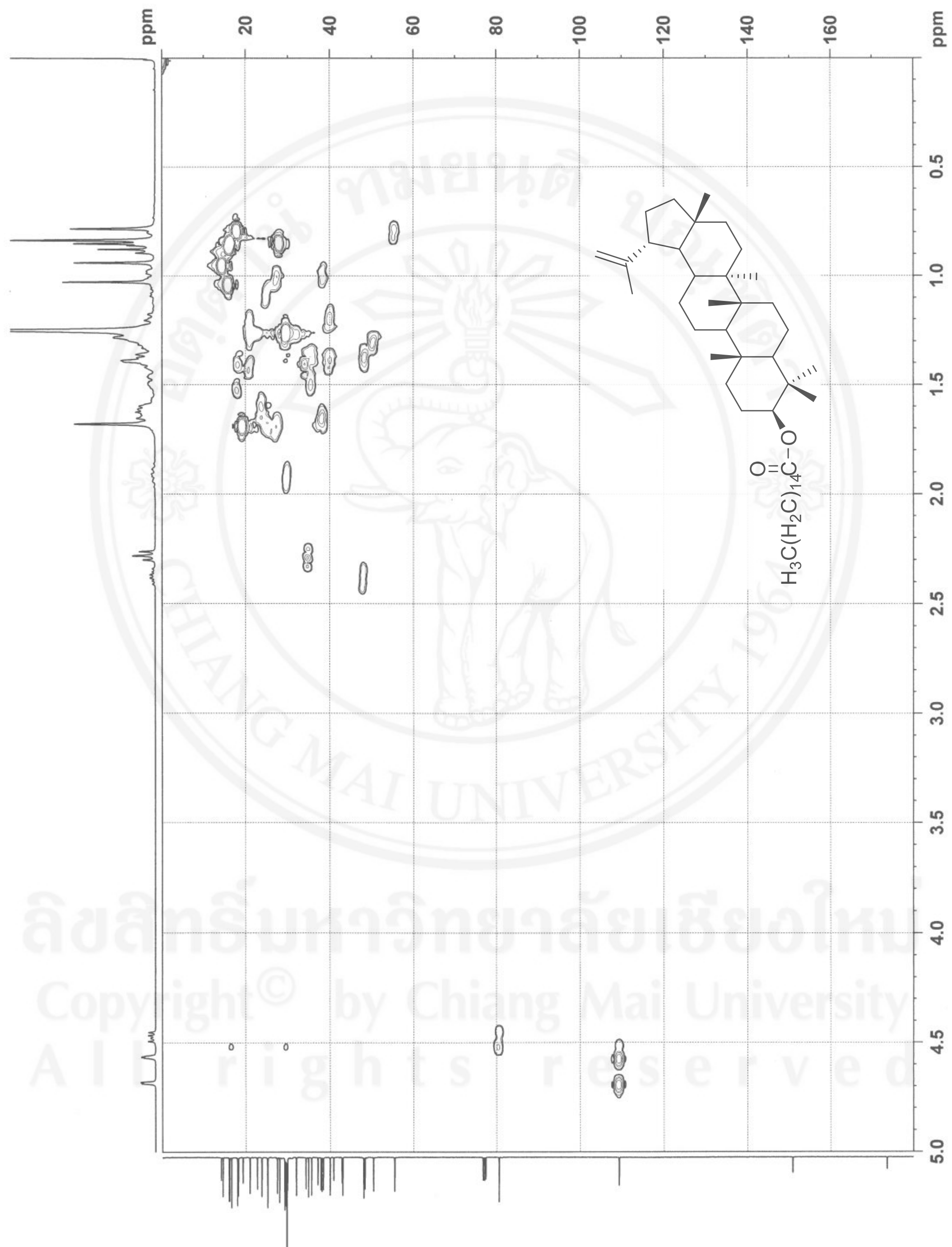

Figure 4 HMQC (in $CDCl_3$) spectrum of lupeol palmitate (**134**)

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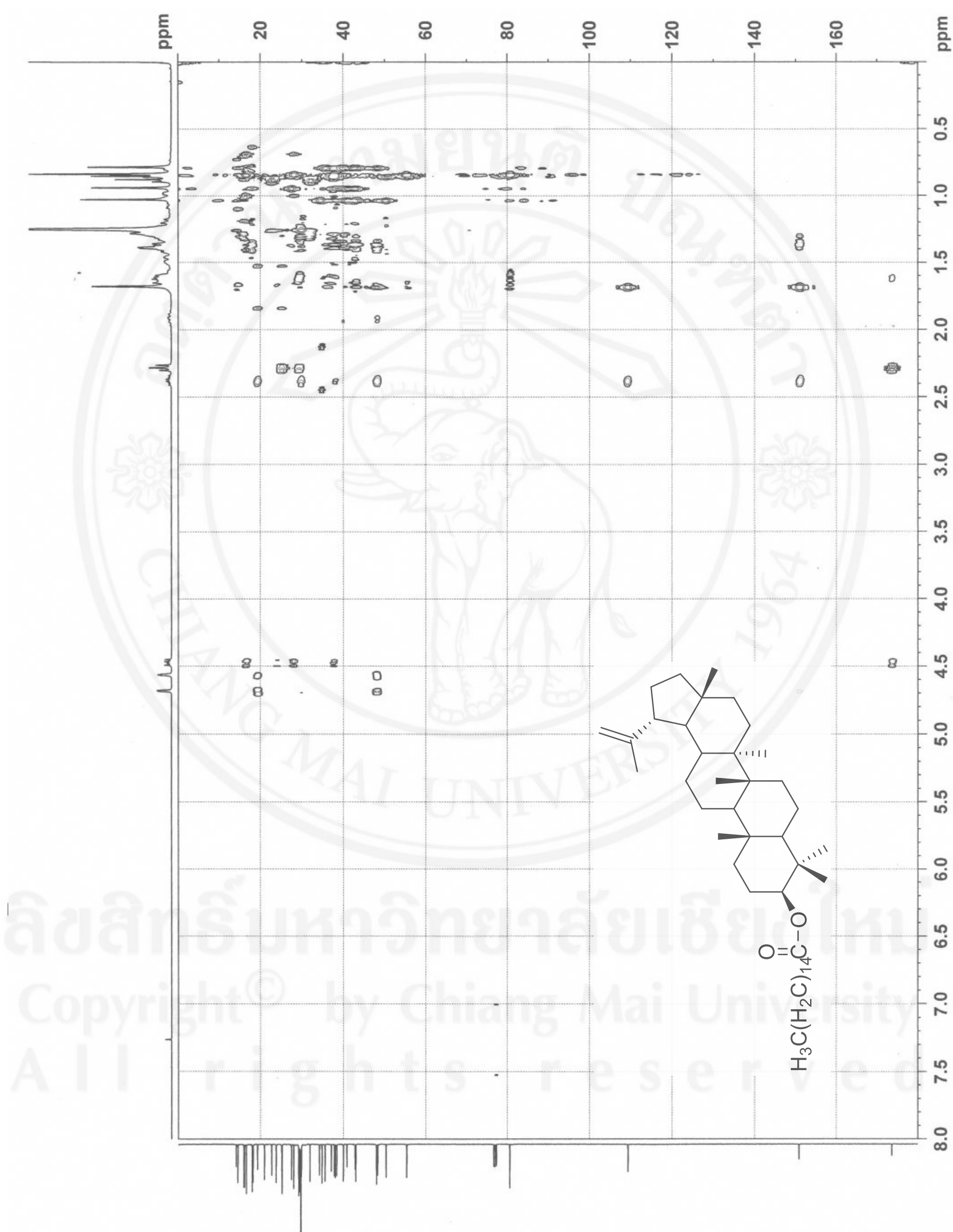

Figure 5 HMBC (in $CDCl_3$) spectrum of lupeol palmitate (**134**)

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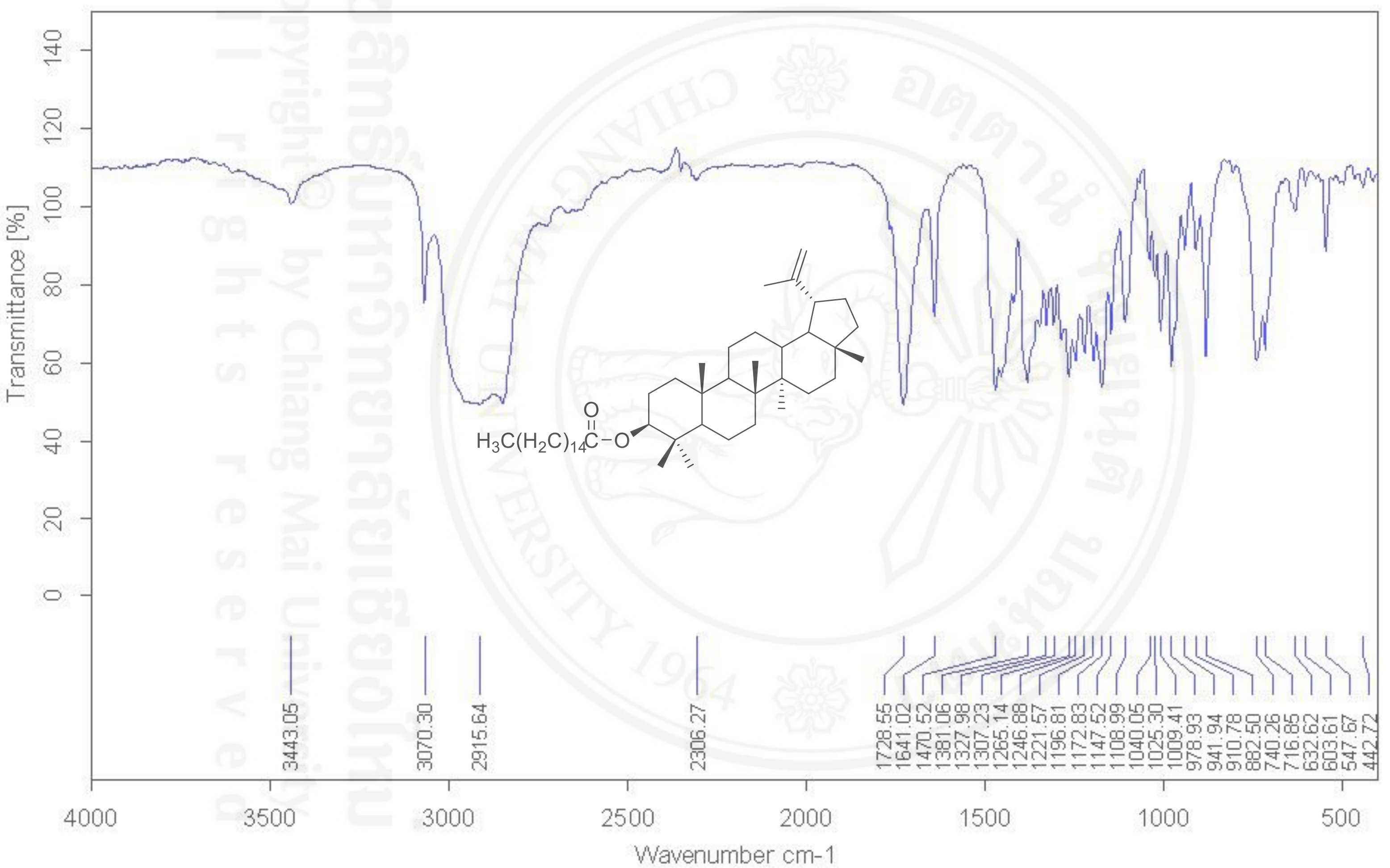

Figure 6 FTIR (evaporated thin film) spectrum of lupeol palmitate (**134**)

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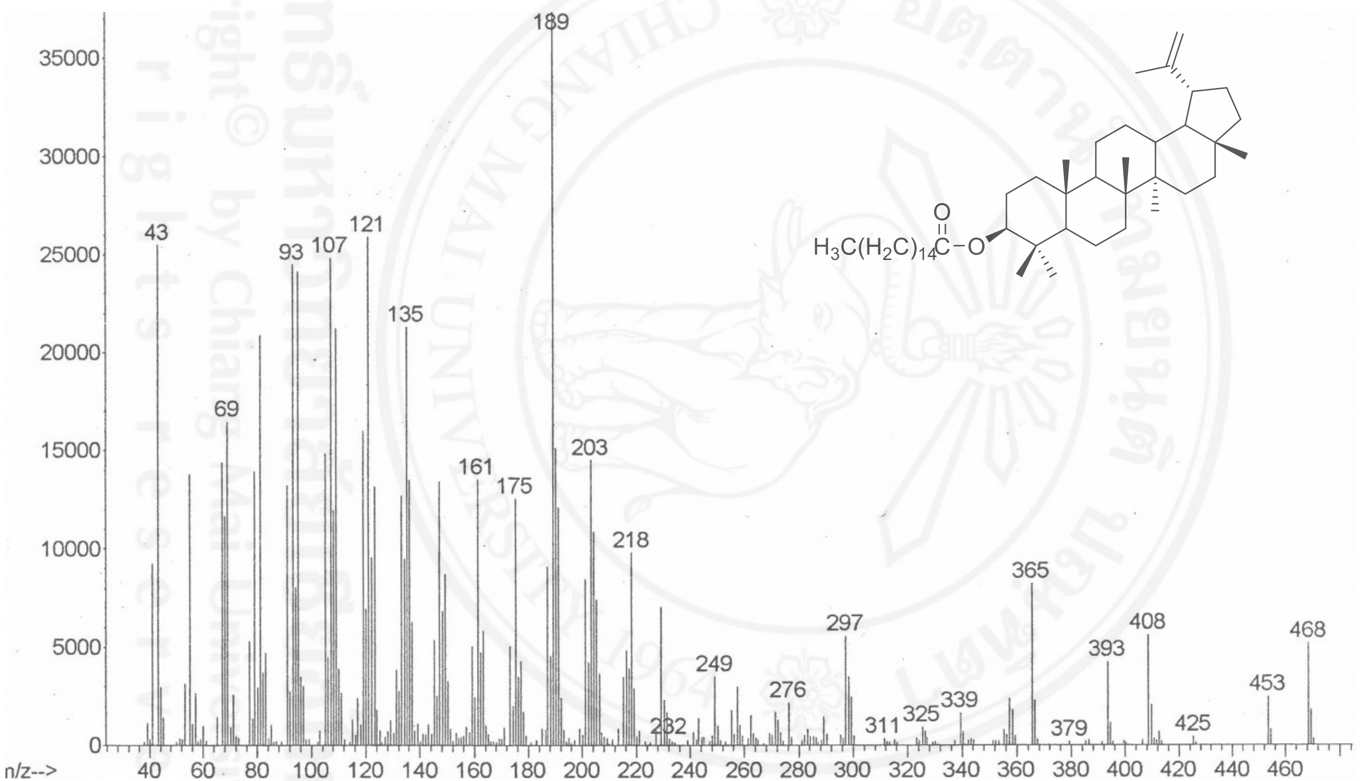

Figure 7 Mass spectrum (GC-MS (EI)) of lupeol palmitate (**134**)

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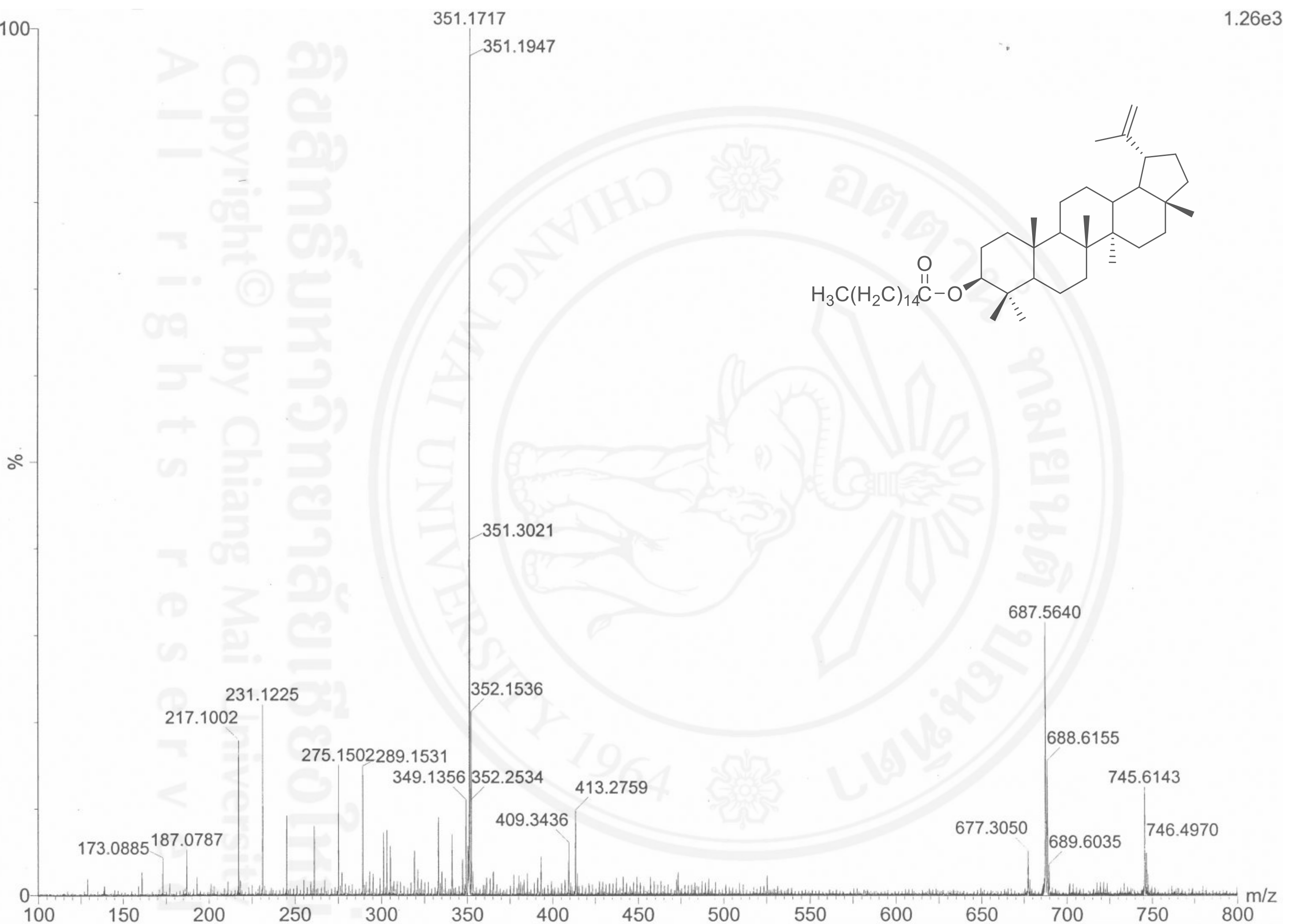

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Figure 8 Mass spectrum (HRMS (ESI)) of lupeol palmitate (**134**)

Figure 9 ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum of lupeol acetate (**135**)

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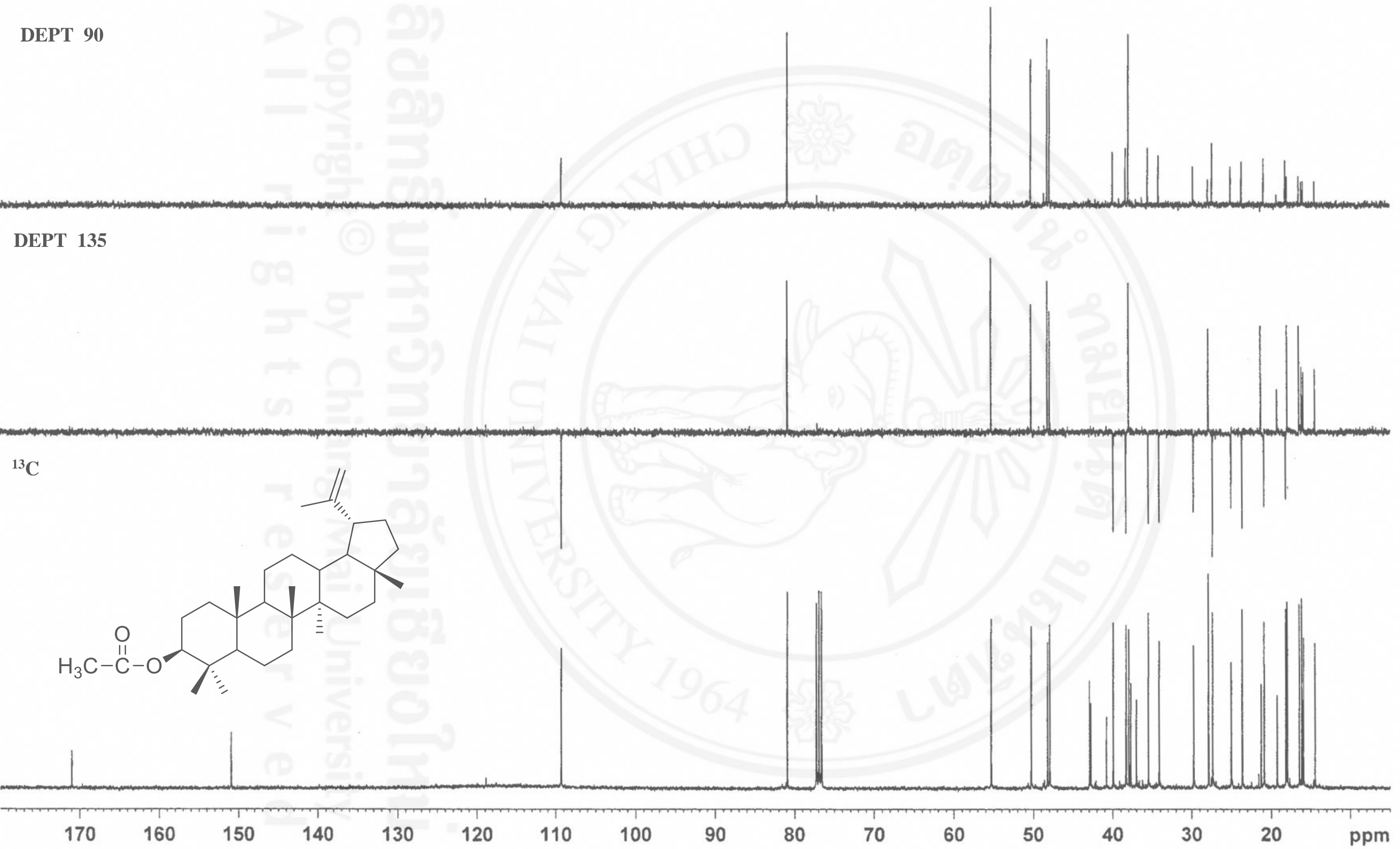

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Figure 10 ^{13}C NMR (100 MHz, in $CDCl_3$) and DEPT spectra of lupeol acetate (**135**)

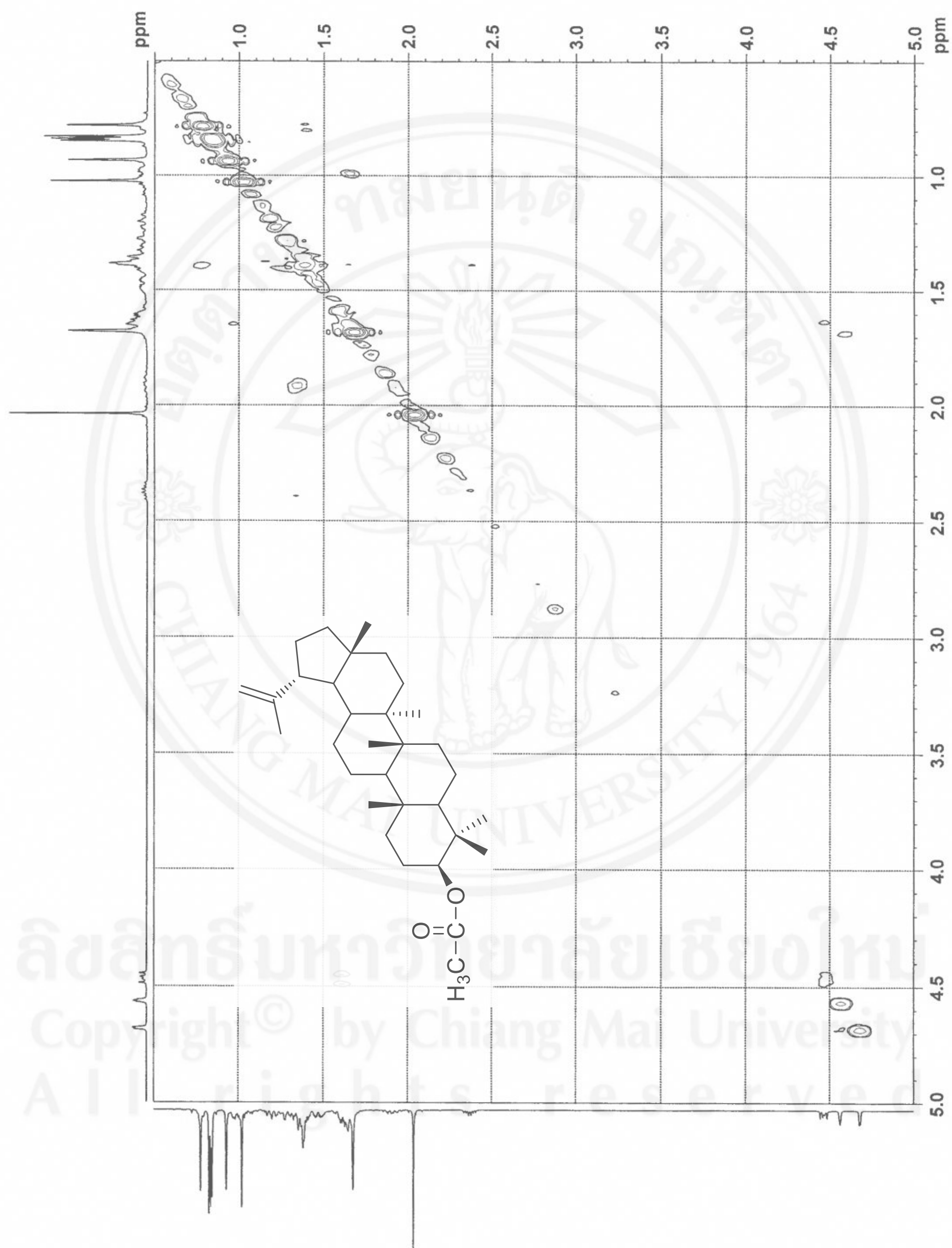

Figure 11 ^{1}H-^{1}H COSY (in $CDCl_3$) spectrum of lupeol acetate (**135**)

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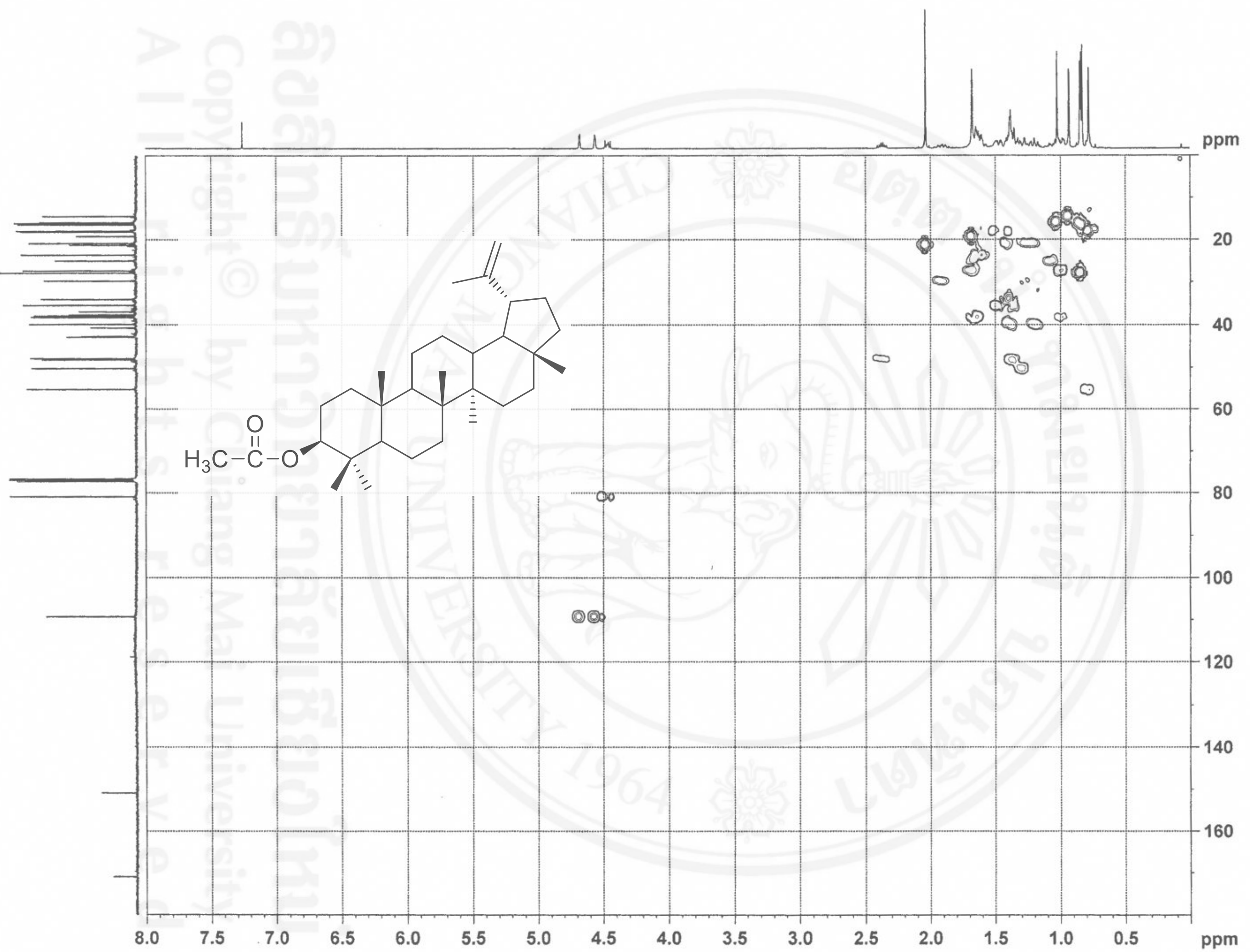

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Figure 12 HMQC (in $CDCl_3$) spectrum of lupeol acetate (**135**)

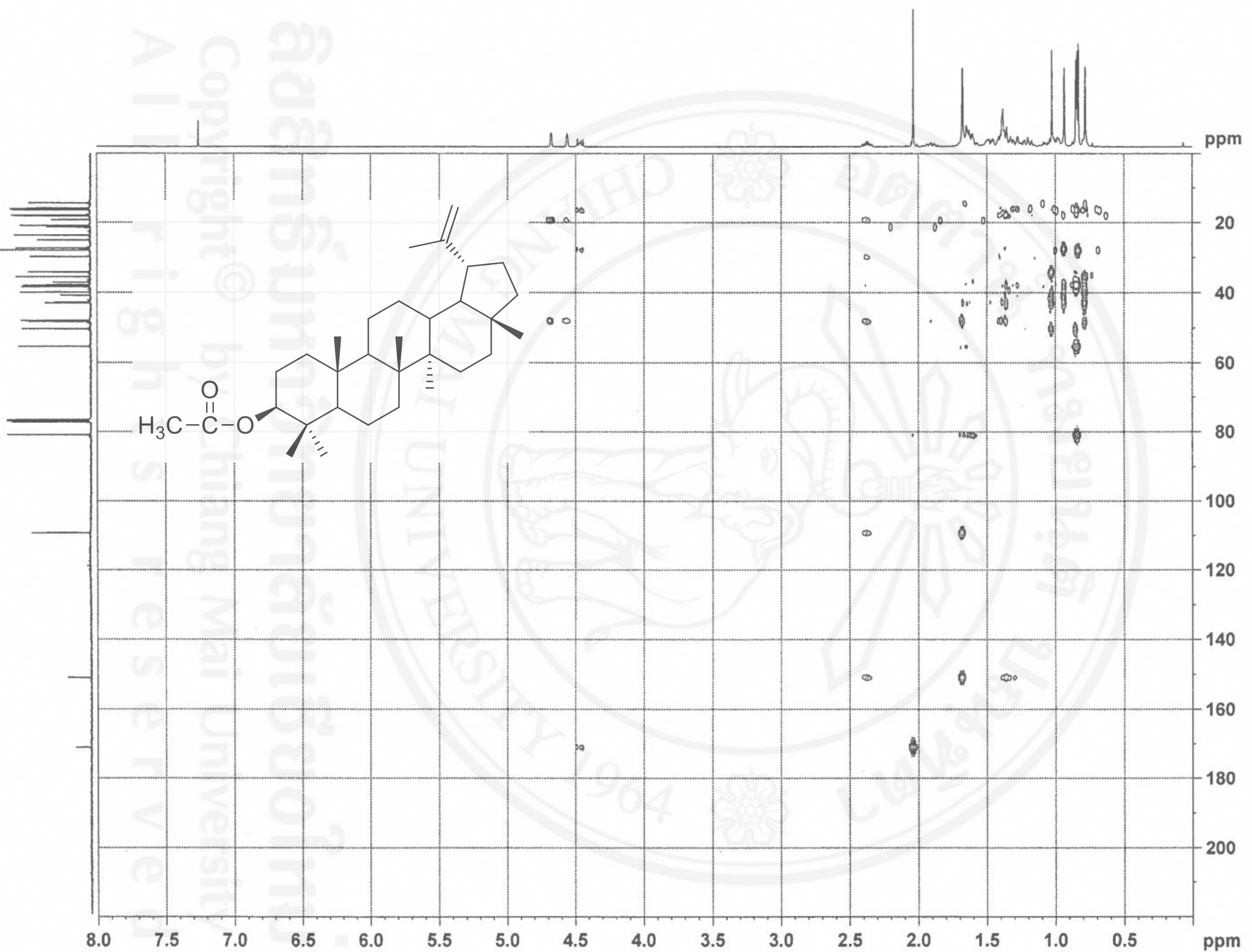

Figure 13 HMBC (in $CDCl_3$) spectrum of lupeol acetate (**135**)

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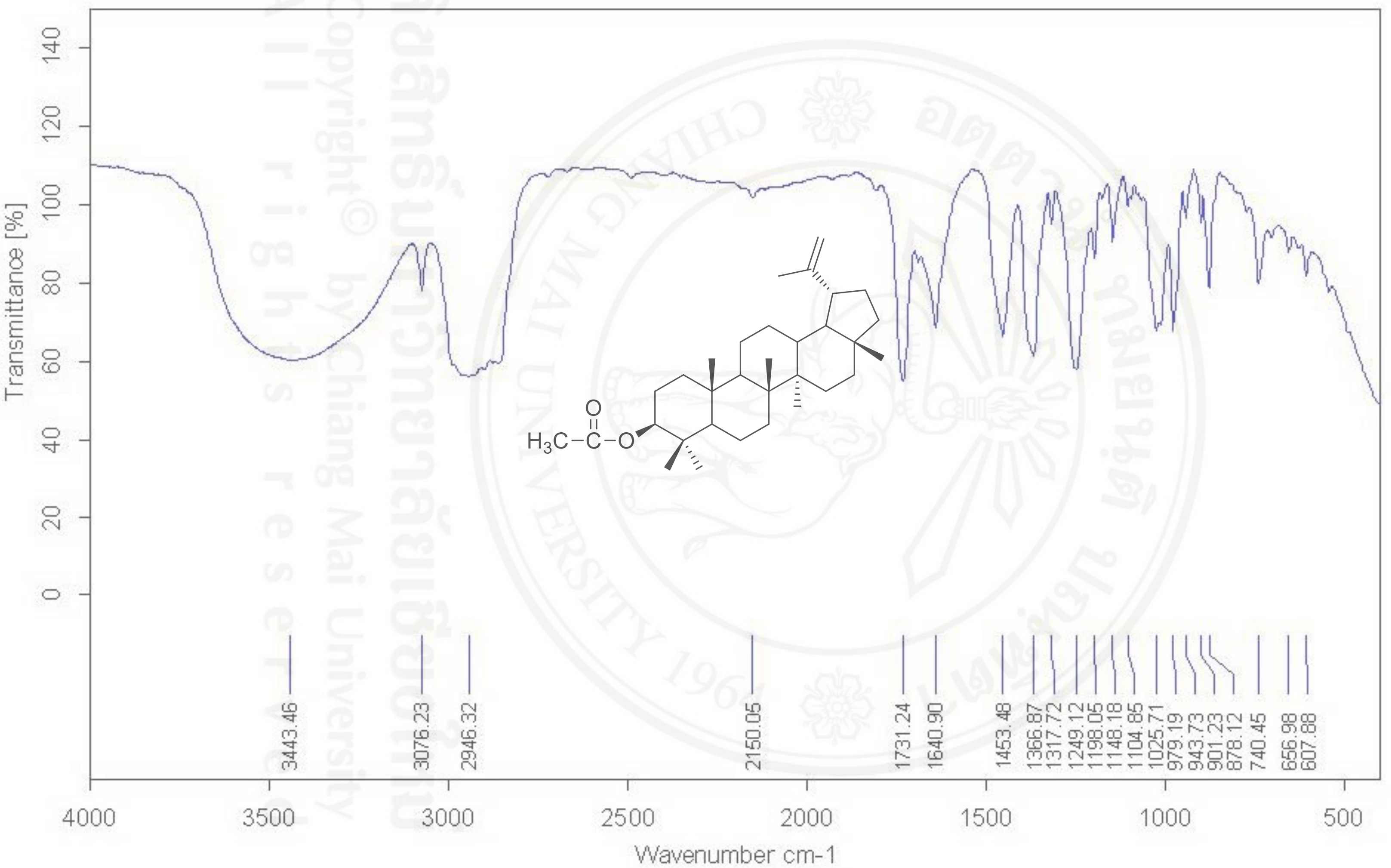

Figure 14 FTIR (evaporated thin film) spectrum of lupeol acetate (**135**)

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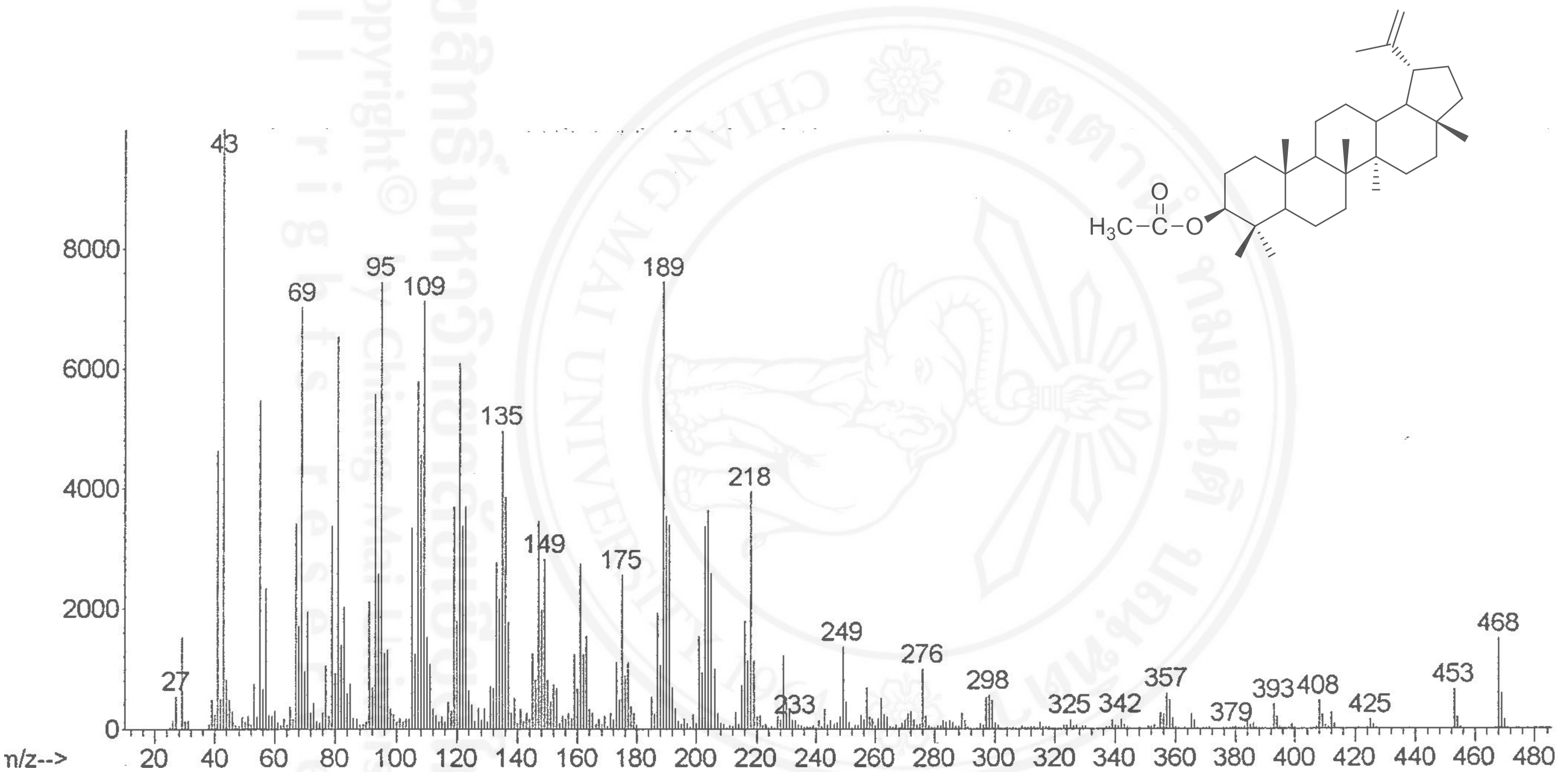

Figure 15 Mass spectrum (GC-MS (EI)) of lupeol acetate (**135**)

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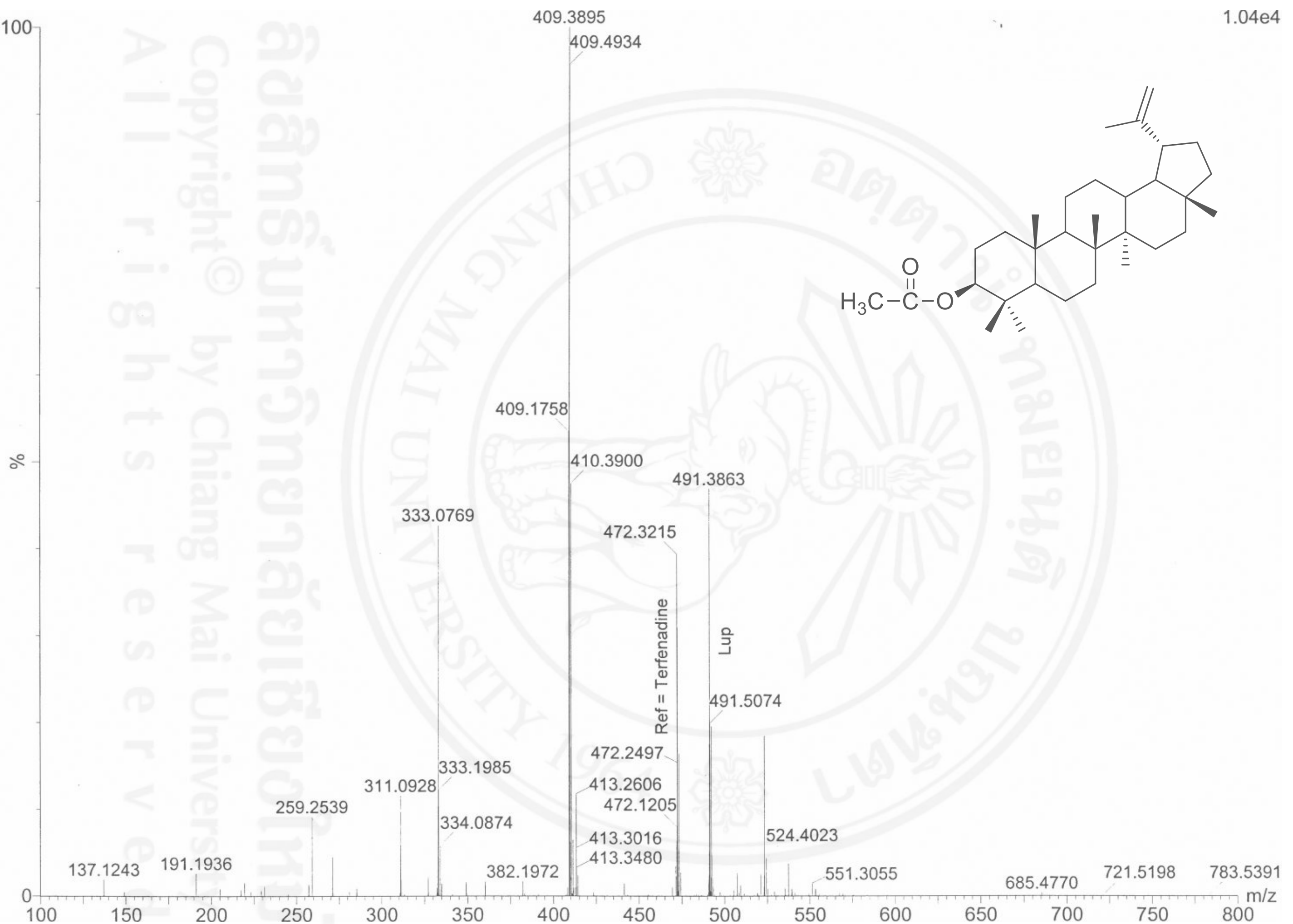

Figure 16 Mass spectrum (HRMS (ESI)) of lupeol acetate (**135**)

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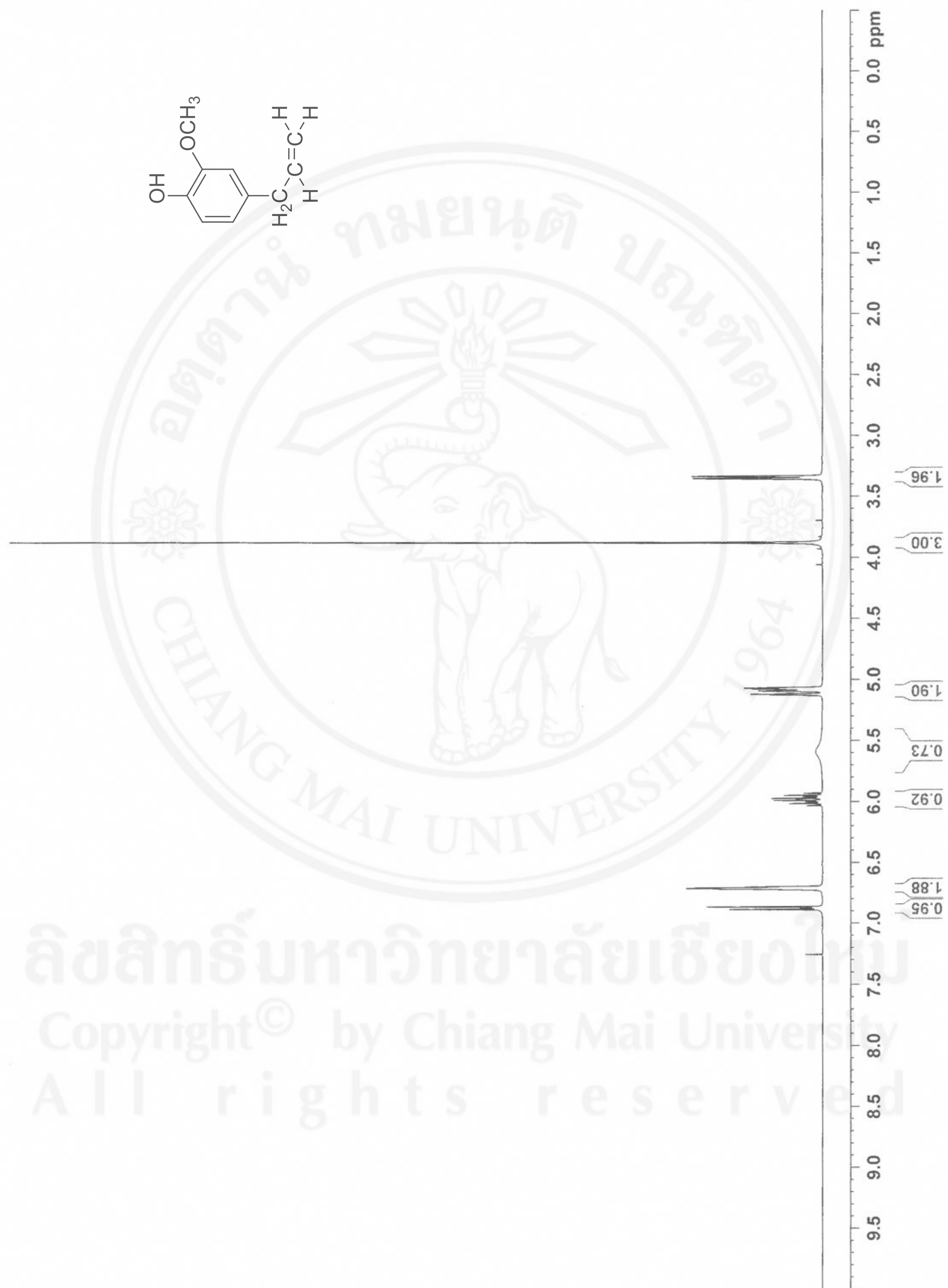

Figure 17 ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum of eugenol (**98**)

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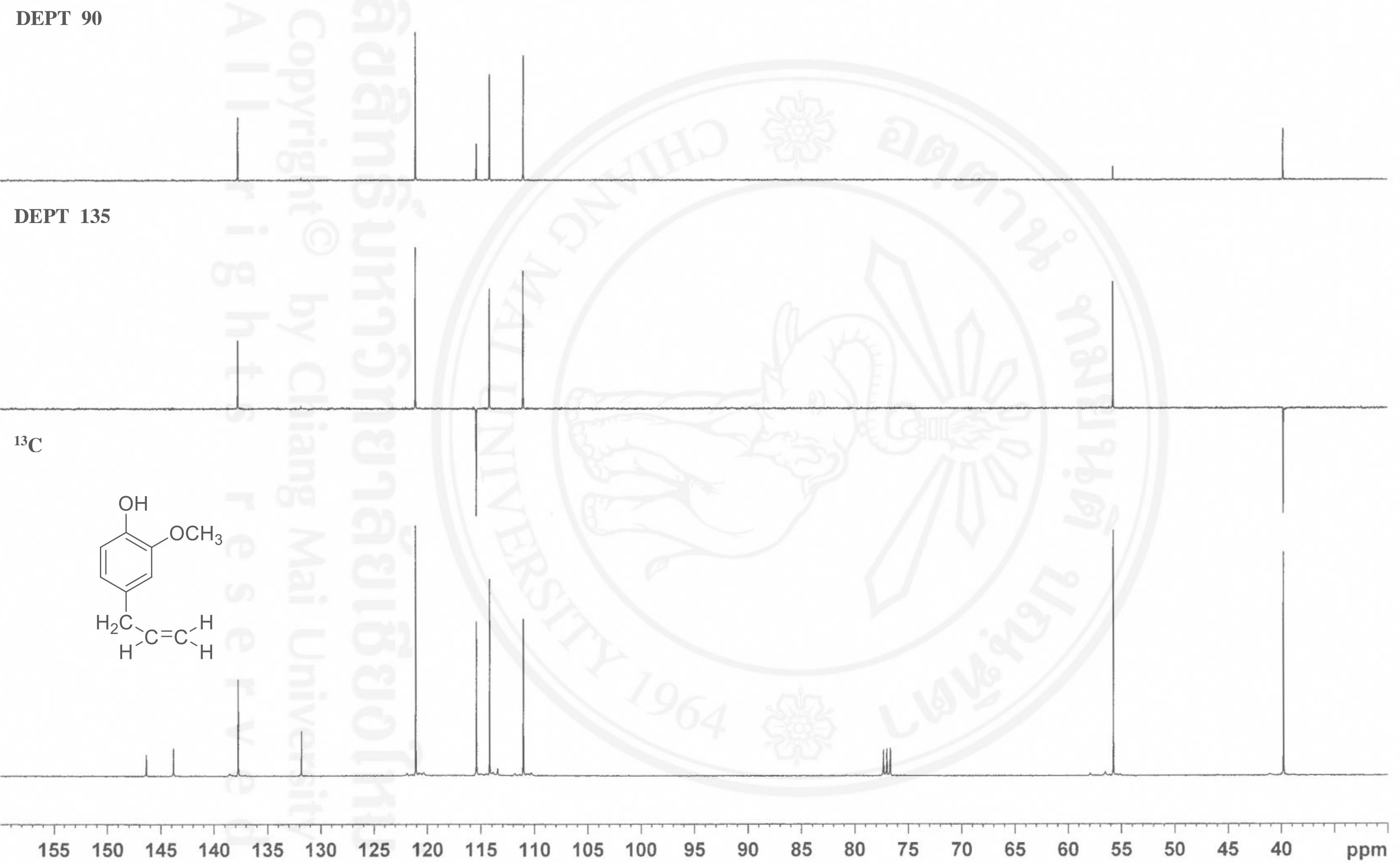

Figure 18 ^{13}C NMR (100 MHz, in $CDCl_3$) and DEPT spectra of eugenol (**98**)

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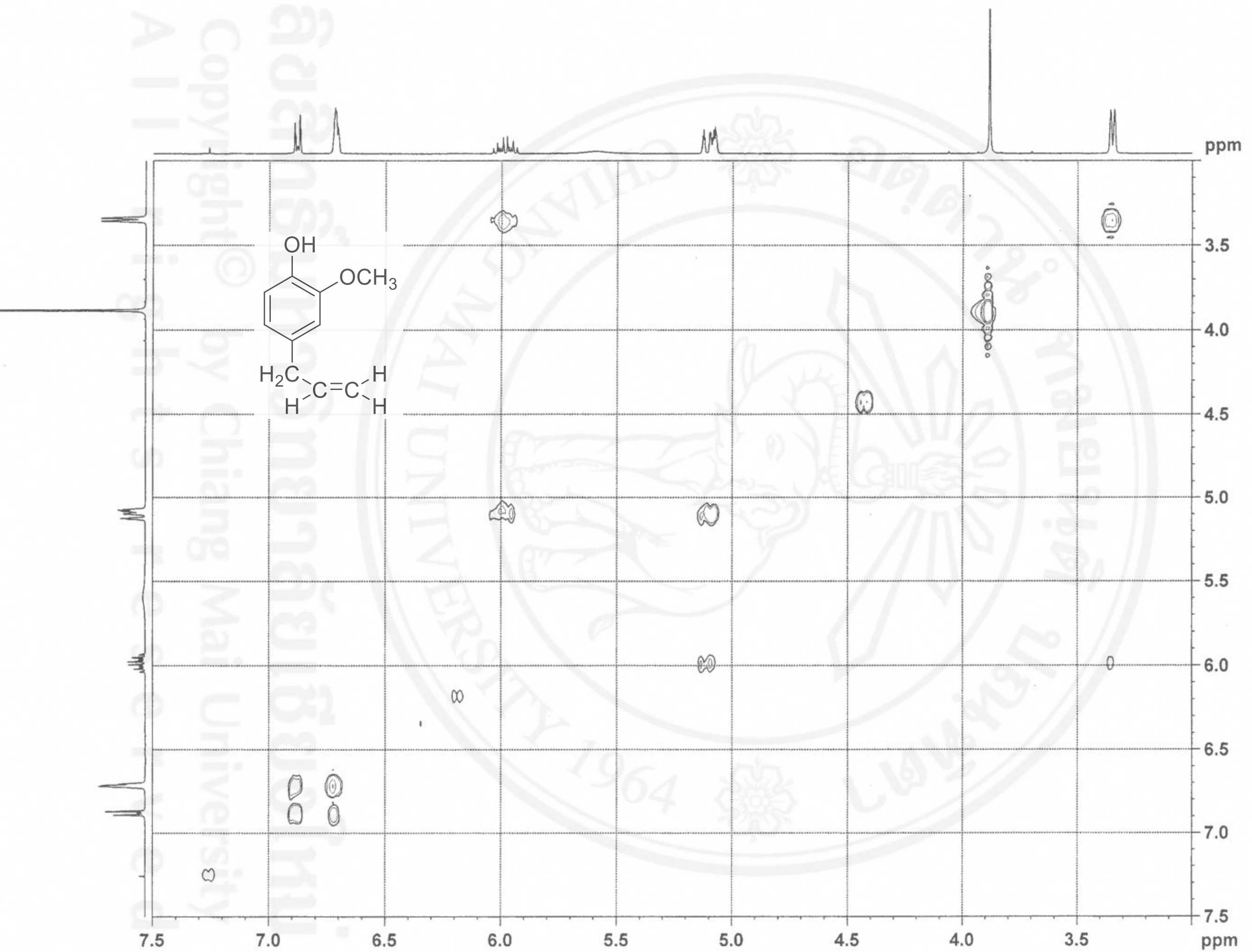

Figure 19 ^{1}H-^{1}H COSY (in $CDCl_3$) spectrum of eugenol (**98**)

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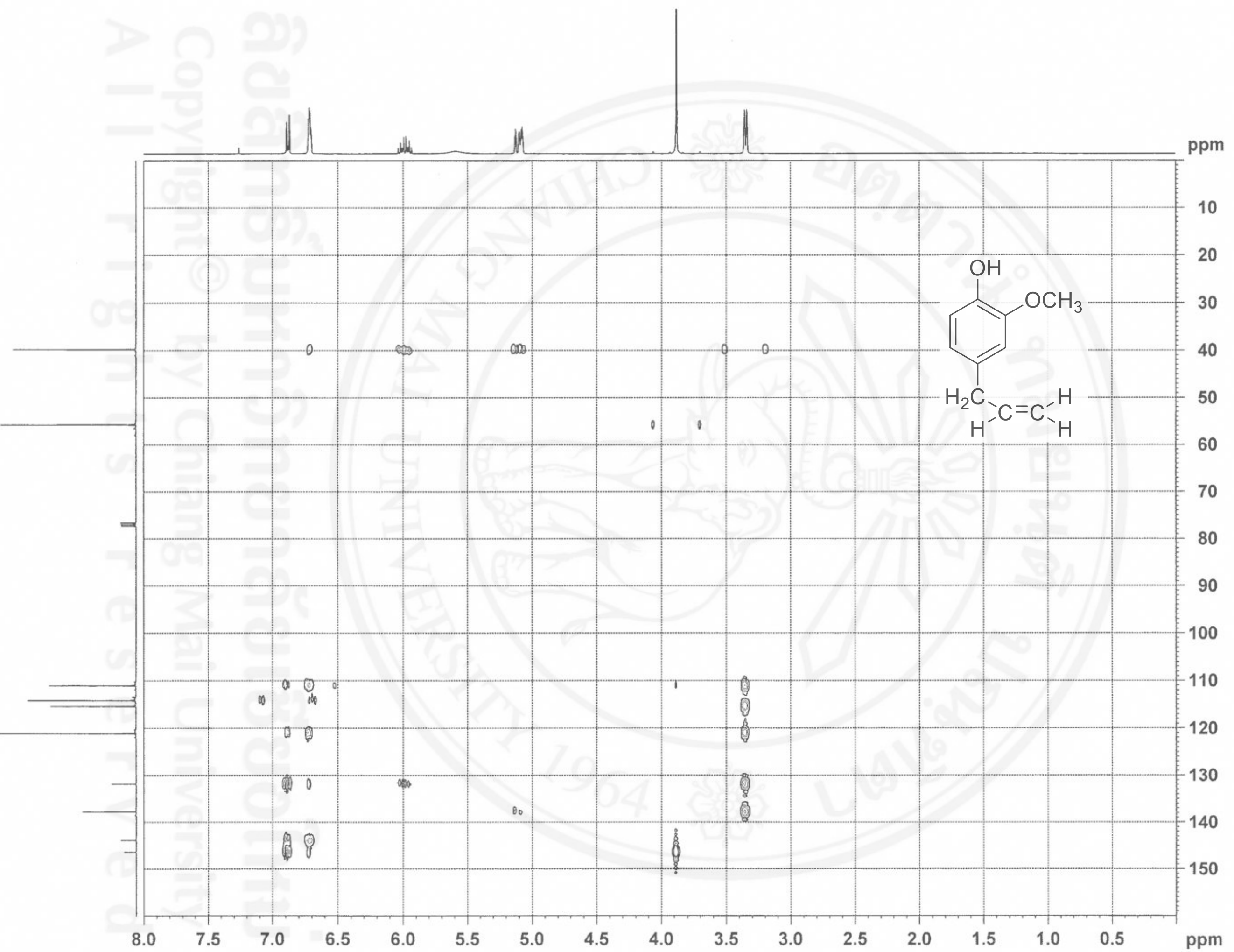

Figure 20 HMQC (in $CDCl_3$) spectrum of eugenol (**98**)

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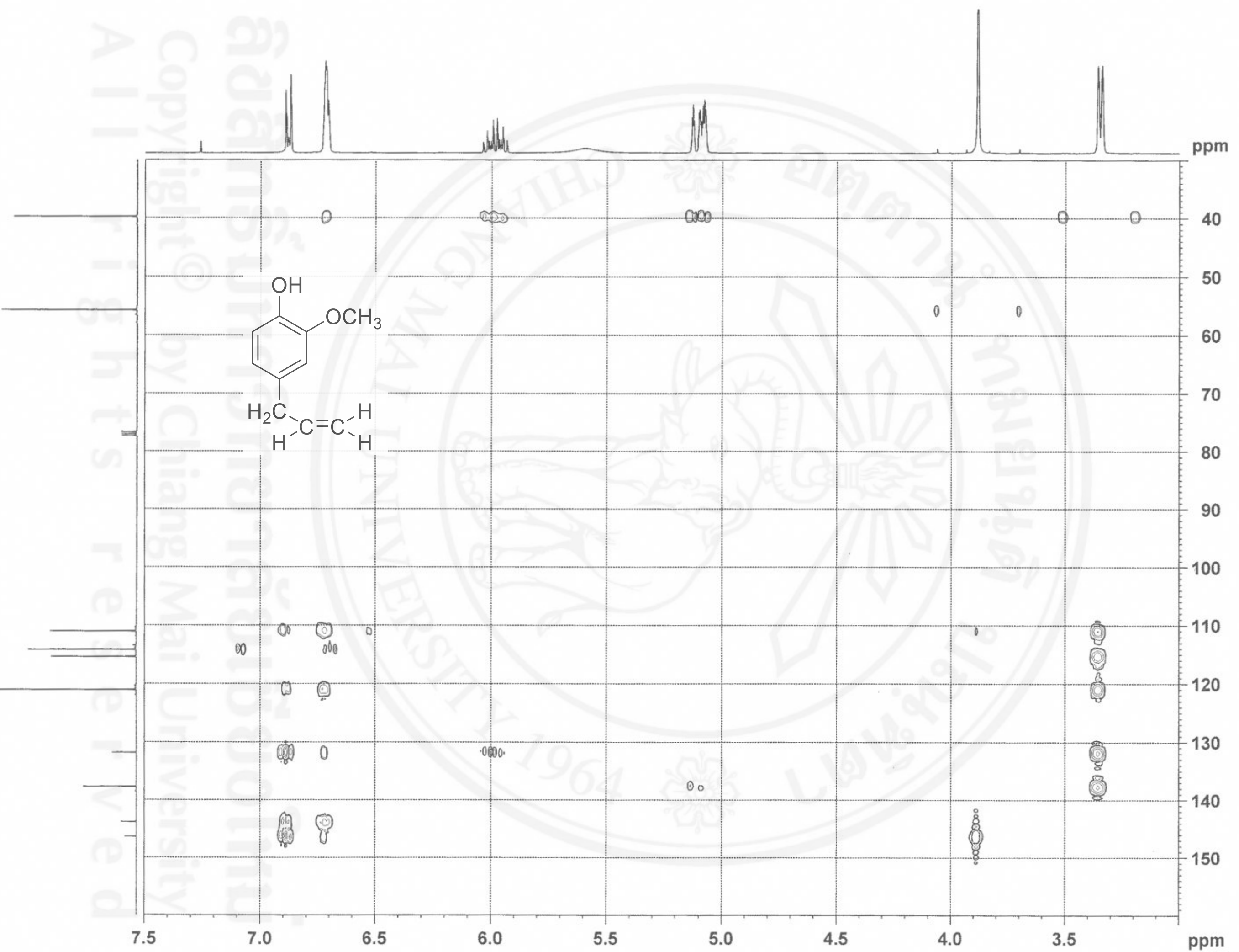

Figure 21 HMBC (in $CDCl_3$) spectrum of eugenol (**98**)

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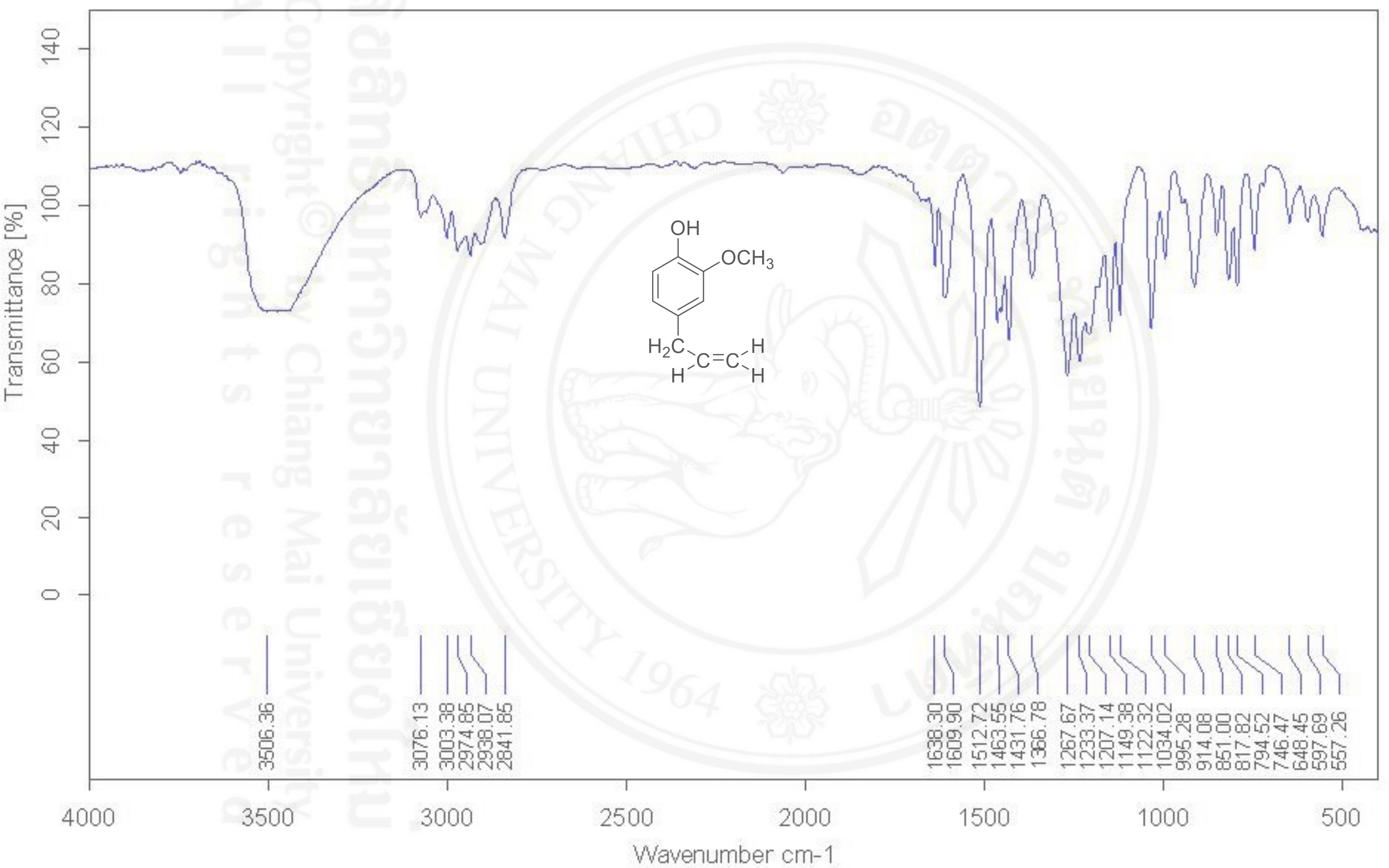

Figure 22 FTIR (evaporated thin film) spectrum of eugenol (**98**)

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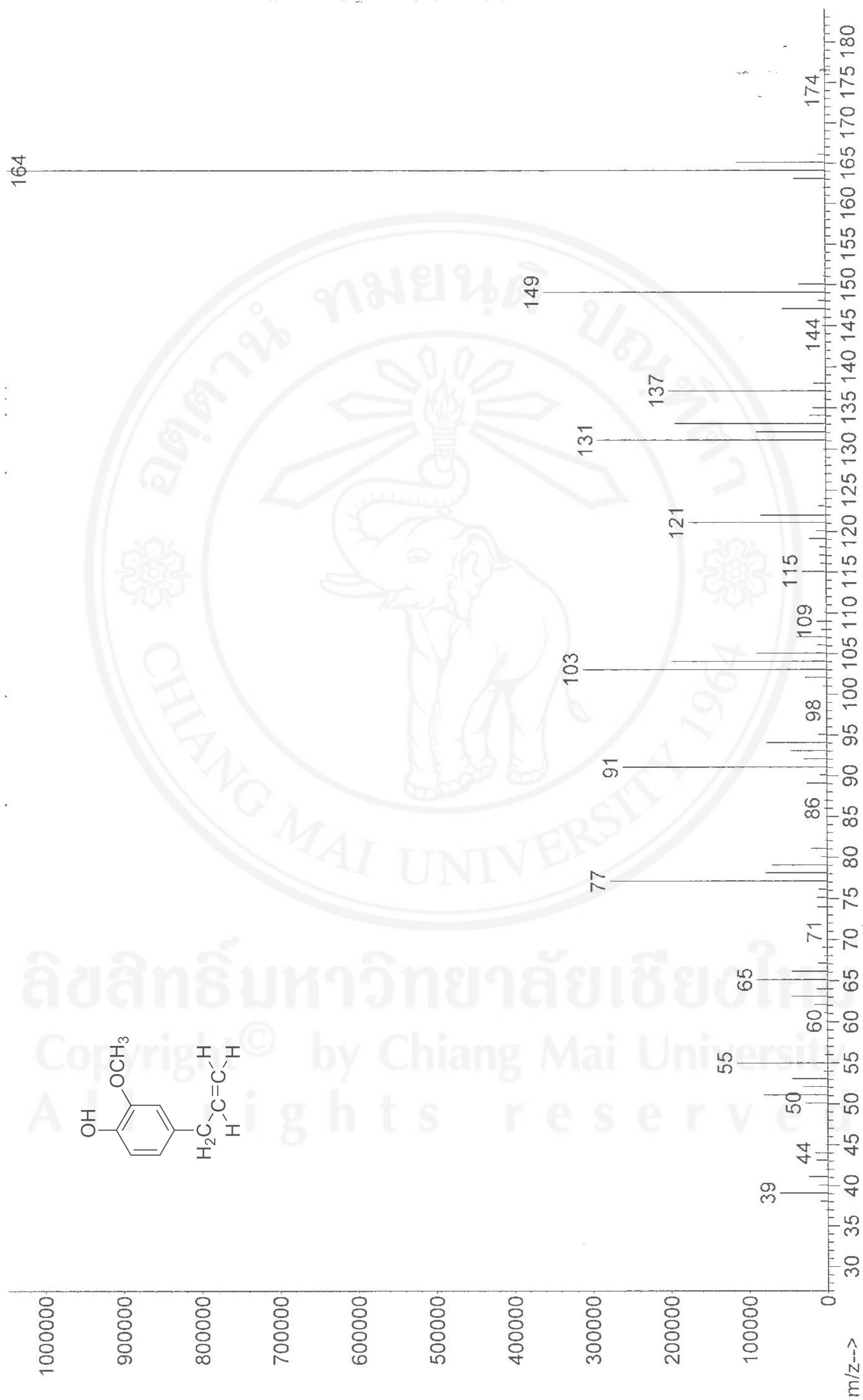

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Figure 23 Mass spectrum (GC-MS (EI)) of eugenol (**98**)

Figure 24 Mass spectrum (HRMS (ESI)) of eugenol (**98**)

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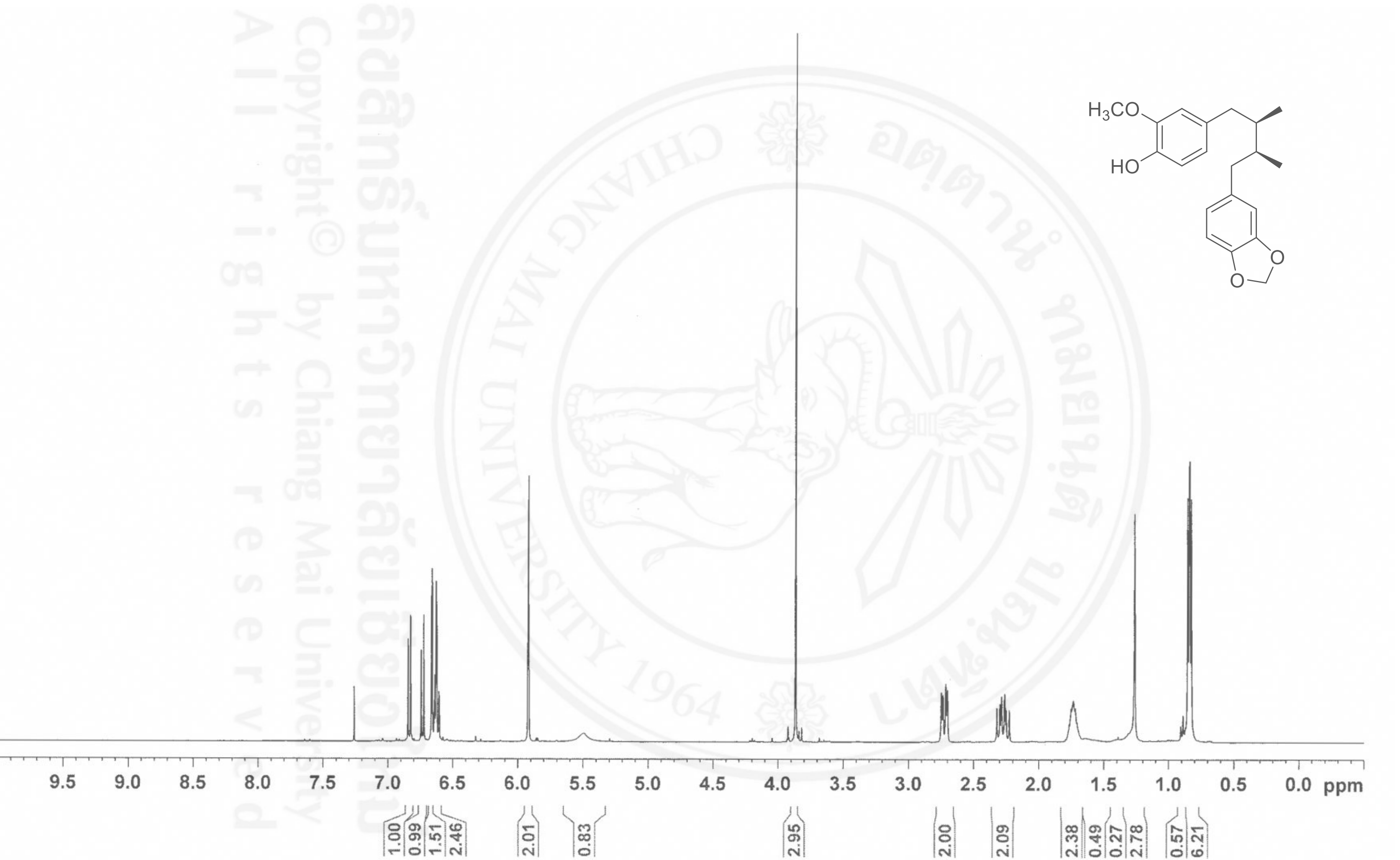

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Figure 25 ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum of macelignan (**136**)

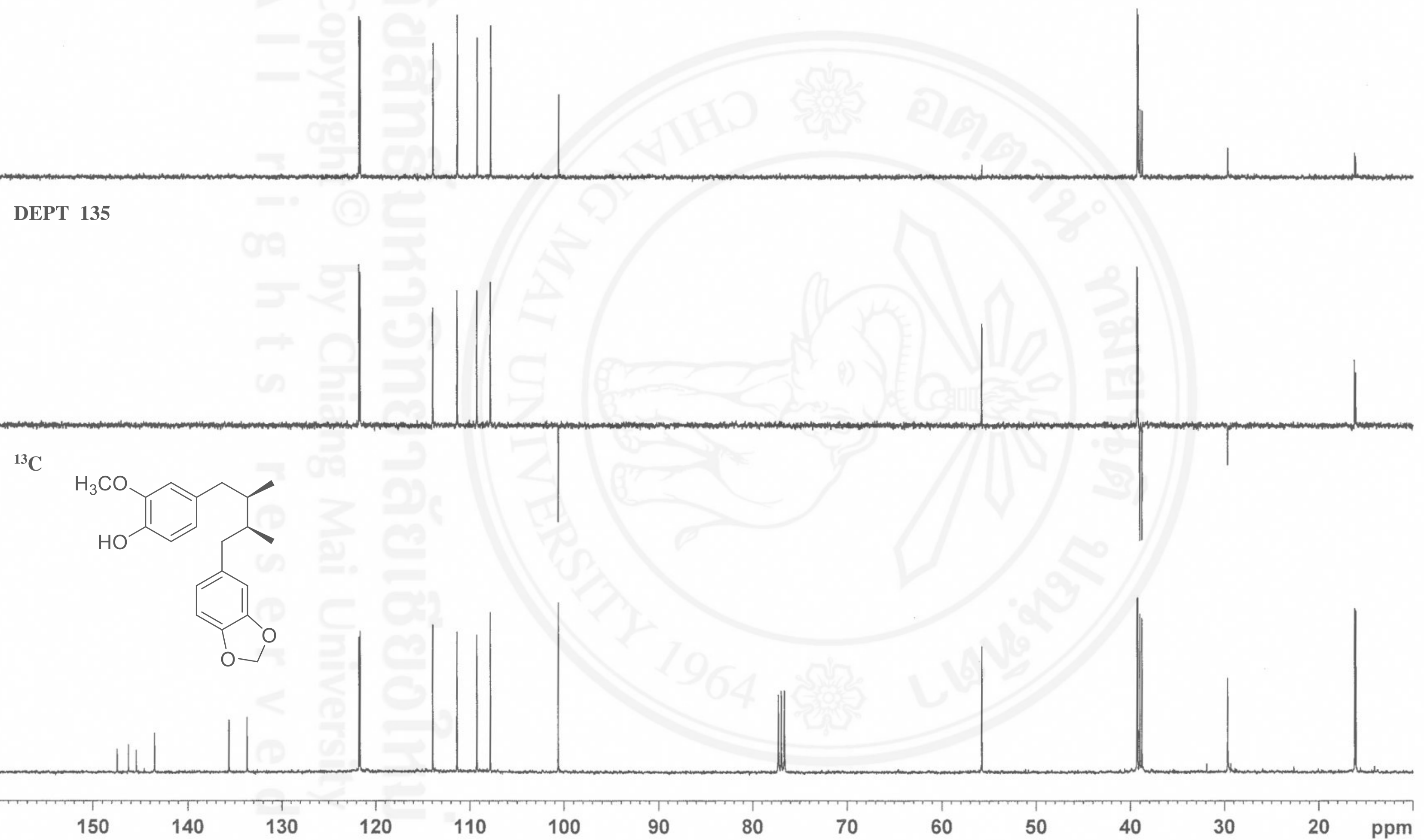

Figure 26 ^{13}C NMR (100 MHz, in $CDCl_3$) and DEPT spectra of macelignan (**136**)

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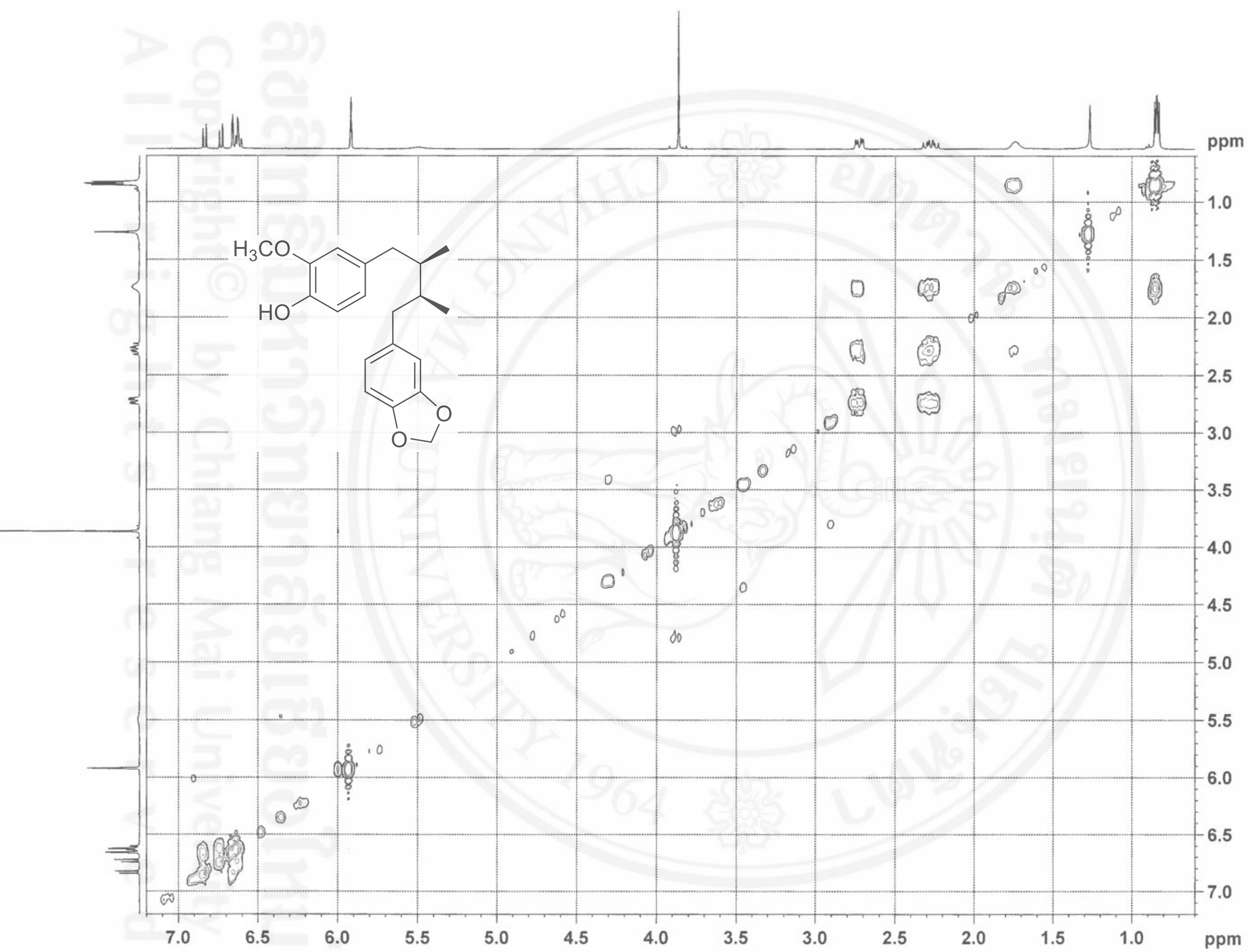

Figure 27 ^{1}H-^{1}H COSY (in $CDCl_3$) spectrum of macelignan (**136**)

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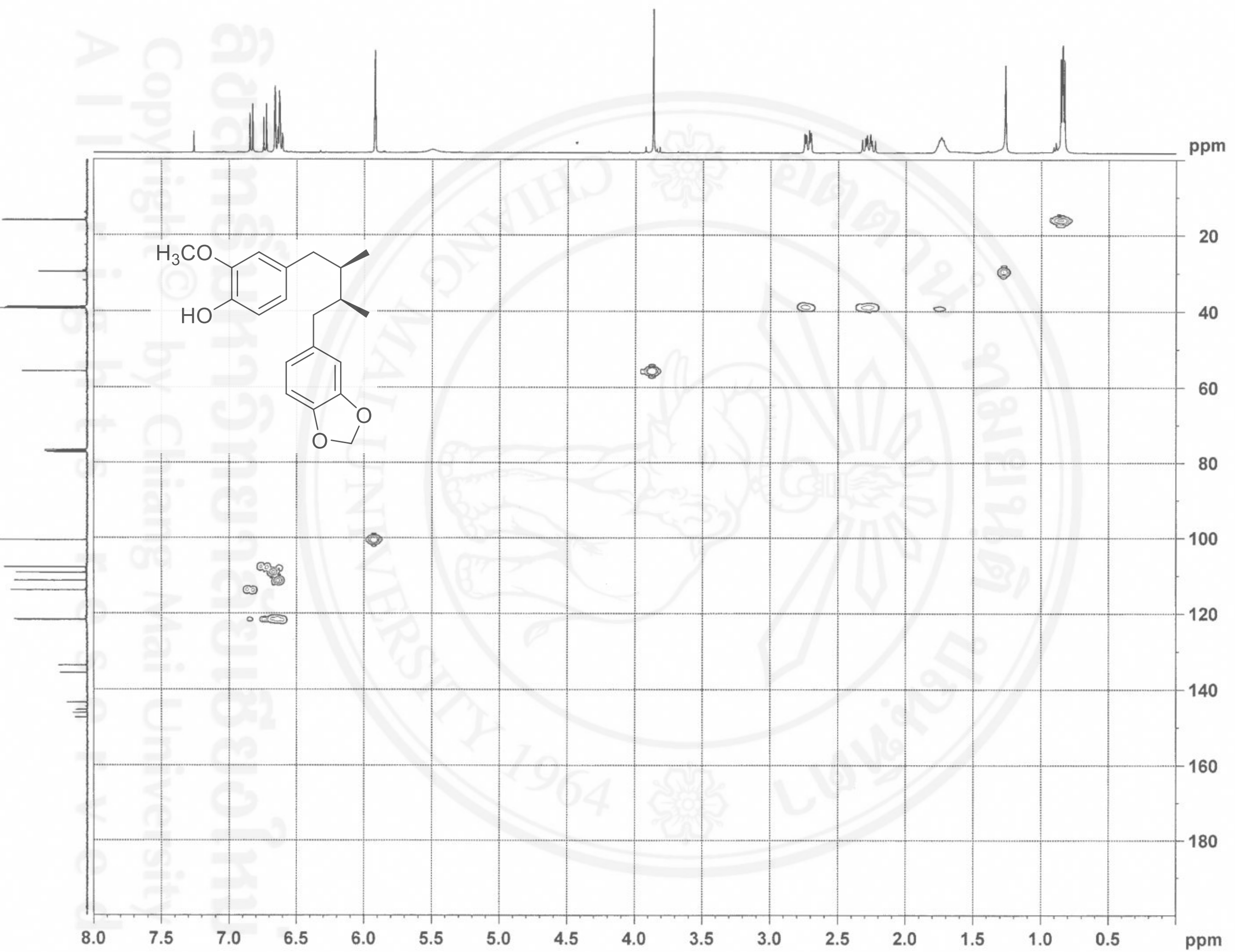

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Figure 28 HMQC (in $CDCl_3$) spectrum of macelignan (**136**)

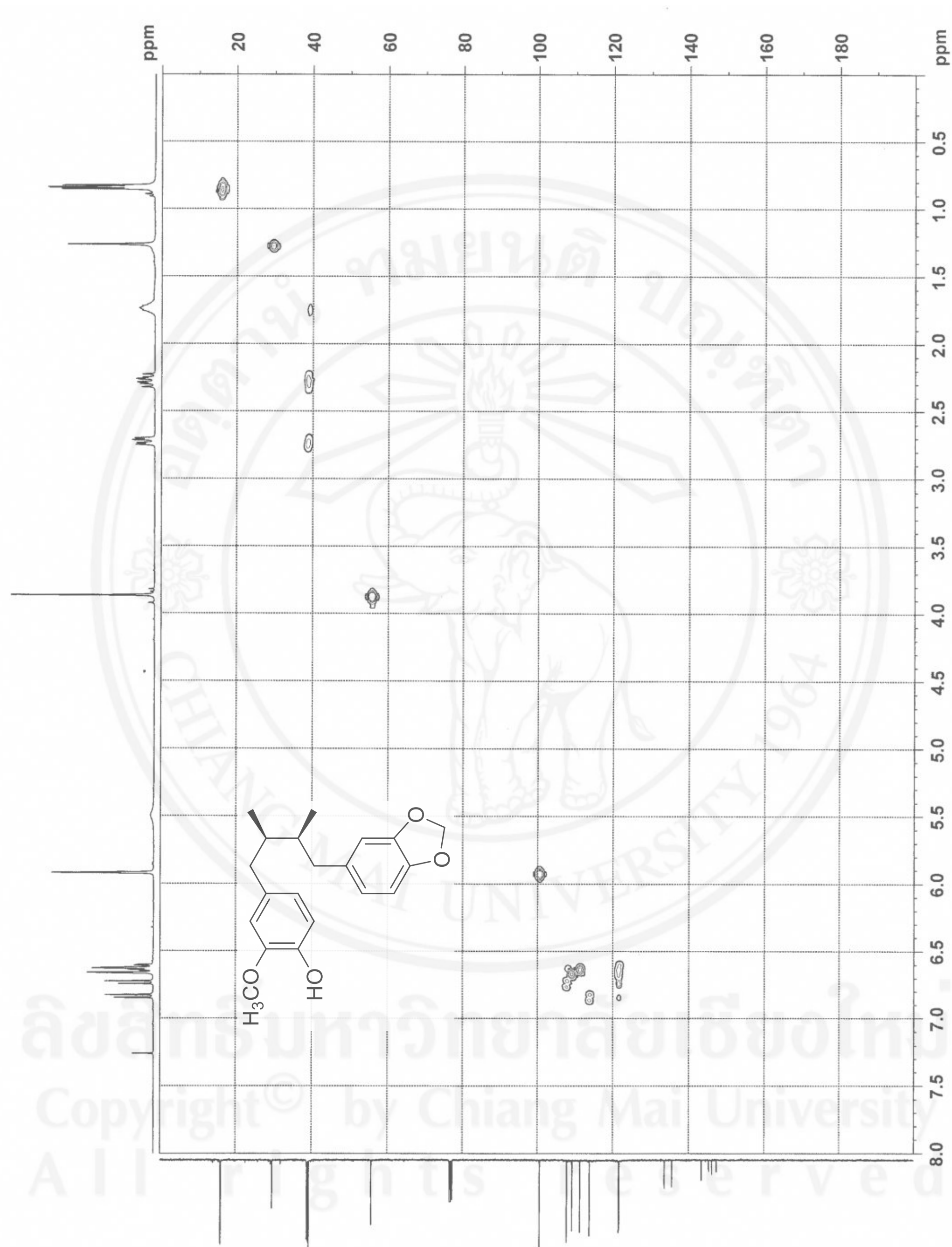

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Figure 29 HMBC (in $CDCl_3$) spectrum of macelignan (**136**)

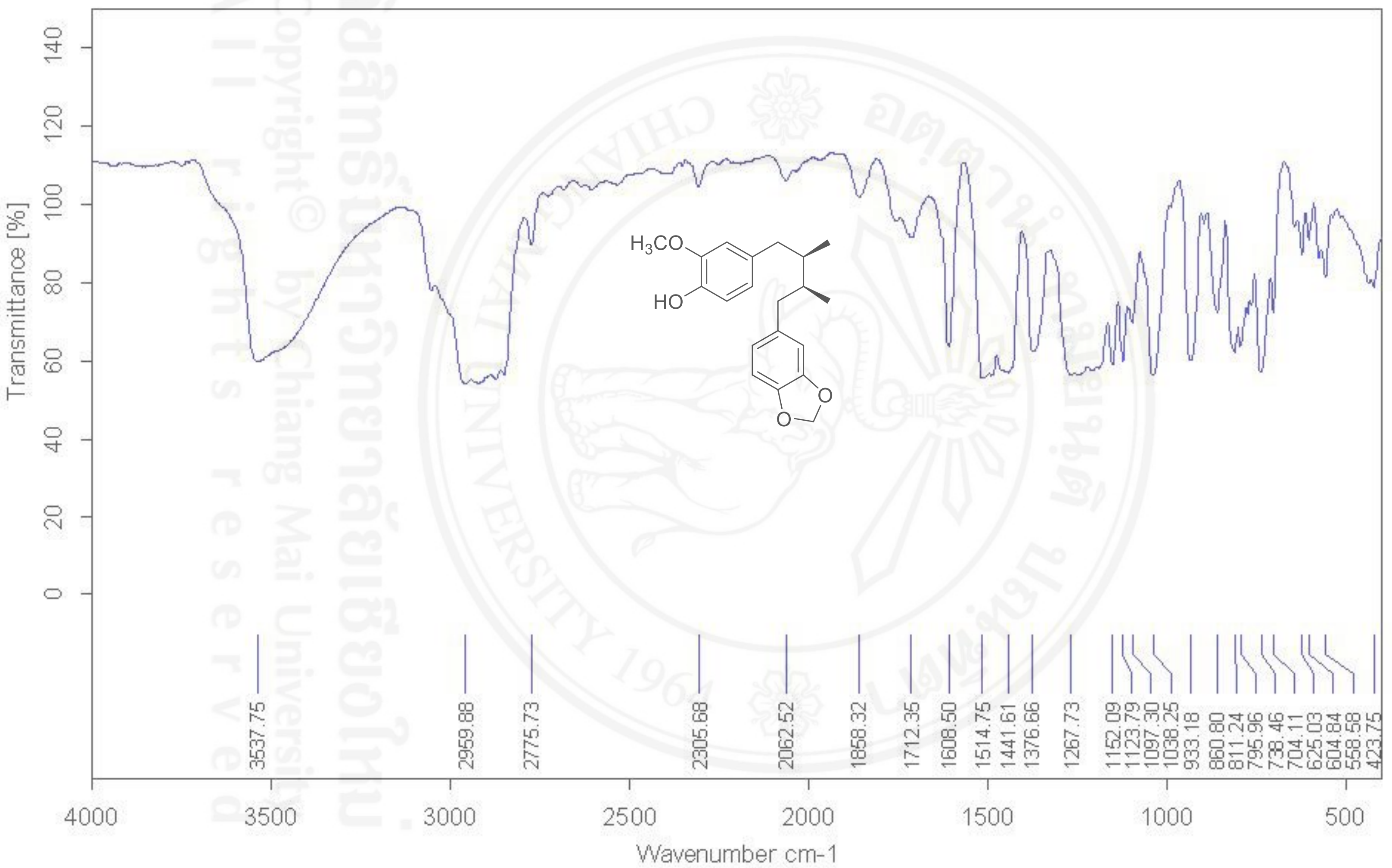

Figure 30 FTIR (evaporated thin film) spectrum of macelignan (**136**)

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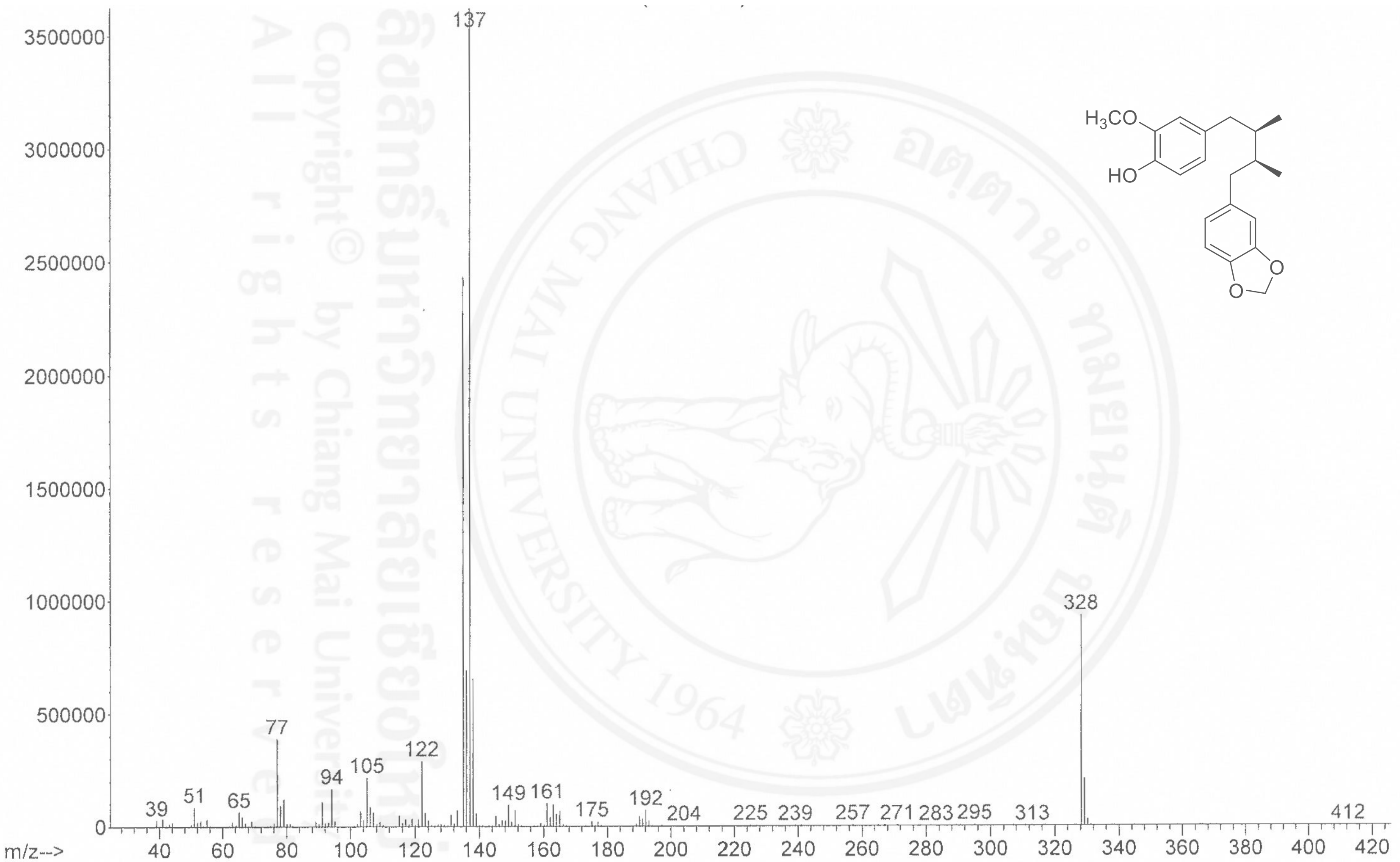

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Figure 31 Mass spectrum (GC-MS (EI)) of macelignan (**136**)

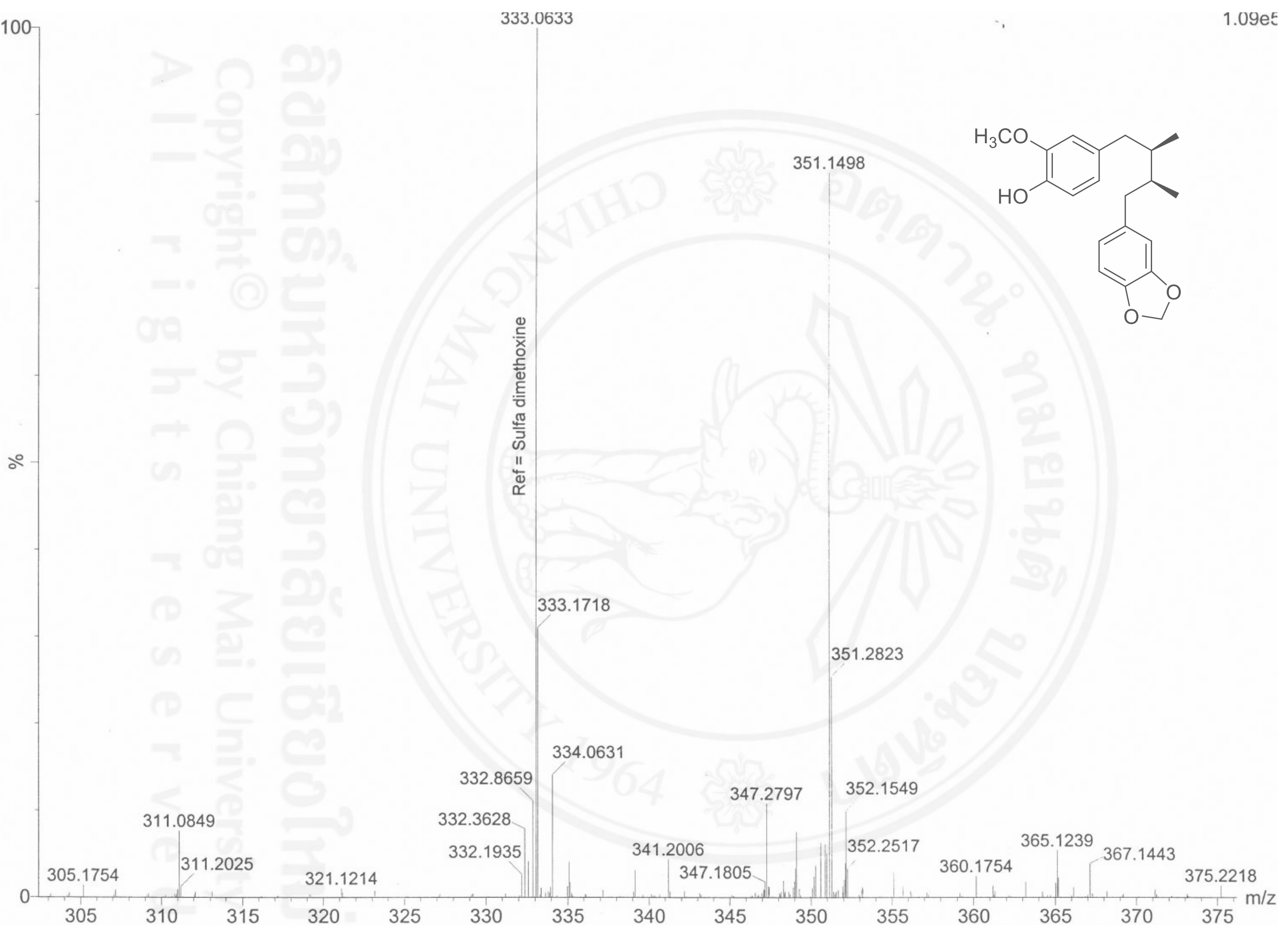

Figure 32 Mass spectrum (HRMS (ESI)) of macelignan (**136**)

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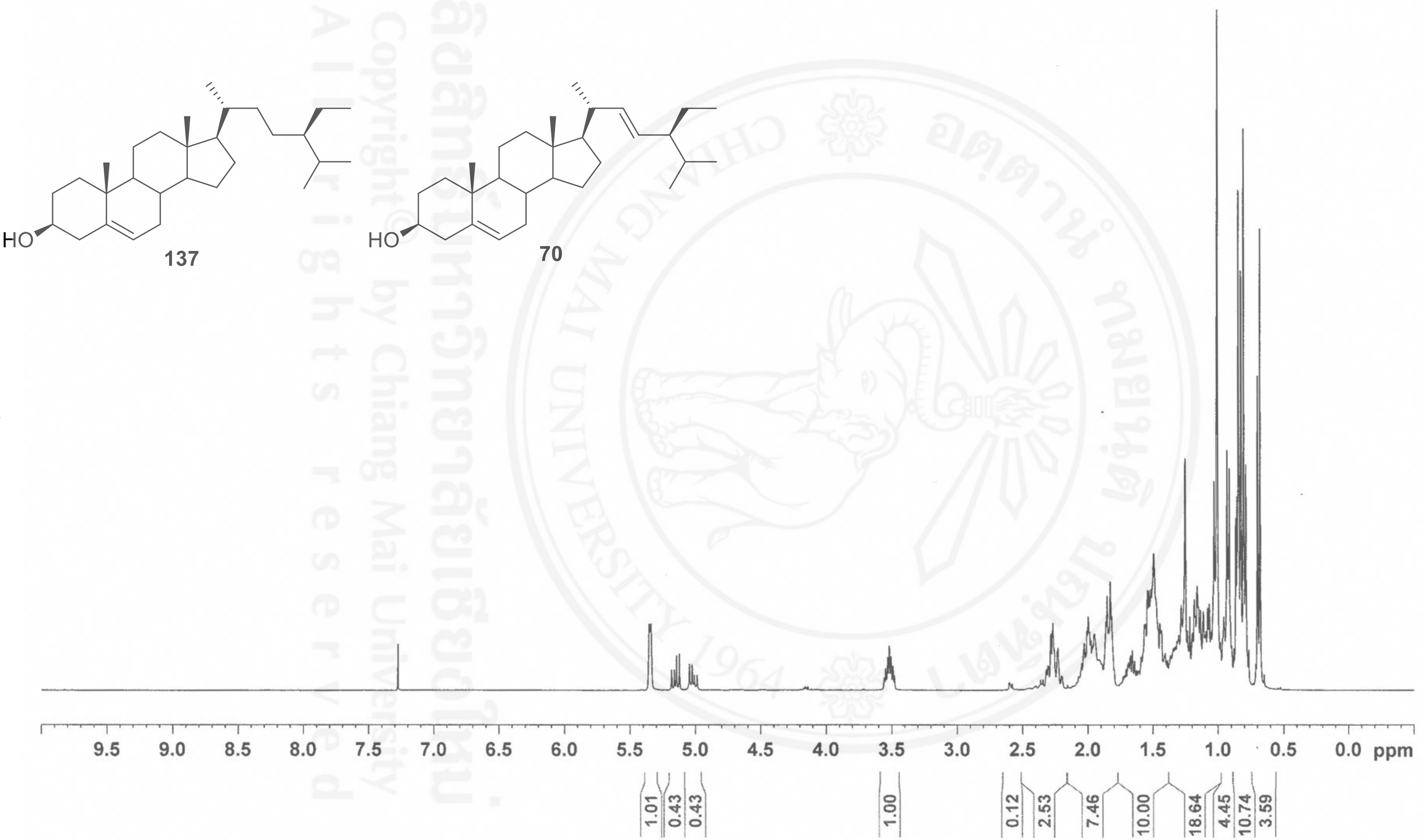

Figure 33 ^{1}H NMR (400 MHz, in $CDCl_3$) spectra of *β*-Sitosterol (**137**) and stigmasterol (**70**)

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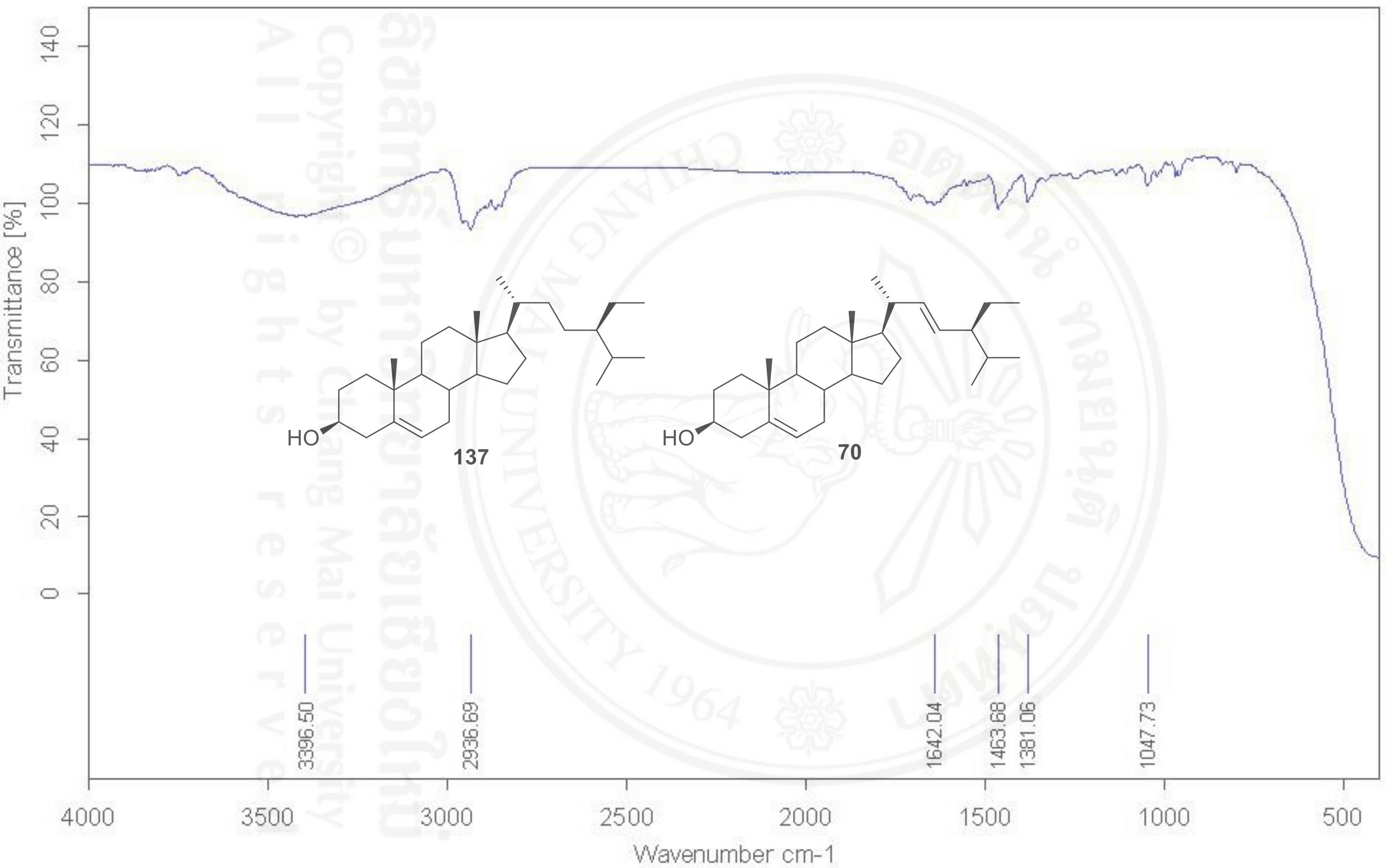

Figure 34 FTIR (evaporated thin film) spectra of β-Sitosterol (**137**) and stigmasterol (**70**)

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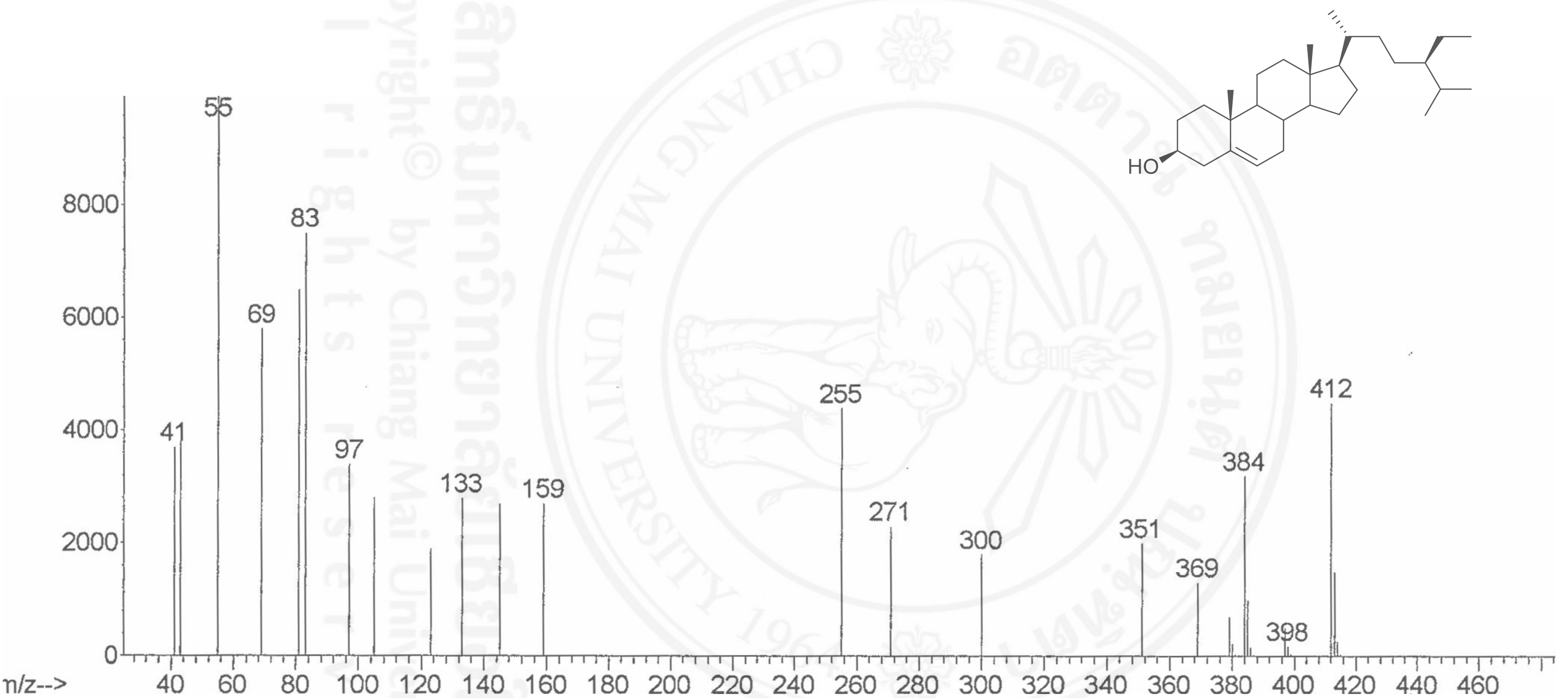

Figure 35 Mass spectrum (GC-MS (EI)) of β-Sitosterol (**137**)

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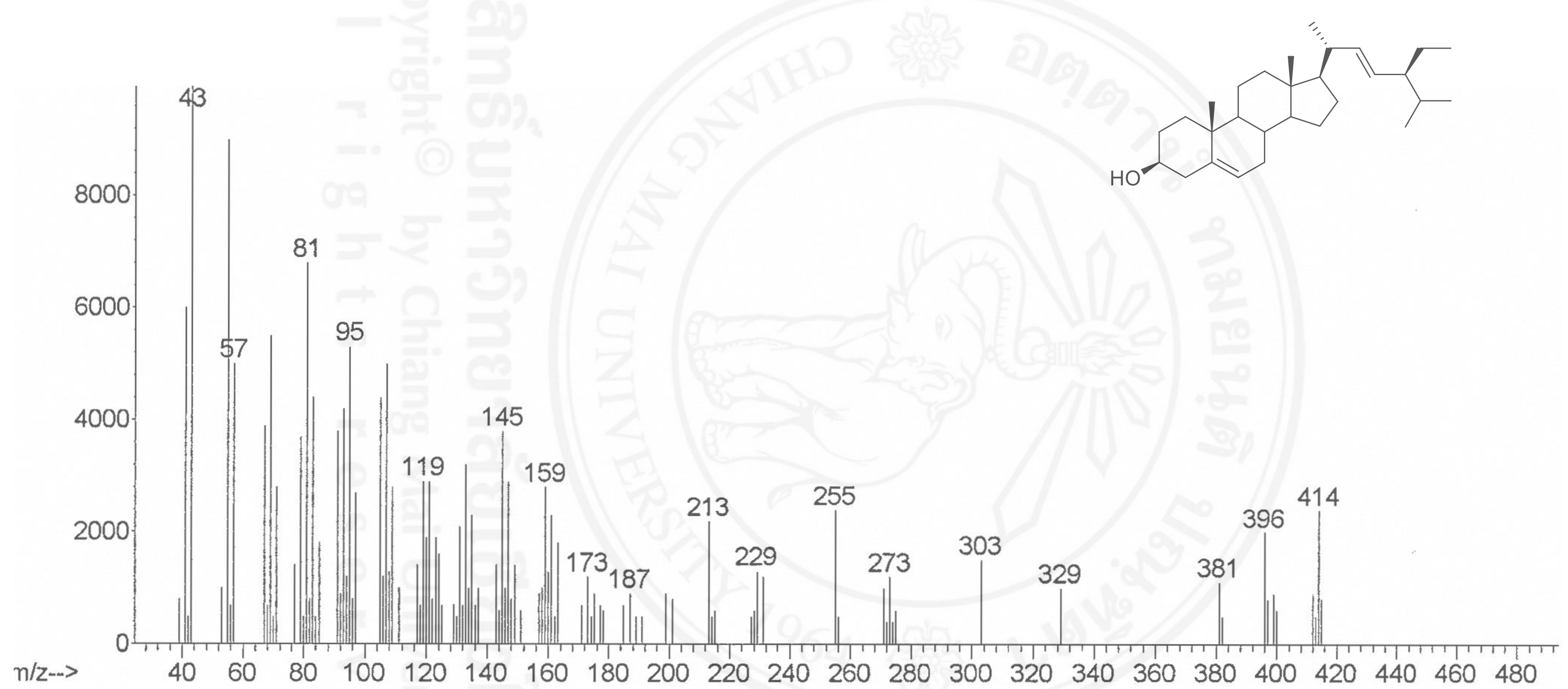

Figure 36 Mass spectrum (GC-MS (EI)) of stigmasterol (**70**)

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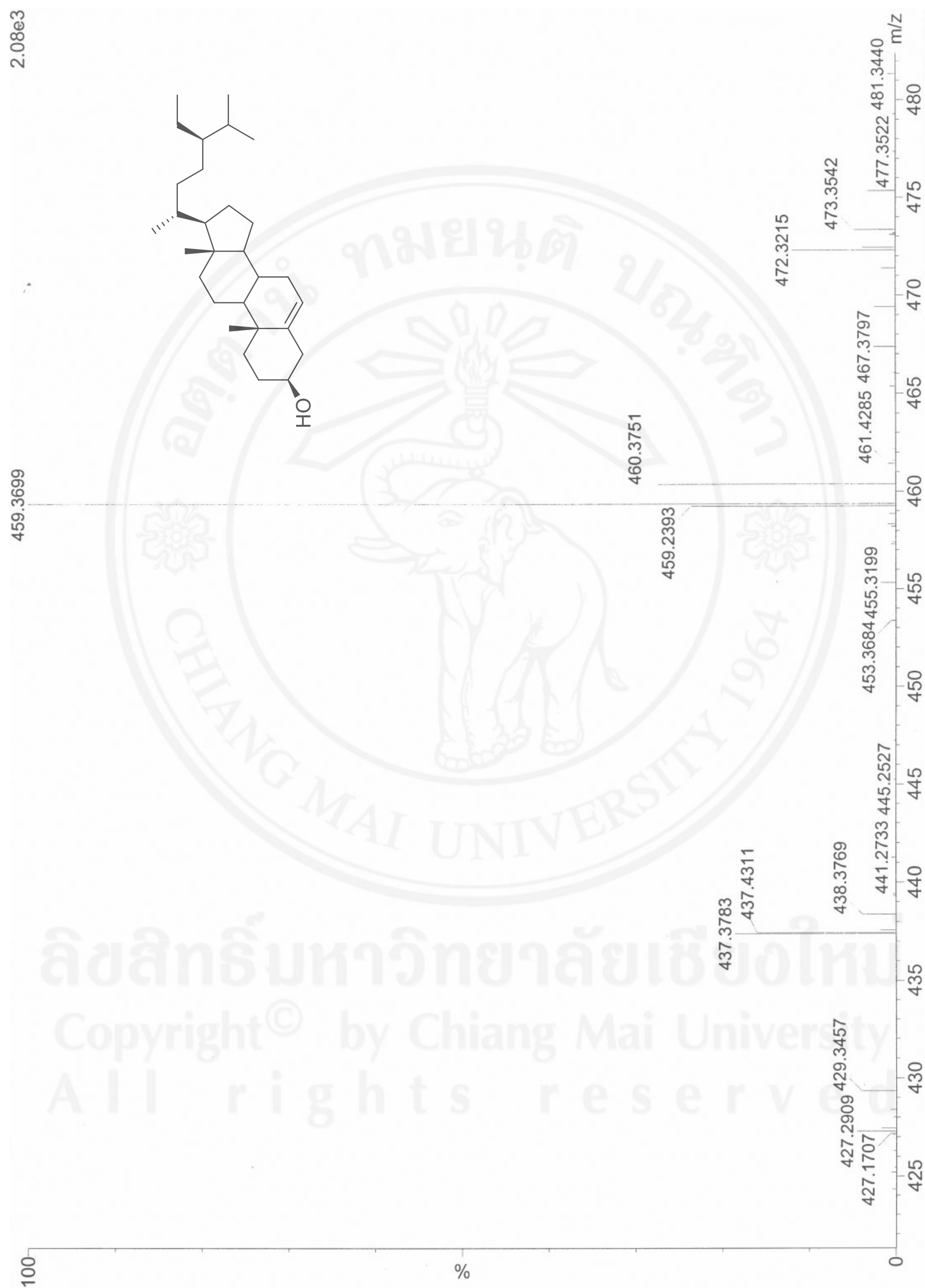

Figure 37 Mass spectrum (HRMS (ESI)) of stigmasterol (**70**)

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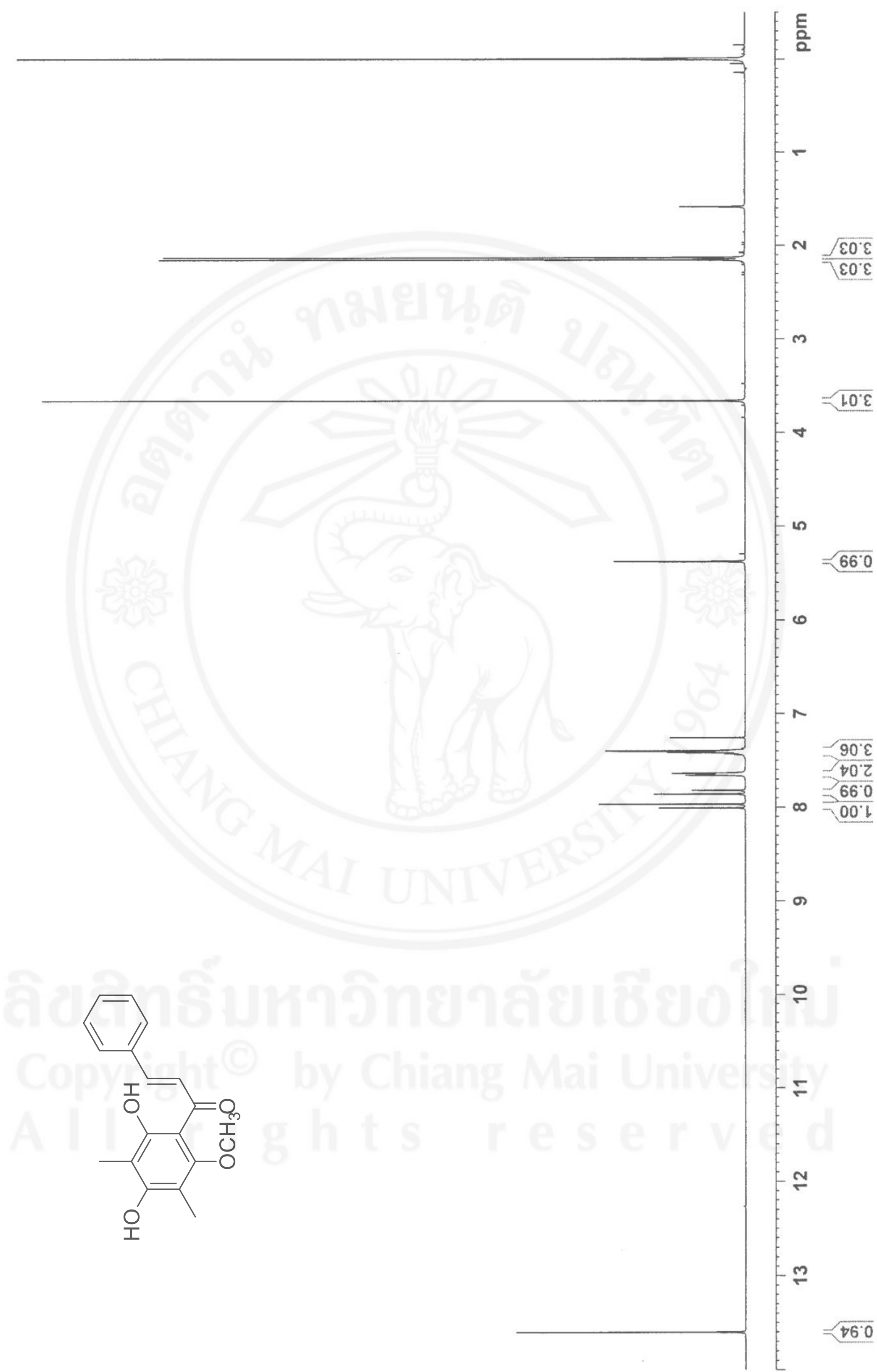

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Figure 38 ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

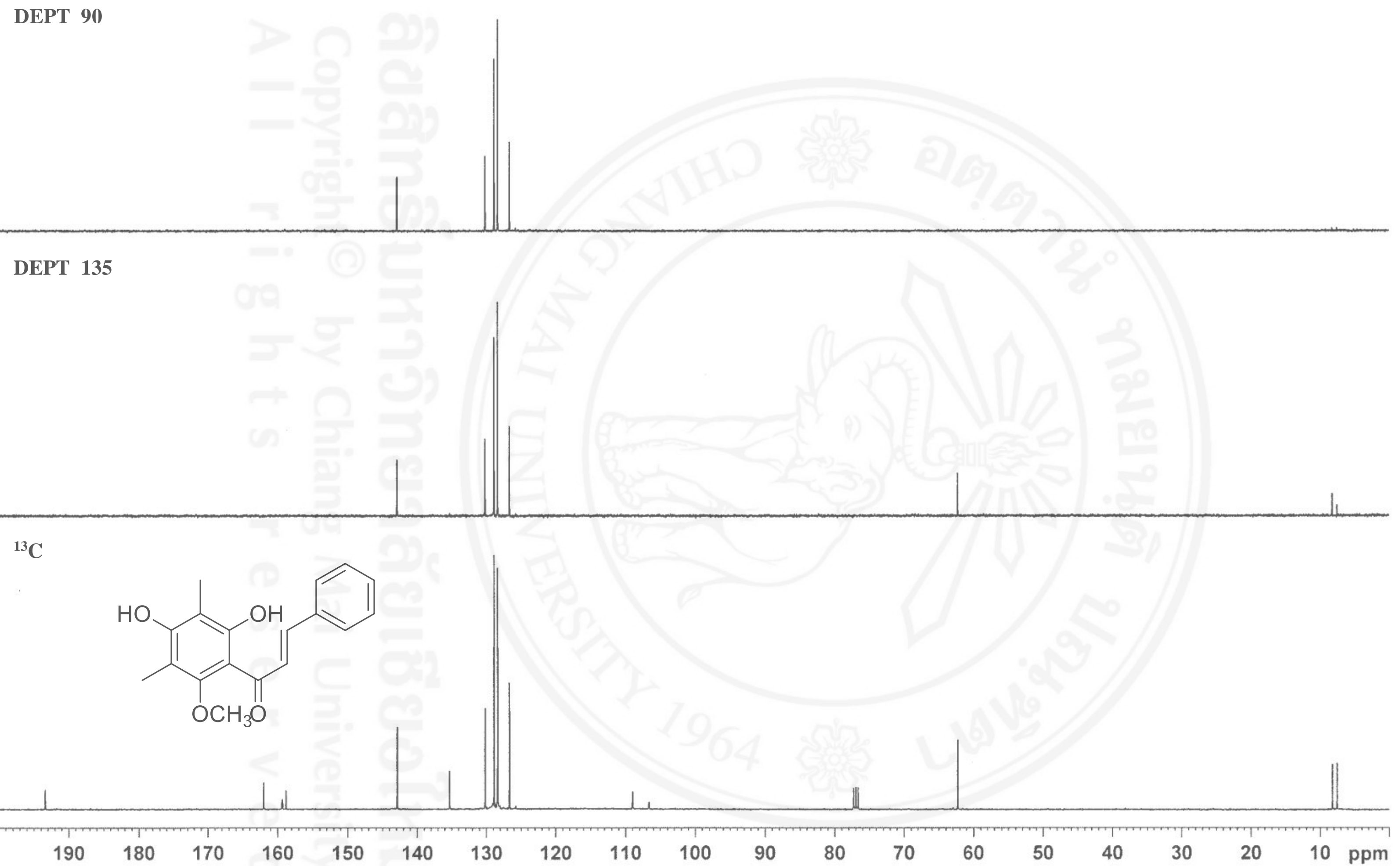

Figure 39 ^{13}C NMR (100 MHz, in $CDCl_3$) and DEPT spectra of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

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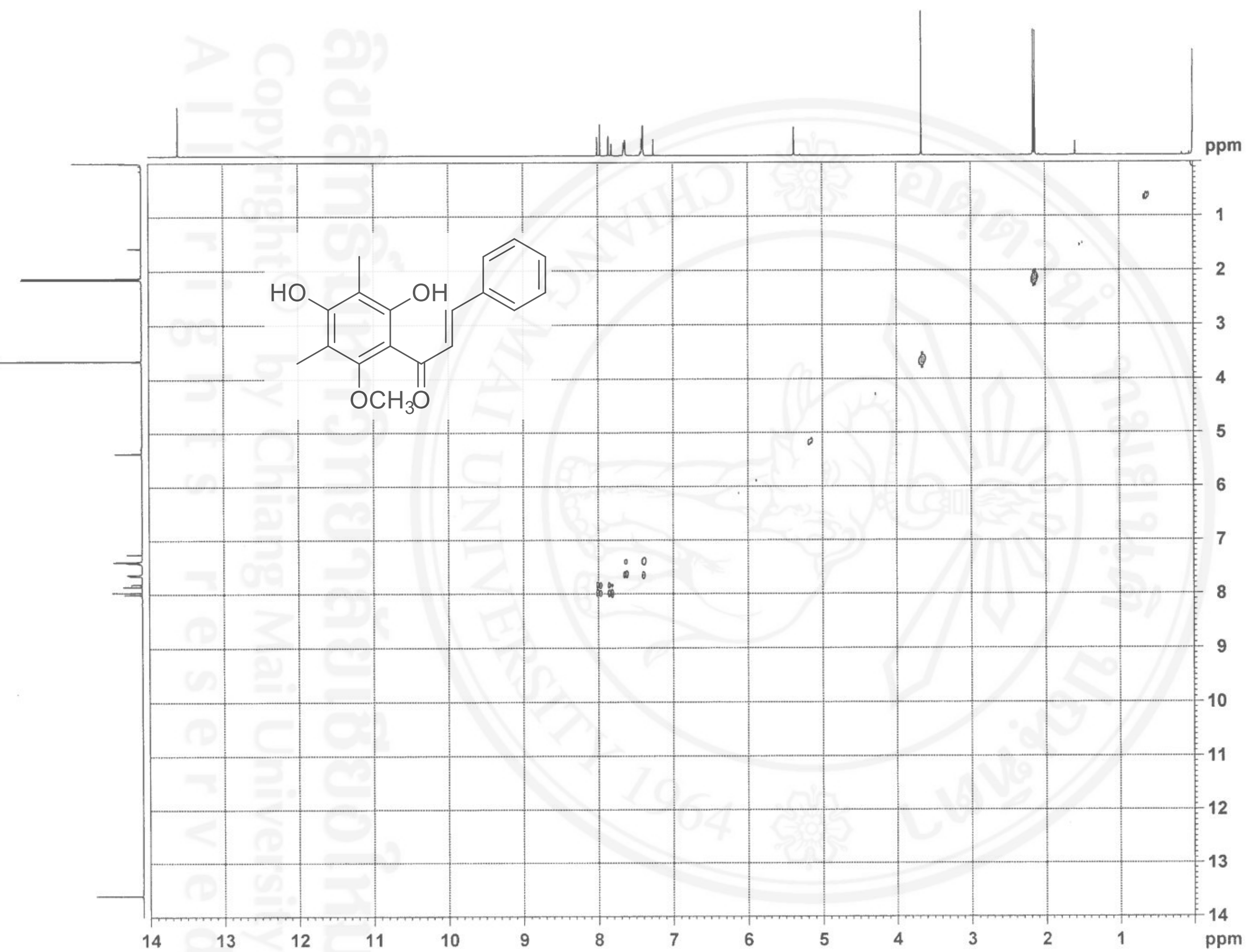

Figure 40 ^1H-^1H COSY (in $CDCl_3$) spectrum of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

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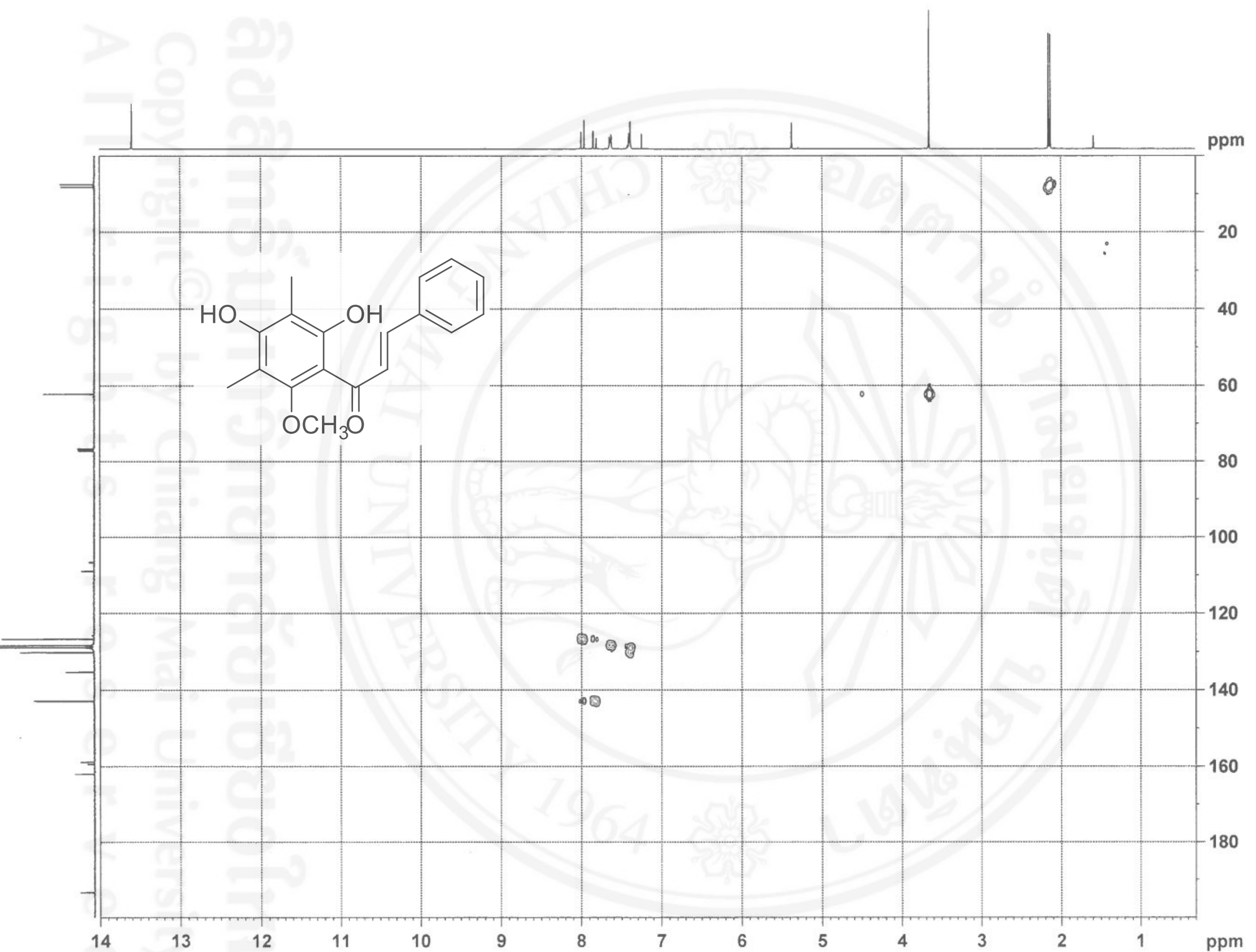

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Figure 41 HMQC (in $CDCl_3$) spectrum of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

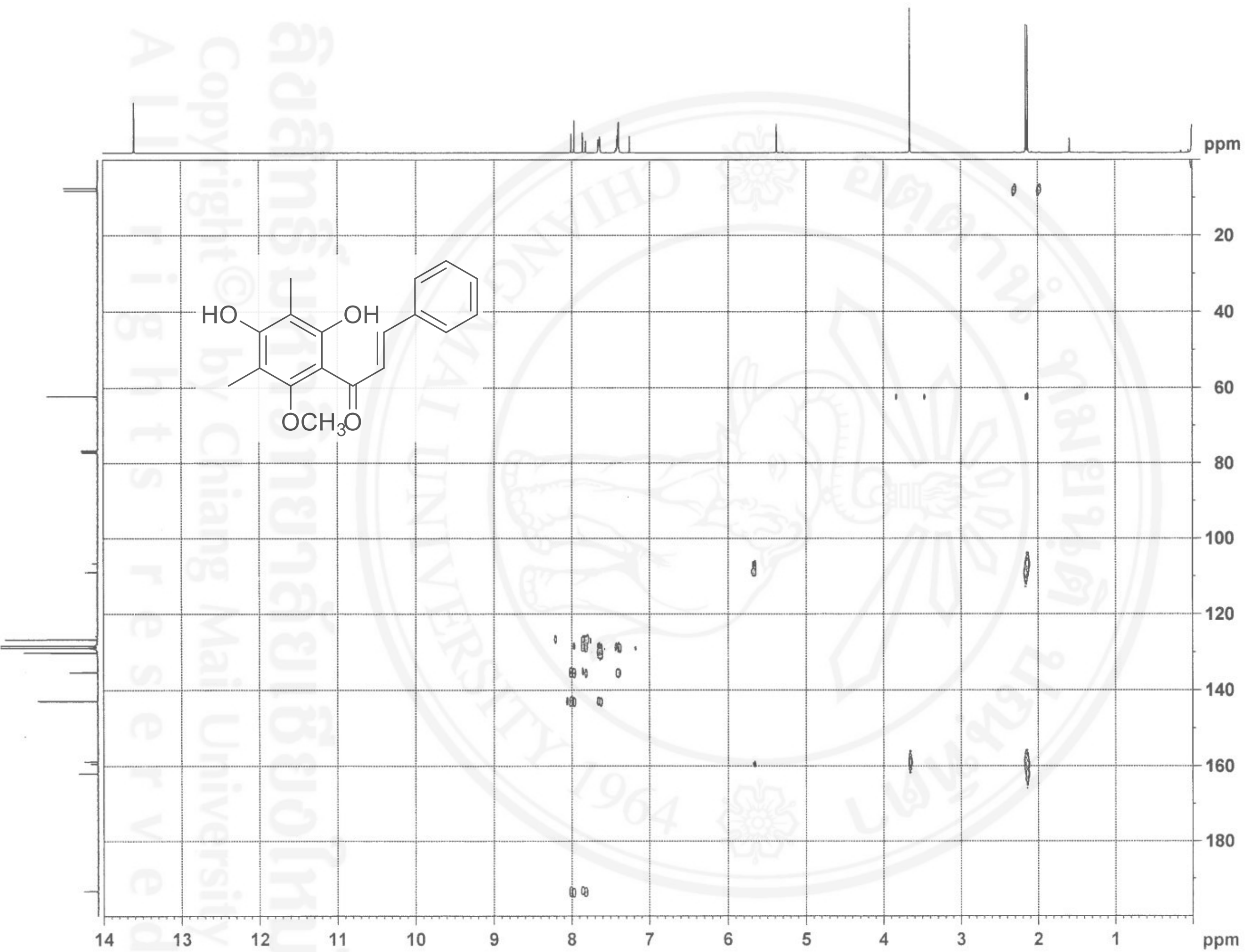

Figure 42 HMBC (in $CDCl_3$) spectrum of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

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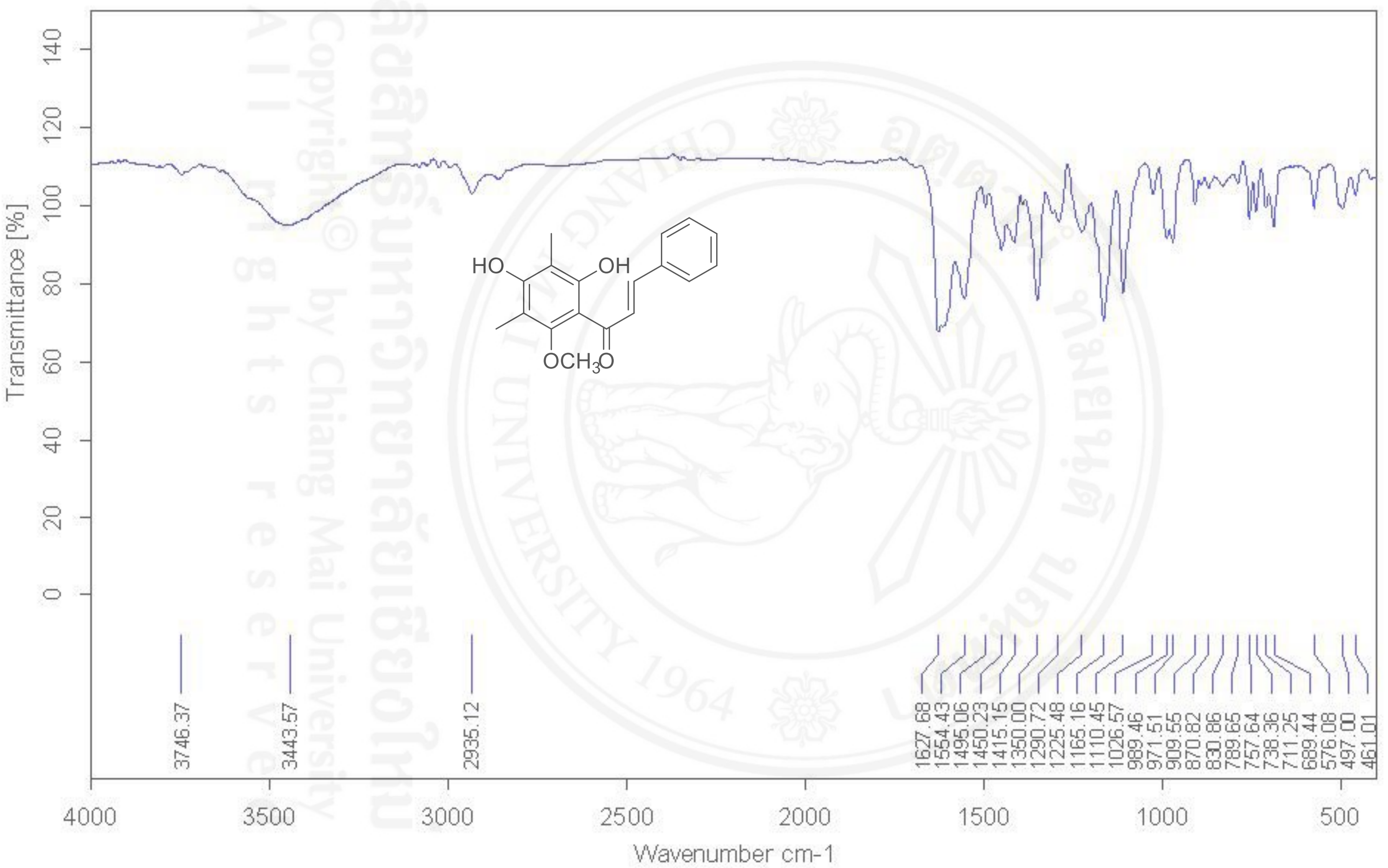

Figure 43 FTIR (evaporated thin film) spectrum of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

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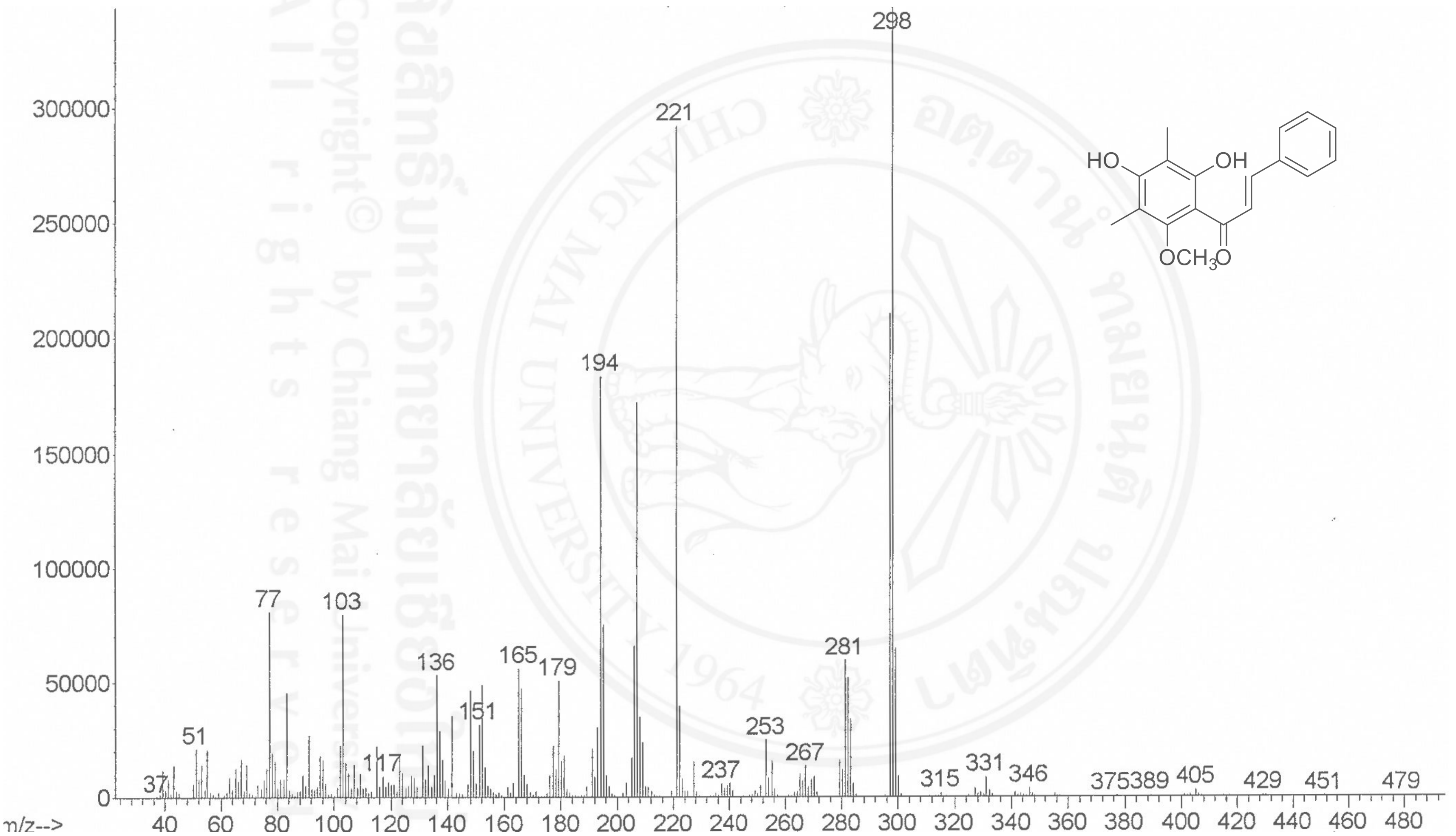

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Figure 44 Mass spectrum (GC-MS (EI)) of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

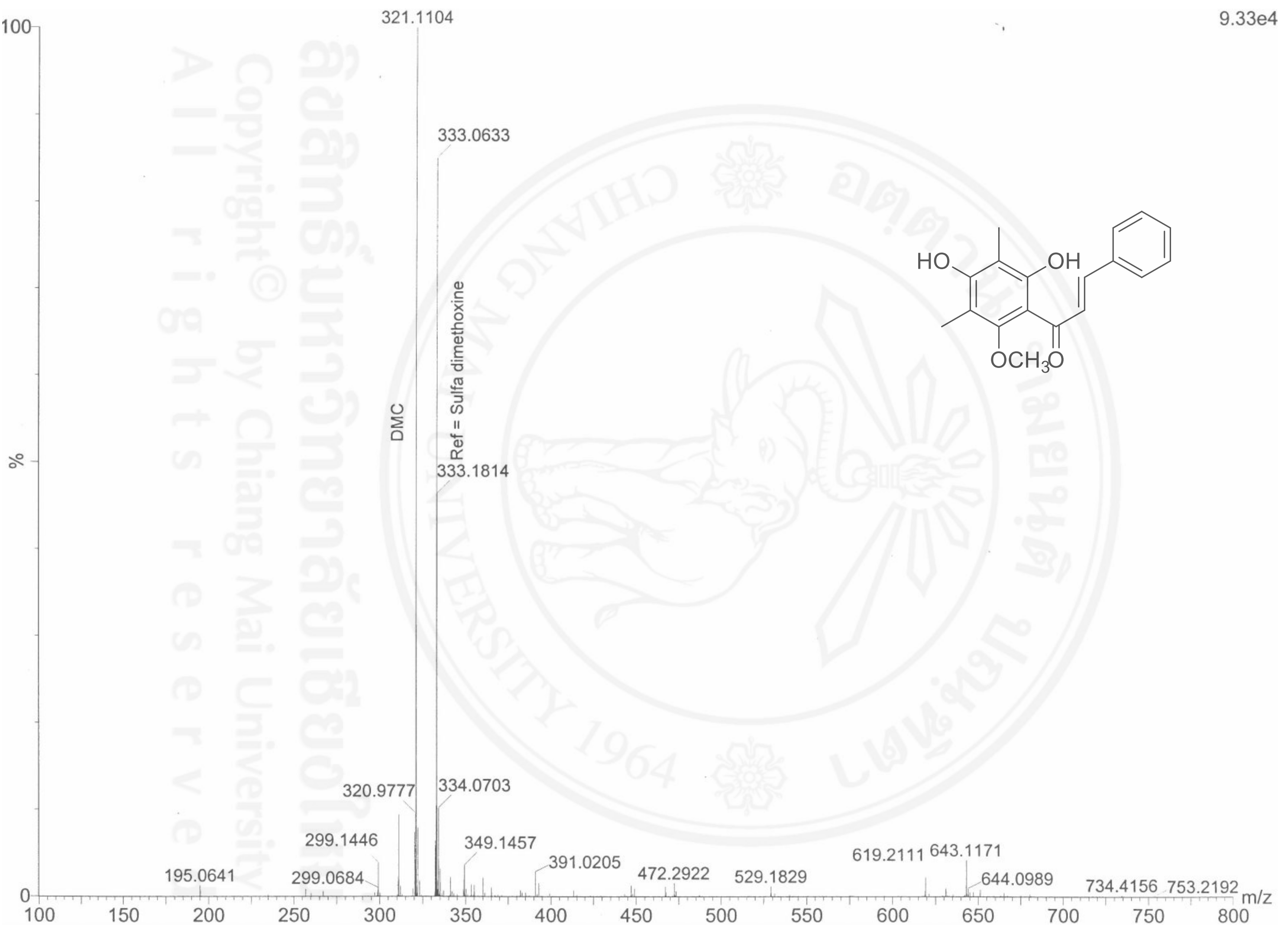

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Figure 45 Mass spectrum (HRMS (ESI)) of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

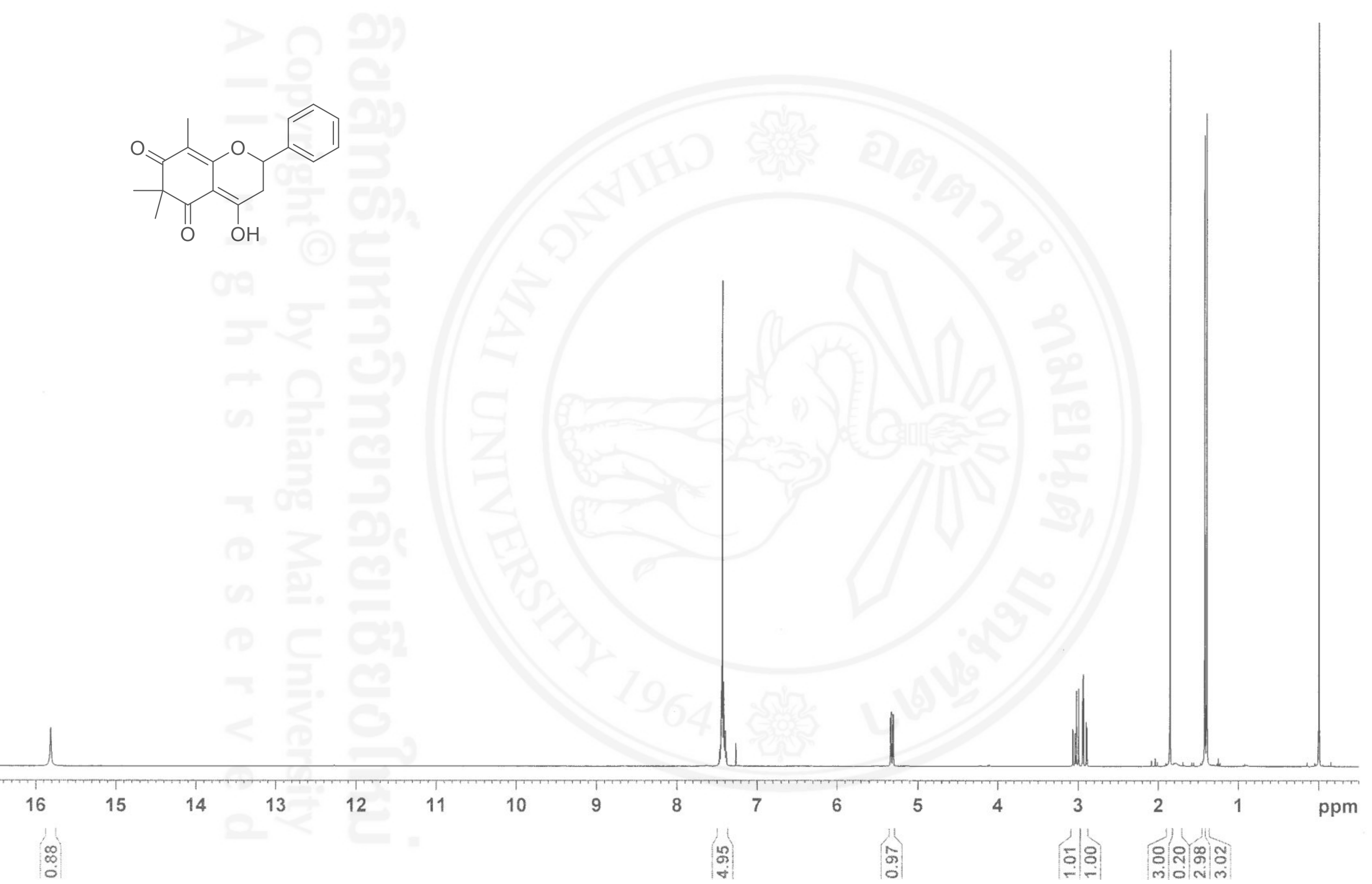

Figure 46 ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum of hariganetin (**138**)

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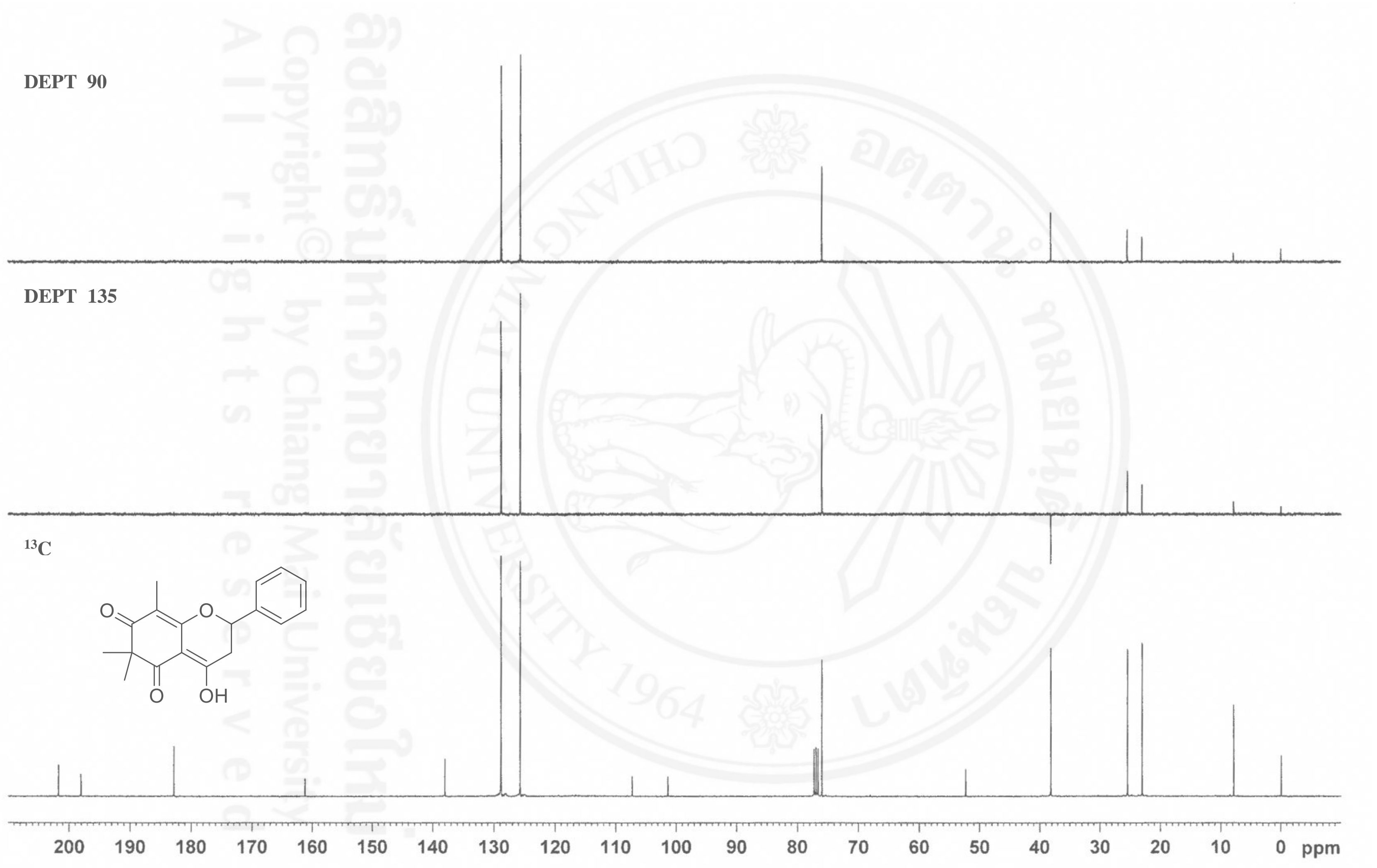

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Figure 47 ^{13}C NMR (100 MHz, in $CDCl_3$) and DEPT spectra of hariganetin (**138**)

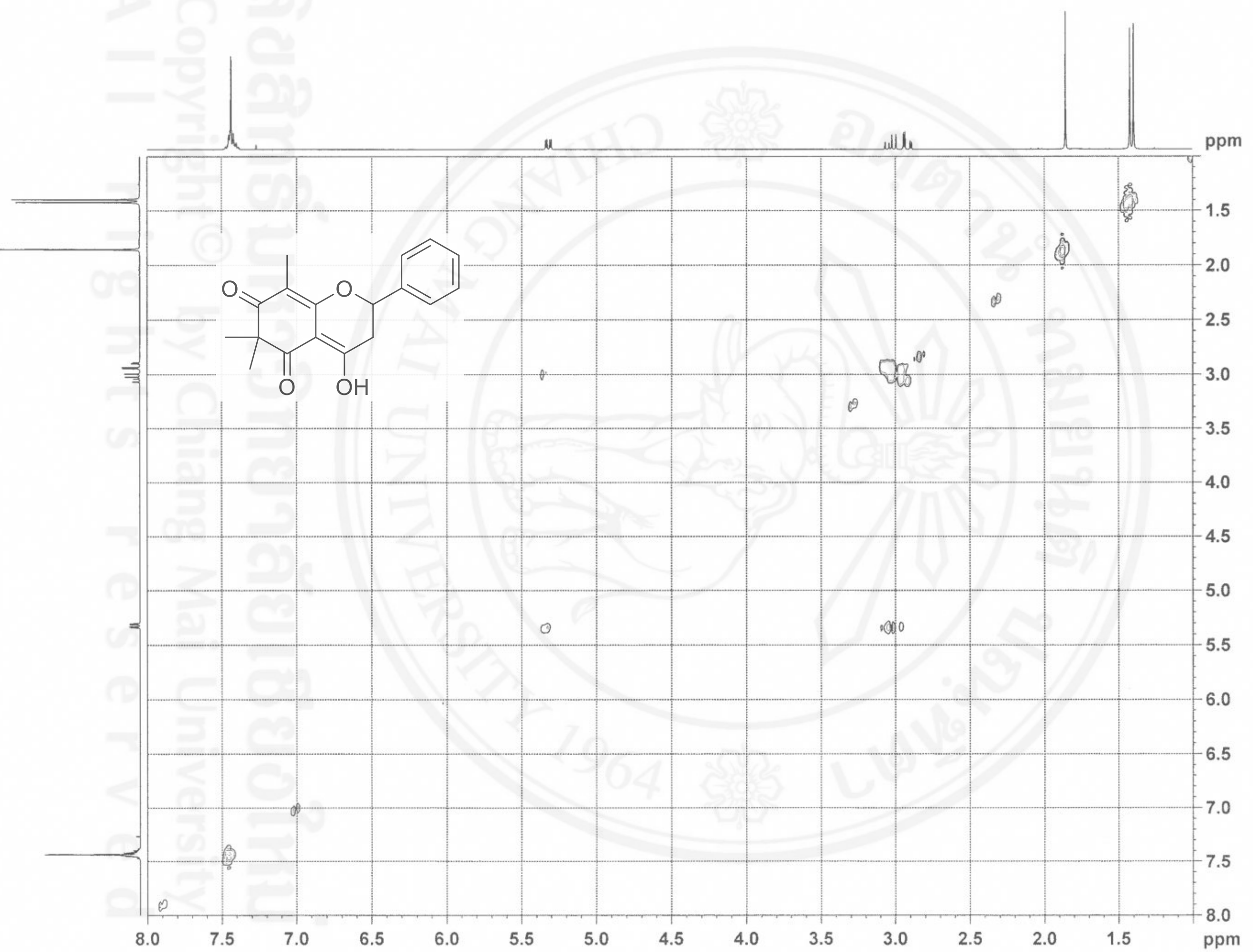

Figure 48 ^{1}H-^{1}H COSY (in $CDCl_3$) spectrum of hariganetin (**138**)

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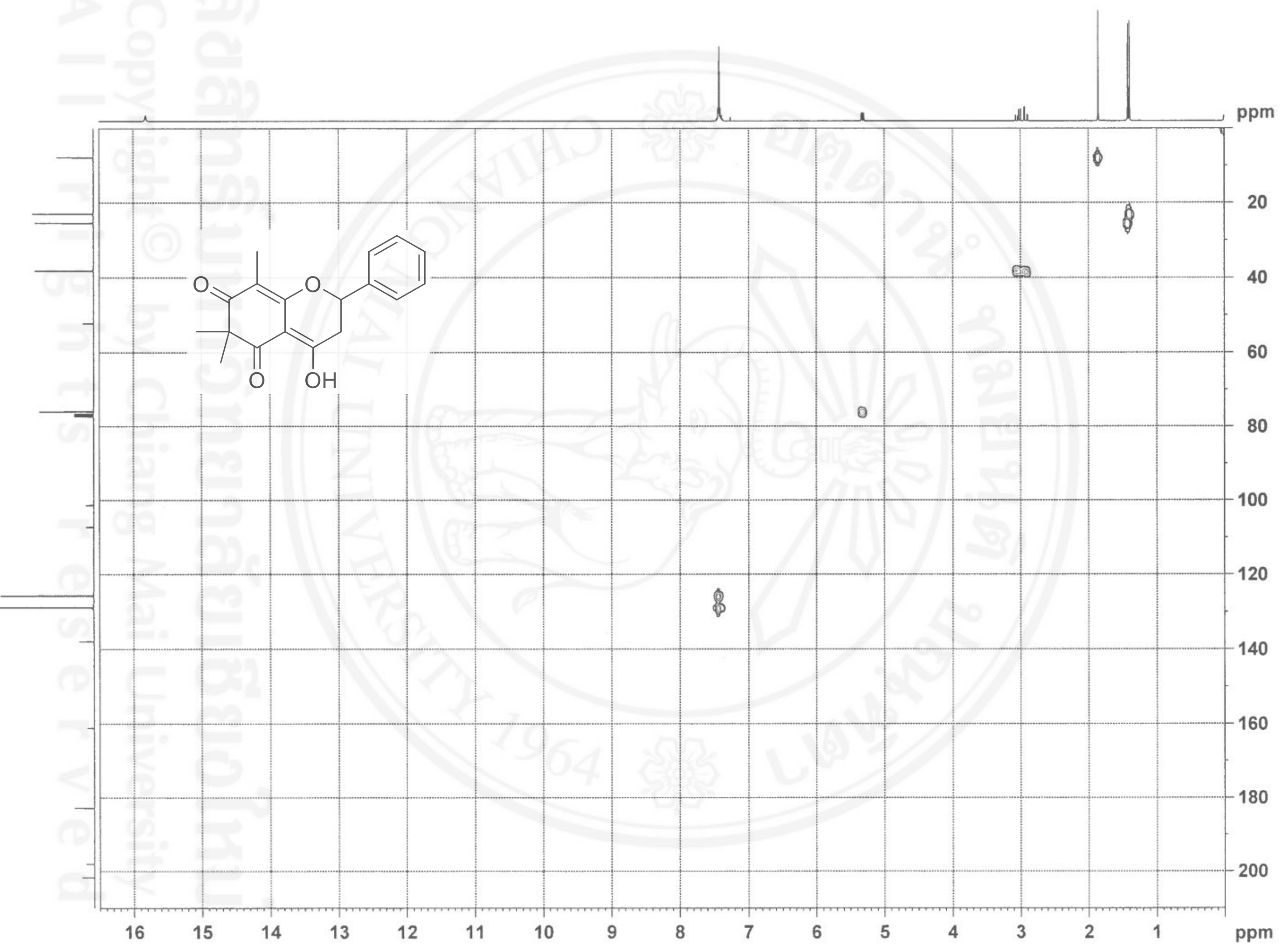

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Figure 49 HMQC (in $CDCl_3$) spectrum of hariganetin (**138**)

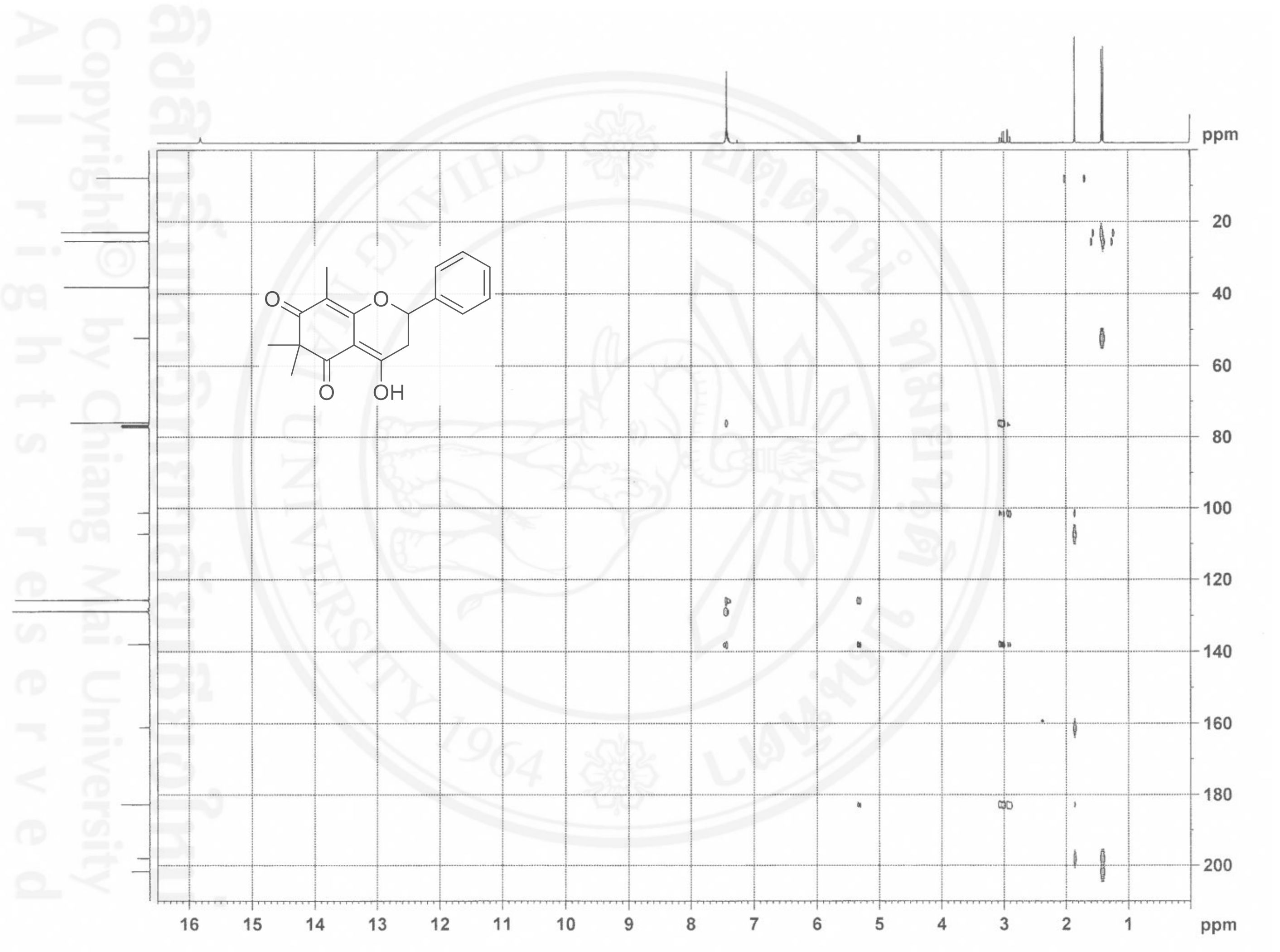

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Figure 50 HMBC (in $CDCl_3$) spectrum of hariganetin (**138**)

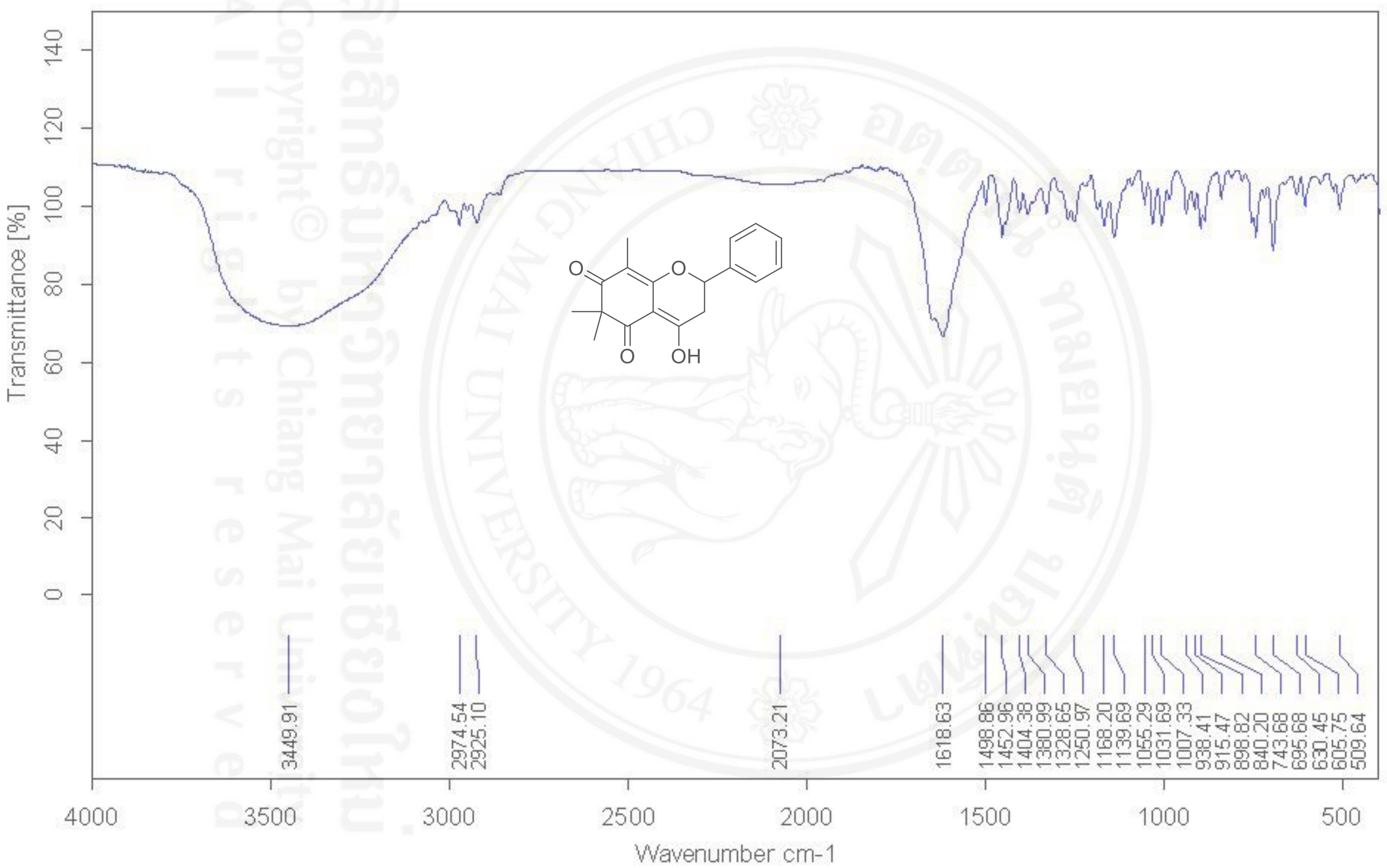

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Figure 51 FTIR (evaporated thin film) spectrum of hariganetin (**138**)

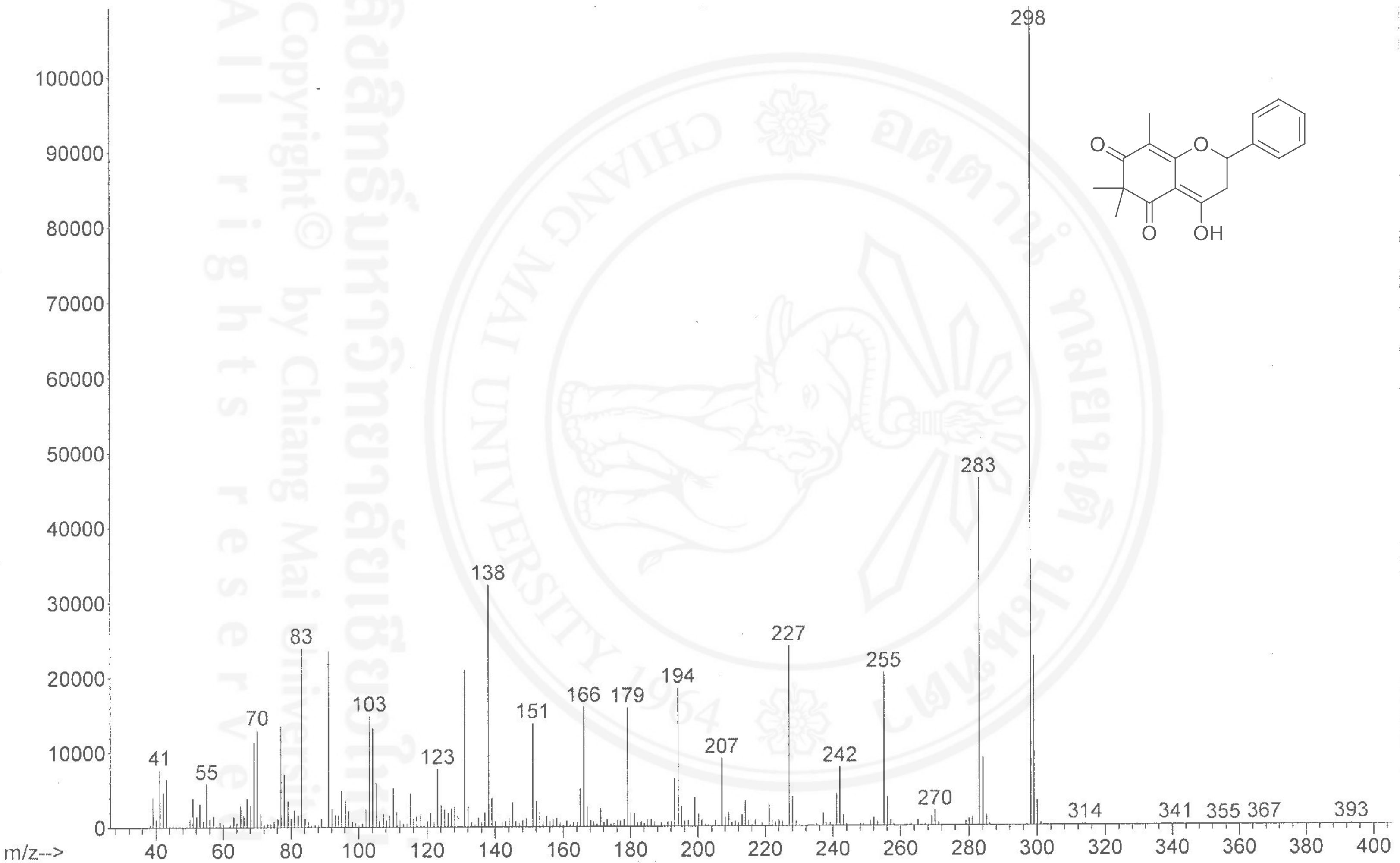

Figure 52 Mass spectrum (GC-MS (EI)) of hariganetin (**138**)

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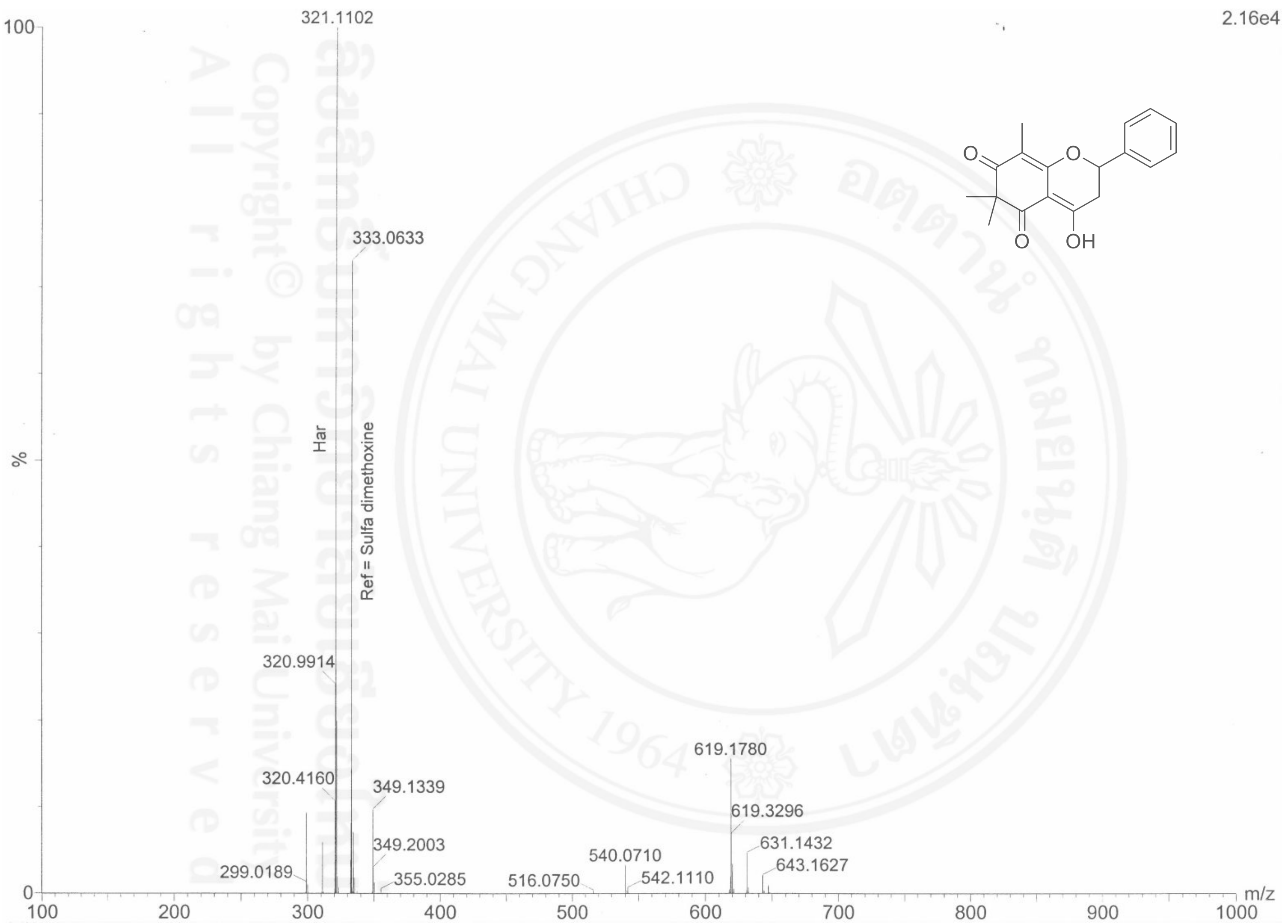

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Figure 53 Mass spectrum (HRMS (ESI)) of hariganetin (**138**)

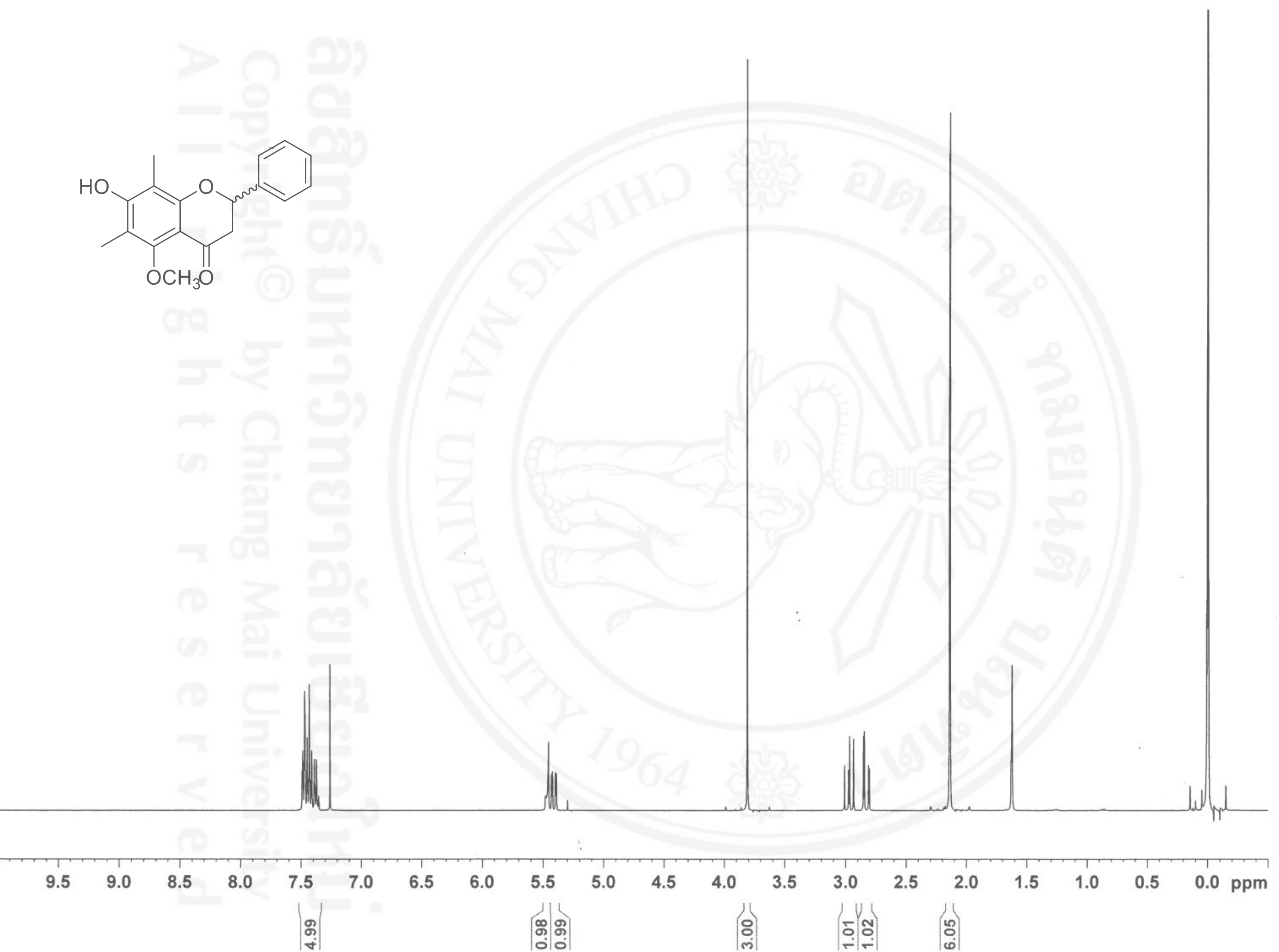

Figure 54 ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum of 7-hydroxy-5-methoxy-6,8-dimethylflavanones (**139**)

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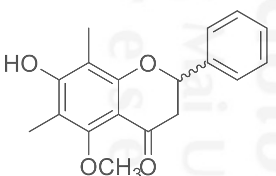
DEPT 90

DEPT 135

^{13}C



190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

Figure 55 ^{13}C NMR (100 MHz, in $CDCl_3$) and DEPT spectra of 7-hydroxy-5-methoxy-6,8-dimethyl flavanones (**139**)

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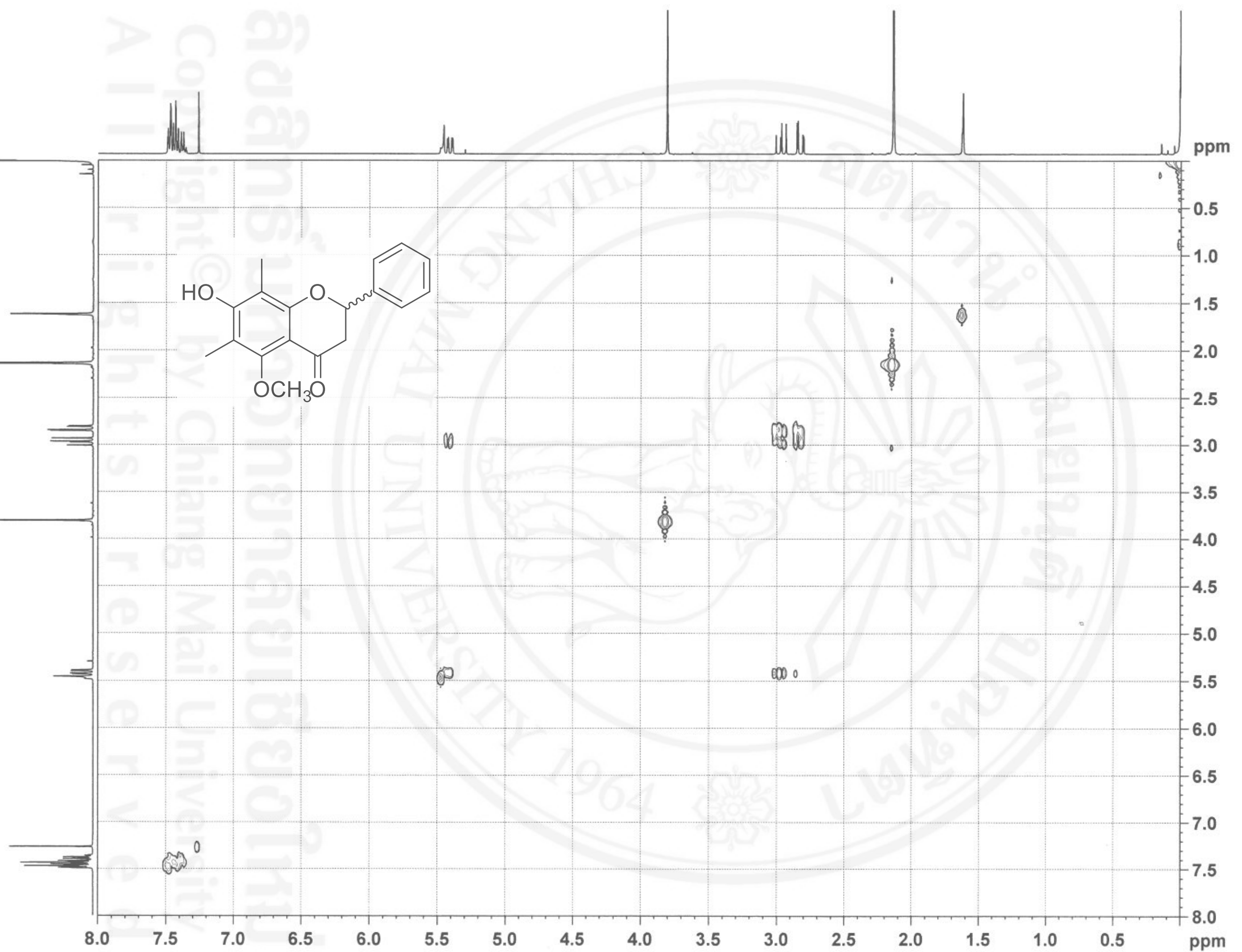

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Figure 56 ^{1}H-^{1}H COSY (in $CDCl_3$) spectrum of 7-hydroxy-5-methoxy-6,8-dimethylflavanones (**139**)

Figure 57 HMQC (in $CDCl_3$) spectrum of 7-hydroxy-5-methoxy-6,8-dimethylflavanones (**139**)

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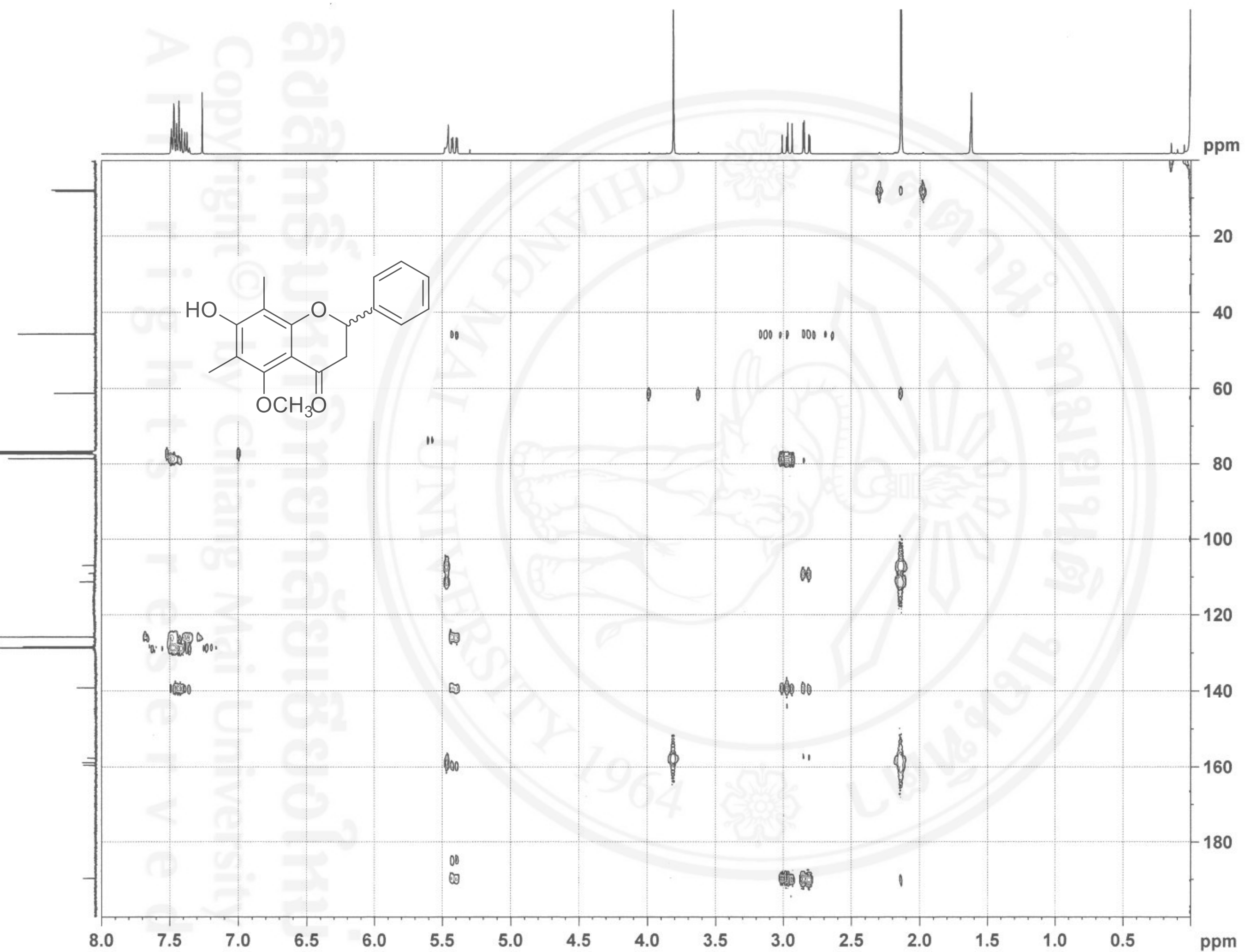

Figure 58 HMBC (in $CDCl_3$) spectrum of 7-hydroxy-5-methoxy-6,8-dimethylflavanones (**139**)

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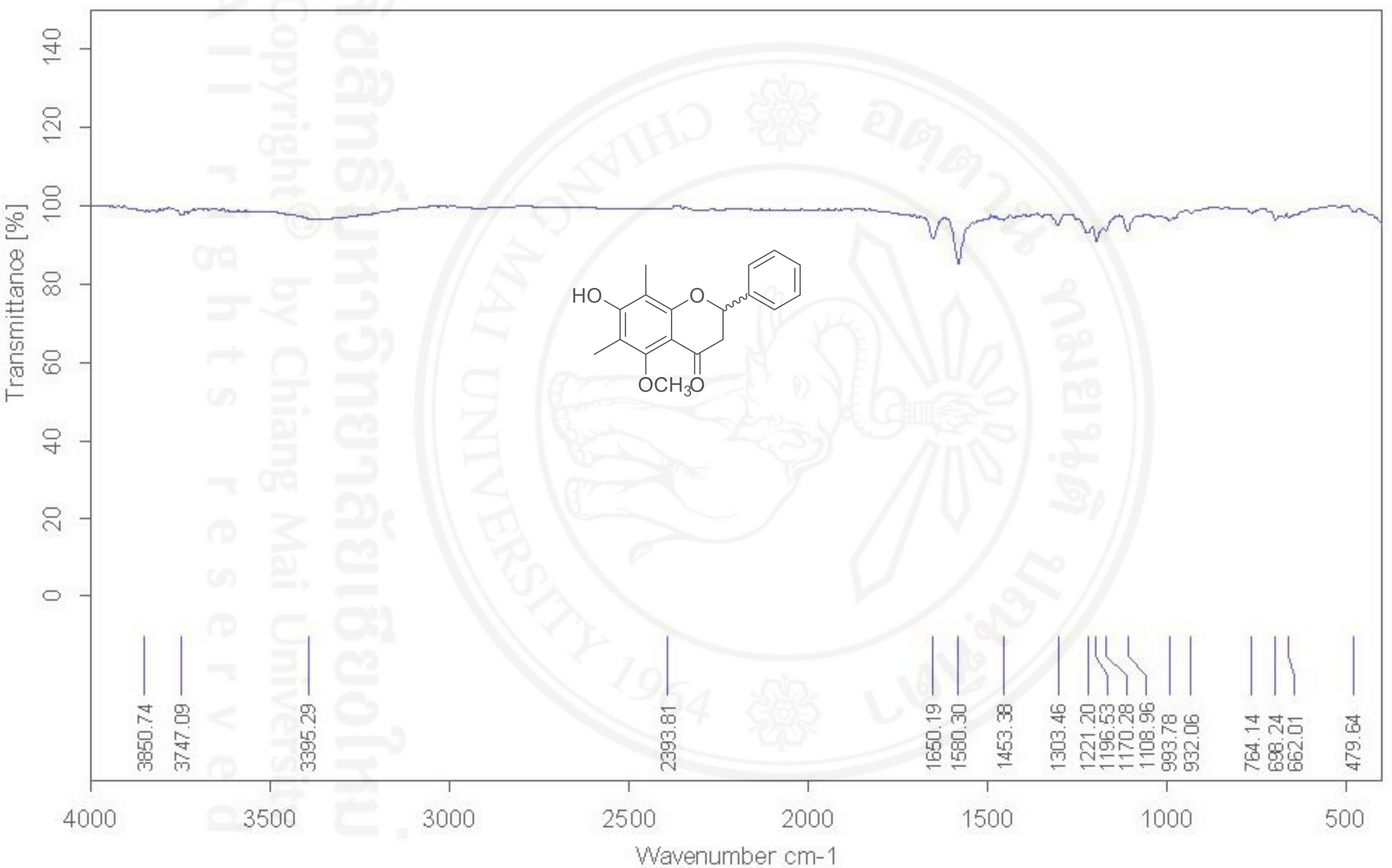

Figure 59 FTIR (evaporated thin film) spectrum of 7-hydroxy-5-methoxy-6,8-dimethylflavanones (**139**)

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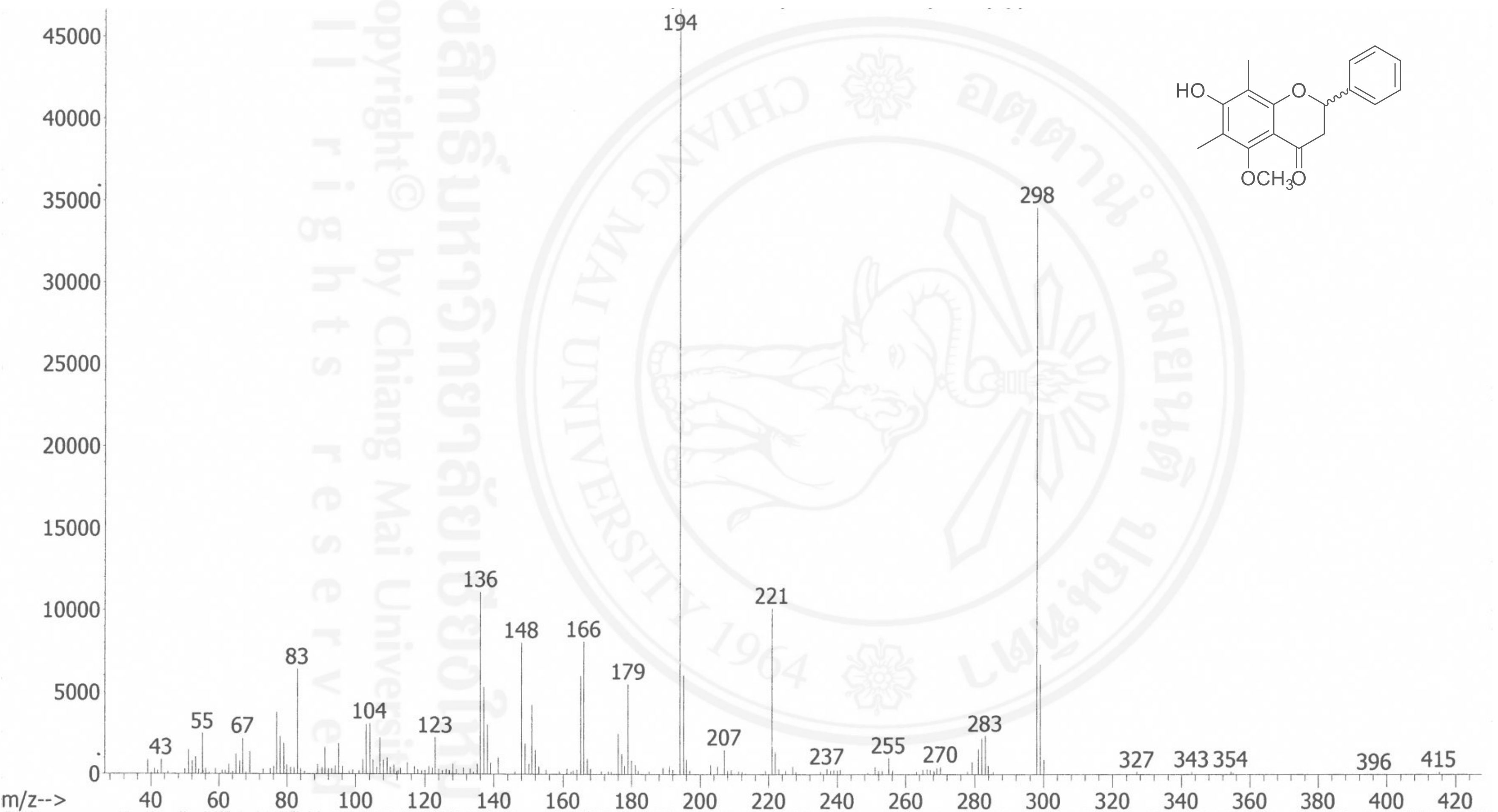

Figure 60 Mass spectrum (GC-MS (EI)) of 7-hydroxy-5-methoxy-6,8-dimethylflavanones (**139**)

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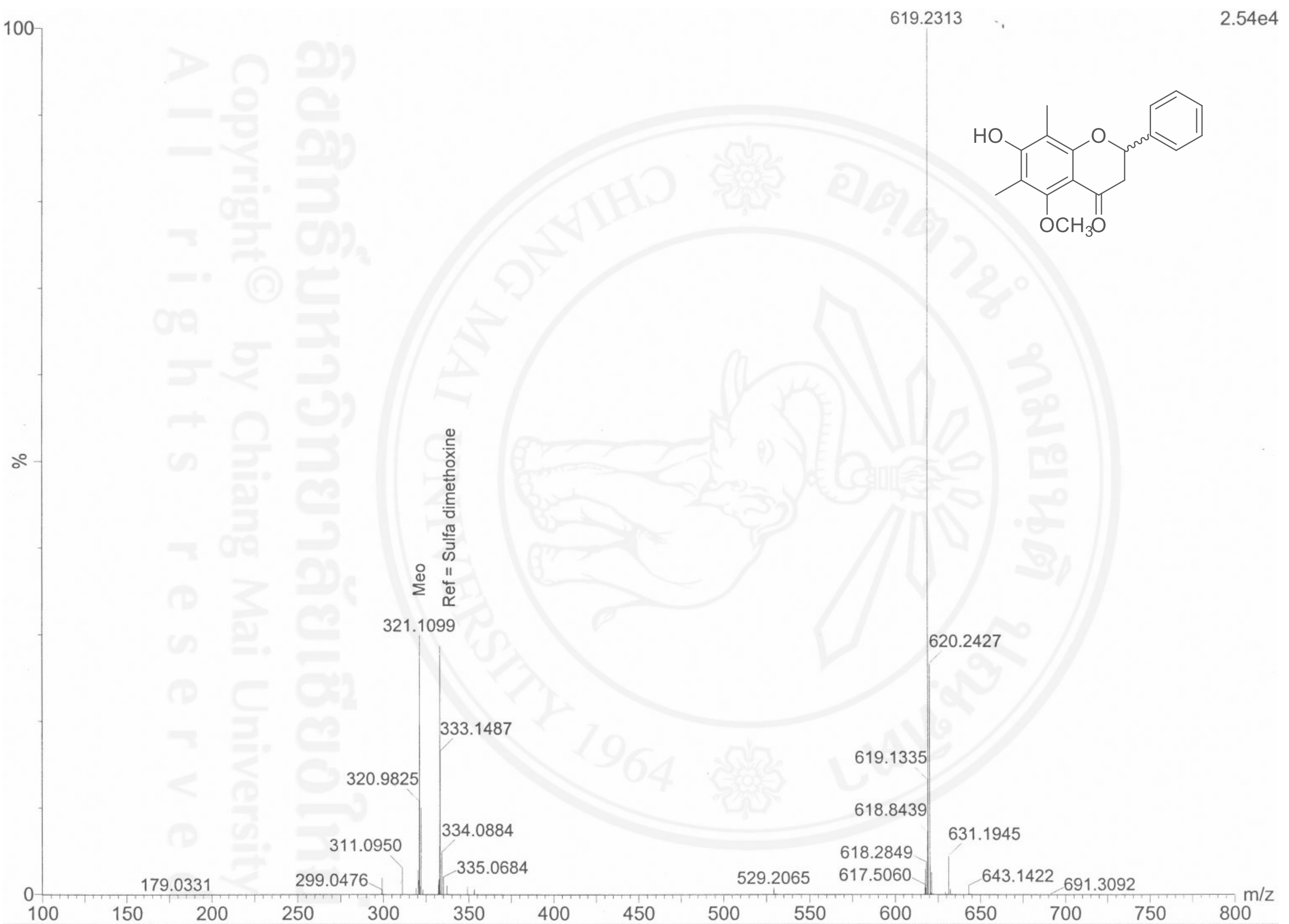

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Figure 61 Mass spectrum (HRMS (ESI)) of 7-hydroxy-5-methoxy-6,8-dimethylflavanones (**139**)

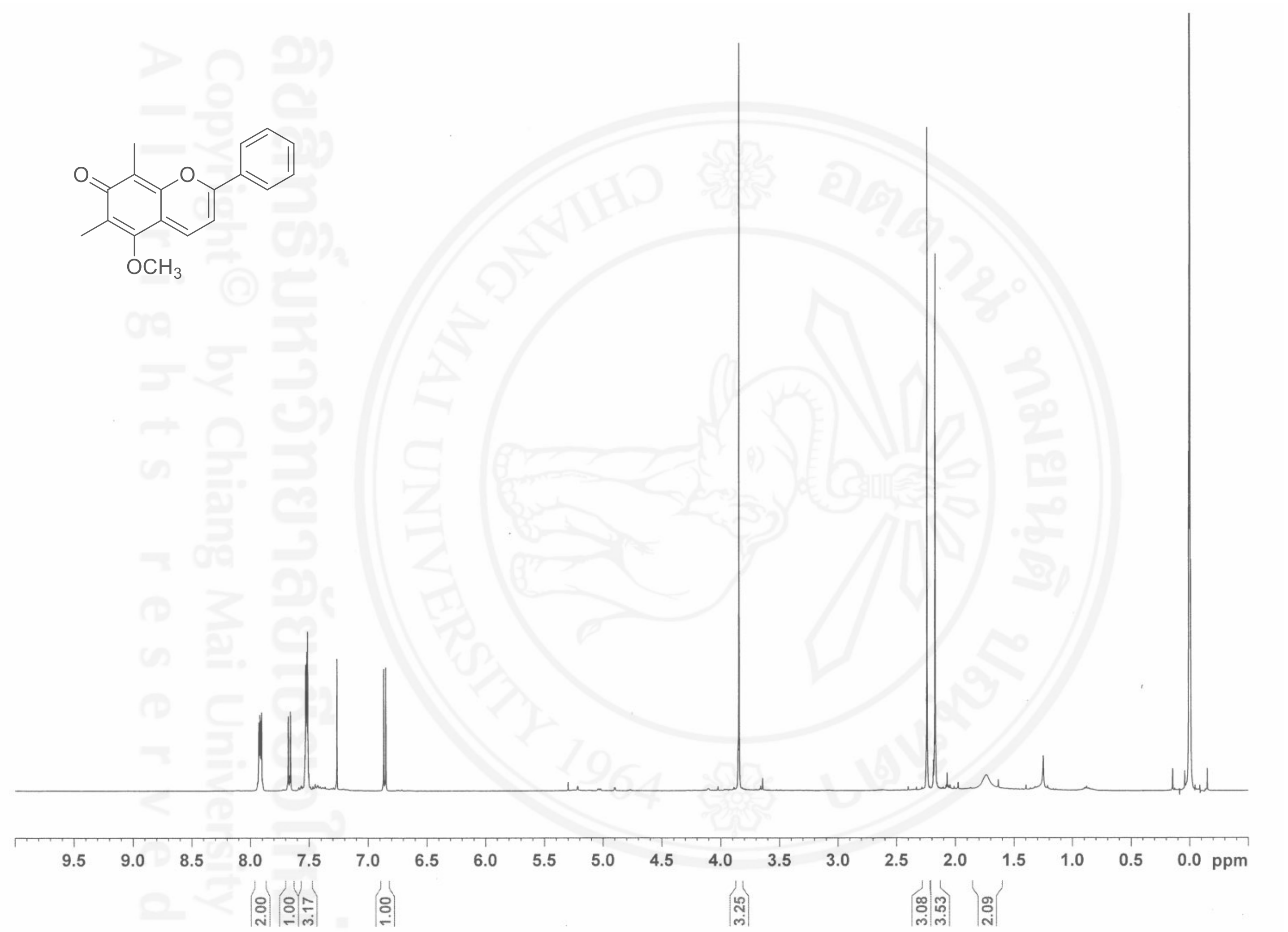

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Figure 62 ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum of 5-methoxy-6,8-dimethyl-2-phenyl-7*H*-1-benzopyran-7-one (**140**)

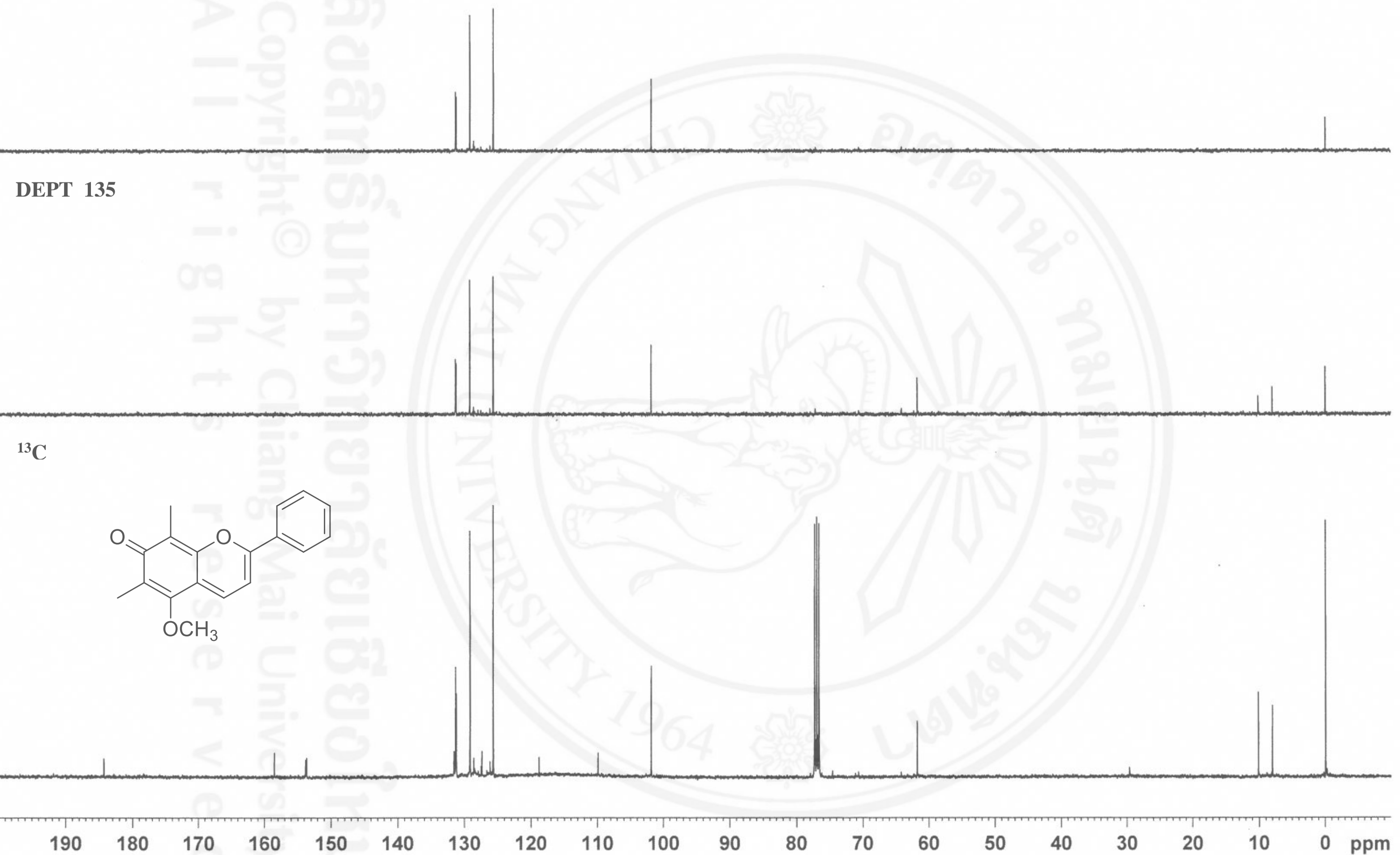

Figure 63 ^{13}C NMR (100 MHz, in $CDCl_3$) and DEPT spectra of 5-methoxy-6,8-dimethyl-2-phenyl-7*H*-1-benzopyran-7-one (**140**)

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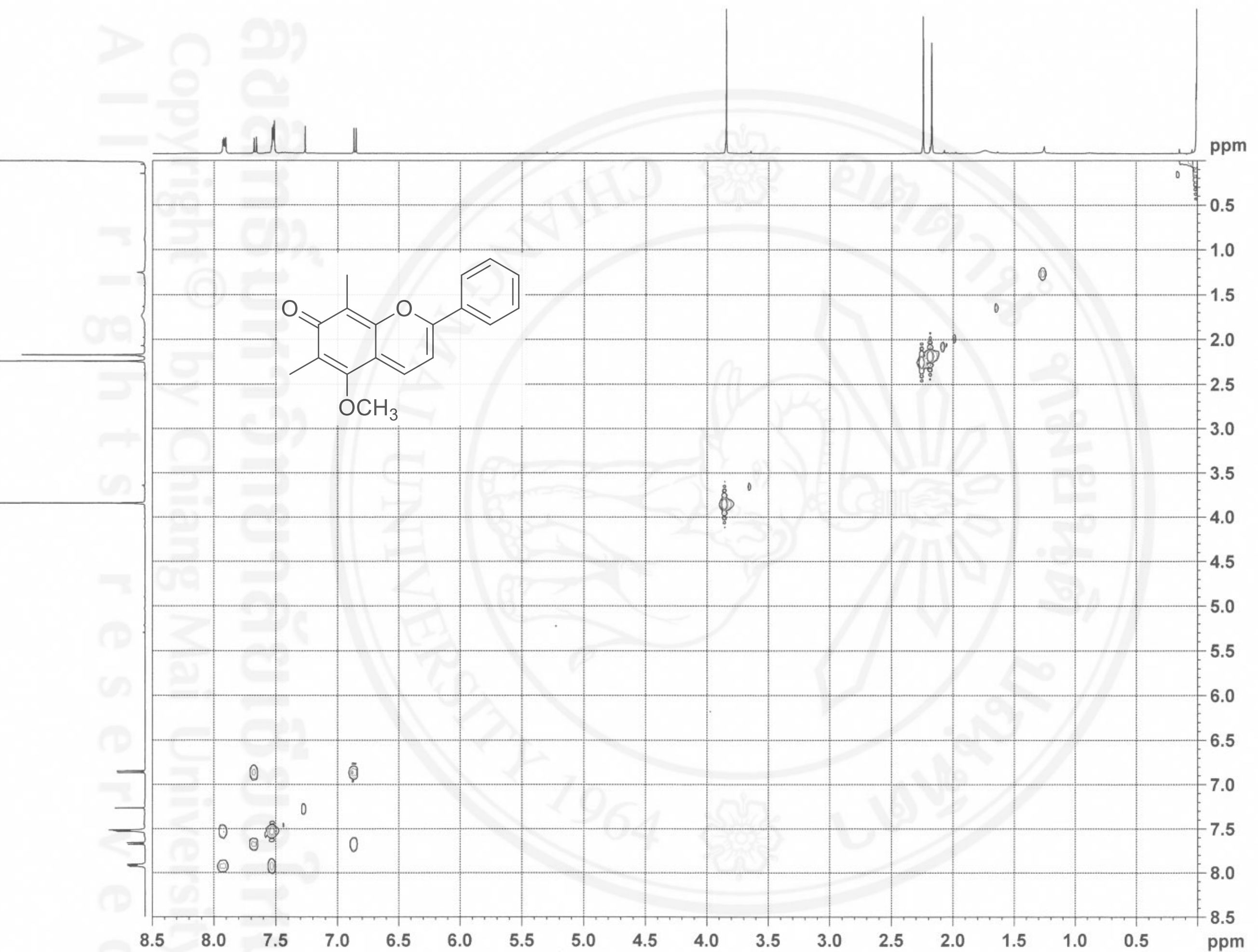

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Figure 64 ^{1}H-^{1}H COSY (in $CDCl_3$) spectrum of 5-methoxy-6,8-dimethyl-2-phenyl-7*H*-1-benzopyran-7-one (**140**)

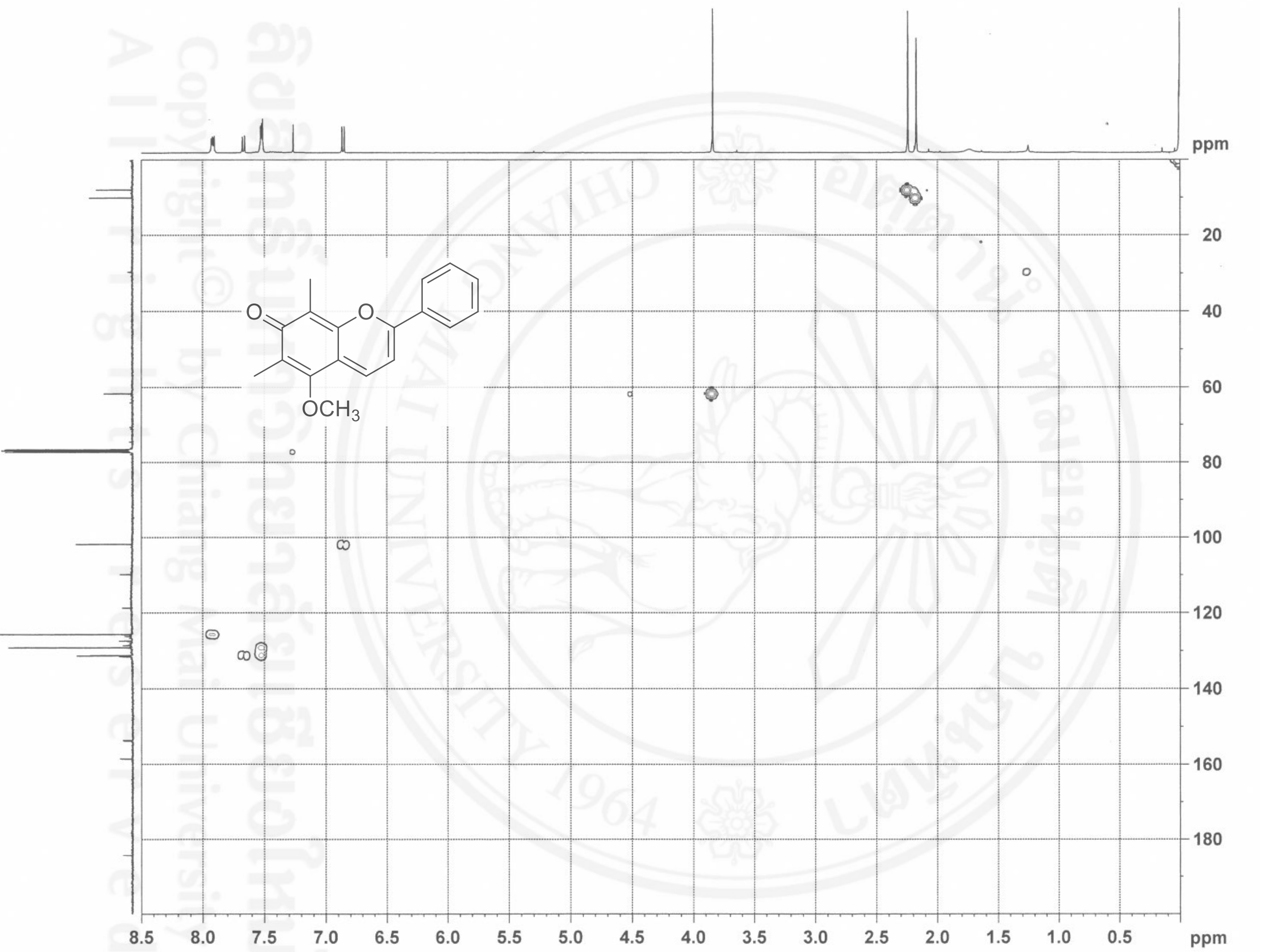

Figure 65 HMQC (in $CDCl_3$) spectrum of 5-methoxy-6,8-dimethyl-2-phenyl-7*H*-1- benzopyran-7-one (**140**)

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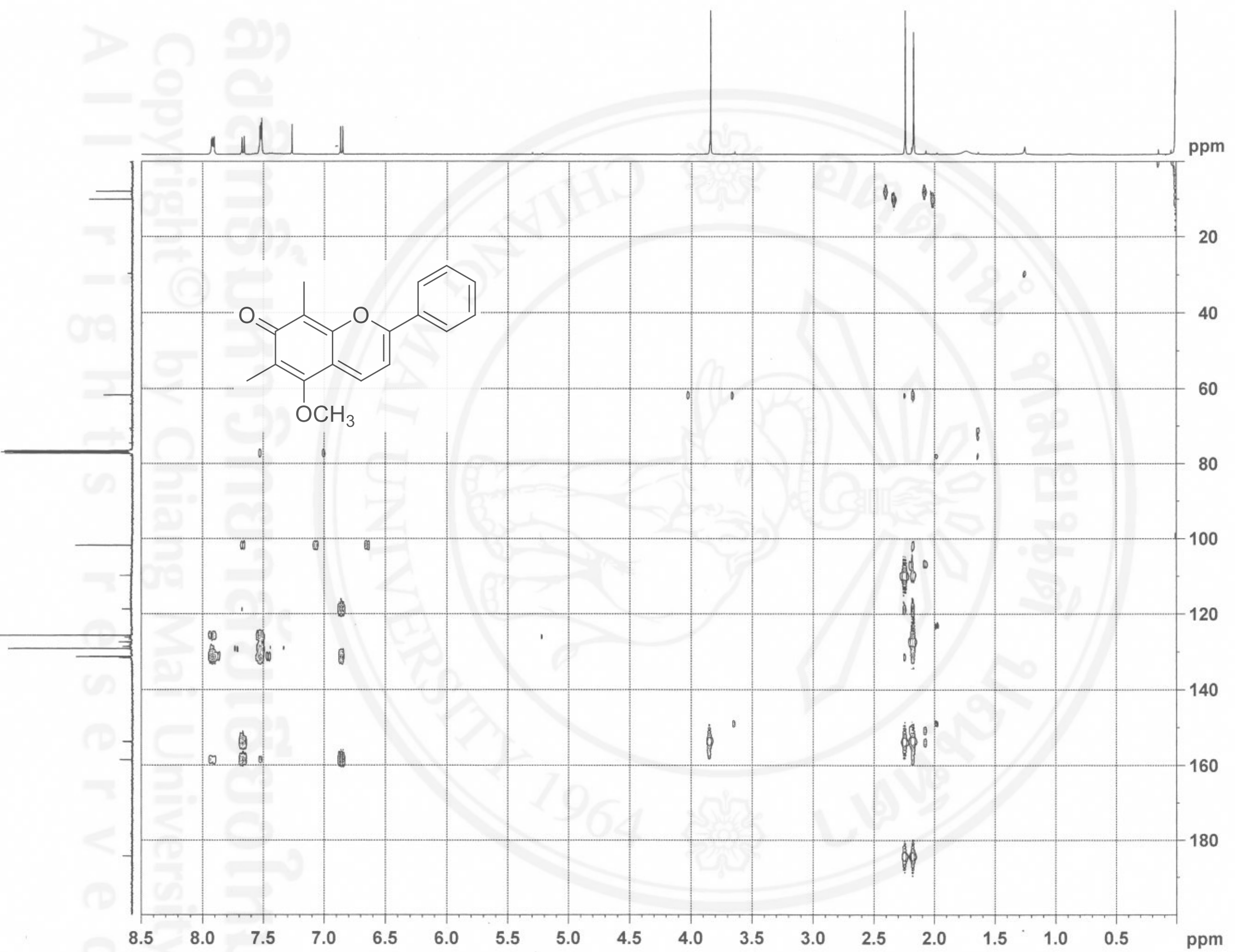

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Figure 66 HMBC (in $CDCl_3$) spectrum of 5-methoxy-6,8-dimethyl-2-phenyl-7*H*-1-benzopyran-7-one (**140**)

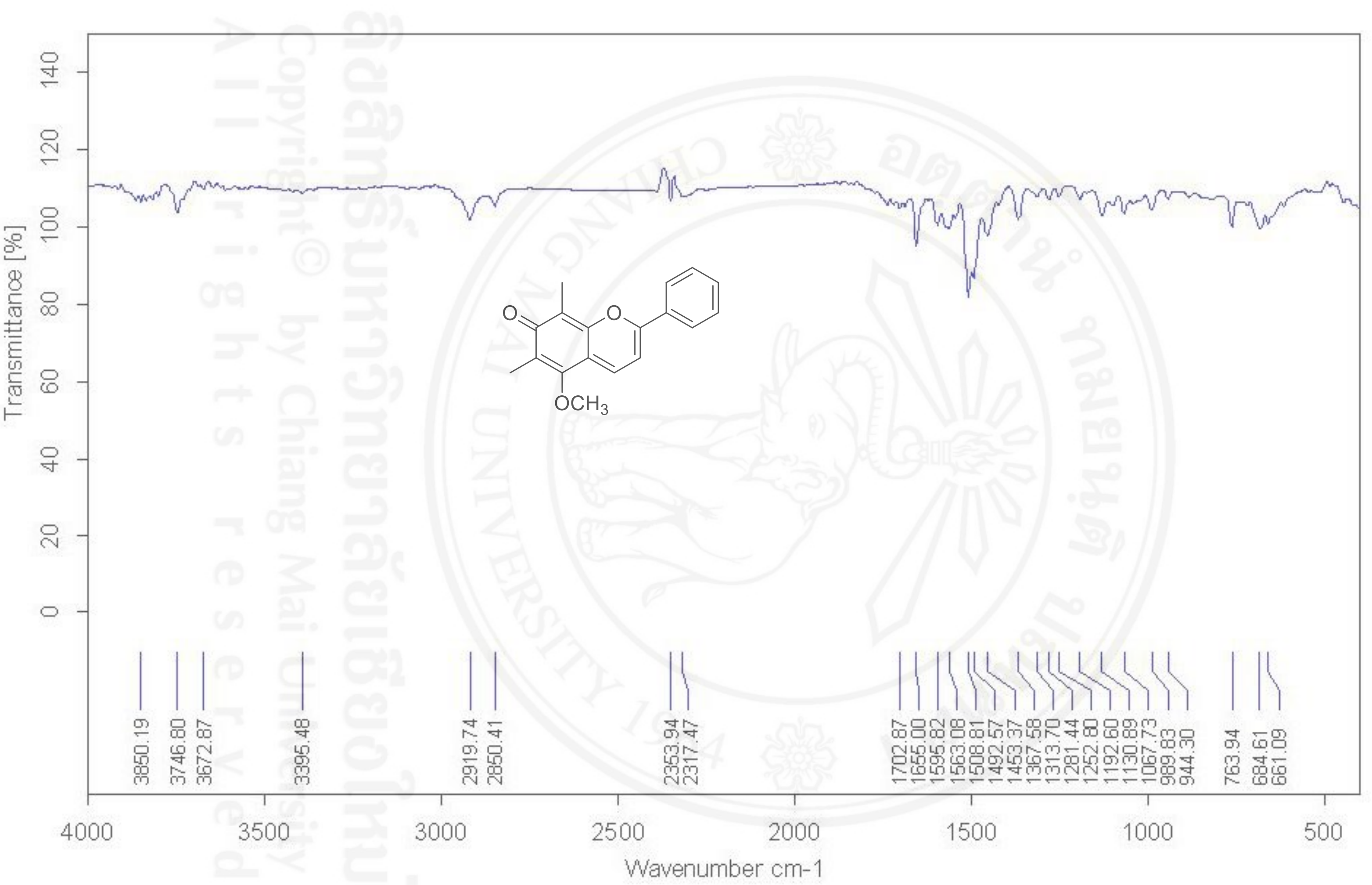

Figure 67 FTIR (evaporated thin film) spectrum of 5-methoxy-6,8-dimethyl-2-phenyl-7*H*-1-benzopyran-7-one (**140**)

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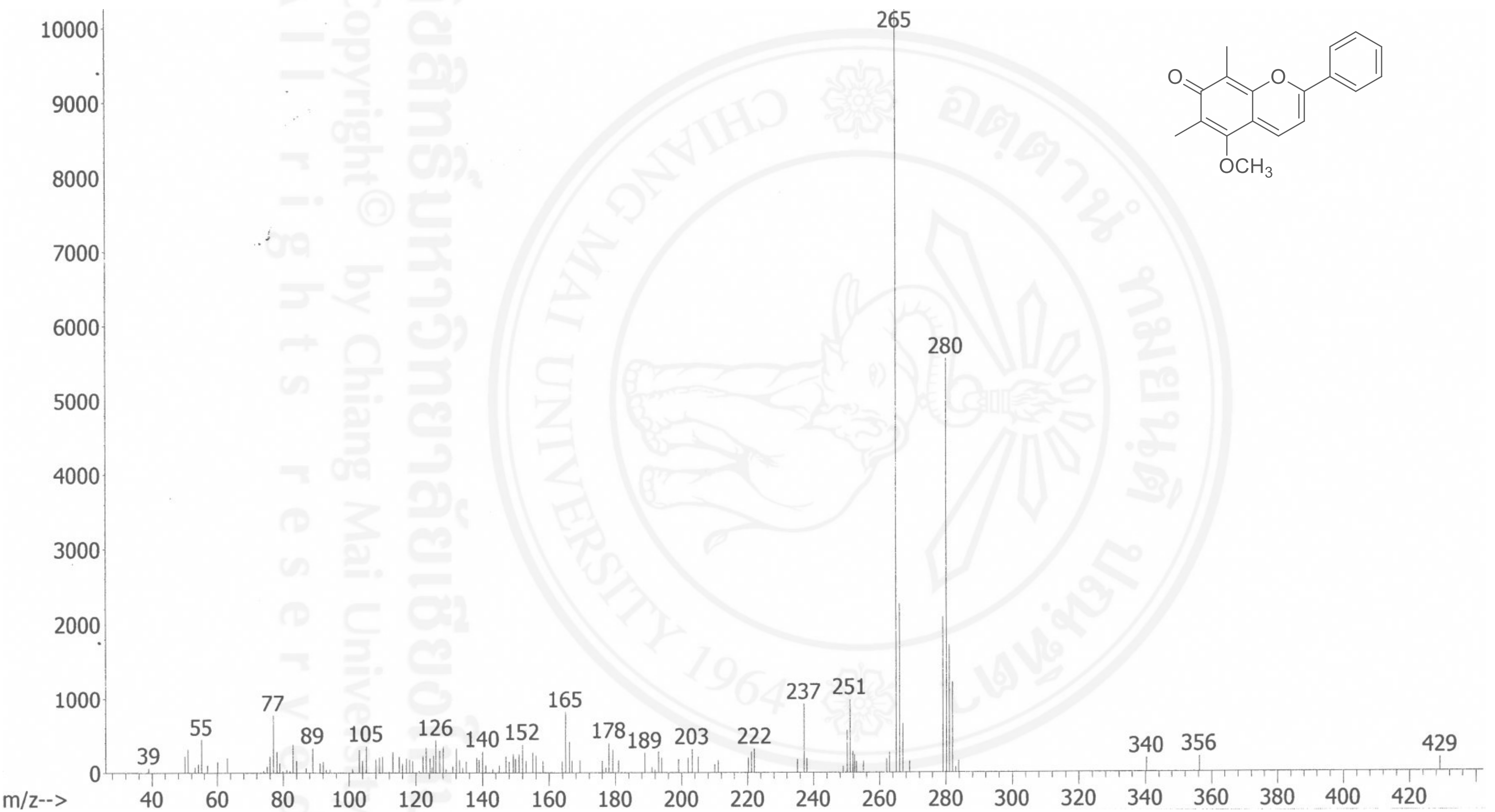

Figure 68 Mass spectrum (GC-MS (EI)) of 5-methoxy-6,8-dimethyl-2-phenyl-7*H*-1-benzopyran-7-one (**140**)

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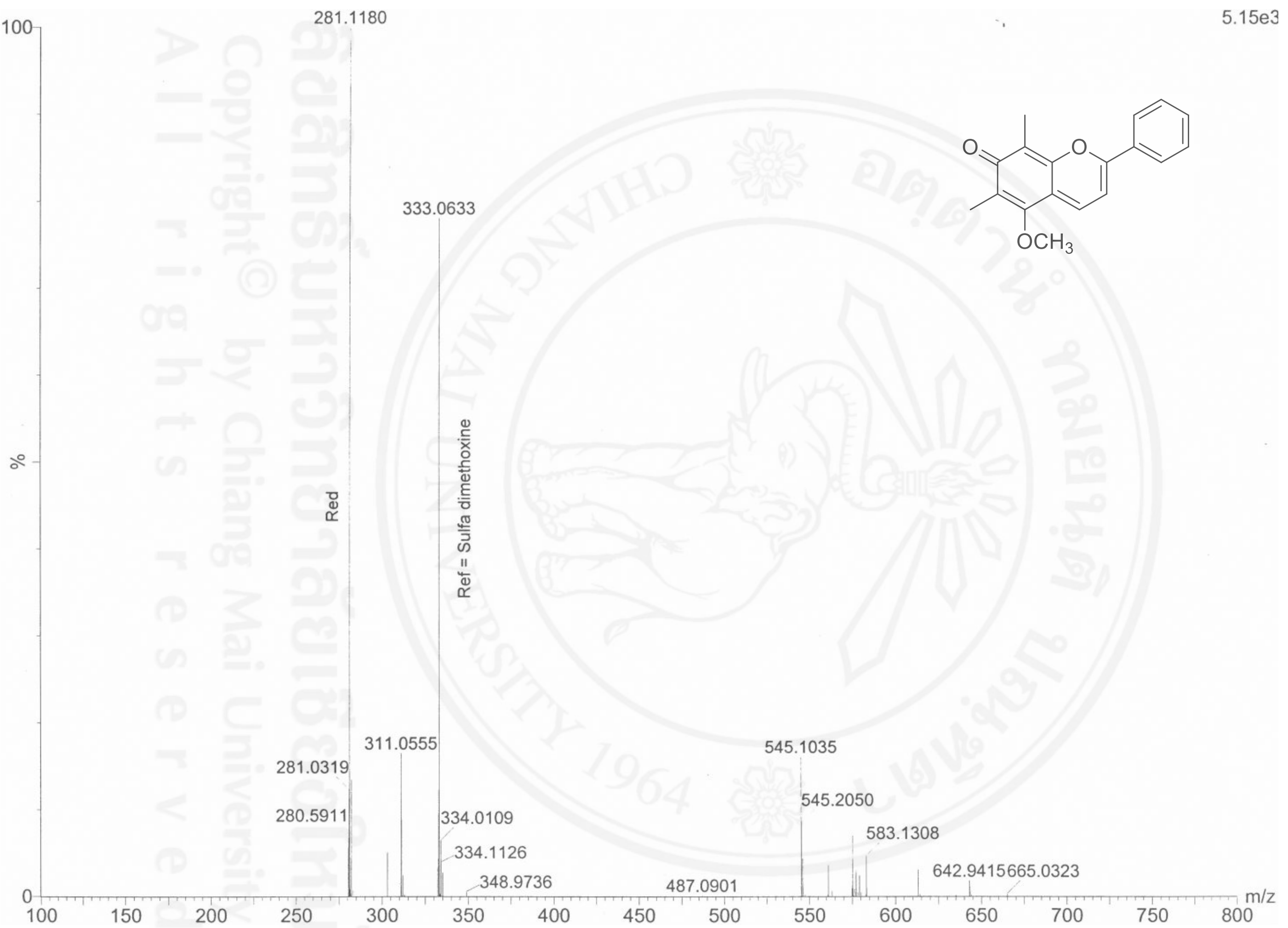

Figure 69 Mass spectrum (HRMS (ESI)) of 5-methoxy-6,8-dimethyl-2-phenyl-7*H*-1-benzopyran-7-one (**140**)

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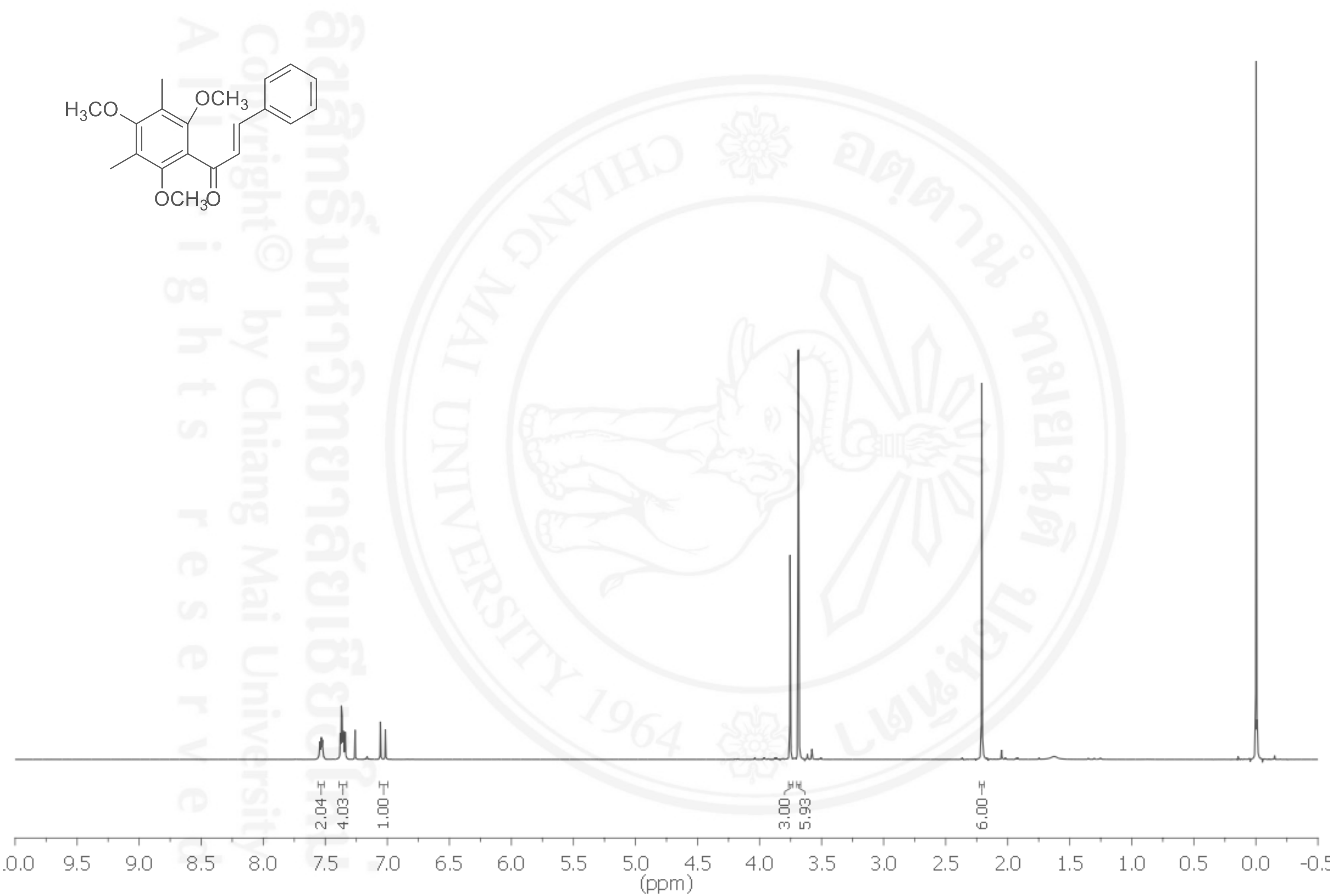

Figure 70 ^1H NMR (400 MHz, in $CDCl_3$) spectrum of 2',4',6'-trimethoxy-3',5'-dimethylchalcone (**141**)

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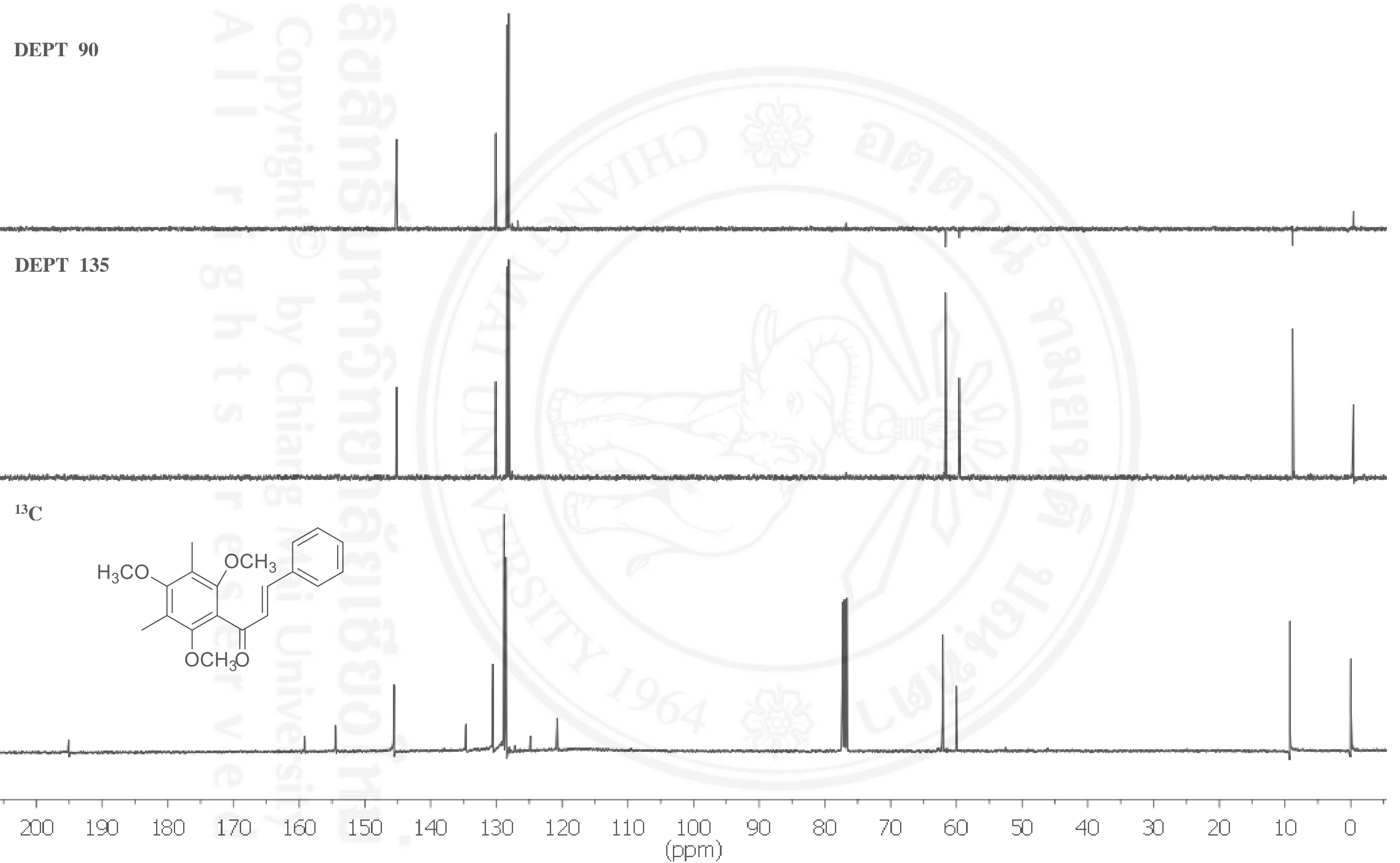

Figure 71 ^{13}C NMR (100 MHz, in $CDCl_3$) and DEPT spectra of 2',4',6'-trimethoxy-3',5'-dimethylchalcone (**141**)

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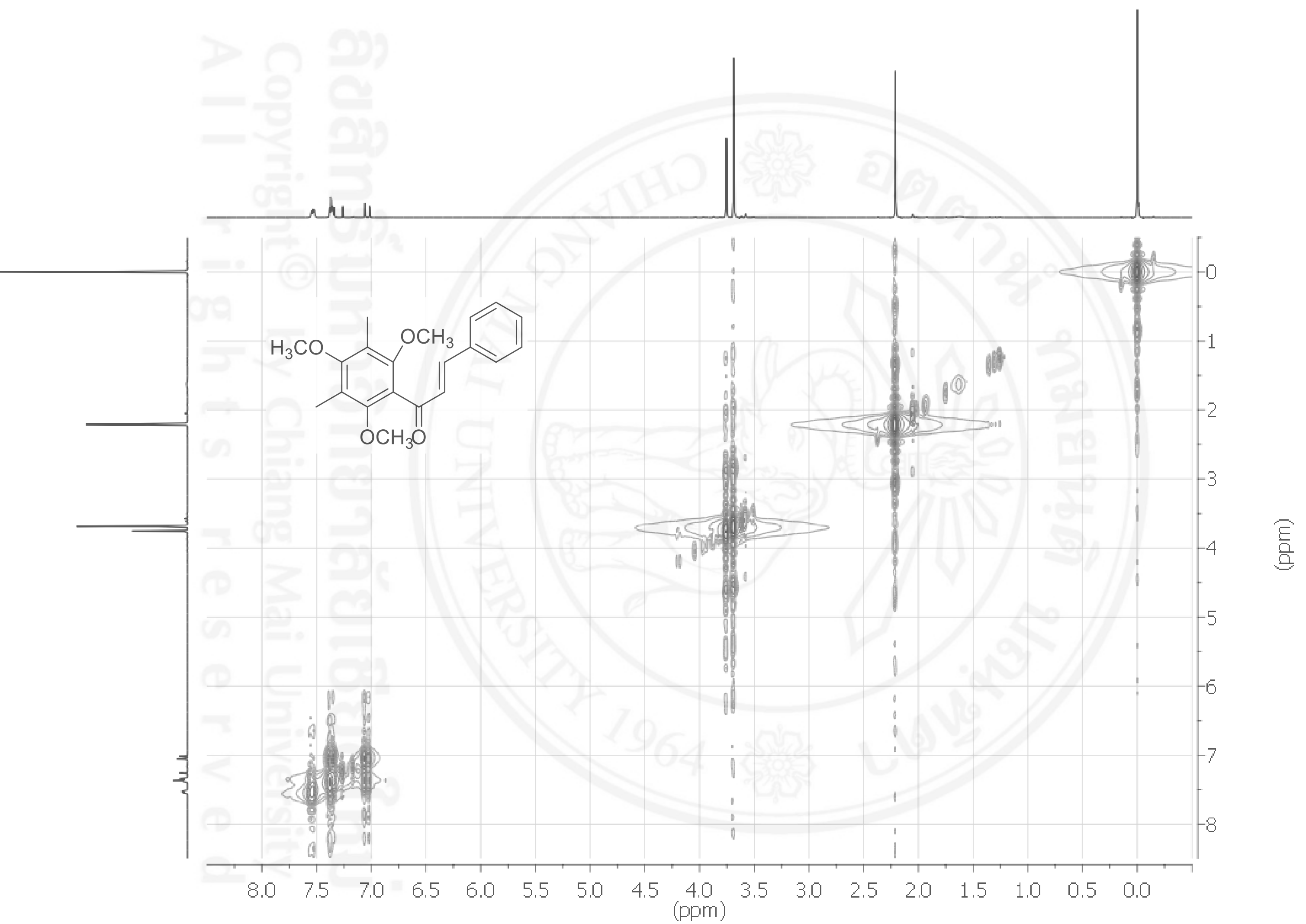

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Figure 72 ^{1}H-^{1}H COSY (in $CDCl_3$) spectrum of 2',4',6'-trimethoxy-3',5'-dimethylchalcone (**141**)

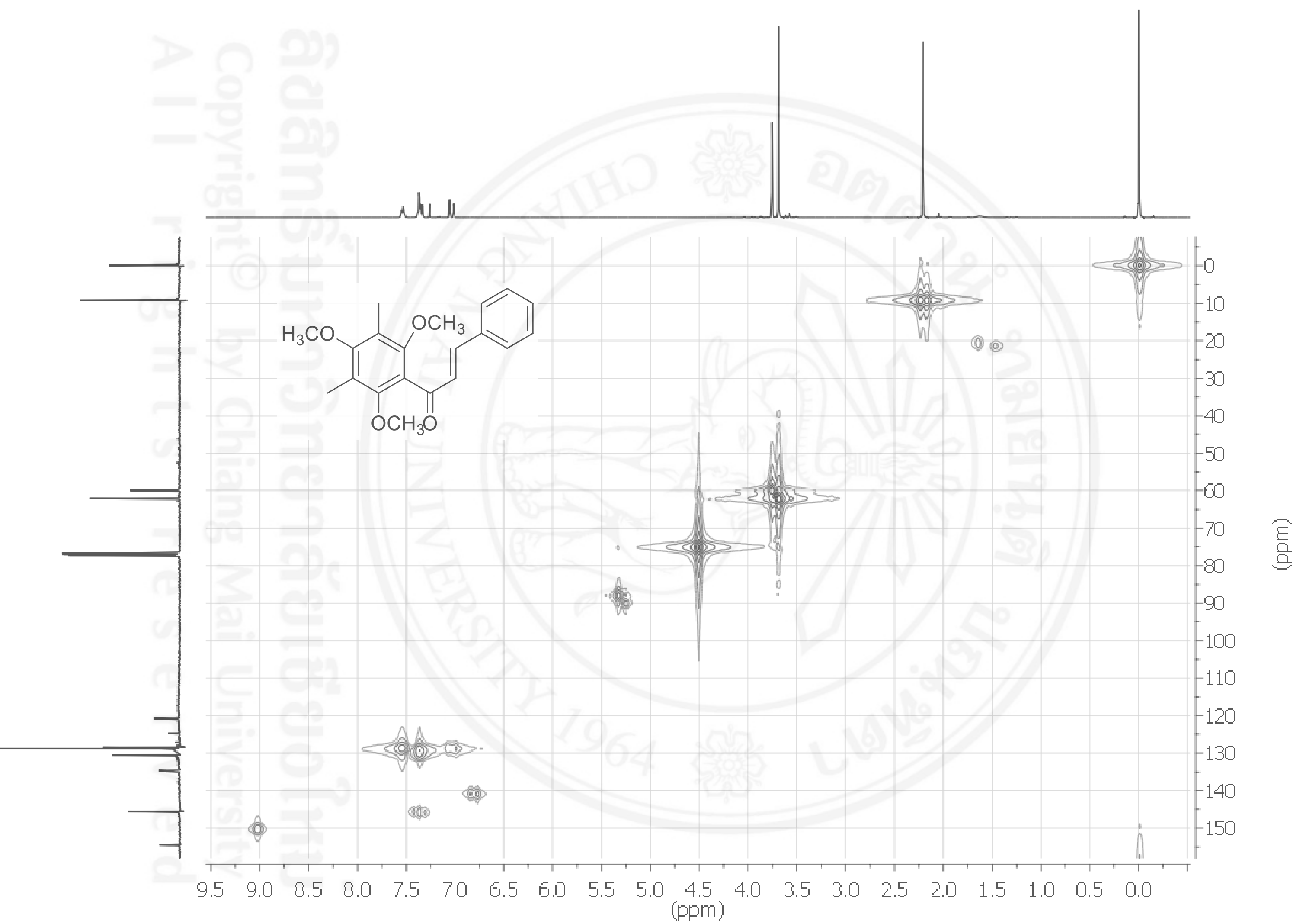

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Figure 73 HMQC (in $CDCl_3$) spectrum of 2',4',6'-trimethoxy-3',5'-dimethylchalcone (**141**)

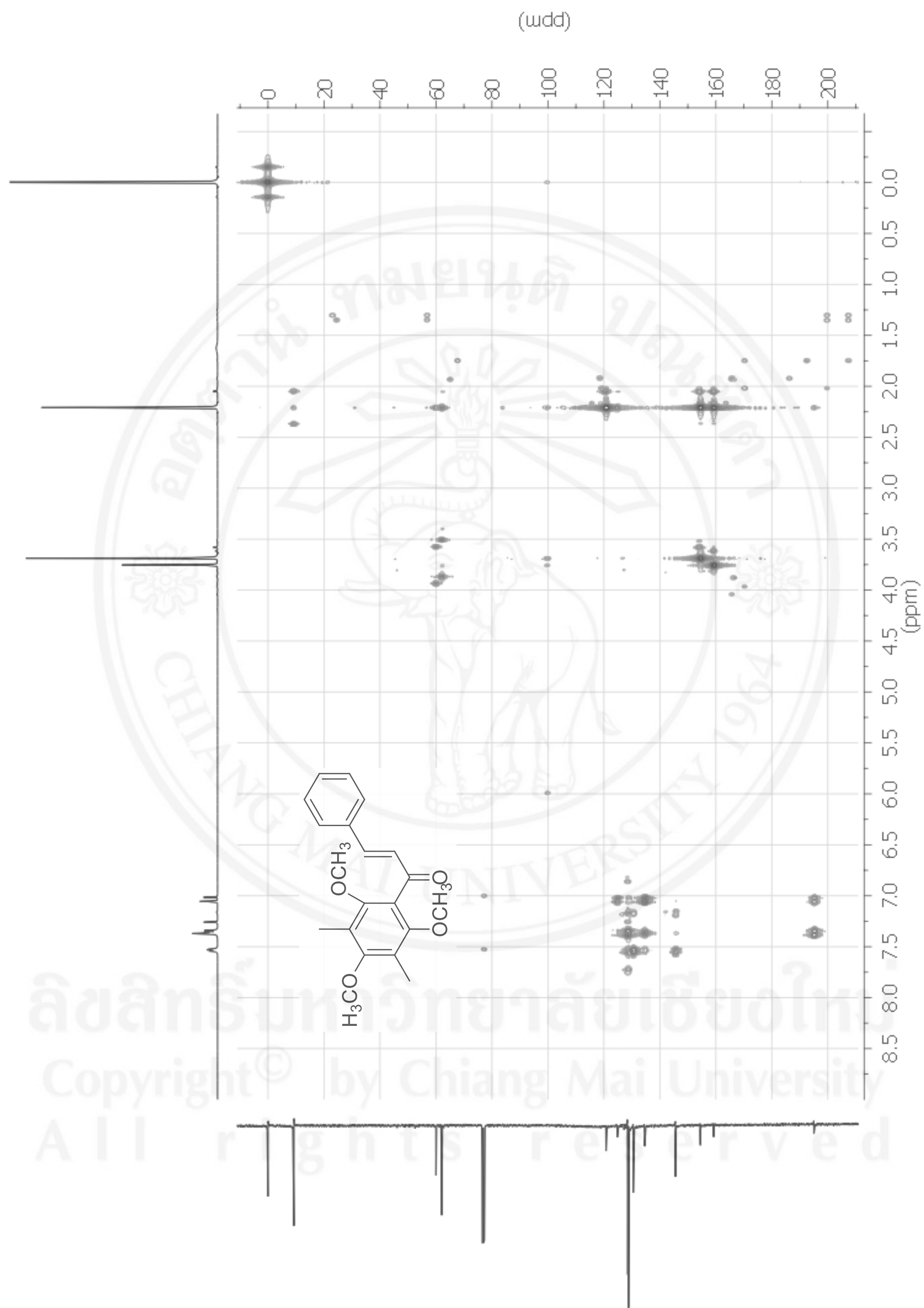

Figure 74 HMBC (in $CDCl_3$) spectrum of 2',4',6'-trimethoxy-3',5'-dimethylchalcone (**141**)

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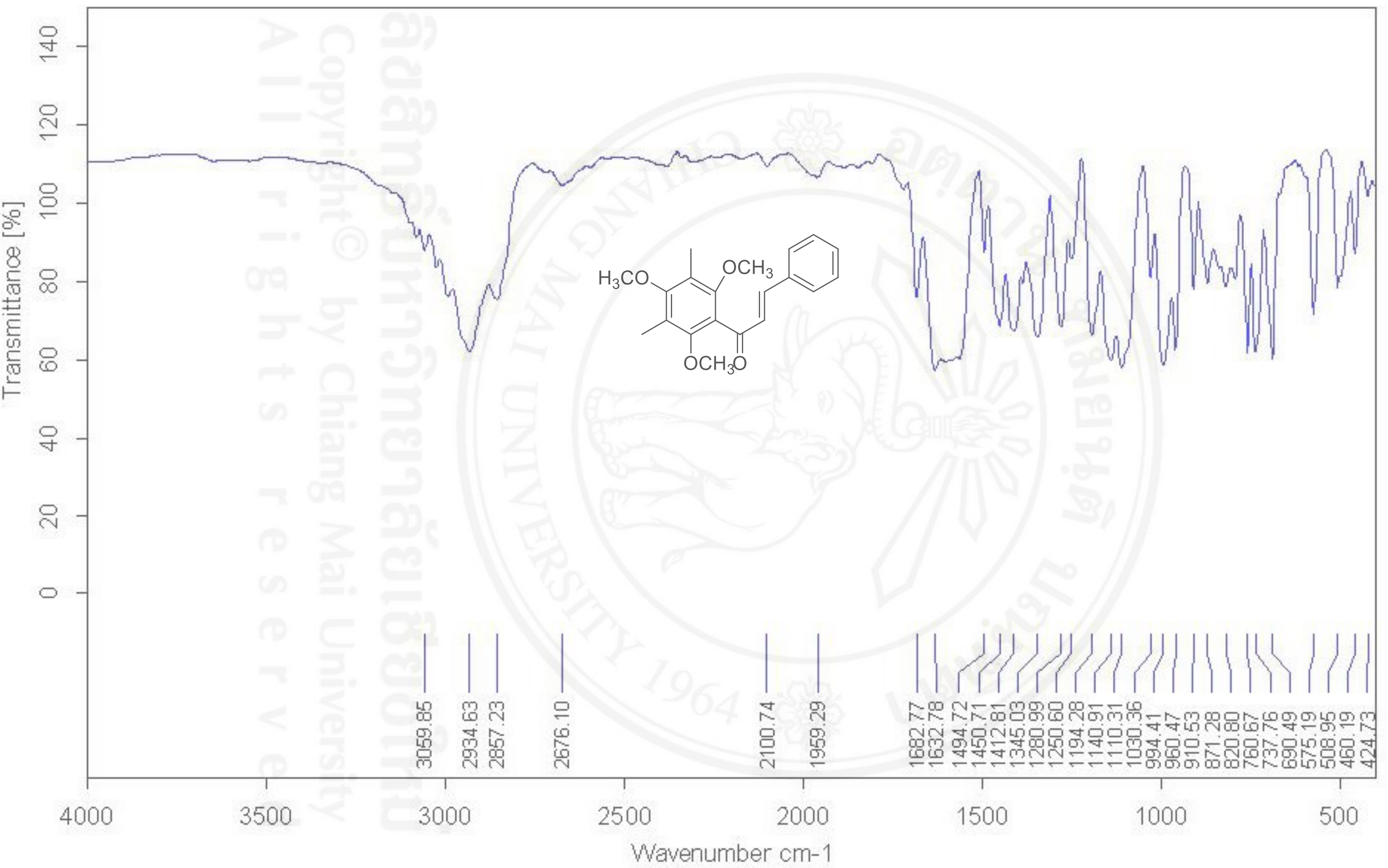

Figure 75 FTIR (evaporated thin film) spectrum of 2',4',6'-trimethoxy-3',5'-dimethylchalcone (**141**)

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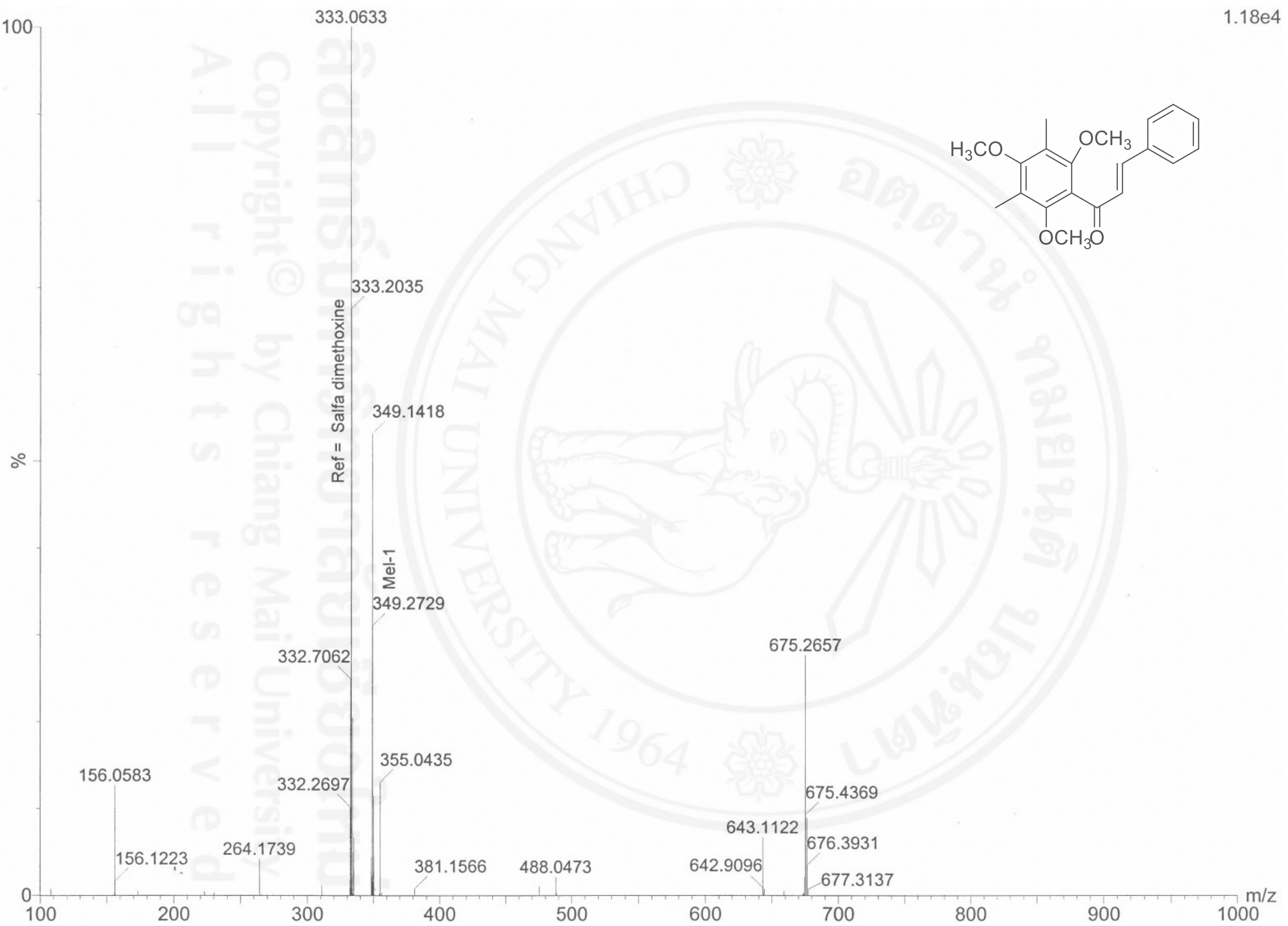

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Figure 76 Mass spectrum (HRMS (ESI)) of 2',4',6'-trimethoxy-3',5'-dimethylchalcone (**141**)

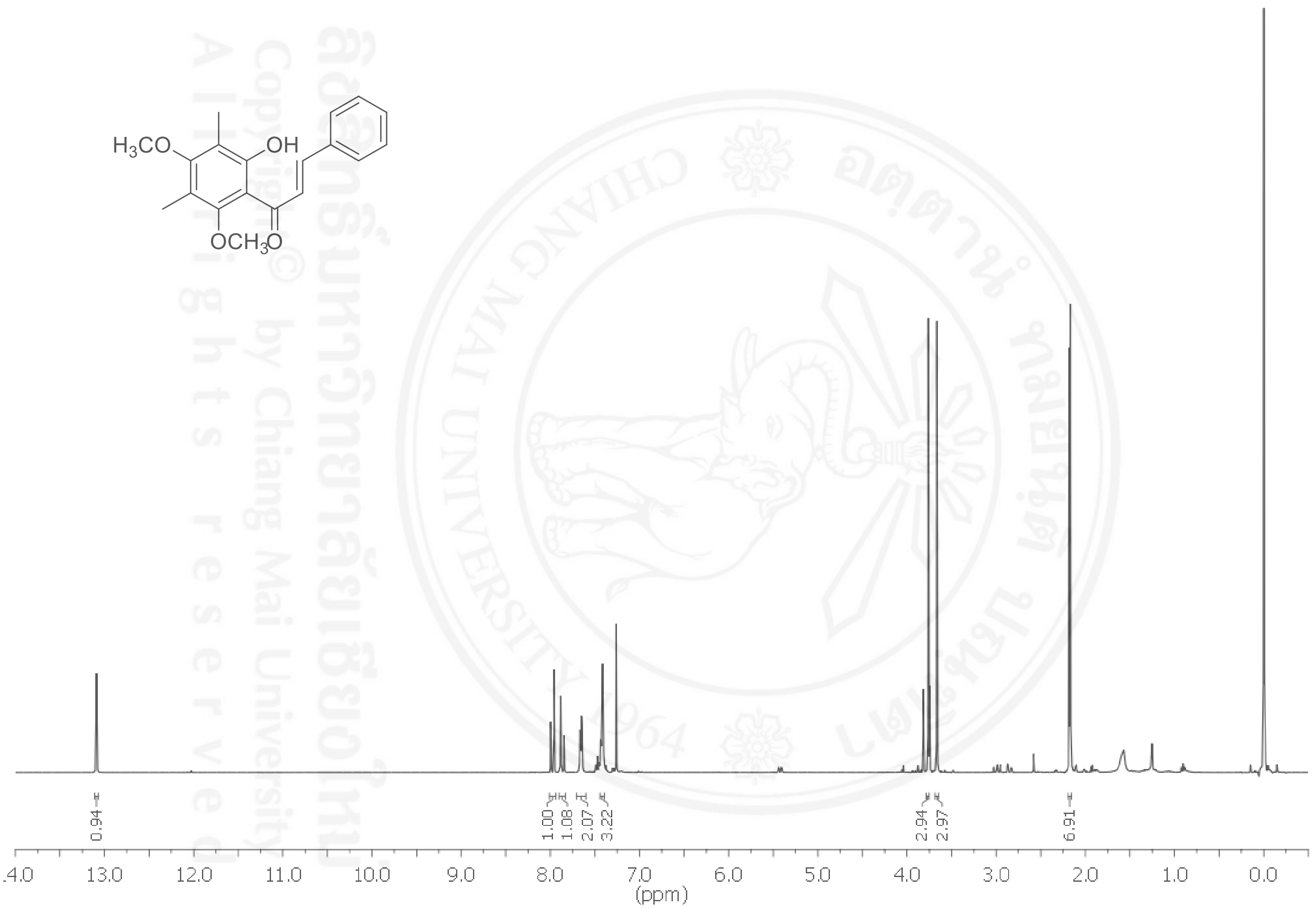

Figure 77 ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum of 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethylchalcone (**142**)

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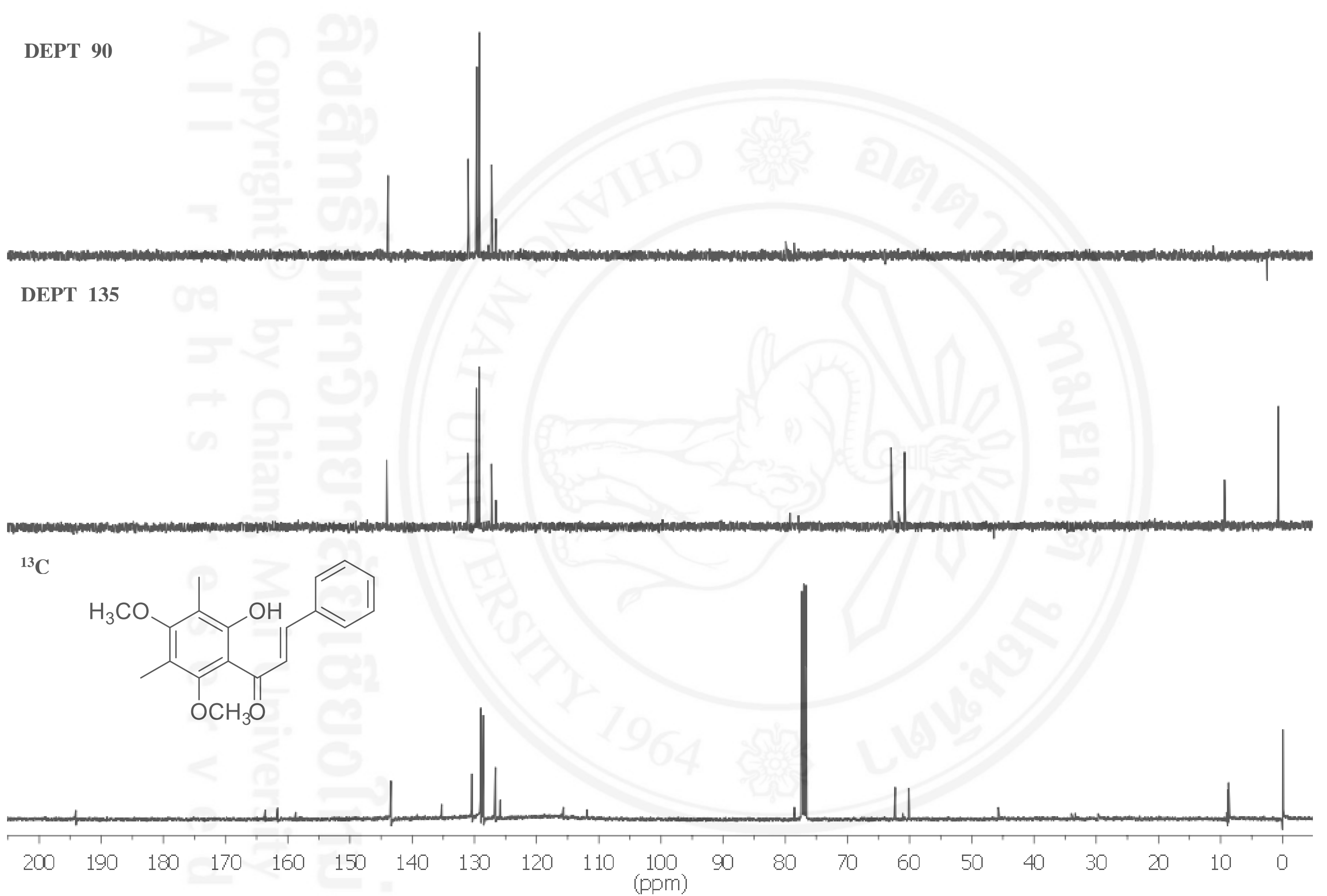

Figure 78 ^{13}C NMR (100 MHz, in $CDCl_3$) and DEPT spectra of 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethyl chalcone (**142**)

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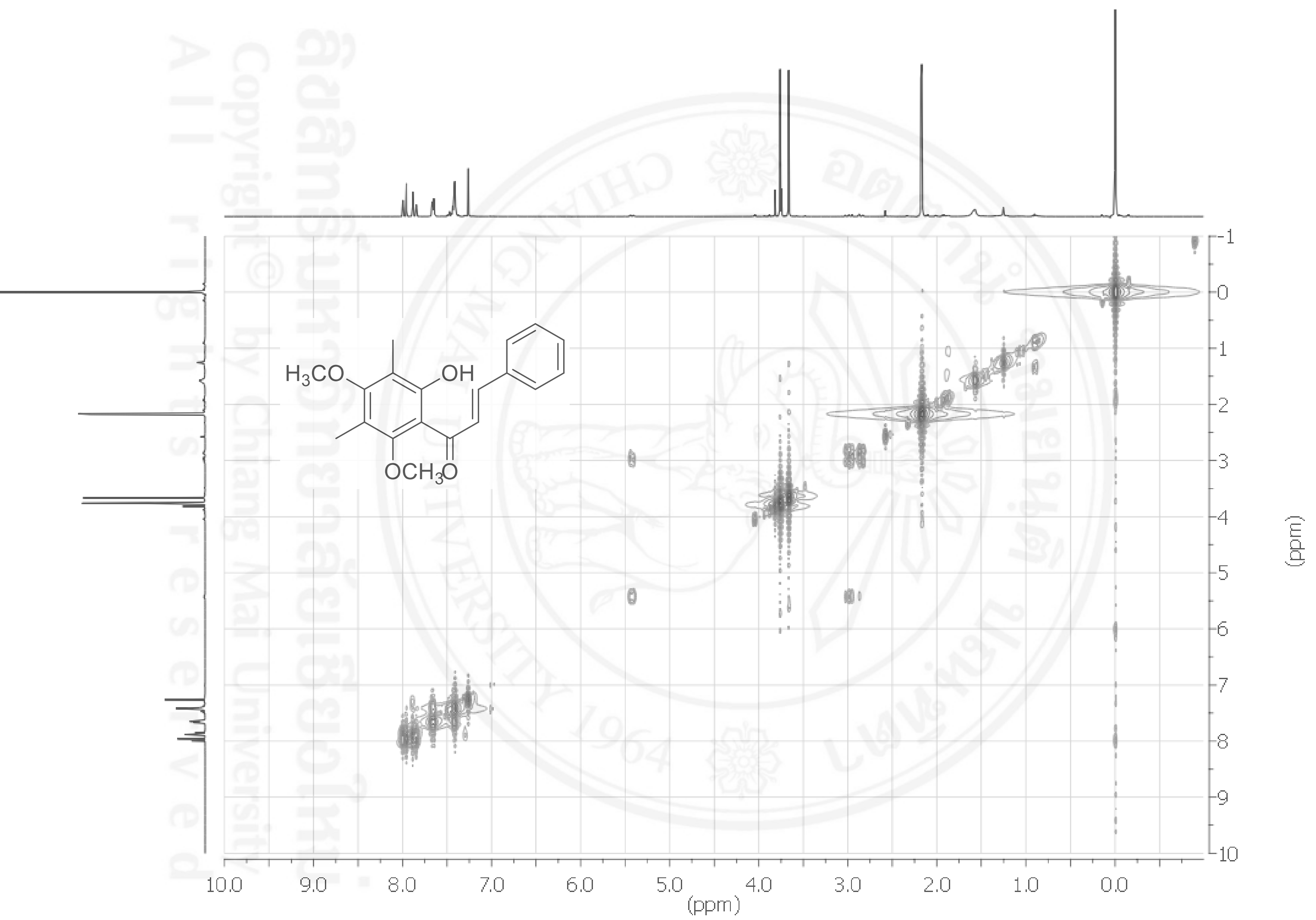

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Figure 79 ^{1}H-^{1}H COSY (in $CDCl_3$) spectrum of 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethylchalcone (**142**)

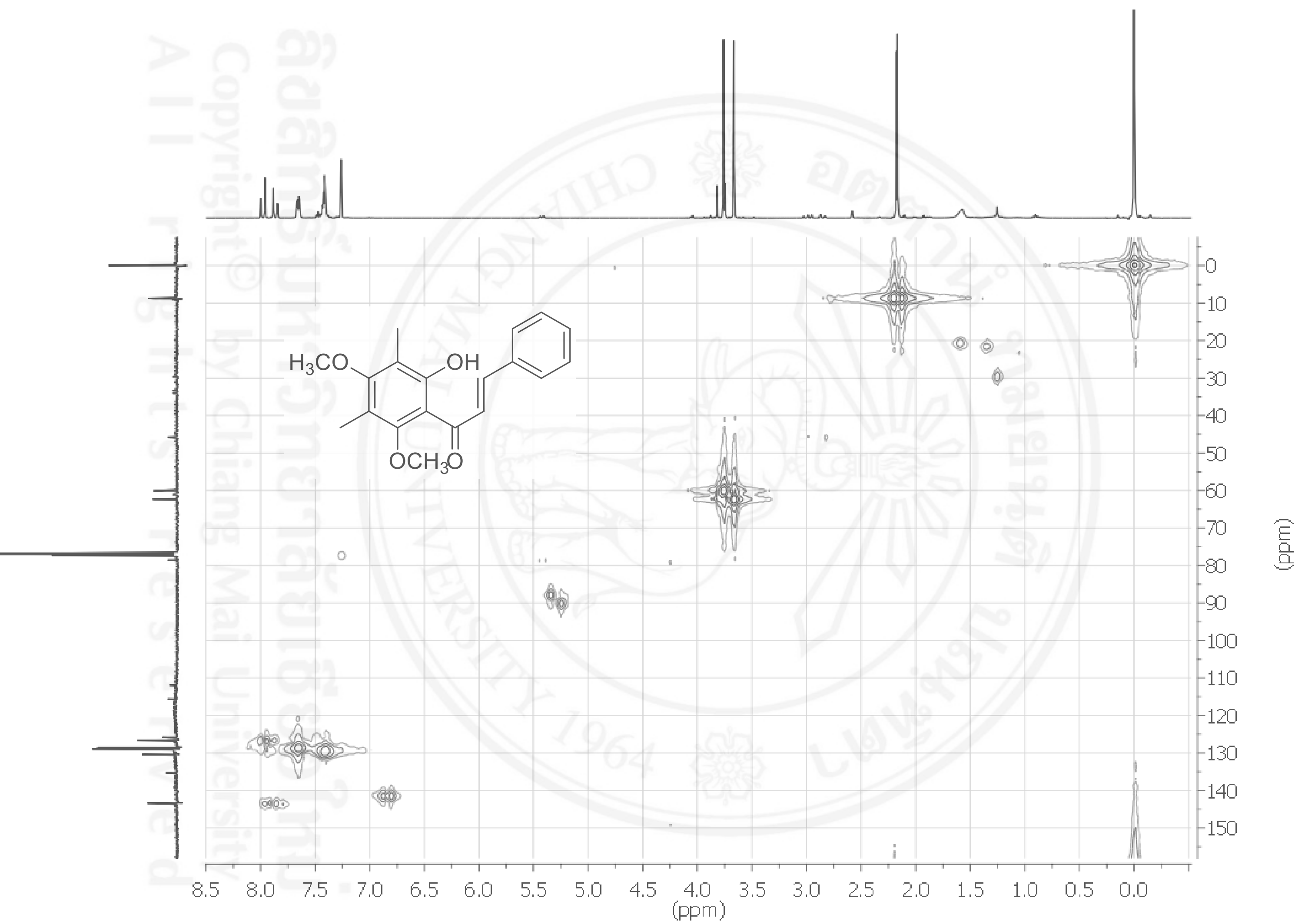

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Figure 80 HMQC (in $CDCl_3$) spectrum of 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethylchalcone (**142**)

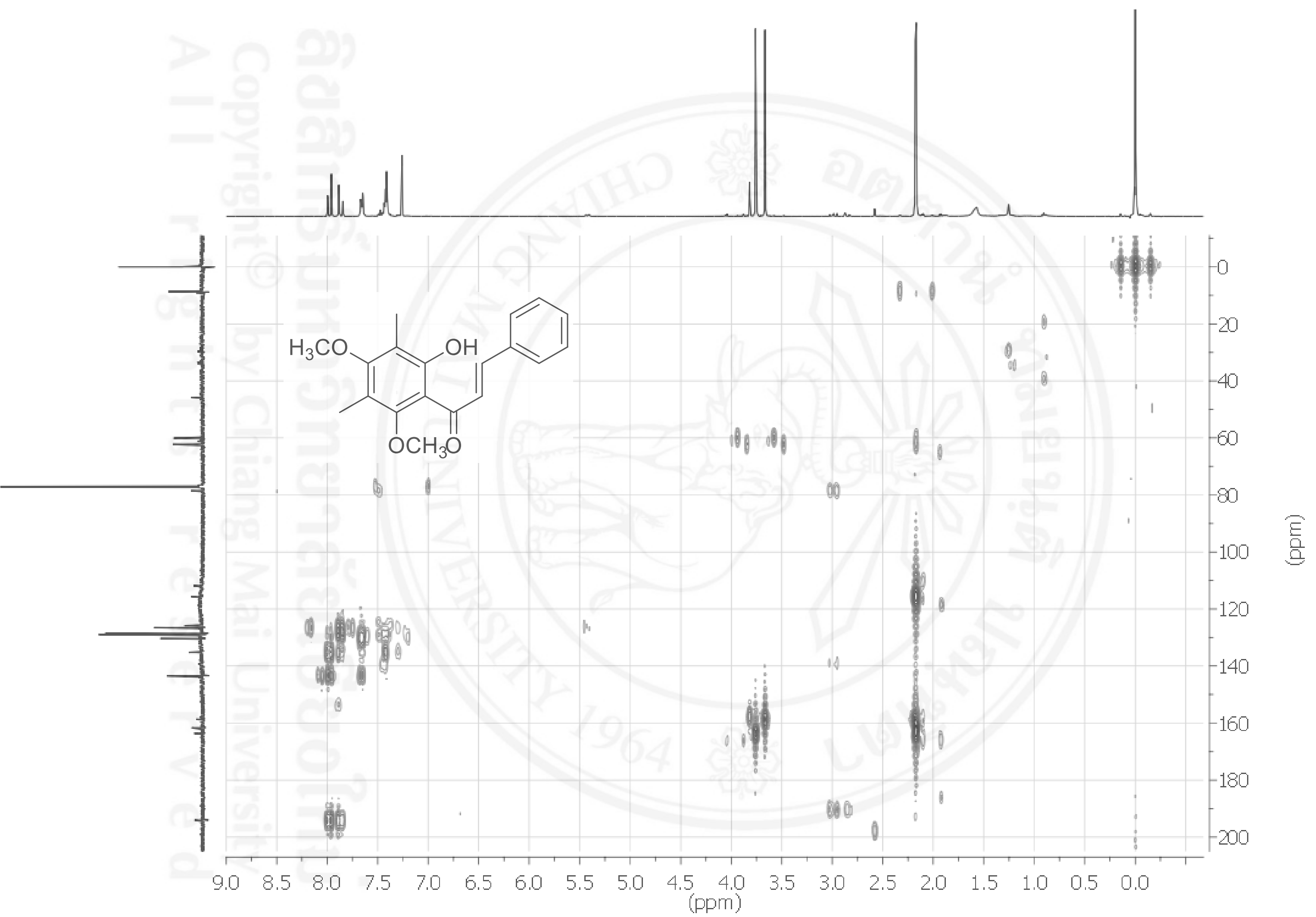

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Figure 81 HMBC (in $CDCl_3$) spectrum of 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethylchalcone (**142**)

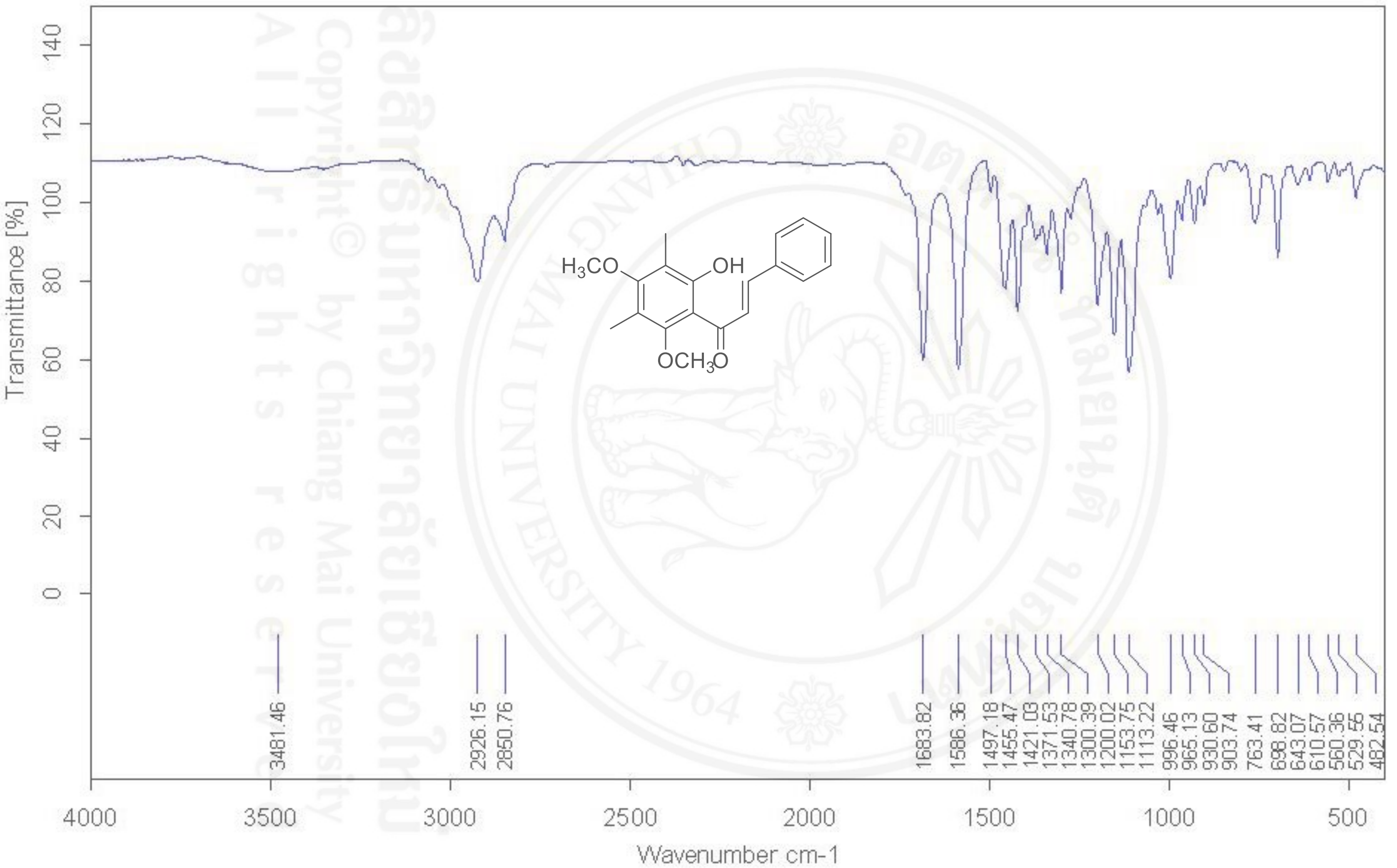

Figure 82 FTIR (evaporated thin film) spectrum of 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethylchalcone (**142**)

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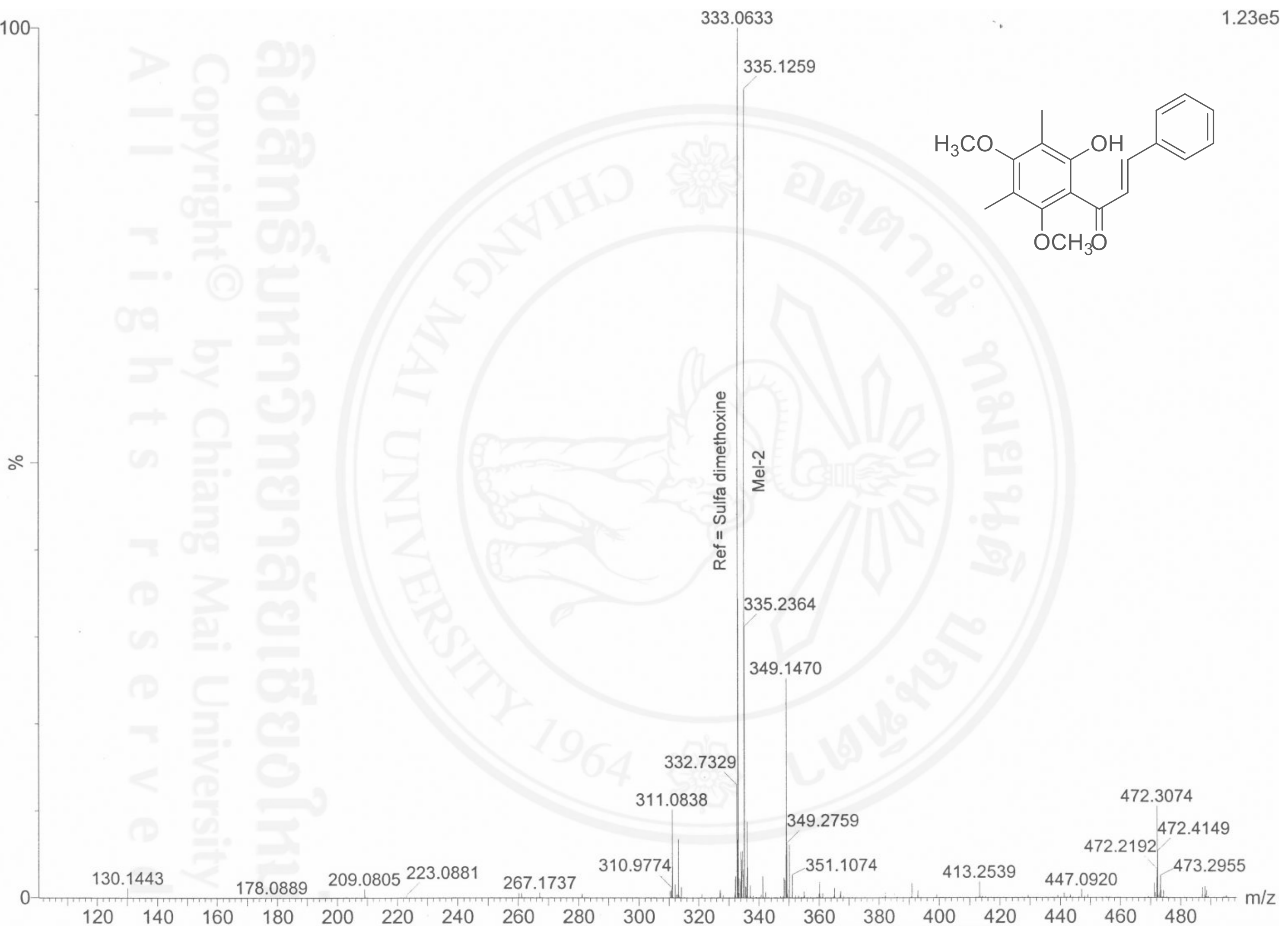

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Figure 83 Mass spectrum (HRMS (ESI)) of 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethylchalcone (**142**)

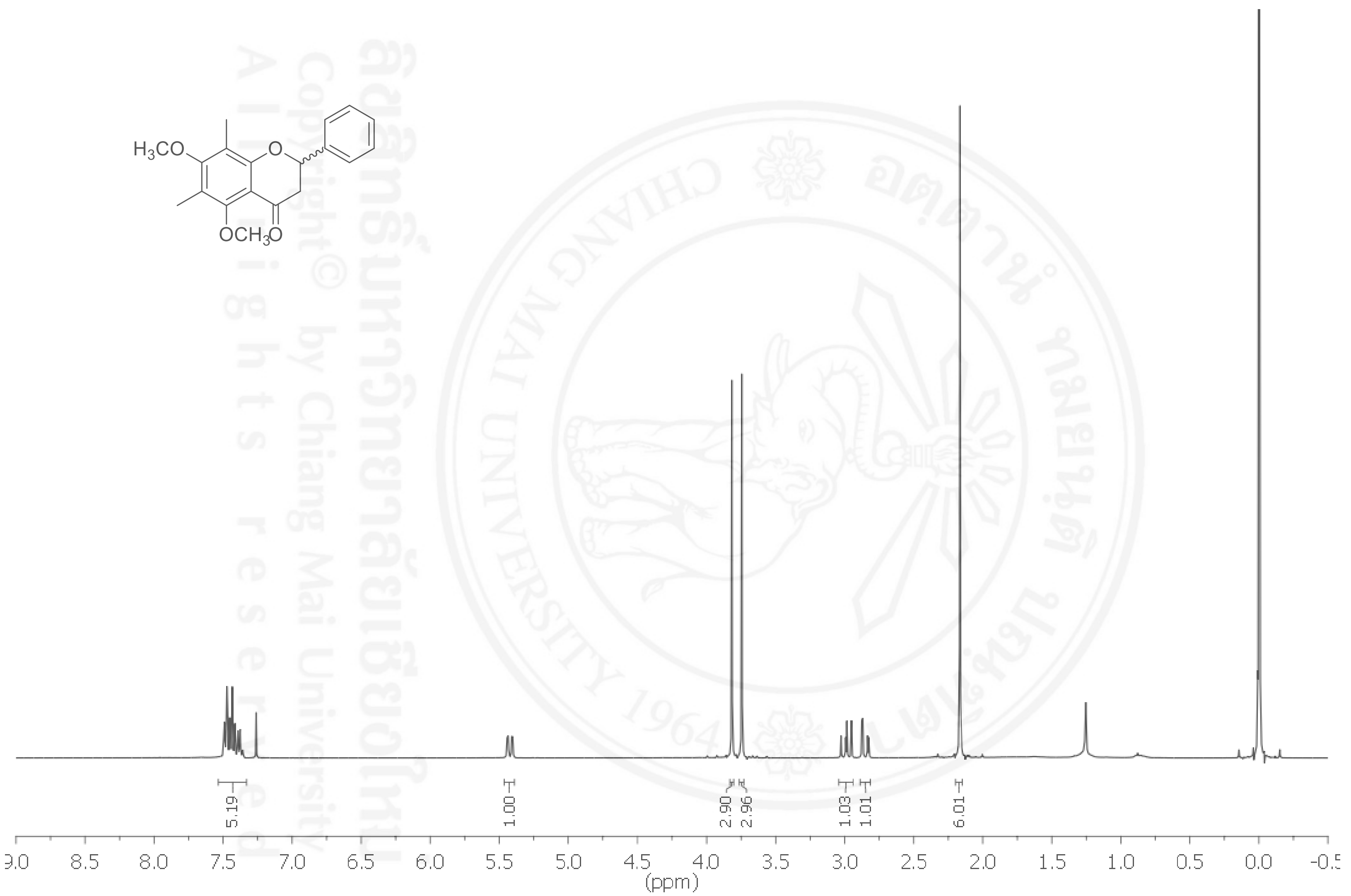

Figure 84 ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum of 5,7-dimethoxy-6,8-dimethylflavanone (**143**)

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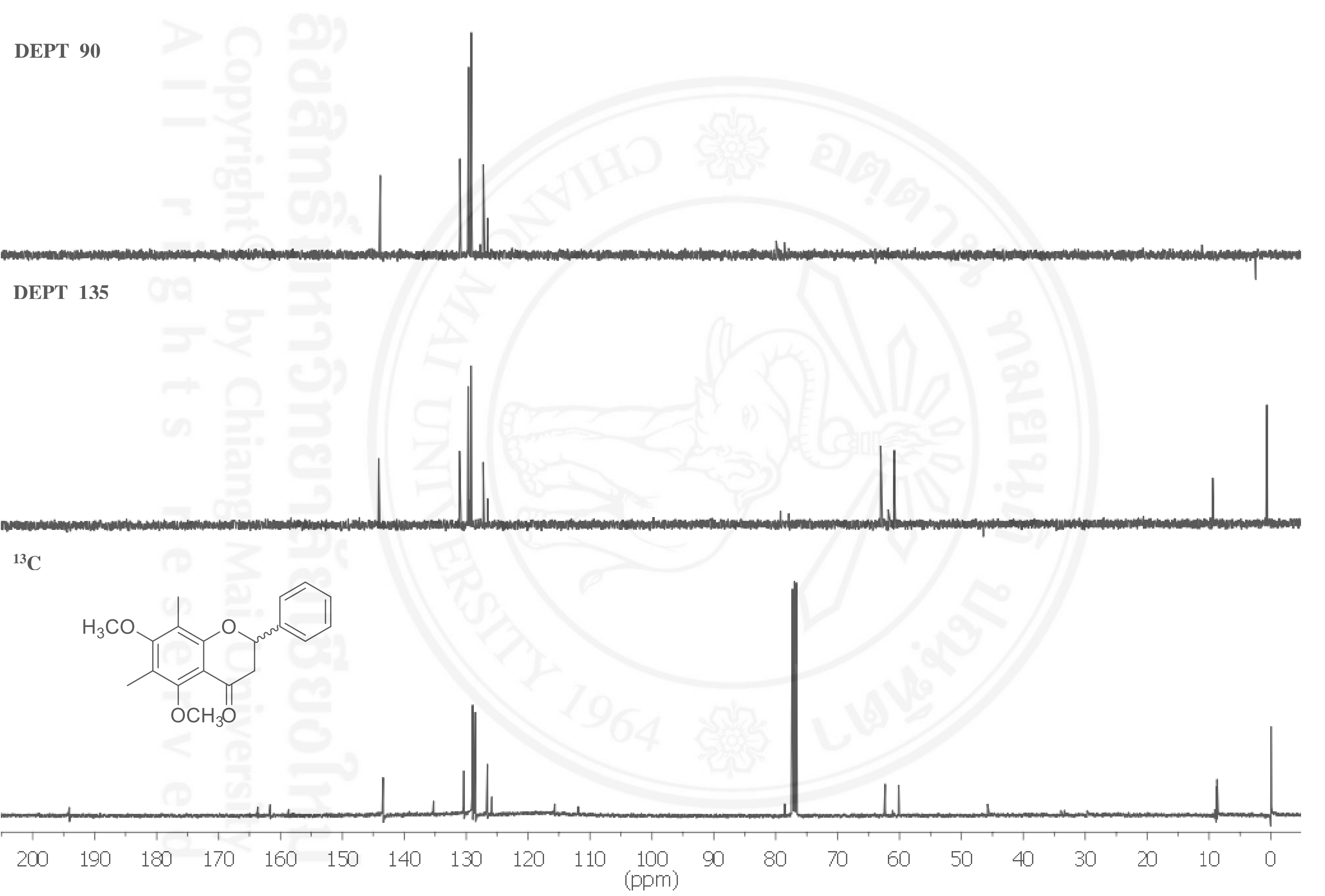

Figure 85 ^{13}C NMR (100 MHz, in $CDCl_3$) and DEPT spectra of 5,7-dimethoxy-6,8-dimethylflavanone (**143**)

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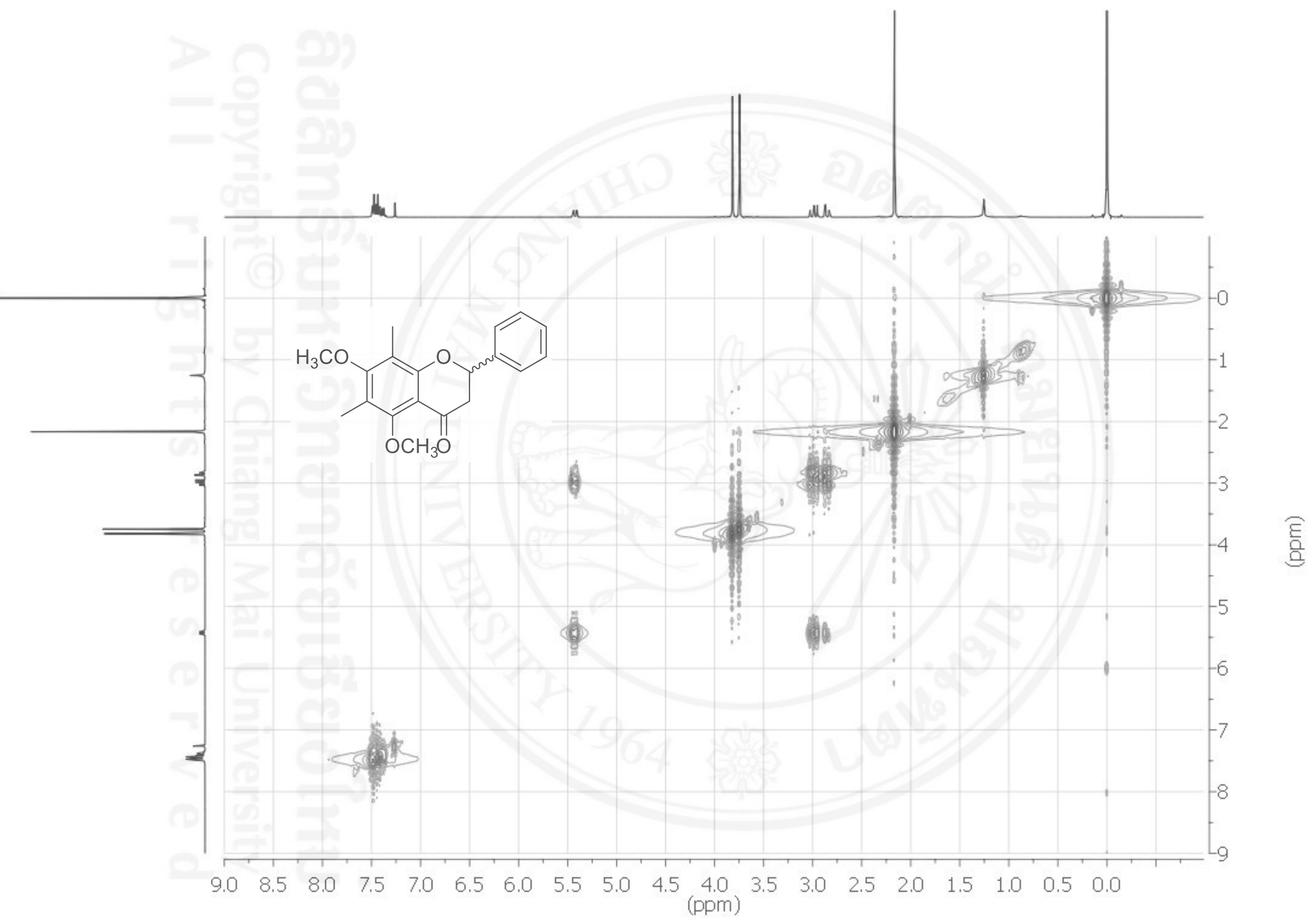

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Figure 86 ^{1}H-^{1}H COSY (in $CDCl_3$) spectrum of 5,7-dimethoxy-6,8-dimethylflavanone (**143**)

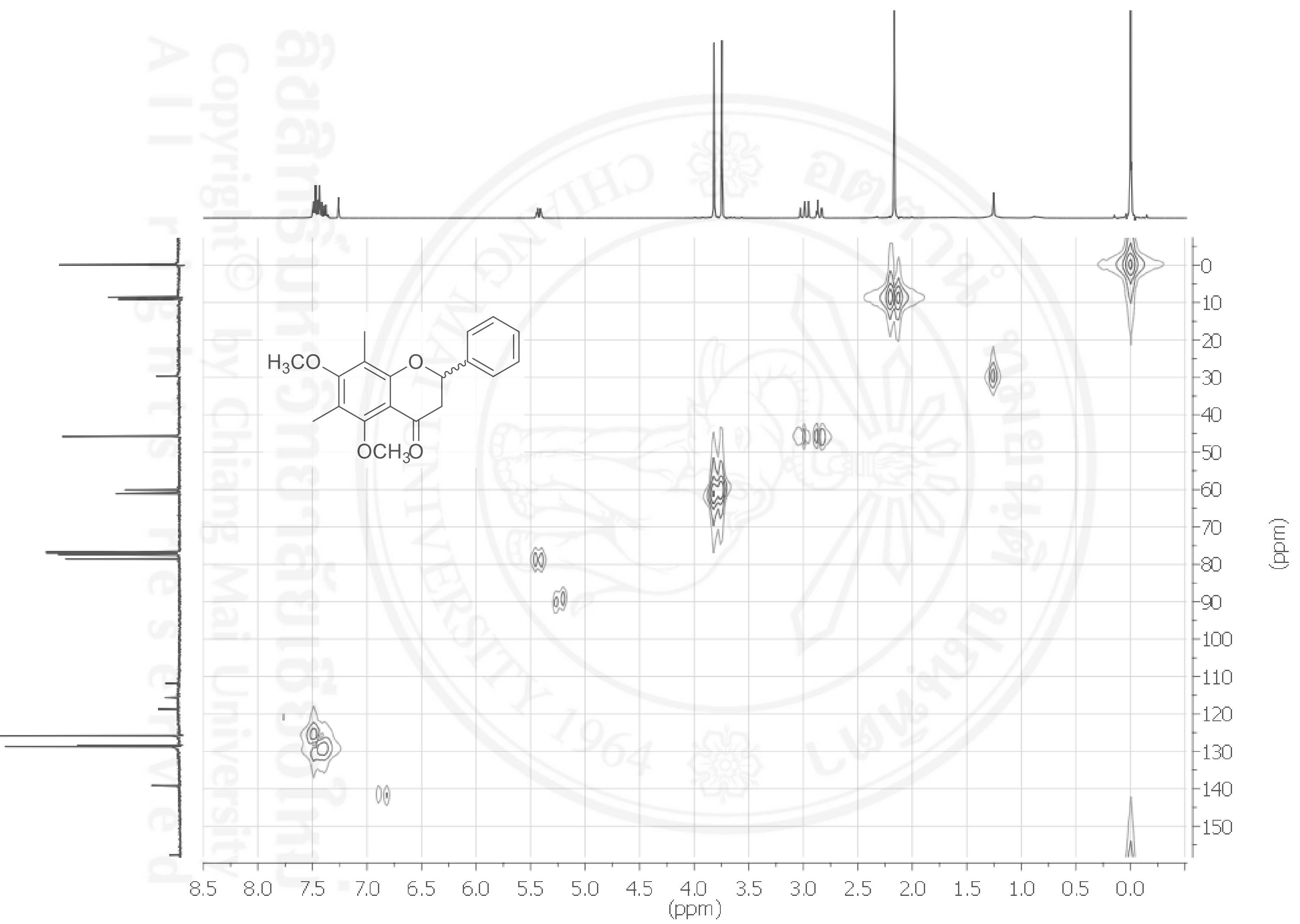

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Figure 87 HMQC (in $CDCl_3$) spectrum of 5,7-dimethoxy-6,8-dimethylflavanone (**143**)

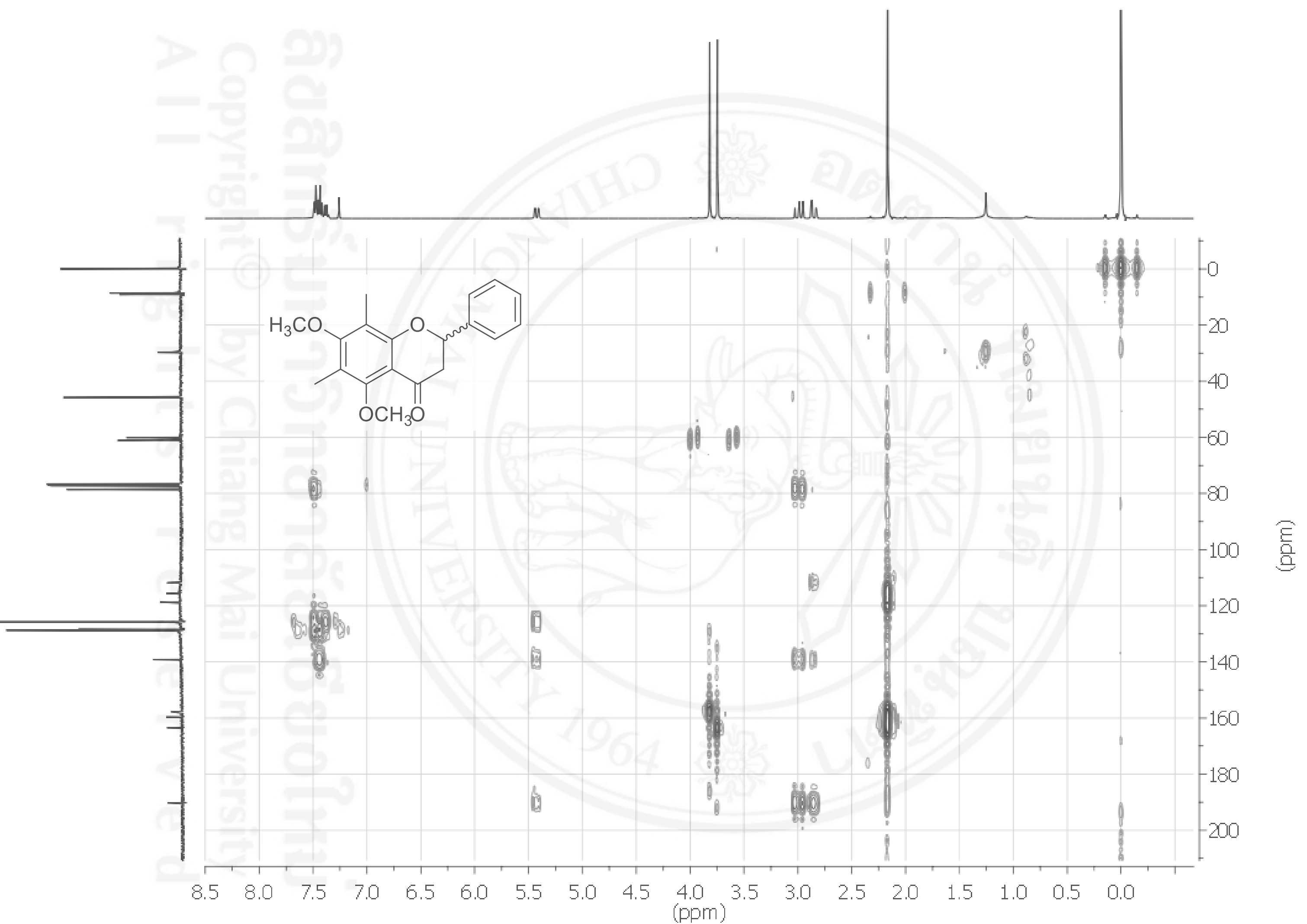

Figure 88 HMBC (in $CDCl_3$) spectrum of 5,7-dimethoxy-6,8-dimethylflavanone (**143**)

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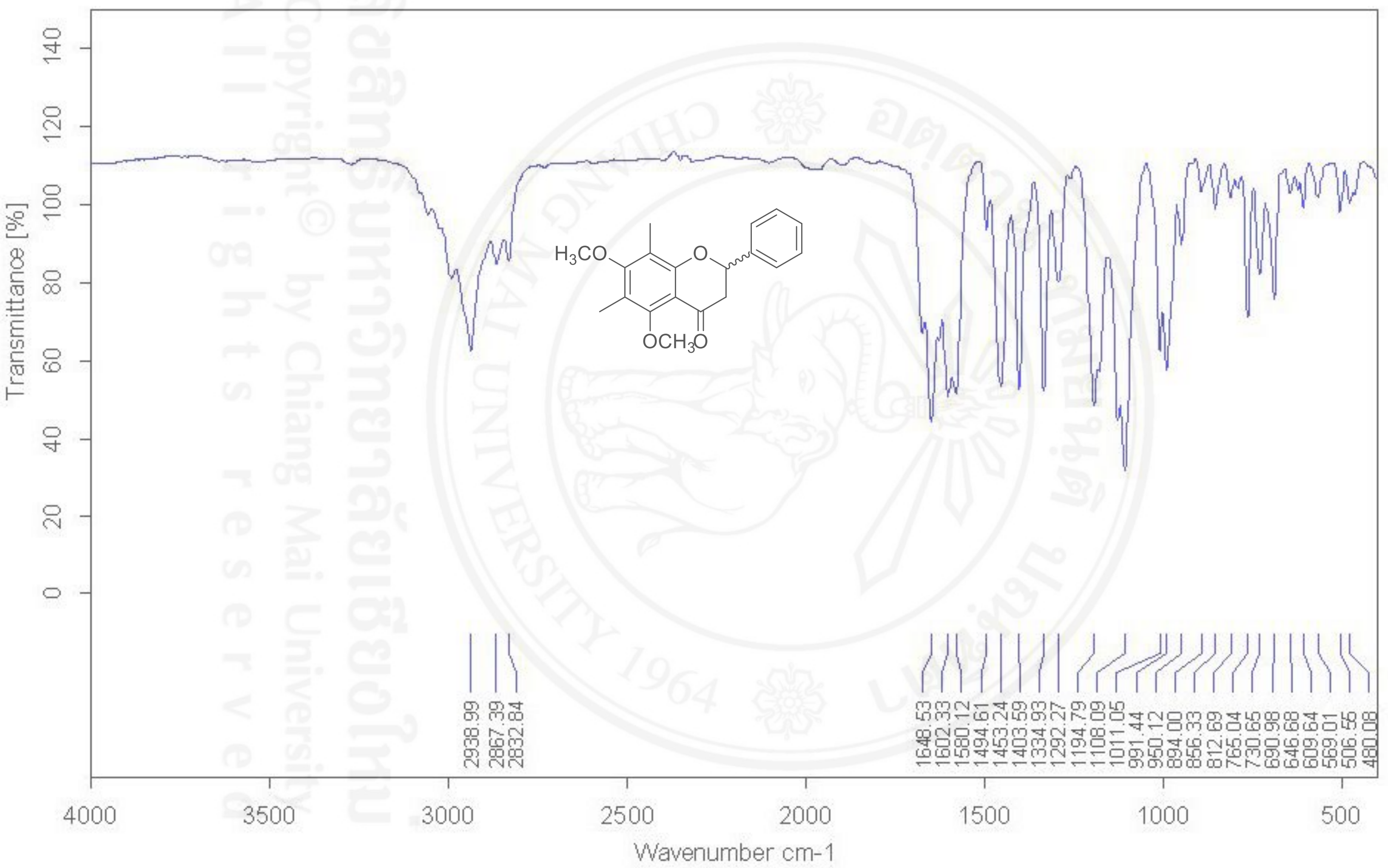

Figure 89 FTIR (evaporated thin film) spectrum of 5,7-dimethoxy-6,8-dimethylflavanone (**143**)

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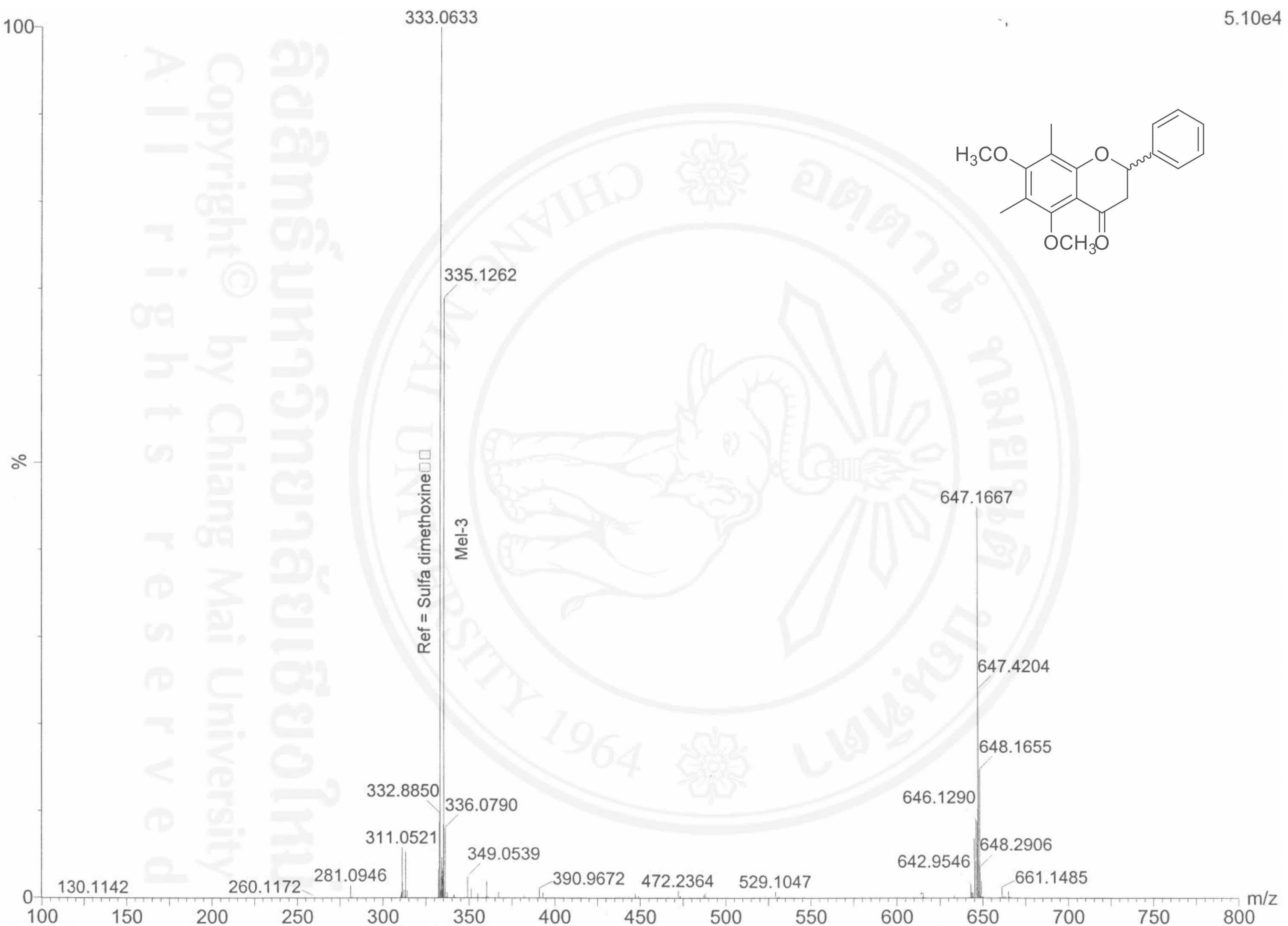

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Figure 90 Mass spectrum (HRMS (ESI)) of 5,7-dimethoxy-6,8-dimethylflavanone (**143**)

CURRICULUM VITAE

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Presentations and Conferences:

National Conference:

Anuruk Chailungka and Puttinan Meepowpan*, "*Chemical Constituents from Dichloromethane Extract of Vernonia scandens Twigs*", PERCH-CIC Congress VII, at Pattaya, Chonburi, Thailand (2011).

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Anuruk Chailungka, Wanida Inboot, Rawiwan Wongpoomchai and Puttinan Meepowpan*, "*Two Flavonoids from the Seeds of Cliestocalyx nervosum var. paniala*", PERCH-CIC Congress VIII, at Pattaya, Chonburi, Thailand (2013).

International Conference:

Anuruk Chailungka and Puttinan Meepowpan*, "*Chemical Investigations of The Dichloromethane Extract of Vernonia scandens Twigs*", The Sixth Pure and Applied Chemistry International Conference (PACCON VI), at Chiang Mai, Thailand (2012), pp. 767−768.

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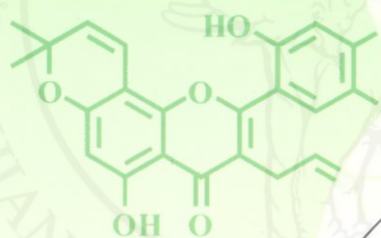
PERCH-CIC
CONGRESS VII

Theme: Chemistry, Environment and Society

การประชุมวิชาการ
ศูนย์ความเป็นเลิศด้านนวัตกรรมทางเคมี ครั้งที่ 7



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4-7 May 2011
Jomtien Palm Beach Hotel & Resort Pattaya, Chonburi

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Chemical Constituents from Dichloromethane Extract of *Vernonia scandens* Twigs

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Abstract **:** Triterpenes, lupeol acetate (**1**), phenylpropene, eugenol (**2**), lignan, *neo*-lignan (**3**), and two steroids, β-sitosterol (**4**) and stigmasterol (**5**), can be obtain from crude CH_2Cl_2 extract of *Vernonia scandens* twigs. Chemical elucidation of compounds **1-5** were identified by ^1H NMR, ^{13}C NMR, DEPT, COSY, HMQC, HMBC, NOE, IR and Mass spectrometry.

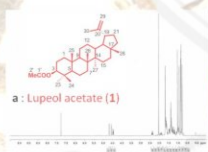
Keywords **:** *Vernonia scandens*, lupeol acetate, eugenol, *neo*-lignan, β-sitosterol, stigmasterol

Introduction

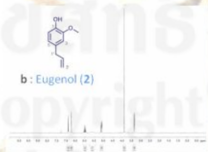
The genus *Vernonia*, in Compositae family, comprising about 1000 species is mainly distributed in the torrid zones of America, Asia, and Africa. Flavonoids, sesquiterpenoids, diterpenes, alkaloids, triterpenoids and cardiac glycosides have also been reported from this genus. This class of compounds has been reported to be insect antifeedant, antifungal, cytotoxic and antitumor activities. However, phytochemical and biological study has not been done on *Vernonia scandens*. Therefore, we are interesting investigation of chemical compositions and bioactive substances.

Method, Results and Discussion

The air dried twigs of *Vernonia scandens* (2,583.16 g) was successively extracted with CH_2Cl_2 and MeOH, respectively. The solvents were filtered and evaporated to dryness by rotary evaporator and follow by high vacuum pump to yield the crude CH_2Cl_2 (28.6457 g, 1.12 %yield) and MeOH (16.3184 g, 0.63 %yield). Chromatographic technique (silica gel) separation of the crude CH_2Cl_2 extract resulted lupeol acetate (**1**) (0.1626 g), eugenol (**2**) (0.1185 g), neo-lignan (**3**) (0.0860 g) and mixture of β-sitosterol (**4**) (0.6977 g) and stigmasterol (**5**) (0.5623 g) which were identified by ^1H NMR (Figure **1a-d**), ^{13}C NMR, DEPT, COSY, HMQC, HMBC, NOE, IR, mass spectrometry and comparison of spectroscopic data with those reported in the literatures.

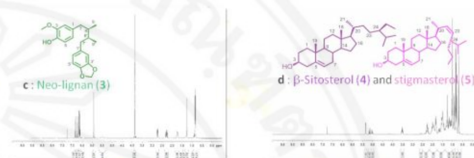


Position	δ ¹H [mult., J in Hz]	δ ¹³C
1		38.3
2		23.7
3	4.47 [1H, dd, 10.4, 5.4]	80.9
4	-	37.7
5	0.79 [1H, m]	55.3
6		18.1
7		34.2
8	-	40.2
9		50.3
10		37.0
11		20.9
12		25.0
13		38.0
14	-	42.8
15		27.4
16		35.5
17	-	42.8
18		48.0
19	2.37 [1H, dt, 11.1, 5.8]	48.2
20	-	150.9
21	1.86-1.96 [2H, m]	29.8
22		39.9
23	0.84 [3H, s]	27.9
24	0.84 [3H, s]	15.9
25	0.84 [3H, s]	16.1
26	1.02 [3H, s]	16.4
27	0.93 [3H, s]	14.5
28	0.83 [3H, s]	17.9
29	4.56 [1H, dd, 2.4, 1.3] 4.68 [1H, d, 2.3]	109.3
30	1.68 [3H, s]	19.2
1'	-	171.0
2'	2.04 [3H, s]	26.1



Position	δ ¹H [mult., J in Hz]	δ ¹³C
1	-	143.84
2	-	146.39
3	6.88 [1H, m]	114.22
4	-	131.85
5	6.70 [1H, m]	121.12
6	6.72 [1H, m]	111.07
1′	3.35 [2H, d, 6.7]	39.82
2′	5.93-6.04 [1H, m]	137.77
3′	5.09-5.14 [1H, m] 5.07-5.10 [1H, m]	115.43
OMe	3.88 [3H, s]	55.78
OH	5.59 [1H, br s]	-

Figure 1 : ^1H NMR data of compounds **1**-**5** were isolated from crude CH_2Cl_2 extract of *Vernonia scandens* twigs



Position	δ ¹H [mult., J in Hz]	δ ¹³C
1	-	133.72
2	6.66 [1H, s]	111.41
3	-	147.44
4	-	143.52
5	6.73 [1H, d, 7.9]	113.99
6	6.61 [1H, d, 1.5]	121.79
7	2.29 [1H, dd, 13.6, 9.1] 2.72 [1H, dd, 13.6, 5.0]	38.81
8	1.73 [1H, m]	39.33
9	0.83 [3H, d, 6.6]	16.08
1'	-	135.66
2'	6.63 [1H, s]	109.32
3'	-	146.26
4'	-	145.44
5'	6.83 [1H, d, 7.8]	107.90
6'	6.64 [1H, d, 1.5]	121.66
7'	2.25 [1H, dd, 13.6, 9.1] 2.72 [1H, dd, 13.6, 5.0]	39.05
8'	1.73 [1H, m]	39.25
9'	0.85 [3H, d, 6.6]	16.19
3-OMe	3.87 [3H, s]	55.79
4-OH	5.49 [1H, s]	-
OCH_2O	5.92 [2H, br]	100.67

Position	δ ¹H [mult., J in Hz]	
	β-Sitosterol	Stigmasterol
1	1.08(α) [1H, m], 1.85(β) [1H, m]	1.08(α) [1H, m], 1.85(β) [1H, m]
2	1.83(α) [1H, m], 1.51(β) [1H, m]	1.83(α) [1H, m], 1.51(β) [1H, m]
3	3.52(α) [1H, m]	3.52(α) [1H, m]
4	2.30(α) [1H, m], 2.23(β) [1H, m]	2.30(α) [1H, m], 2.23(β) [1H, m]
5	-	-
6	5.43 [1H, m]	5.43 [1H, m]
7	1.50(α) [1H, m], 1.97(β) [1H, m]	1.50(α) [1H, m], 1.97(β) [1H, m]
8	1.46(β) [1H, m]	1.46(β) [1H, m]
9	0.94(α) [1H, m]	0.94(α) [1H, m]
10	-	-
11	1.46(α β) [2H, m]	1.46(αβ) [2H, m]
12	1.18(α) [1H, m], 2.00(β) [1H, m]	1.18(α) [1H, m], 2.00(β) [1H, m]
13	-	-
14	1.01(α) [1H, m]	1.01(α) [1H, m]
15	1.56(α) [1H, m], 1.06(β) [1H, m]	1.56(α) [1H, m], 1.06(β) [1H, m]
16	1.72(α) [1H, m], 1.27(β) [1H, m]	1.72(α) [1H, m], 1.27(β) [1H, m]
17	1.15(α) [1H, m]	1.15(α) [1H, m]
18	0.69(β) [3H, s]	0.69(β) [3H, s]
19	1.10(β) [3H, s]	1.10(β) [3H, s]
20	2.06 [1H, m]	2.06 [1H, m]
21	6.61 [1H, d, 1.5]	6.61 [1H, d, 1.5]
22		5.14 [1H, dd, 15.2, 8.6]
23		5.01 [1H, dd, 15.2, 8.6]
24	1.53 [1H, m]	1.53 [1H, m]
25	1.55 [1H, m]	1.55 [1H, m]
26	0.84 [3H, d, 6.5]	0.84 [3H, d, 6.5]
27	0.79 [3H, d, 7.0]	0.79 [3H, d, 7.0]
28	1.43 [1H, m], 1.18 [1H, m]	1.43 [1H, m], 1.18 [1H, m]
29	0.81 [3H, t]	0.81 [3H, t]

Figure 1 : ^1H NMR data of compounds **1**-**5** were isolated from crude CH_2Cl_2 extract of *Vernonia scandens* twigs (continued)

Conclusion

Lupeol acetate (**1**), eugenol (**2**), neo-lignan (**3**), and mixture of β-sitosterol (**4**) and stigmasterol (**5**) can be successfully isolated by column chromatography in the crude CH_2Cl_2 extract of *Vernonia scandens* twigs.

Acknowledgements

We are grateful to the Center of Excellence for Innovation in Chemistry (PERCH-CIC) and North-Chiang Mai University for financial supports, Department of Chemistry, Faculty of Science, Chiang Mai University for facilities supporting this research.

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PERCH-CIC
CONGRESS VIII

Theme: Chemistry for Creative Economy

การประชุมวิชาการ
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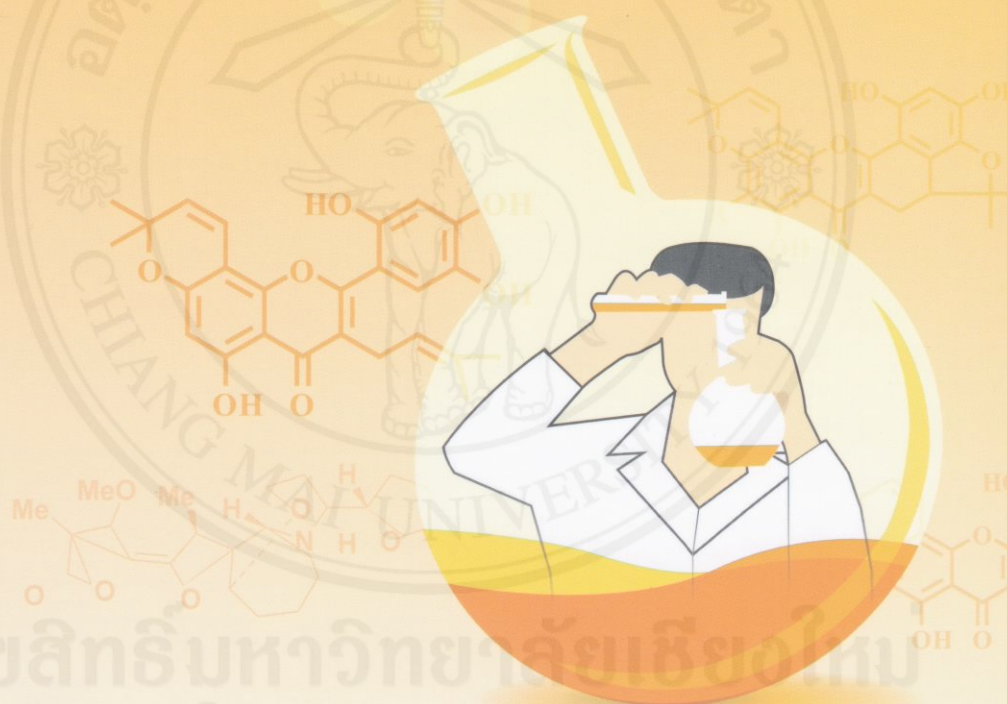

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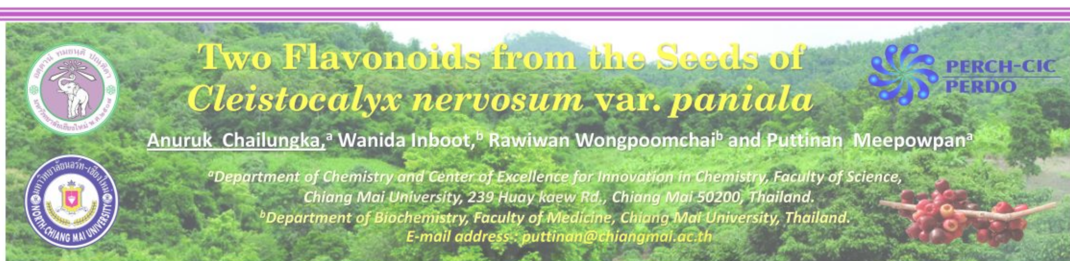

Two Flavonoids from the Seeds of *Cleistocalyx nervosum* var. *paniala*

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Abstract : Two flavonoids, chalcone; 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone or DMC (**1**) and flavone; hariganetin (**2**), can be isolated from the crude CH_2Cl_2 extract of *Cleistocalyx nervosum* var. *paniala* seeds. Chemical elucidation of compounds **1** and **2** were identified by ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC, HMBC, NOE, IR and mass spectrometry. Two flavoniods showed antigenotoxicity against AFB1, MeIQ and AF-2 induced mutagenesis in *S. typhimurium*.

Keywords : *Cleistocalyx nervosum* var. *paniala*, 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone, DMC, hariganetin, flavonoid

Introduction

Cleistocalyx nervosum var. *paniala*, locally known as Makiang, belongs to the family *Myrtaceae* and is found growing in scatter locations in some villages of the northern provinces of Thailand such as Chiang Rai, Chiang Mai, Lamphun, Lumpang and Mae Hong Son. For the *Cleistocalyx* genus, there were a few phytochemical investigations carried out on this genus. Thus we are interested in isolation and structural elucidation of chemical constituents from the seeds of this plant, and also test antigenotoxicity activity.

Method, Results and Discussion

The air dried seeds of *Cleistocalyx nervosum* var. *paniala* (3,217 g) was successively extracted with CH_2Cl_2 and MeOH, respectively. The solvents were filtered and evaporated to dryness by rotary evaporator and follow by high vacuum pump to yield the crude CH_2Cl_2 (97.47 g, 3.03 %yield) and MeOH (33.81 g, 1.05 %yield). Chromatographic technique (silica gel) separation of the crude CH_2Cl_2 extract resulted 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**1**) (7.7998 g, 8.00 %yield) and hariganetine (**2**) (8.8092 g, 9.04 %yield) which were identified by ^{1}H NMR (Figure 1), ^{13}C NMR and DEPT, COSY, HMQC, HMBC, NOE, IR, mass spectrometry and comparison of spectroscopic data with those reported in the literatures.

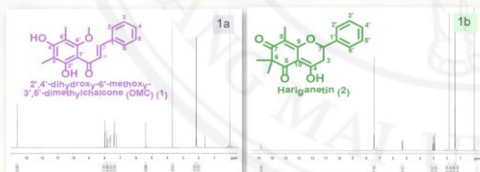

Figure 1 : ^{1}H NMR data of compounds **1** and **2** were isolated from crude CH_2Cl_2 extract of *C. nervosum* var. *paniala*: **1a**) 2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**1**) and **1b**) Hariganetine (**2**)

2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (1): orange solid; mp. 120.8–122.3 °C (from CH_2Cl_2/hexane); IR(thin film): ν_{max} 3444, 1627, 1554, 1165 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.14 (3H, *s*, 5′-CH$_3$), 2.16 (3H, *s*, 3′-CH$_3$), 3.66 (3H, *s*, 6′-OCH$_3$), 5.38 (1H, *s*, 4′-OH), 7.41 (3H, *m*, H-3,4,5), 7.64 (2H, *m*, H-2,6), 7.84 (1H, *d*, *J* = 15.7 Hz, H_β), 7.99 (1H, *d*, *J* = 15.7 Hz, H_α), 13.69 (1H, *s*, 2′-OH); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 7.6 (5′-CH$_3$), 8.2 (3′-CH$_3$), 62.3 (6′-OCH$_3$), 106.6 (C-1′), 109.0 (C-3′), 109.0 (C-5′), 126.7 (C_α), 128.4 (C-5), 128.4 (C-3), 128.9 (C-6), 128.9 (C-2), 130.2 (C-4), 135.3 (C-1), 142.9 (C_β), 158.8 (C-6′), 159.3 (C-4′), 162.0 (C-2′), 193.4 (C=O); HRMS (ESI) calcd for $C_{18}H_{18}O_4Na$ $(M+Na)^+$: m/z 321.1103, found 321.1105.

Hariganetine (2): orange solid; mp. 140.4–141.5°C (from EtOAc/hexane); IR(thin film): ν_{max} 3452, 1617, 1498, 1168 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.40 (3H, *s*, 6-CH$_3$), 1.42 (3H, *s*, 6-CH$_3$), 1.86 (3H, *s*, 8-CH$_3$), 2.92 (1H, *dd*, *J* = 17.9, 3.7 Hz, H-3), 3.03 (1H, *dd*, *J* = 17.9, 10.8 Hz, H-3), 5.32 (1H, *dd*, *J* = 10.8, 3.7 Hz, H-2), 7.37-7.48 (5H, *m*, H-2′,3′,4′,5′,6′), 15.80 (1H, *s*, 4-OH). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 7.9 (8-CH$_3$), 23.1 (6-CH$_3$), 25.5 (6-CH$_3$), 38.2 (C-3), 52.3 (C-6), 76.0 (C-2), 101.4 (C-10), 107.3 (C-8), 125.8 (C-2′,6′), 128.9 (C-3′,4′,5′), 138.0 (C-1′), 161.2 (C-9), 182.8 (C-4), 198.0 (C-7), 201.7 (C-5); HRMS (ESI) calcd for $C_{18}H_{18}O_4Na$ $(M+Na)^+$: m/z 321.1103, found 321.1104.

Two isolated compounds were test using Salmonella mutation assay against AFB1, MeIQ and AF-2. The results of bioactivity tests from *Department of Biochemistry, Faculty of Medicine, Chiang Mai University*, were shown in Table 1.

Table 1 % Inhibition of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**1**) and hariganetin (**2**) using Salmonella mutation assay against AFB1, MeIQ and AF-2 induced mutagenesis

Treatment	% Inhibition		
	AFB1 (5 ng/pl)	MeIQ (12.5 ng/pl)	AF-2 (10 ng/pl)
DMC 2 μg/pl	98.6 ± 6.3	83.6 ± 4.3	N.A.
DMC 10 μg/pl	100.0 ± 5.4	99.5 ± 0.3	56.8 ± 22.0
DMC 50 μg/pl	N.A.	N.A.	70.9 ± 14.1
Hariganetin 2 μg/pl	11.5 ± 8.4	49.3 ± 10.7	N.A.
Hariganetin 10 μg/pl	63.5 ± 6.1	58.4 ± 5.5	40.0 ± 29.4
Hariganetin 50 μg/pl	N.A.	N.A.	56.0 ± 12.3

% Inhibition showed as a Mean ± SD
N.A.: Not analyzed
AFB1: Aflatoxin B1 induced mutagenesis on *S. typhimurium* strain TA98
MeIQ: 2-Amino-3,4-dimethylimidazo [4,5-*f*] quinoline induced mutagenesis on *S. typhimurium* strain TA98
AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)-acrylamide induced mutagenesis on *S. typhimurium* strain TA100

From the result of Salmonella mutation assay against AFB1, MeIQ and AF-2 induced mutagenesis, it was found that, 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**1**) has an active antimutagenic compound represented more than hariganetin (**2**).

Conclusion

2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**1**) and hariganetine (**2**) can be successfully isolated from the crude CH_2Cl_2 extract of *Cleistocalyx nervosum* var. *paniala* seeds. Two compounds showed antigenotoxicity against AFB1, MeIQ and AF-2 induced mutagenesis in *S. typhimurium.*

Acknowledgements

We are grateful to the Center of Excellence for Innovation in Chemistry (PERCH-CIC) and North-Chiang Mai University for financial supports, Department of Chemistry, Faculty of Science, Chiang Mai University for facilities supporting this research.

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THE 6th PURE AND APPLIED CHEMISTRY INTERNATIONAL CONFERENCE 2012

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PROCEEDINGS

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DEPARTMENT OF CHEMISTRY, FACULTY OF SCIENCE, CHIANG MAI UNIVERSITY
& THE CHEMICAL SOCIETY OF THAILAND UNDER THE PATRONAGE OF
HER ROYAL HIGHNESS PRINCESS CHULABHORN MAHIDOL

CHEMICAL INVESTIGATIONS OF THE DICHLOROMETHANE EXTRACT OF *VERNONIA SCANDENS* TWIGS

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Abstract: Lupeol acetate (1) as triterpene, eugenol (2) as phenylpropene and two steroids, the mixture of β-sitosterol (3) and stigmasterol (4), were isolated from the dichloromethane extract from the twigs of *Vernonia scandens* using silica gel on column chromatographic technique and identified by analysis of the IR, Mass, ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HMQC and HMBC spectra and comparison with the literature. Compounds 1-4 were firstly reported in this plant.

1. Introduction

The genus *Vernonia*, in Compositae family, comprising about 1000 species is mainly distributed in the torrid zones of America, Asia, and Africa. About twenty-seven species of this genus grow in the southern part of China, many of which have applications in Chinese folklore medicine [1]. Several *Vernonia* species are used widely in native cultures as folklore remedies for a variety of human ailments. It should be noted that flavonoids, sesquiterpenoids, diterpenes, alkaloids, triterpenoids and cardiac glycosides have also been reported from this genus [2]. This class of compounds has been reported to be insect antifeedant, antifungal, cytotoxic and antitumoral [3].

Vernonia scandens, commonly known as Gu-si-pah-doh (in Lua Language) found in Chiang Mai province, northern part of Thailand. Since there has no report on phytochemical and biological study on this plant, the present study is therefore aimed to investigate the chemical constituents from the twigs of *Vernonia scandens*.

2. Materials and Methods

2.1 General experimental procedures

Melting points of all compounds could measure with Electrothermal Melting Point apparatus (Sanyo, Model Gallenkamp). The temperature is given in degree Celsius. ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) spectra were recorded in $CDCl_3$ on Bruker DRX 400 spectrometers. The chemical shifts are given in δ (ppm) downfield from tetramethylsilane (TMS) and coupling constants (J values) in Hz. Peak multiplicities are indicated as follows: *s* (singlet), *d* (doublet), *t* (triplet), *dd* (doublet of doublets), *dt* (doublet of triplets), *br s* (broad singlet) and *m* (multiplet). Infrared spectra were taken with a FT-IR model TENSOR 27 (Bruker) spectrometer and absorption frequencies were reported in reciprocal centimeters (cm^{-1}). Mass spectra (electrospray ionization mode, ESI-MS) were measured on a Q-TOF-2™ (Waters) spectrometer. Flash column chromatography was performed employing Merck silica gel 60 and Merck silica gel 60H. Preparative thin layer chromatography (PTLC) plates were carried out using Merck silica gel 60 PF_{254}. Analytical thin layer chromatography was performed with Merck silica gel 60 F_{254} aluminum plates.

2.2 Plant material

The twigs of *V. scandens* were collected in March, 2008 at Haw Mai (Lua) village, Bahng Hin Fohn Sub district, Mae Jam District, Chiang Mai, Thailand.

2.3 Extraction

The air dried twigs of *V. scandens* (2,583.16 g) was extracted with dichloromethane 10 L (2 × 3 days), followed by filtration. The filtrates were combined and evaporated in *vacuo* to give a dichloromethane extract (23.9832 g). Similar extraction was conducted using methanol to give methanol extract (5.1012 g).

2.4 Isolation

The extracts were isolated by silica gel column chromatography using gradient elution with solvent mixtures of increasing polarity. Fractions were combined base on TLC and evaporated to dryness in *vacuo*. The dichloromethane extract (23.9832 g) was subjected to a silica gel column chromatography (CC), and then eluted by hexane-dichloromethane, dichloromethane-ethyl acetate and ethyl acetate-methanol to give nine fractions: F1–F9. Fractions 2, 4, and 7 were further purified by repeated silica gel on column chromatography and preparative thin layer chromatography (PLC). Fraction **2** give compound **1** (0.1626 g), fraction 4 give compound **2** (0.1185 g) and fraction 7 to give a mixture of compounds **3** (0.6977 g) and **4** (0.5623 g). The structures of all isolated compounds were identified by interpretation of their spectral data including EIMS, IR, ^{1}H-NMR and ^{13}C-NMR as well as by comparison of spectroscopic data with those of reported values.

3. Results and Discussion

The dichloromethane extract from the twigs of *V. scandens* was purified to afford four compounds (**1**–**4**). Compounds **1**–**4** were identified as lupeol acetate [4], eugenol [5], β-sitosterol and stigmasterol [6,7], respectively, by analysis of the IR, Mass, ^{1}H-NMR,

^{13}C-NMR, ^{1}H-^{1}H COSY, HMQC and HMBC spectra and comparison with the literatures. Their structures are shown in Figure 1.

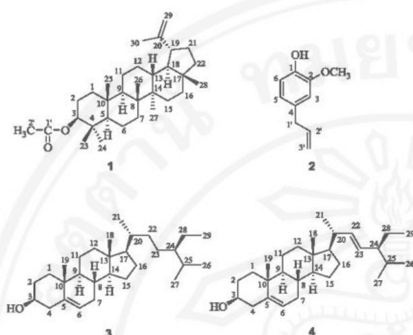

Figure 1. Compounds **1-4** isolated from the twigs of *Vernonia scandens*.

Lupeol acetate (**1**): white needle crystals, mp 210.0–212.0 °C, $C_{32}H_{52}O_2$. EIMS *m/z*: 468 $[M]^+$. IR (CH_2Cl_2) (ν_{max}, cm^{-1}): 2941, 1731, 1641, 1249. ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 0.79 (1H, *m*, H-5), 0.83 (3H, *s*, H-28), 0.84 (9H, *m*, H-23,24,25), 0.93 (3H, *s*, H-27), 1.02 (3H, *s*, H-26), 1.68 (3H, *s*, H-30), 1.86–1.96 (2H, *m*, H-21), 2.04 (3H, *s*, H-2′), 2.37 (1H, *dt*, *J* = 11.1, 5.8 Hz, H-19), 4.47 (1H, *dd*, *J* = 10.4, 5.4 Hz, H-3), 4.56 (1H, *d*, *J* = 2.4 Hz, H-29), 4.68 (1H, *d*, *J* = 2.4 Hz, H-29). ^{13}C-NMR (100 MHz, $CDCl_3$, δ, ppm): 14.5 (C-27), 15.9 (C-24), 16.1 (C-25), 16.4 (C-26), 17.9 (C-28), 18.1 (C-6), 19.2 (C-30), 20.9 (C-11), 21.6 (C-2′), 23.7 (C-2), 25.0 (C-12), 27.4 (C-15), 27.9 (C-23), 29.8 (C-21), 34.2 (C-7), 35.5 (C-16), 37.0 (C-10), 37.7 (C-4), 38.0 (C-13), 38.3 (C-1), 39.9 (C-22), 40.8 (C-8), 42.8 (C-14,17), 48.0 (C-18), 48.2 (C-19), 50.3 (C-9), 55.3 (C-5), 80.9 (C-9), 109.3 (C-29), 150.9 (C-20), 171.0 (C-1′).

Eugenol (**2**): yellow oil, $C_{10}H_{12}O_2$. EIMS *m/z*: 164 $[M]^+$. IR (CH_2Cl_2) (ν_{max}, cm^{-1}): 3441, 2850, 1641, 1172. ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 3.35 (2H, *d*, *J* = 6.7 Hz, H-1′), 3.88 (3H, *s*, OCH_3), 5.07-5.14 (1H, *m*, H-3′), 5.59 (1H, *br s*, OH), 5.93-6.04 (1H, *m*, H-2′), 6.70 (1H, *m*, H-5), 6.72 (1H, *m*, H-6), 6.88 (1H, *m*, H-3). ^{13}C-NMR (100 MHz, $CDCl_3$, δ, ppm): 39.8 (C-1′), 55.8 (OCH_3), 111.1 (C-6), 114.2 (C-3), 115.4 (C-3′), 121.1 (C-5), 131.8 (C-4), 137.8 (C-2′), 143.8 (C-1), 146.4 (C-2).

***β*-Sitosterol** (**3**) and **stigmasterol** (**4**): white solid, mp 138.7–144.8 °C (Lit.[6], 144–146 °C), IR (CH_2Cl_2) (ν_{max}, cm^{-1}): 3395, 2936, 1642. ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 0.69, 0.79, 0.81, 0.84, 0.94, 1.01 (each 3H, Me × 6), 3.52 (1H, *m*, H-3), 5.01* (1H, *dd*, *J* = 15.2, 8.6 Hz, H-23), 5.14* (1H, *dd*, *J* = 15.2, 8.6 Hz, H-22), 5.43 (1H, *m*, H-6).
*found that in stigmasterol

4. Conclusions

Four compounds (Fig. 1) were isolated from the dichloromethane extract of *Vernonia scandens* twigs. They were identified to lupeol acetate (**1**), eugenol (**2**), and a mixture of *β*-sitosterol (**3**) and stigmasterol (**4**). All compounds were reported for first time from *Vernonia scandens*.

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ORIGINAL PAPER

Genotoxicity and antigenotoxicity of the methanol extract of *Cleistocalyx nervosum* var. *paniala* seed using a Salmonella mutation assay and rat liver micronucleus tests

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Abstract *Clesitocalyx nervosum* var. *paniala*, an edible fruit found in some parts of Southeast Asia including Thailand, contains high amounts of polyphenols and has multiple biological activities. The purposes of this study were to evaluate the genotoxic and antigenotoxic effects of methanol extracts of *C. nervosum* seeds via a Salmonella mutation assay and a rat liver micronucleus test. *C. nervosum* extract was not mutagenic to *Salmonella typhimurium* strains TA98 and TA100 in both the presence and absence of metabolic activation. Furthermore, *C. nervosum* seed extract presented antigenotoxicity against aflatoxin B1, MeIQ and AF-2 induced mutagenesis. Clastogenicity and anticlastogenicity of *C. nervosum* seed extracts were determined in rat livers. Male wistar rats were divided into 6 groups. Groups 1 and 3 were treated with 5% tween-80 as a vehicle control. Group 2 received 1,000 mg/kg bw of methanol seed extract and groups 4-6 were fed with 20, 100 and 1,000 mg/kg bw of seed extracts, respectively for 21 days. At day 15 and 18 of the experiment, treated rats in groups 3-6 were intraperitoneally injected with 30 mg/kg bw of diethylnitrosamine to initiate hepatocarcinogenesis. At day 22, all rats were partially hepatectomized to amplify mutated hepatocytes. *C. nervosum* seed extract did not affect micronucleus formation in rat livers, but did slightly decrease the frequencies of micronucleated hepatocytes of diethylnitrosamine treated rats. In conclusion, the methanol extract of *C. nervosum* seed may contain chemopreventive compounds against carcinogenesis.

Keywords Antimutagenicity, Clastogenicity, *Cleistocalyx nervosum*, Liver micronucleus test, Mutagenicity, Salmonella mutation assay

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Cancer is a major leading cause of death worldwide. Chemically induced carcinogenesis consists of three distinct stages: initiation, promotion and progression[1,2]. DNA mutation plays a major role in the initiation stage, and is usually both rapid and irreversible[3]. Both reduction of carcinogen exposure in the environment and increase in chemopreventive agent intake are of prime importance for reducing cancer incidence in humans. An effective chemopreventive agent should inhibit, delay or reverse carcinogenesis by either protecting normal cells from transforming to premalignant and malignant cells, or by eliminating preneoplastic cells before they become neoplasia[4]. Epidemiological studies have consistently shown that increased consumption of fruits and vegetables is associated with reduced risk of developing cancer[5]. Potential antimutagens and anticarcinogens from natural products include flavonoids, a common group of polyphenolic compounds that are ubiquitous in nature[6]. They have been reported to have antiviral, anti-allergic, anti-platelet, anti-inflammatory, antitumor and antioxidant activities.

Cleistocalyx nervosum var. *paniala* is an edible fruit belonging to the Myrtaceae family and is found in some areas of Southeast Asia, including the northern parts of Thailand[7]. Because of the high content of poly-

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phenols and flavonoids in *C. nervosum* var. *paniala*, the species is known to have *in vitro* antioxidant properties[8]. Our previous studies have found that the aqueous extract of *C. nervosum* had no acute or subacute toxic effects on rats, and also significantly enhanced activity of heme oxygenase-1 and reduced oxidative stress in rat liver[9]. Furthermore, *C. nervosum* extracts significantly stimulated human lymphocyte proliferative responses and significantly enhanced NK cells activity[10]. Its seeds also presented antibacterial activity against *Propionibacterium acnes* and *Staphylococcus aureus*[11]. In the present study, the genotoxicity and antigenotoxicity effects of *C. nervosum* seed extract were determined by Salmonella mutation assay and rat liver micronucleus tests.

Chemical constituents of methanol extract of *Clesitocalyx nervosum* var. *paniala*

The contents of chemical constituents in methanol extract of *C. nervosum* seed were analyzed by spectrophotometric methods. The total phenolic compounds, total flavonoids and condensed tannins were 1,515.8 ± 645.5 mg GAE/100 g fresh weight, 148.2 ± 28.7 mg CE/100 g fresh weigh and 396.0 ± 161.0 mg CE/100 g fresh weight, respectively.

Mutagenic and antimutagenic properties of *Clesitocalyx nervosum* seed extract in *Salmonella typhimurium*

The mutagenicity of the methanol extract of *C. nervosum* seed was studied in *Salmonella typhimurium* strain TA98, indicating a frame-shift mutation, and strain TA100, which carries a base-pair mutation. The methanol extract of *C. nervosum* seed, 100-1,000 μg/plate, had no mutagenic effects on *S. typhimurium* strains TA98 and TA100 both with and without metabolic activation (Table 1). It did not induce the number of revertant colonies more than 2-fold when compared to a negative control that produced spontaneous colonies. However, the highest concentration, 1,000 μg/plate, of the extract had a lethal effect on *S. typhimurium* strain TA98 in the absence of metabolic activation. Mutagenicity tests, including the Ames test, have been modified to assess the antimutagenic activities of various compounds. The antimutagenicity of the methanol crude extract obtained from *C. nervosum* seed was evaluated by using AFB1 and MeIQ induced mutagenesis in strain TA98 under metabolic activation and

Table 1. Mutagenic effect of methanolic extract of seeds of *C. nervosum* in *S. typhimurium* strains TA98 and TA100 with or without S9 mix.

Treatment	Average of His⁺ revertant colonies			
	TA98 (−S9)	TA98 (+S9)	TA100 (−S9)	TA100 (+S9)
DMSO (50 μL/plate)	18.7 ± 0.7	24.5 ± 1.3	79.7 ± 13.0	105.3 ± 5.4
AF-2 (0.01 μg/plate)	N.A.	N.A.	416.8 ± 107.3	N.A.
AF-2 (0.1 μg/plate)	334.0 ± 24.9	N.A.	N.A.	N.A.
2-AA (0.5 μg/plate)	N.A.	615.0 ± 63.1	N.A.	562.3 ± 155.6
Methanol extract 100 μg/plate	14.9 ± 1.9	24.4 ± 1.9	101.1 ± 5.8	112.2 ± 0.8
Methanol extract 250 μg/plate	16.1 ± 3.4	30.1 ± 6.2	96.3 ± 16.7	94.8 ± 14.6
Methanol extract 500 μg/plate	12.2 ± 3.6	19.7 ± 1.7	79.0 ± 8.9	100.9 ± 6.6
Methanol extract 1000 μg/plate	9.8 ± 2.4 (K)	17.1 ± 0.7	61.8 ± 4.2	82.9 ± 10.7

AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide; 2-AA: 2-amino anthracene; K: Killing effect ; N.A.: Not analyze
The data show the number of His⁺ revertant colonies as a Mean ± SEM

Table 2. Antimutagenicity of methanol extract of seeds of *C. nervosum* against AFB1, MeIQ and AF-2 induced mutagenesis in *S. typhimurium*.

Treatment	Average of His⁺ revertant colonies (% Inhibition)		
	AFB1 (8.0 ng/plate)	MeIQ (12.5 ng/plate)	AF-2 (20 ng/plate)
DMSO (50 μL/plate)	23.5 ± 0.82	27.2 ± 1.8	133.6 ± 24.3
Standard mutagen	747.5 ± 100.02	556.4 ± 56.3	754.1 ± 68.6
Methanol extract 100 μg/plate	154.5 ± 71.17* (86.1%)	132.3 ± 15.8* (80.1%)	699.0 ± 57.2 (8.9%)
Methanol extract 250 μg/plate	34.3 ± 0.82* (97.5%)	31.2 ± 6.5* (99.2%)	668.2 ± 57.7 (13.8%)
Methanol extract 500 μg/plate	28.7 ± 11.98* (97.7%)	20.6 ± 1.9* (100.0%)	540.6 ± 71.8* (34.4%)

AFB1: Aflatoxin B1; MeIQ; 2-amino-3,4 dimethylimidazo [4,5-*f*] quinoline; AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide; N.A.: Not analyze
The data show the number of His⁺ revertant colonies as a Mean ± SEM
*Significantly different compare to control ($P < 0.05$)

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Table 3. Clastogenic and anticlastogenic effects of methanol extract seeds of *C. nervosum* on DEN-induced rat micronucleated hepatocyte formation.

Treatment	N	Initial bw (g)	Final bw (g)	MNHEPs/1,000	% Inhibition	Mitotic index
5% Tween 80	5	93.0±8.4	228.0±11.5	1.0±1.0		3.4±0.7
Methanol extract 1000 mg/kg bw	5	93.0±4.5	209.0±20.4	0.5±0.6		3.5±0.7
DEN+5% Tween 80	5	93.8±4.8	237.5±12.6	13.2±3.0*	0	4.6±1.3
DEN+Methanol extract 20 mg/kg bw	6	99.2±4.9	231.7±6.8	12.0±0.7	8.7±5.5	3.6±1.1
DEN+Methanol extract 100 mg/kg bw	6	95.0±9.4	222.0±13.0	10.4±0.3	21.4±2.3	3.6±0.5
DEN+Methanol extract 1000 mg/kg bw	6	97.5±2.9	223.8±18.4	10.7±1.0	18.6±7.8	3.3±0.1

DEN: Diethylnitrosamine (30 mg/kg bw); MNHEPs/1,000: number of micronucleated hepatocytes per 1,000 hepatocytes
The results are given as Mean±SD
*Significantly different from control group ($P<0.05$)

AF-2 induced mutagenesis in strain TA100 without metabolic activation. The concentrations of methanol extract used in these studies ranged from 100 to 500 μg/plate; these were not toxic to bacteria. The methanol extract showed strong antimutagenicity against AFB1 and MeIQ (80-100%) ($P<0.05$). The mild antimutagenic effect on AF-2 induced mutagenesis was only found at the highest dose of methanol extract, 500 μg/plate (Table 2).

Clastogenic and anticlastogenic activities of *Clesitocalyx nervosum* seed extract in rat liver

The *in vivo* clastogenicity and anticlastogenicity of methanol seed extract were examined using a micronucleus assay in regenerating liver tissue of male wistar rats. The concentrations of the seed extract in this study were 20, 100 and 1,000 mg/kg bw. The treatments with methanol extract were safe for the male rats. The food and water consumption throughout the experiment and the rat body weights of all methanol extract treated groups were not significantly different from those of the vehicle control group. The administration of 1,000 mg/kg bw of methanol extract, which was the highest soluble concentration used, did not increase the number of micronucleated hepatocytes and hepatocytes presenting mitosis when compared to the control group (Table 3). This indicates that the methanol extract of *C. nervosum* seed extract did not have a clastogenic effect on rat liver tissue. The analysis for anticlastogenicity was performed in rats initiated by diethylnitrosamine, a hepatomutagen and a hepatocarcinogen (Table 3). The injection of diethylnitrosamine significantly increased the number of micronucleated hepatocytes. The treatment with methanol extract, however, slightly reduced micronucleus formation in rat liver tissue treated with diethylnitrosamine. This result demonstrated that the methanol extract of *C. nervosum* seed extract did not possess *in vivo* anticlastogenicity against diethylnitrosamine.

Discussion

Since grape seeds have various biological and pharmacological effects, including anticancer activity, many laboratories have analyzed seeds of other medicinal plants for cancer chemopreventive ingredients[12,13]. In this study, we evaluated genotoxicity and antigenotoxicity both *in vitro* and *in vivo* of seed extracts derived from *Cliestocalyx nervosum*, an edible fruit from Southeast Asia.

The methanol extract of *C. nervosum* seed used in this research contained polyphenolic compounds, including flavonoids, in amounts comparable to those from grape seed extracts, as described elsewhere[14]. Furthermore, it contained high amounts of condensed tannins, which play a crucial role in cancer prevention.

The mutagenic and antimutagenic activities of phytochemicals such as phenolics, flavonoids and tannins have been extensively described in the literature. Their biological actions may depend on the dosage, structural and chemical affinity, and on the potential to act as inhibitors or inductors of enzymatic pathways, or their metabolites may be involved in mutagen deactivation[15-17]. This seed extract did not show mutagenicity toward *Salmonella typhimurium* strains TA98 and TA100, which represent frameshift and base-pair substitution mutations, respectively, both in the presence and absence of metabolic activation. These results indicated that methanol seed extract of *C. nervosum* may not contain any mutagenic compounds. Notably, the high dose of *C. nervosum* seed extract was cytotoxic to TA98 only in the absence of metabolic activation. This suggests that metabolizing enzymes in the S9 mix may alter some toxic ingredients in the seed extract to be nontoxic.

Furthermore, the *C. nervosum* seed extract demonstrated significantly inhibited AFB1 and MeIQ-induced mutagenesis in a dose-dependent manner. AFB1 is a natural mycotoxin mainly occurring in agricultural grains and MeIQ is one of the pyrolysate products pre-

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sent in fried and grilled meat. These indirect-acting mutagens are predominately metabolized to be ultimate mutagens by CYP1A and CYP3A families for AFB1 and CYP1A2 family for MeIQ[18-20]. The methanol extract at high dosage slightly modulated AF-2 induced mutagenesis. AF-2 is a nitrofuran derivative that is used as a food preservative. It is a common direct mutagen used in Salmonella mutation tests as it can directly bind to DNA without metabolic activation[21]. Our results indicate that the antimutagenic compounds in *C. nervosum* seed extracts act so as to inhibit enzymes involved in carcinogen bioactivation rather than directly binding to mutagens.

The treatment with the methanol extract of *C. nervosum* seeds did not produce clastogenicity in male wistar rats, as determined by the liver micronucleus test. However, it tended to reduce rat body weight, although food and water intake were similar to those of the control group, suggesting that the methanol extract did not affect the appetite of the rats. High tannin content in the extract might interfere with some enzymes in the gastrointestinal tract functioning in digestion and absorption of nutrients[22].

The methanol extract of *C. nervosum* seed slightly reduced micronucleus formation in hepatocytes of rats initiated by diethylnitrosamine. Diethylnitrosamine is metabolized by CYP2E1 to unstable intermediates that break down spontaneously to form alkyldiazonions, which are the ultimate carcinogen species[23]. These electrophiles commonly conjugate with glutathione by gluatathione S-transferase preventing them from forming DNA adducts. The methanol extract had no anticlastogenic effects in rats. Since high doses of the extract have a limited absorption, the prolong administration of methanol seed extract might increase its anticlastogenic activity.

In conclusion, the methanol extract of *C. nervosum* seed has no genotoxic effect on the bacterial or animal systems studied. It demonstrated antigenotoxicity against some environmental mutagens in the bacterial mutation assay.

Materials & Methods

Chemicals

Folin-Ciocalteu phenol reagent and Oxoid Nutrient Broth No. 2 were purchased from Fluka Biochemika, Fluka A.G. (Buchs, Switzerland). β-dihydronicotinamide adenine dinucleotide (β-NADH) and β-dihydronicotinamide adenine phosphasedinucleotide (β-NADPH) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). 2-aminoanthracene (2-AA), 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (AF-2) and 2-amino-3,4 dimethylimidazo [4,5-*f*] quinoline (MeIQ) were purchased from Wako Pure Chemicals (Osaka, Japan). Aflatoxin B1 (AFB 1), diethylnitrosamine (DEN) and histidine were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Gallic acid, catechin, biotin, glucose-6-phosphate were purchased from Sigma-Aldrich Co. (St. Louis, Mo, USA). Bacto agar was from Difco (Detroit, MI, USA). Collagenase type IV and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), were purchased from Invitrogen (Carlsbad, CA, USA).

Preparation of methanol extract from *Clesitocalyx nervosum* var. *paniala*

The fruit of *C. nervosum* was collected from Chiang Mai Horticulture Research Center, Office of Agricultural Research and Development Region 1, Department of Agriculture, Lampang, Thailand, in July-August, 2009. The seed was separated from ripe fruit and dried with a hot air dryer at 40°C, overnight. Dried seeds were powdered and extracted with methanol. The extract was filtered and concentrated under an evaporator and lyophilizer to obtain the crude extracts. The crude extract was stored at -20°C until analysis.

Determination of total phenolic, flavonoid and condensed tannin contents

The total phenolic contents were determined using the Folin-Ciocalteu method according to Singelton *et al.*[24]. The extract was oxidized with Folin-Ciocalteu reagent and neutralized by 7% Na_2CO_3. Then the solution mixture was incubated for 15 min at 45°C. The absorbance of the resulting blue color was measured at 760 nm. The phenolic contents were determined using a standard curve obtained from various concentrations of gallic acid. The total phenolic contents were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight.

The total flavonoid contents were determined using the aluminum chloride colorimetric method[25] with several modifications. After the extract was incubated with 5% $NaNO_2$ for 10 min, 10% $AlCl_3 \cdot 6H_2O$ was added and the mixture was incubated at room temperature. Then 1 M NaOH was added and the absorbance of the reaction mixture was measured at 532 nm. The flavonoid contents were determined using a standard curve obtained from various concentrations of catechin. Total flavonoid contents were expressed as mg of catechin equivalents (CE) per 100 g of fresh weight.

The condensed tannin contents were determined using the Vanillin-HCl assay according to Sun *et al.*[26]. The extract was added into the mixture containing methanol and vanillin solution and incubated for 30 min at 30°C. The absorbance of the reaction mixture

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was measured at 500 nm. The condensed tannins were determined using a standard curve obtained from various concentrations of catechin. Condensed tannins expressed as mg of catechin equivalents (CE) per 100 g of fresh weight.

Mutagenicity and antimutagenicity of *C. nervosum* seed extract using the Salmonella mutation assay

Mutagenicity was performed using the *Salmonella* mutation assay with pre-incubation technique according to Matsushima *et al.*[27] on *S. typhimirium* strains TA98 and TA100, with or without metabolic activation. The S9 fraction was prepared from the supernatant at 9,000 g of male rat liver treated by sodium phenobarbital and β-naphthoflavone as described elsewhere[28]. The bacterial tester strain was cultured overnight at 37°C for 14 hr.

After adding 500 μL of S9 mix (metabolic activation condition) or sodium phosphate buffer, pH 7.4 (non-metabolic activation condition) into a test tube containing 50 μL of vehicle control, or various concentrations of test sample (range 2-20 mg/mL), or a standard mutagen, 100 μL of bacterial culture were added. The mixture was shaken in a shaker water bath at 37°C for 20 min. After that, 2 mL of top agar, at 45°C, containing 0.5 mM of Histidine-Biotin were added and poured onto a minimal agar plate. The plate was incubated at 37°C for 48 hr. The number of revertant His^+ colonies on the plate was counted and examined for toxic effects under a stereomicroscope. DMSO was used as a negative control. The standard mutagens, 2-AA and AF-2, were used as positive controls for metabolic activation and without metabolic activation condition, respectively.

Antimutagenicity test was performed similar to the mutagenicity assay developed by Matsushima *et al.*[27]. The mutagens, 160 ng/mL of AFB1 and 250 ng/mL of MeIQ, were tested in strain TA98 with metabolic activation and 12 ng/mL of AF-2, was tested in strain TA100 without metabolic activation. The 50 μL of mutagen and 50 μL of test sample or DMSO were mixed with bacterial culture and S9 mix or phosphate buffer before incubating in a shaker water bath at 37°C for 20 min. The calculation of % inhibition was done according to the formula:

$$\% \text{ Inhibition} = [[(\text{Std. Mutagen} - \text{BG}) - (\text{Test compound-BG})]/(\text{Std. Mutagen} - \text{BG})] \times 100$$

Where BG was the number of revertant colonies per plate of DMSO alone, Std. Mutagen was the number of revertant colonies per plate of standard mutagen alone and Test compound was the number of revertant colonies per plate of test sample and mutagen.

The criteria for a positive mutagenic response in the Salmonella mutation assay were a clear dose-dependent increase in the number of revertants within the non-toxic range[27]. Tests on methanolic extract-induced antimutagenicity were performed at a nontoxic dose interval, and the criteria for the presence of an antimutagenic effect was a substantial (30% or higher) and dose-dependent.

Clastogenicity and anticlastogenicity of *C. nervosum* seed extract using liver micronucleus test

Male Wistar rats, weight ranged 60-80 g were obtained from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. They were housed under standard environmental conditions of temperature at 24°C under a 12 hr dark-light cycle, and allowed free excess to drinking water and pelleted diet. An experimental protocol was approved by The Animal Ethics Committee of Faculty of Medicine, Chiang Mai University.

Rats were divided into 6 groups. Groups 1 and 3 were treated by 4 mL/kg bw of 5% Tween-80 as a vehicle control. Groups 2 and 4 received 1,000 mg/kg bw of methanol extract orally and groups 5 and 6 received 20 and 100 mg/kg bw of methanol extract, respectively for 21 days. At day 15 and 18 of an experiment, rats in groups 3-6 were intraperitoneally injected with 30 mg/kg bw of diethylnitrosamine to initiate hepatocarcinogenesis. All rats were partially hepatectomized at day 22 of the experiment and were sacrificed under light anesthesia 4 days after the operation. Single hepatocytes were isolated by the 2-steps collagenase perfusion method according to Charoensin *et al.*[29]. The hepatocytes were stained with DAPI and counted under a fluorescent microscope. The number of micronucleated hepatocytes and mitotic cells was scored based on analysis of 2,000 hepatocytes of each animal. The criteria for micronucleated hepatocytes followed the method of Cliet *et al.*[30].

Statistical analysis

Results were expressed as mean ± SD. Statistical significance of differences between groups were determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) tests. P values < 0.05 were regarded as significant. The results for the Salmonella mutation assay were expressed as mean number of His^+ revertant colonies ± SEM. Triplicate plates were tested per dose per experiment.

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