

**ISOLATION AND STRUCTURE ELUCIDATION OF SOME
COMPOUNDS FROM *HYDNOPHYTUM FORMICARUM* JACK.**



PRASIT BURAPARUANGSANG

**Presented in partial fulfillment of the requirements
for the Master of Science degree in Biological Chemistry**

May 2000

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ศึกษาการแยก และวิเคราะห์โครงสร้างสารประกอบบางชนิดจากหัวร้อยรู
(*Hydnophytum formicarum* Jack.)

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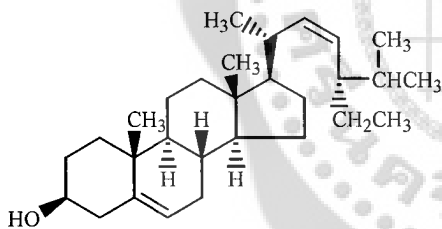
เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา
ตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต วิชาเอกเคมีชีวภาพ

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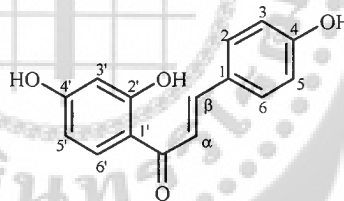
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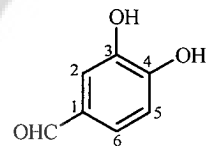
หัวร้อยรูเป็นพืชสมุนไพรไทย ใช้บำรุงหัวใจ ขับพยาธิ แก้อักเสบ จากการนำสารสกัดหยาบเอทิลแอลกอฮอล์ของหัวร้อยรูมาแยกและวิเคราะห์โครงสร้างเป็นครั้งแรก พบสารประกอบ 4 ชนิดได้แก่ isoliquiritigenin (2',4,4'-trihydroxychalcone; 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one), **34**, protocatechualdehyde (rancinamycin IV; 3,4-dihydroxybenzaldehyde), **35**, (-)-butin (3',4',7-trihydroxyflavanone; 2-(3,4-dihydroxyphenyl)-2,3-dihydro-7-hydroxy-4H-1-benzopyran-4-one), **36**, และ butein (2',3,4,4'-tetrahydroxychalcone; 1-(2,4-dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)-2-propen-1-one), **37** นอกจากนั้นยังพบ stigmasterol ปริมาณเล็กน้อยในสารสกัดเฮกเซนและไดคลอโรมีเทน การศึกษาโครงสร้างของสารเหล่านี้ใช้วิธีทางสเปกโตรสโกปี (COSY, HMQC และ HMBC)



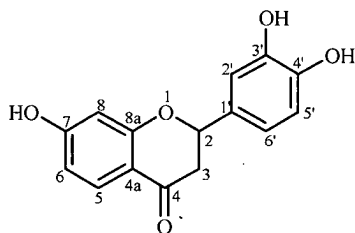
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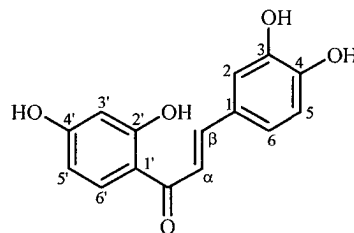
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**ISOLATION AND STRUCTURE ELUCIDATION OF SOME
COMPOUNDS FROM *HYDNOPHYTUM FORMICARUM* JACK.**



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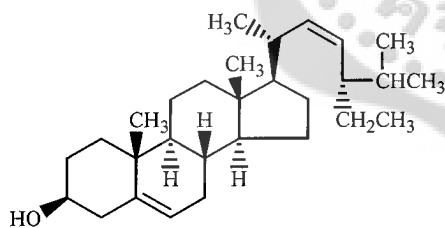
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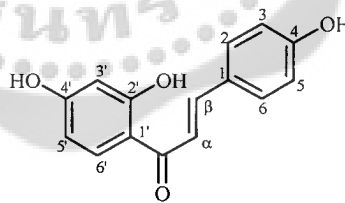
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Prasit Buraparuangsang. (2000). *Isolation and Structure Elucidation of some compounds from Hydnohytium formicarum* Jack.. Master Thesis, M.Sc. (Biological Chemistry). Bangkok : Graduate School, Srinakharinwirot University. Advisor Committee: Assoc. Prof. Dr. Supaluk Prachayasittikul, Assoc. Prof. Dr. Somsak Ruchirawat, Asst. Prof. Dr. Sunan Chainakul.

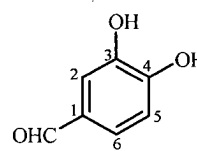
Hydnophytum formicarum Jack., a Thai medicinal plant has long been used as traditional medicine, possesses cardiac effect, antiparasitic and anti-inflammatory activities. Our first investigation on its tubers crude ethyl acetate extracts led to the isolation of four compounds, isoliquiritigenin (2',4,4'-trihydroxychalcone; 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one), **34**, protocatechualdehyde (rancinamycin IV; 3,4-dihydroxybenzaldehyde), **35**, (-)-butin (3',4',7-trihydroxy flavanone; 2-(3,4-dihydroxyphenyl)-2,3-dihydro-7-hydroxy-4*H*-1-benzopyran-4-one), **36**, and butein (2',3,4,4'-tetrahydroxychalcone; 1-(2,4-dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)-2-propen-1-one), **37**. In addition, stigmasterol was also found in crude hexane and crude dichloromethane extracts. Their structures were elucidated on the basis of spectral analysis, including homonuclear and heteronuclear correlation NMR experiments (COSY, HMQC and HMBC).



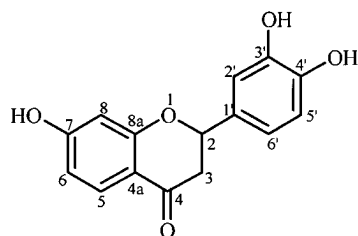
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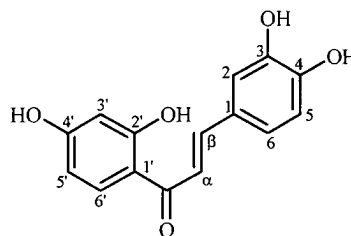
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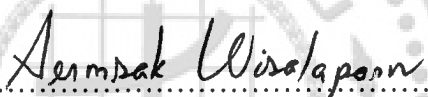
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MR.PRASIT BURAPARUANGSANG

was submitted to the Faculty of Graduate School, Srinakharinwirot University
for the Master of Science degree in Biological Chemistry



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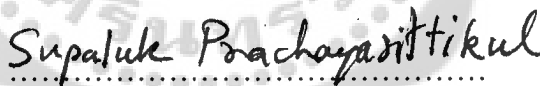
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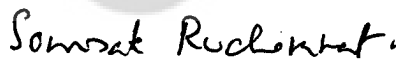
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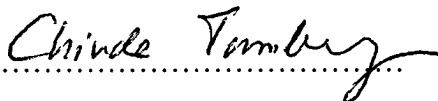
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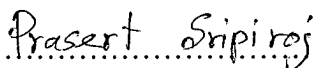
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Chapter 1

Introduction

Background

In the paleolithic era, man made weapons for protecting and hunting. They used herbs which were written on the wall of cave for treatment of ailment. Later, when science especially ethnobotany has been developed, the use of plants has become widespread both in the New World and Old World.

However, Thailand is rich in resource of tropical plants which have long been used as traditional medicine. For example, *Gloriosa superba* (dong dueng or climbing lily: colchicine) has been used for treatment of gout¹. Recently, a number of plants have been investigated in chemistry and pharmacology. Although enormous literatures have verified the validity of the plants. However, still many plants remain to be studied in chemical aspect.

A pharmacopoeia of Phrawai jata tarnyo of Wat Teppanom Hinkong Saraburi, has been used for diabetic treatment. It contains 8 Thai medicinal plants of *Smilax corbolaria* Benth. (khaoyennuar), *S. china* Linn. (khaoyen tai), *Derris seandens* Benth (chuag khoa nang), *Rhinacanthus nasutus* Kurz. (thongpunchang), *Tectona grandis* Linn. F. (maaisak), *Phylla nodiflora* Greene. (nguag pla mo, or yaa ket pla), *Diospyros viriegata* Kurz. (phaya rakdum) and *Hydnophytum formicarum* Jack. (hua roi ruu)².

Hydnophytum formicarum Jack., a medicinal plant whose tuber possessed cardiovascular effect, antiparasitic action, antiinflammatory effect on gout as well as relief of skin rash, legache³ and neurotonic. In addition, it has also been used for treatment of hepatitis (Malaysia) headache (Malaysia-Java)⁴ and liver complaints (Philippines). (NAPRALERT)

Besides being obtained from natural sources, tubers of *Hydnophytum formicarum* Jack. have been produced by tissue culture and investigated for alkaloids content. The results have shown that tubers of tissue culture produce more alkaloid than the natural one⁵.

However, our previous chemical investigation of *Hydnophytum formicarum* Jack. has shown that β -sitosterol and ketone compound, $C_{18}H_{16}O_2$, were found in crude hexane and crude chloroform respectively⁶.

It is interesting to study in detail of *Hydnophytum formicarum* Jack. (**Fig 1.1**) in order to isolate pure compounds for structure elucidation as well as study of pharmacological activity.



Fig 1.1 Picture of *Hydnophytum formicarum* Jack.⁷

Objective

1. To isolate and purify some compounds from *Hydnophytum formicarum* Jack.
2. To elucidate structure of pure compounds using spectroscopic methods
3. To investigate the biological activity of the obtained compounds

Benefits

1. To provide more data of *Hydnophytum formicarum* Jack. regarding natural product chemistry and plant taxonomy
2. To further investigate toxicity of the so obtained compounds
3. To utilize the isolated compounds and their synthetic derivatives as “lead compounds” for design and development of new potential medicinal agents

Scope

1. Plant materials, the tubers of *Hydnophytum formicarum* Jack., were purchased from “Chao krom pur” Wat Sampluem, Samphanthawong, Bangkok.
2. Sequential extraction of plant materials was carried out in hexane, dichloromethane, and ethyl acetate respectively.
3. Isolation and purification were carried out using chromatographic techniques together with recrystallization.
4. Structure elucidation of pure compounds was performed using spectroscopic techniques e.g. IR (Infrared Spectroscopy), NMR (Nuclear Magnetic Resonance Spectroscopy) and MS (Mass Spectroscopy).
5. Biological activity of the obtained compounds was tested on HuCCA-1 and KB cells.

Chapter 2

Literature Review

Introduction

Hydnophytum formicarum Jack. (Hua roi ruu) is commonly found in the east and south of Thailand, South-East Asia, Malaysia, Philippines, Indonesia, Papua New Guinea, Pacific Islands⁸, the Andamans, Tenasserim, Cochin-China, Peninsula on the coast and in the mountains⁹.

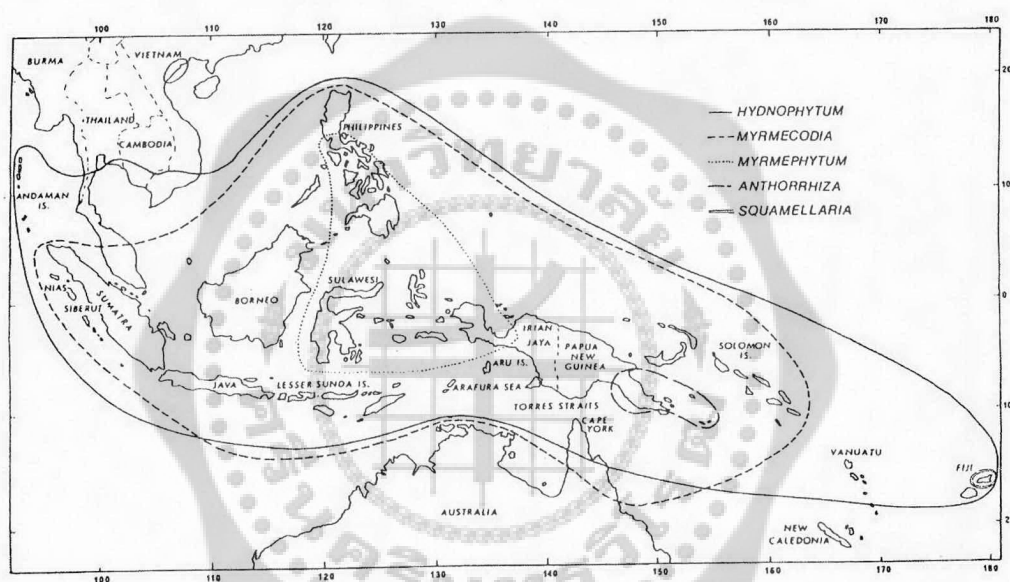


Fig 2.1 Distribution of five genera of Hydnophytinae⁸

Common name of *Hydnophytum formicarum* Jack. is an ant plant⁷. Names in Thai are varied from province to province, for example kra chao pheemot (Surat thani), daa-luu-puu-taa-li-maa (Malay-Pattani), pum pao or pum pha (Trat), roi ruu (Pattani), hua roi ruu (Central)¹⁰. In Malaysia and Java *Hydnophytum formicarum* Jack. is known as kepala berok or baboon's head⁷.

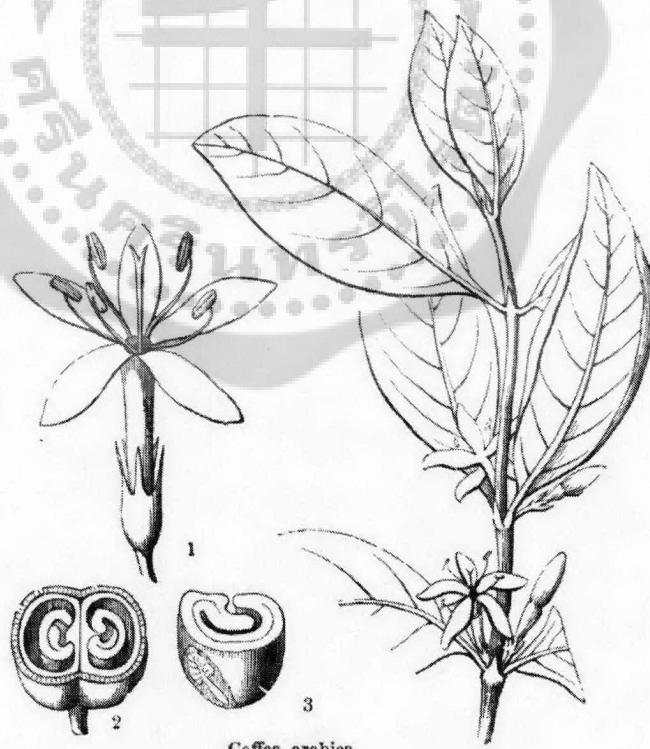
Based on Cronquist⁷, *Hydnophytum formicarum* Jack. has been classified as the following.

- Division Magnoliophyta
- Class Magnoliopsida

- Subclass Asteridae
- Order Rubiales
- Family Rubiaceae (Madder)⁹
- Subfamily Rubioideae
- Tribe Psychotrieae

Hydnophytum formicarum Jack. belongs to family Rubiaceae¹¹ consisting of nearly 400 genus 5000 species. They are chiefly tropical and subtropical as illustrated below.

1. *Ixora congesta* Roxb. (Khem setthee or West Indian Jusmine)
2. *Ixora subsessilis* Wall. (Khem lek)
3. *Ixora spectabilis* Wall. (Khem luang)
4. *Ixora coccinea* Linn. (Khem farung)
5. *Coffea arabica* Linn.
6. *Morinda citrifolia* Linn. (Yo baan)



Coffea arabica.

1. a flower magnified ; 2. a section across a ripe fruit ;
3. a portion of a seed, showing the small embryo laid bare in the end of convolute albumen.

Fig 2.2 Characteristic feature of *Coffea arabica*¹²

7. *Mussaenda crythrophylla* Schum. & Thonn. (Baitaangdok)
8. *Anthocephalus cadamba* Miq. (Krathum)
9. *Mitragyna speciosa* Korth. (Krathom)
10. *Gardenia jasminoides* Ellis. (Phutson)
11. *Randia siamensis* Craib. (Khatkhao)
12. *Pavetta wallichiana* Steud. (Khem paa)
13. *Cinchona* spp.
14. *Morinda coreia* Ham. (Yo paa)

The plants of family Rubiaceae have been characterized as herbs, shrubs, trees, and sometimes climbers. In addition, they have been shown to be economically important as ornament, medicinal plants, and herb tea¹³.

Early in 1891 Schumann¹⁴ classified Hydnophytum as tribe Psychotrieae, subfamily Coffeoidae. Later in 1958 Verdcourt¹⁴ classified such plant as the same tribe Psychotrieae but subfamily Rubioideae instead of Coffeoidae. This agreed to the same results of the rbcL phylogeny¹⁴. For example, many plants of tribe Psychotrieae are Hydnophytum, Lasianthus, Ixora, Myrmecodia, Morinda, Paederia, Pavetta, Psychotria, and Rubia¹³.

Hydnophytum, a rubiaceae ant-plant, is closely related to Myrmecodia, Anthorrhiza, Myrmephytum, and Squamellaria, and is characterized as subtribe Hydnophytinae.

Among 50 species, Hydnophytum is the largest genus in the subtribe Hydnophytinae¹⁵. *Hydnophytum formicarum* Jack. has several branches with opposite fleshy, oval leaves and small sessile, axillary white flowers and red berries⁷. Typical size of *Hydnophytum formicarum* Jack. varies from 10-12 cm (as big as coconut) to 30-50 cm¹⁵. The tubers are derived from the swollen hypocotyl. Tuber cavities are made by phellogens and meristematic layers that arise de novo in the parenchyma of the swollen hypocotyl and enclose volumes of parenchyma⁸.

These cavities have a hole or many entrance holes to outside. The initial cavity is usually warted. Generally, warts¹⁵ are small (approximately 1 mm diameter) and are described as lenticel-like. In 1911, Miehe^{8,15} shown that these warts absorbed both water and stains, besides gaining a source of essential nutrients from faecal

material and debris brought in by the ants. But, however, the plants provide food for the ants in the form of nectar or protein-rich source.

In general, the shapes of tubers of the same genera *Hydnophytum* are globular, elongate, and ridge. But in nature they do not form a tuber growing itself, this explanation remains uncertain.

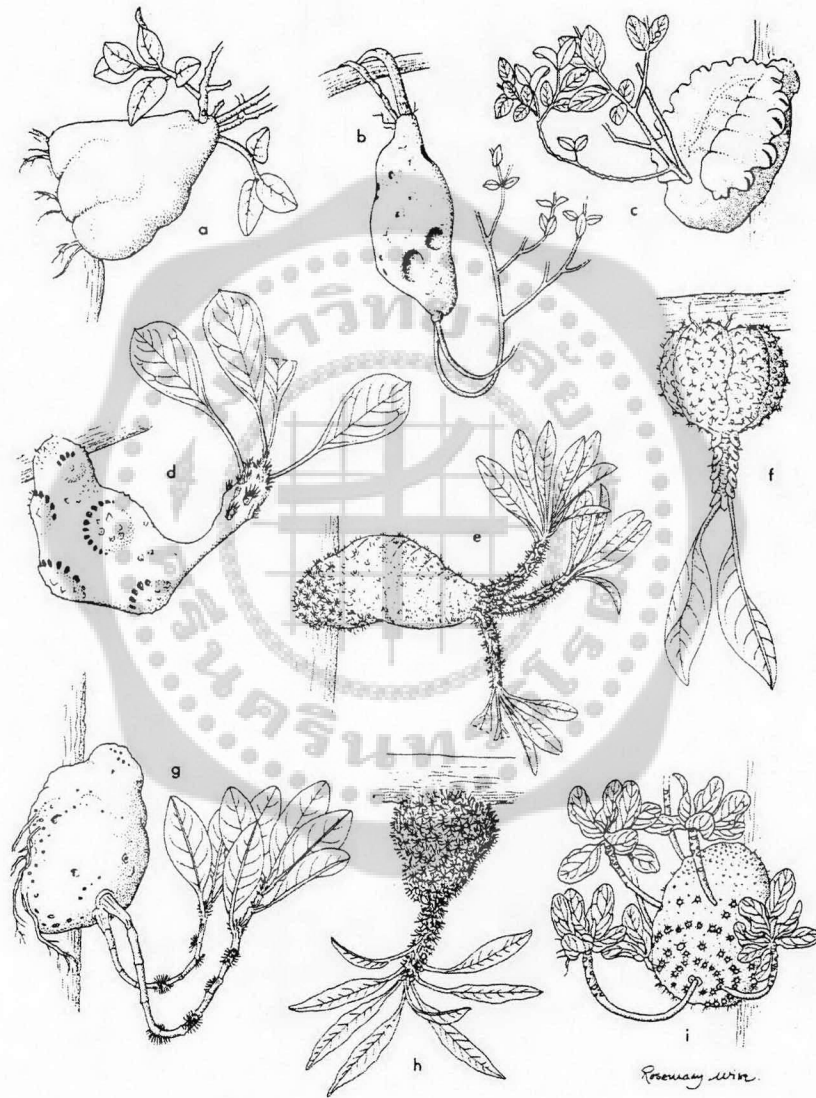


Fig 2.3 External form of the Hydnophytinae. a. *Hydnophytum moseleyanum* Becc.; b. *H. myrtifolium* Merr. & Perry; c. *H. guppyanum* Becc.; d. *Myrmecodia tuberosa* Jack; e. *M. brassii* Merr. & Perry; f. *M. platytyrea* Becc.; g. *Anthorrhiza recurvispina* Huxley & Jebb; h. *A. caerulea* Huxley & Jebb; i. *Squamellaria major* A.C. Smith. Drawn by Rosemary Wise⁸.



Fig 2.4 *Hydnophytum* sp. (left top) *Myrmecodia* sp. (right top) *Myrmecodia tuberosa* seedlings (left bottom) and *Myrmecodia tuberosa* adult (right bottom) Photograph by Wilson, H.P.C., Sarawak, East Malaysia¹⁶.

Root arising from around the base of the swollen hypocotyl grows and attaches the plant to this host tree. Spine, modified from root, can not be found in *Hydnophytum formicarum* Jack., but usually presents on the tuber and stem of *Myrmecodia* and other plants in this group^{8,15}.

Previously in 1976, upon the phytochemical screening by Boonyarattvech¹⁷, it was not found alkaloids, cardiac glycosides, flavonoids, saponins and coumarins from stems of *Hydnophytum formicarum* Jack..

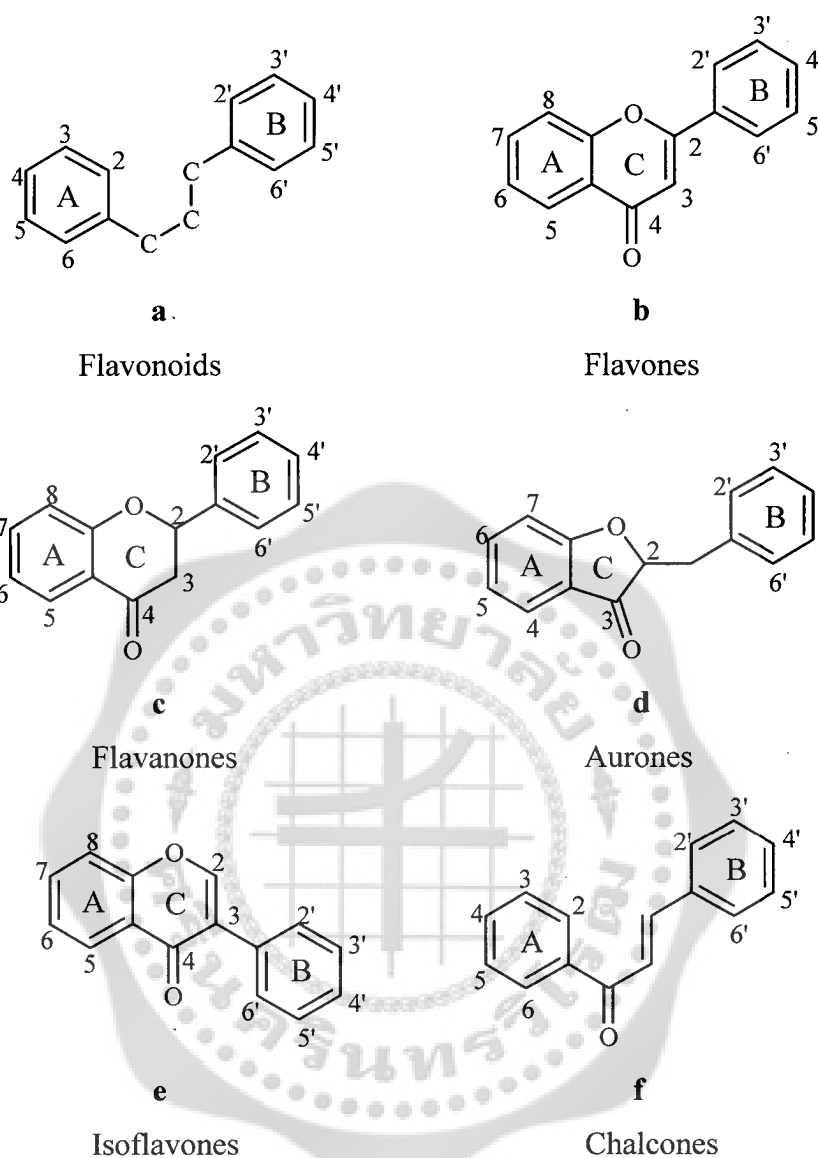
In 1988, quantitative analysis of crude extracts of *Hydnophytum formicarum* Jack. using Inductively Coupled Plasma Atomic Emission Spectrometry was reported by Prachayasittikul and Yoshida¹⁸. It was observed that *Hydnophytum formicarum* Jack. from crude hexane extract contained 9 elements (in ppm) such as Be(0.145) Al(1.47) Ca(0.741) Cr(0.231) Mn(0.135) Fe(0.202) Zn(0.0469) Ba(0.092) P(7.57), from crude chloroform extract contained 10 elements (in ppm) such as Li(0.341) Sr(0.0258) Rb(8.31) Hg(1.18) Tl(9.67) In(9.42) Pb(2.59) Cd(0.0771) As(4.99) Cs(1910) and from crude methanol extract contained 5 elements (in ppm) such as Na(35.6) K(40.6) Mg(0.49) Mn(0.022) Fe(0.192).

In addition, in 1990 Sakorn⁵ reported the alkaloid content of tubers and leaves of *Hydnophytum formicarum* Jack. derived from tissue culture. The results showed that the higher alkaloid quantity was found in tissue culture compared to natural *Hydnophytum formicarum* Jack..

In 1991, Khumsap⁶ reported the isolation of some major chemical constituents from *Hydnophytum formicarum* Jack.. They isolated β -sitosterol from crude hexane extract, ketone compound, $C_{18}H_{16}O_2$, from crude chloroform extract and sodium, potassium salts from crude methanol extract.

Numerous phenolic compounds (polyphenols) are of plant origin and possess various biological activity. However, a small number of phenolic compounds are found in animals (arthropods) and microorganism¹⁹.

Flavonoids are colouring polyphenolic compounds of $C_6-C_3-C_6$ skeleton possessing 15 carbon atoms. They are structurally characterized as having two hydroxylated aromatic rings A and B joined by a linear three carbon chain, thus the flavonoids are 1,3-diaryl propanes²⁰ shown as structure **a**.



Examples of flavonoids are flavones **b**, the most common group of compounds which an oxygen bridge between the *ortho*-position of ring A and the benzylic carbon atom of ring B forming a new 4-pyrone type ring, and flavanone **c** which corresponds to the lowest oxidation level of the ring C^{20, 21}.

In addition, an oxygen bridge joining the central carbon atom of the C₃-unit occurs in a rather limited number of cases resulting in furan type, C₁₅-skeleton of 2-benzyl-coumarane for example, aurones **d**. Isoflavone **e** contains aryl ring B at C-3 position. In the case of chalcones **f** oxygen bridge is absent^{20, 21}.

First flavonoid was found from parsley seed, apiin, in 1845. Structural studies on flavonoids were later carried out by W.H. Perkin in the latter half of the

19th century and by Sir Robert Robinson in the first half of the 20th century. The studies depended on derivatization (acetylation, methylation) to establish the number of phenolic groups. Later, in the latter half of the 20th century the structures of flavonoids and various organic compounds were clearly elucidated by modern instruments (NMR, FAB-MS and x-ray crystallography)¹⁹.

Generally, flavonoids are present in the cells of flower tissues. The wide range of colours and shades in flowers are principally due to the presence of flavonoids. The flavones give yellow or orange colours, the anthocyanins are red, violet or blue colours. The occurrence of this numerous class of oxygen heterocycles is restricted to higher plants and ferns. Mosses contain a few flavonoid types but they are absent in algae, fungi and bacteria. Biologically, the flavonoids play a major role in relation to insects pollinating or feeding on plants, but some flavonoids have a bitter taste^{20,21}.

The C₁₅-skeleton of flavonoids bearing certain oxygen substituents, mostly as hydroxyl groups in the required positions derived from the connection of shikimate (**Fig 2.5**), origin of ring B (L-phenylalanine→cinnamic acid), and with the formation of ring A (polyketide pathway, **Fig 2.6**). The origin of all the carbon atoms in the flavonoid skeleton has been experimentally checked. Now, most chemists believe that structure of the phenyl propane unit derived from cinnamic acids (or *p*-coumaric). Ring A was incorporated into phenyl propane unit *via* polyketide pathway resulting in the biosynthesis of most flavonoids^{19,20}.

Biosynthesis of shikimic and chorismic acids began with the condensation of D-Erythrose-4-phosphate **1** (**Fig 2.5**) coming from the pentosephosphate cycle, and phosphoenolpyruvic acid (PEP) arising from glycolysis. A typical nucleophilic addition of the PEP methylene group to carbonyl group of **1** followed by hydrolytic cleavage of phosphate bond to form enol **1a** which again underwent intramolecular nucleophilic addition of enol group to keto carboxylic resulting in formation of the cyclohexane ring of 5-dehydroquinic acid, **2**. Enzymatic catalysed reaction of 5-dehydroquinic acid **2** producing first route guinic acid **3** and second route 3-dehydroshikimate **4** then shikimate **6** respectively. Rearrangement of shikimate **6** to chorismic **7** then prephenic acid **10** which finally formed L-tyrosine **13** and L-

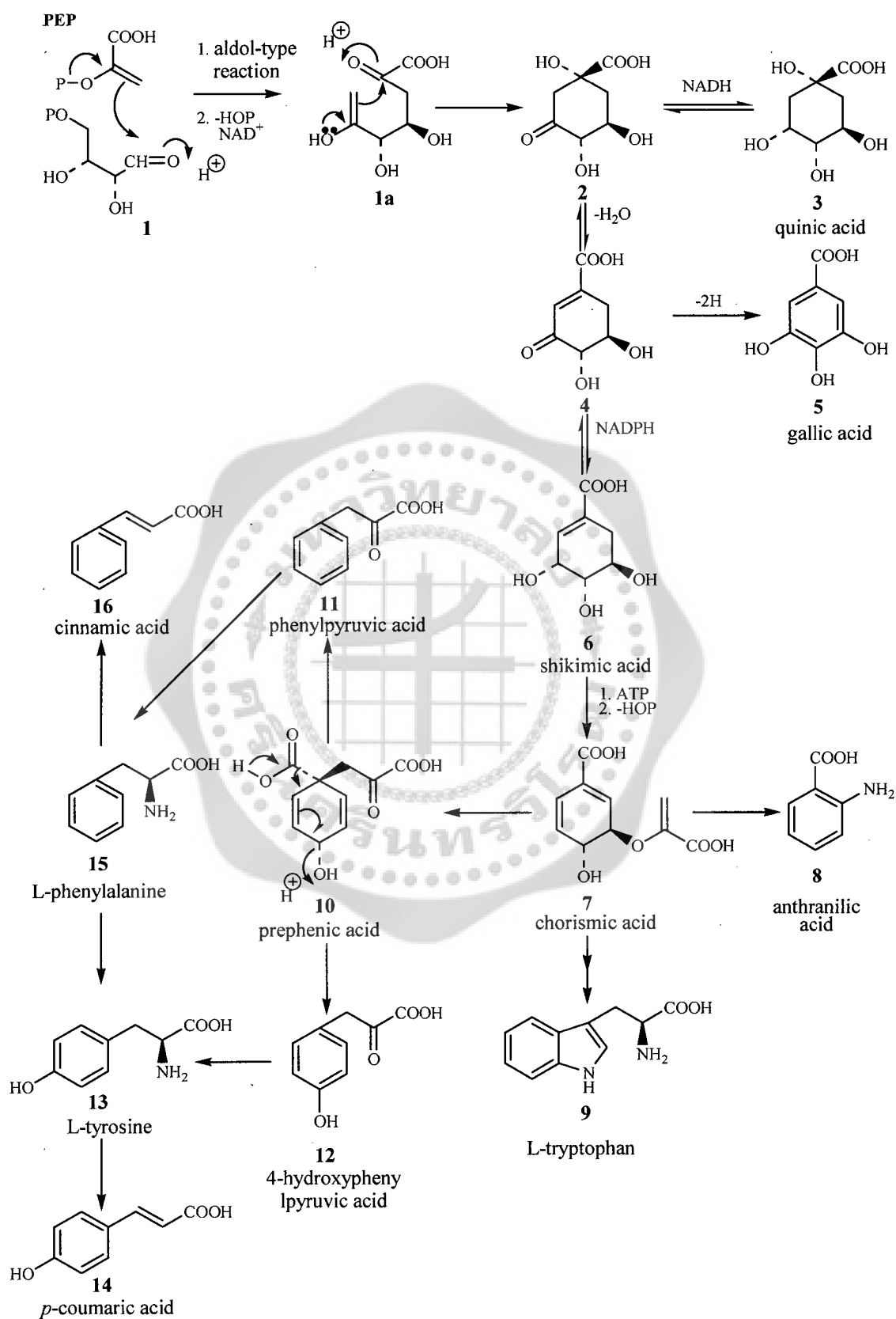


Fig 2.5 The Shikimic Acid Pathway (Ring B)

phenylalanine **15**²¹.

Shikimic and polyketide pathways are important process to produce several compounds in the nature. Further conversion of compounds **13** and **15** is necessary to produce various oxygen heterocyclic compounds such as chalcones and flavanones existed in equilibrium. For example, biosynthesis of chalcone and flavanone is illustrated in **Fig 2.7**. The biosynthesis began with the conversion of L-phenylalanine **15** into cinnamic acid **16** catalyzed by L-phenylalanine ammonia-lyase (PAL: EC 4.3.1.5). Subsequent hydroxylation of cinnamic acid **16** by cinnamate 4-hydroxylase (EC 1.14.13.11) then followed by 4-cinnamic CoA ligase (EC 6.2.1.12) led to the formation of 4-cinnamic acid CoA ester **18** which is the ring B-C₃ unit. Condensation of the 4-cinnamoyl CoA **18** with three acetate units deriving from malonyl-CoA was catalyzed by chalcone synthase (EC 2.3.11.74), the key enzyme of flavonoid biosynthesis, to form ring A giving rise to chalcone **20**, isoliquiritigenin. Isomerization of **20** was ultimately taken place by chalcone isomerase (EC 5.5.1.6) to produce flavanone **21**, liquiritigenin²².

Biosynthetic pathway of polyketides is shown in **Fig 2.6**. The pathway involved the enzymatic catalyzed condensation of acetyl CoA, active form of acetic acid and two molecules of malonyl CoA to form phloroglucinol **17**¹⁹.

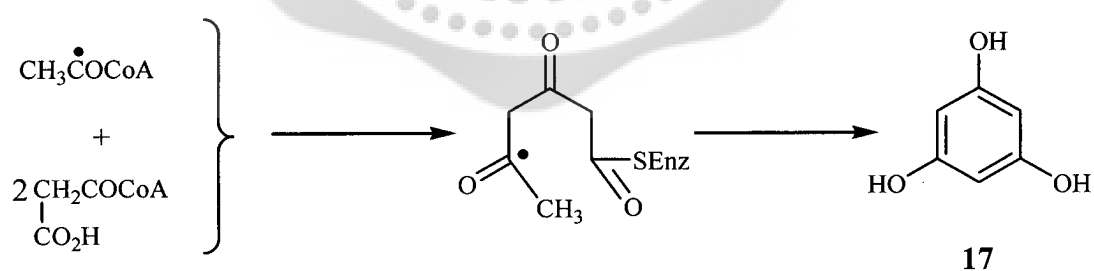


Fig 2.6 Biosynthesis of phloroglucinol by the polyketide pathway (Ring A)¹⁹

3,3',4'-Trihydroxyflavone **28** inhibited epinephrine oxidation due to its capability to form metal complex or chelation²³.

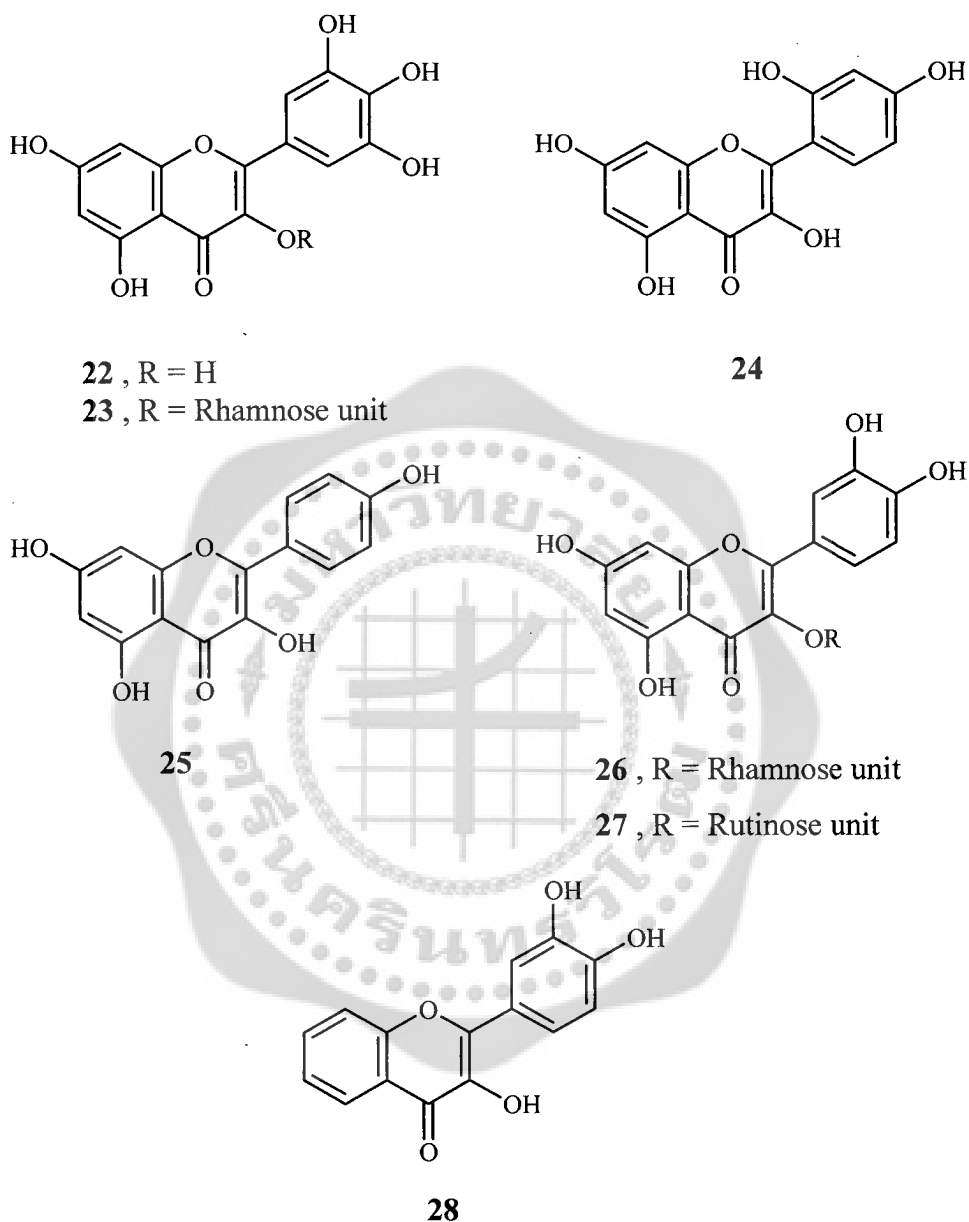


Fig 2.8 Some flavonoids in health and nutrition²³

Some flavonoids were cardiac stimulants and vasoconstrictors and that they increase blood pressure. They seem to be absorbed in the intestinal canal and from the subcutaneous tissue and excreted unchanged in the urine and in the bile. It was found that action of quercitrin **26** on the blood content of abdominal organs reduced

the blood pressure and affected the kidney and mesenteric vessels as well. Moreover, dietary flavonoids have definite influence in frost-bite in rats and rabbits²³.

The effects of flavonoids have also been studied on such clinical conditions as hypertension, diabetes, rheumatic fever, arthritis and pregnancy. In addition, flavonoids have also been studied in the treatment of allergy, protection against x-ray and other radiation injury, hemorrhage after dicoumarol therapy, dermatosis and albuminuric diseases²³.

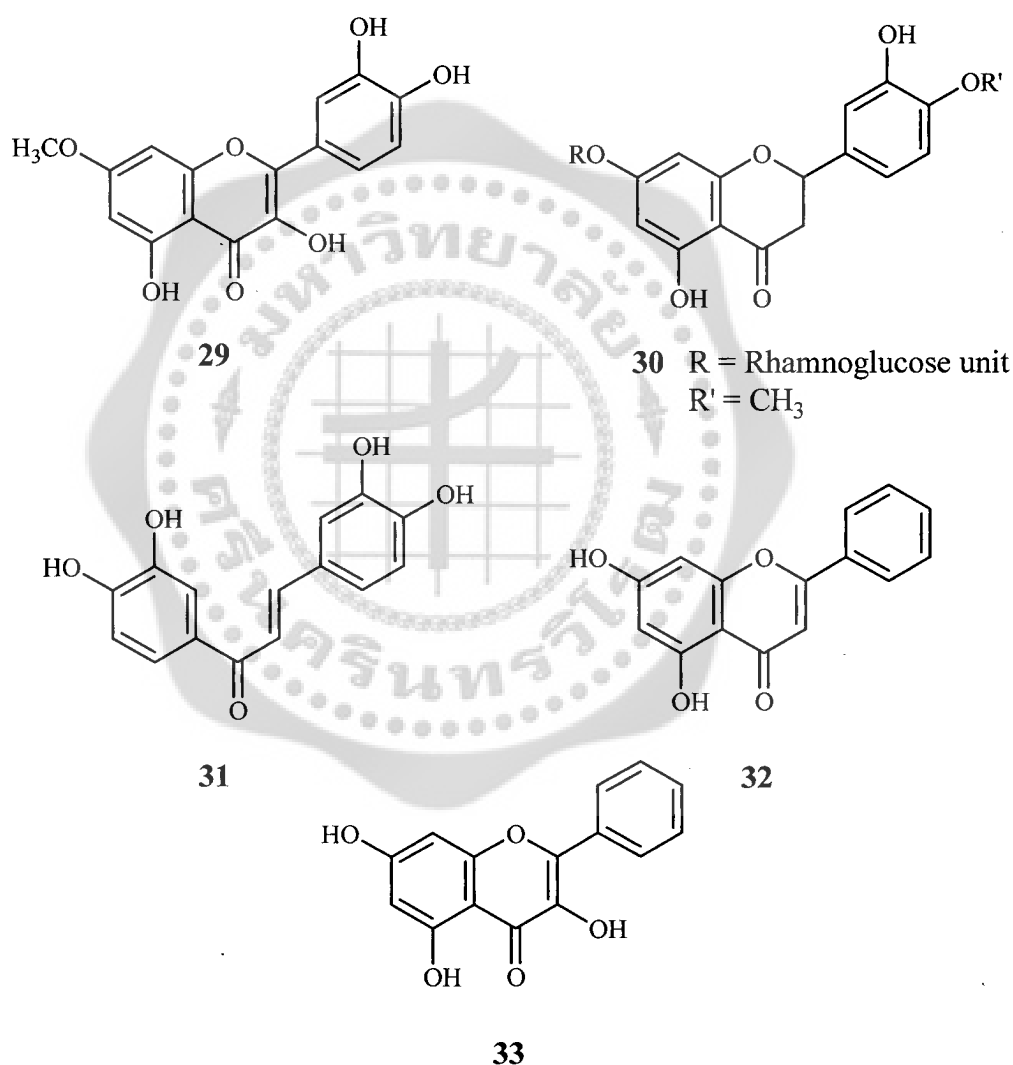


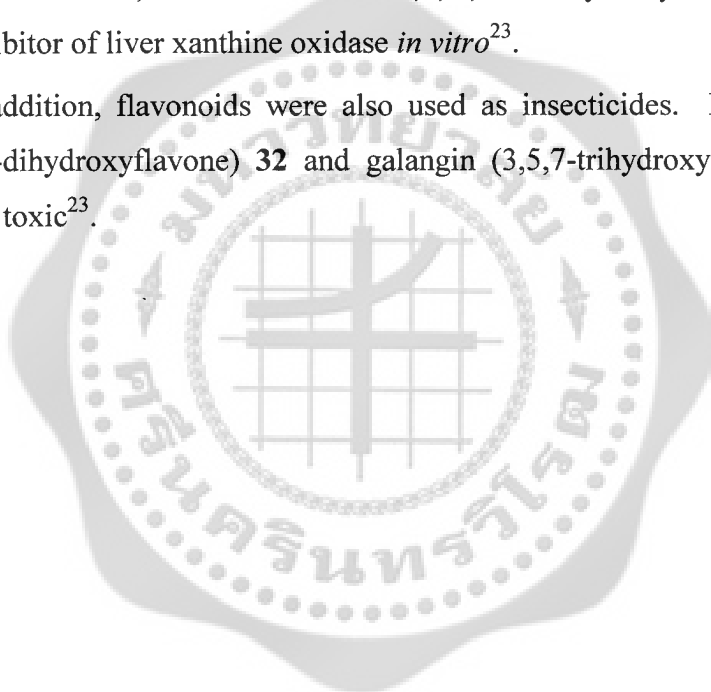
Fig 2.9 Some flavonoids in health and nutrition²³

It was reported that quercetin possessed considerable toxicity towards *Staphylococcus aureus*, thus completely inhibited their growth. Furthermore, rutin

or aureomycin in rats before and after splenectomy reduced the degree of anemia and leukocytosis. Investigation on antibacterial activity of many naturally occurring flavonoids and numerous synthetic chalcone derivatives found that chalcones were more active against *S. aureus* than the flavonoids²³.

Hesperidin derivatives were reported to act as inhibitors of hyaluronidase. It was found that solubilized phosphorylated hesperidin was shown to suppress fertility. Due to hesperetin **29** and its 7-rhamnoglucoside, hesperidin **30** are extremely insoluble unless phosphorylated, thus their presence in foodstuffs may not exhibit any such action. Moreover, it was shown that 3,4,3',4'-tetrahydroxy chalcone **31** was an effective inhibitor of liver xanthine oxidase *in vitro*²³.

In addition, flavonoids were also used as insecticides. It was found that chrysin (5,7-dihydroxyflavone) **32** and galangin (3,5,7-trihydroxyflavone) **33** were considerably toxic²³.



Chapter 3

Experimental

General methods

Melting points were determined on an electrothermal melting point apparatus (Electrothermal 9100) and reported without correction.

^1H Nuclear magnetic resonance (^1H NMR) spectra were recorded on a Varian EM30L with a 60 MHz operating frequency, a Bruker AM 400 with a 400 MHz operating frequency by using a solution in deuteriochloroform and deuteromethanol with tetramethylsilane as internal standard. Infrared spectra (IR) were obtained on Perkin Elmer System 2000 FT IR. Mass spectra were determined using a Finnigan MAT INCOS 50 mass spectrometer. ^{13}C Nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker AM400 by using a solution in deuteriochloroform and deuteromethanol with tetramethylsilane as internal standard. The UV spectra were measured with Milton Roy Spectronic 3000 Array. Optical rotation was measured in methanol solution with sodium D line (590 nm) on JASCO DIP370 digital polarimeter.

Column chromatography was carried out by using Silica gel 60 (0.063-0.200 mm) and silica gel 60 (particle size less than 0.063 mm). Thin Layer Chromatography (TLC) and preparative Thin Layer Chromatography were carried out on silica gel 60 PF₂₅₄ (cat. No. 7747 E. Merck).

Plant materials

Tubers of *Hydnophytum formicarum* Jack. were purchased from traditional drug store “Chao krom pur” Wat Sampluem, Samphanthawong, Bangkok.

Cell cultures²⁴

HuCCA-1 were established from cholangiocarcinomas experimentally induced in hamsters. The cell lines were characterized and have been maintained in CRI laboratory ever since 1994 in Ham's F12 culture medium (GIBCO Laboratories, Grand Island NY) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), 100 U/mL penicillin and 100 µg/mL streptomycin. The KB

cell lines, originally derived from epidermoid carcinoma of the floor of the oral cavity and commonly used as a reference laboratory standard for cytotoxicity assay, has been maintained in CRI laboratory in DMEM (Dulbecco's modified Eagle medium).

Extraction and Isolation

The air-dried tubers of *Hydnophytum formicarum* Jack (approximately 20 cm diameter, 4 kg) were ground and extracted with hexane (10 L $\times$ 7 days $\times$ 3), followed by filtration. The filtrates were combined and evaporated to dryness under reduced pressure to obtain crude hexane extract. Similarly, the extraction was carried out using dichloromethane and ethyl acetate respectively.

Crude hexane extract (15 g) was isolated by column chromatography on silica gel. Elution was conducted initially with hexane gradually enriched with acetone. All fractions were collected, monitored by TLC and combined as approximate. The solvents were evaporated to dryness to afford 14 fractions. Fractions **9** and **10**, which were eluted by 3-5% hexane-acetone, were combined and evaporated to give green gum with white crystal (2.7 g). This mixture was further separated by column chromatography over silica gel using gradient mixture of hexane and acetone as eluent to give white crystal. Recrystallization of all semi-solid from hexane and ethyl acetate to give compound **g** (496 mg).

Crude dichloromethane extract (20 g) was subjected to silica gel column chromatography. Initial elution with hexane then gradually increasing with acetone and finally enriched with methanol gave fractions which were collected and combined as approximate according to TLC monitoring. The solvents were evaporated to dryness to afford 18 fractions. Fraction **9** and **10** (2.31 g) was repeatedly isolated by column chromatography using silica gel. Elution with increasing gradient hexane in acetone afforded white amorphous compound. Recrystallization of white amorphous compound from hexane and ethyl acetate gave compound **h** (77 mg).

Crude ethyl acetate extract (59 g) obtained from 10 kg of *H. formicarum* Jack. which was extracted with hexane (20 L $\times$ 7 days $\times$ 3), dichloromethane and ethyl acetate respectively) was isolated by silica gel column chromatography. Elution was conducted initially with hexane, then gradually increasing with acetone and

finally enriched with methanol. All fractions were collected and combined as approximate according to TLC monitoring. The solvents were evaporated to dryness to give 11 fractions. Three selected main fractions were isolated and purified.

Fraction **1** (13.51 g), as yellowish green, was obtained from 20% acetone-hexane elution. The mixture was subjected to silica gel column eluting by 2% methanol-dichloromethane, and then further purified by preparative TLC using 5% methanol-dichloromethane as mobile phase to give oily yellow compound **34**.

Fraction **2** (6.75 g) obtaining from 30% acetone-hexane elution was further purified by silica gel column which was eluted with increasing methanol in dichloromethane. Purification by preparative TLC using 3 % methanol-dichloromethane as developing solvent to afford a semi-solid which was recrystallized from methanol-ethyl acetate to give compound **35**.

Fraction **3** (2.26 g), brown solid obtained from 40% acetone-hexane fractions, was rechromatographed on silica gel column. Elution with dichloromethane then increasing amounts of methanol and finally methanol to provide the following compounds.

-Elution with 1% methanol-dichloromethane gave white crystal and some yellow gum which was further purified by preparative TLC on silica gel using 1% methanol-dichloromethane as mobile phase of double developing. The obtained compound was crystallized in dichloromethane-ethanol to give compound **i**.

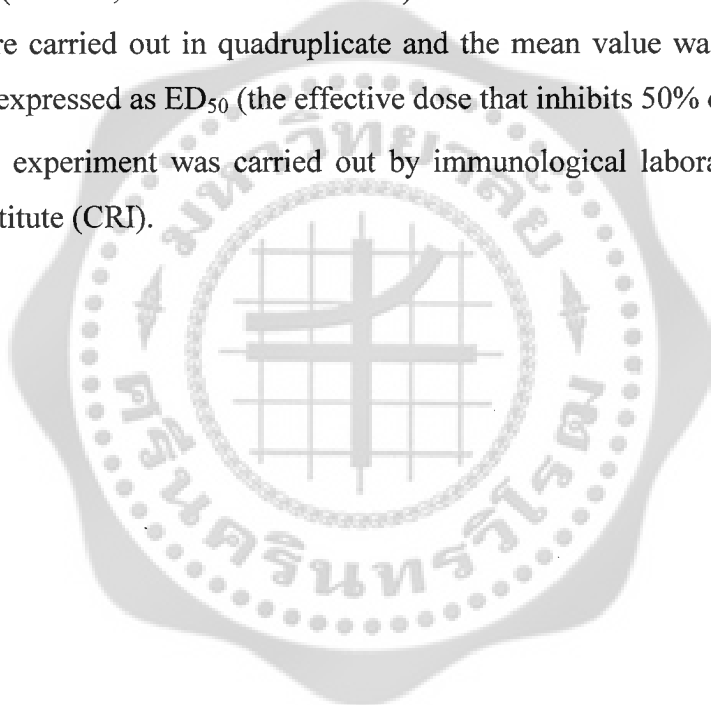
-Elution with 6% methanol-dichloromethane afforded brown gum which was further purified by preparative TLC on silica gel using 6% methanol-dichloromethane for 5 times to afford 2 bands. The lower band ($R_f = 0.40$) was crystallized in acetone-methanol to give compound **36** and the higher band ($R_f = 0.44$) was crystallized in dichloromethane to give compound **37**.

Cytotoxic assay²⁴

Cytotoxic activity was determined by a quantitative cytotoxic assay slightly modified from other investigators. Briefly, the confluent cell monolayers were trypsinized and diluted with appropriate culture medium to a final concentration of 3×10^5 cells/mL. One hundred microliter portions containing approximately 3×10^4

cells were distributed into 96-well flat-bottomed tissue culture plates and incubated overnight at 37°C in a humidified 5% CO₂ incubator. One hundred microliters of solution containing different concentrations of compounds **35-37** (0.001-10 µg/mL) or taxol (0.012-1.2 µg/mL) were added to each well and the plates were incubated as above for an additional 48 h. After the incubation, each well was washed three times with phosphate-buffered saline (PBS) (pH 7.2) and then stained with crystal violet. After the excess dye was removed, the stained cells were lysed with 100 µL of 100 mM HCl in absolute methanol and the optical density was determined by a microtitre plate reader (Titertek, Multiskan MCC/340) set to read at a wavelength of 540 nm. All tests were carried out in quadruplicate and the mean value was calculated. The activity was expressed as ED₅₀ (the effective dose that inhibits 50% of cell growth).

The experiment was carried out by immunological laboratory, Chulabhorn Research Institute (CRI).



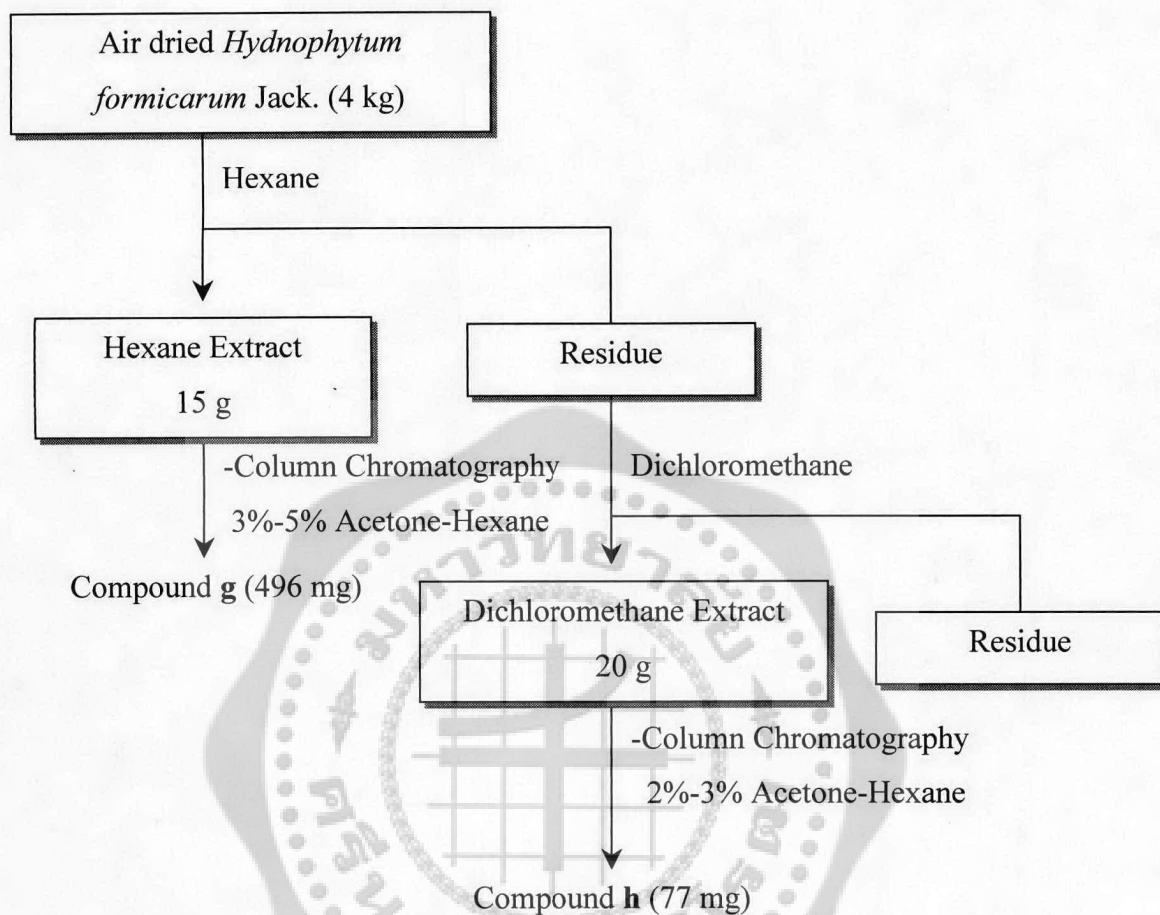


Fig 3.1 Extraction and isolation of *Hydnophytum formicarum* Jack.

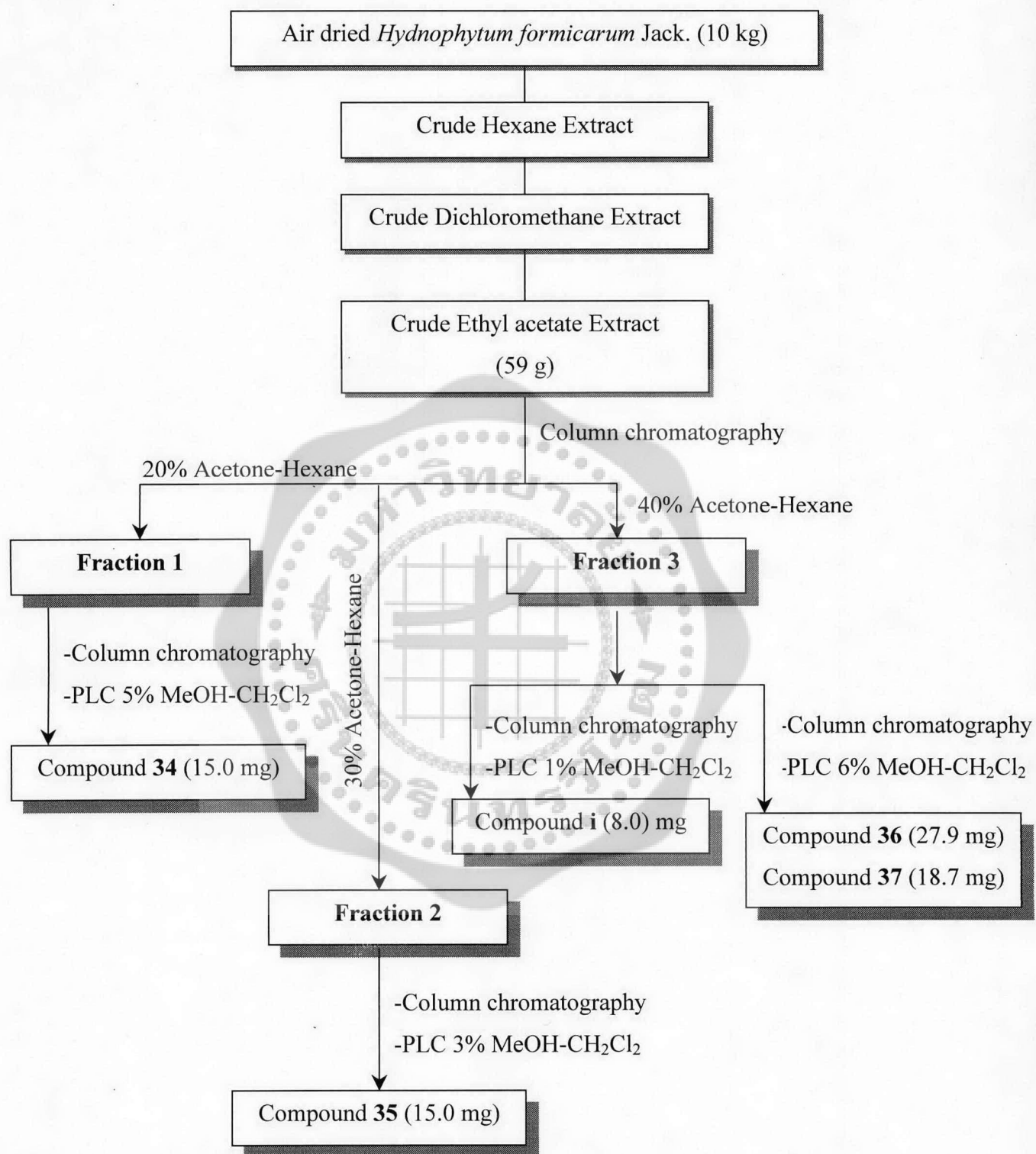
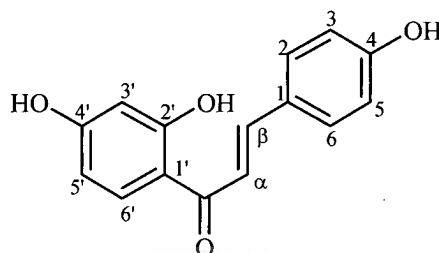


Fig 3.2 Extraction and isolation of *Hydnophytum formicarum* Jack. (Continued)

Experimental Data of Compounds 34-37

Compound 34

Isoliquiritigenin (2',4,4'-trihydroxychalcone; 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one)



Physical characteristic: yellow solid from dichloromethane-methanol,

m.p. 182-183°C (lit²⁵ 202°C, lit²⁶ 193.5-195°C)

UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ):

369 (7.42) (lit²⁷ in methanol 258sh, 298sh, 367)

FT IR $\nu_{\max}^{\text{KBr}}$ cm^{-1} :

3514, 3288, 1634, 1589, 1514, 1371, 1229, 1175 (lit²⁶ 3380, 1626, 1607, 1585, 1543, 1370, 1343, 1320, 1287)

¹H NMR (CD_3OD , 400 MHz) 7.75 (1H, *d*, $J = 15.4$ Hz, H- β), 7.74 (1H, *d*, $J = 8.8$ Hz, H-6'), 7.47 (2H *d*, $J = 8.6$ Hz, H-2,6), 7.36 (1H, *d*, $J = 15.4$ Hz, H- α), 6.80 (2H, *d*, $J = 8.6$ Hz, H-3,5), 6.36 (1H, *dd*, $J = 8.8, 2.4$ Hz, H-5'), 6.31 (1H, *d*, $J = 2.4$ Hz, H-3')

¹³C NMR (CD_3OD , 100 MHz) δ 191.92(CO), 165.71(C-4'), 164.36(C-2'), 159.50(C-4), 144.40(CH- β), 131.72(CH-6'), 130.48(CH-2,6), 126.58(C-1), 117.20(CH- α), 115.90(CH-3,5), 113.61(C-1'), 108.18(CH-5'), 103.08(CH-3')

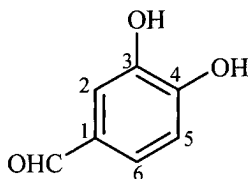
MS m/z (% relative intensity):

256(M^+ , 65), 255(60), 239(15), 163(15), 150(27), 137(29), 28(100)

(lit²⁸ 256 (M^+ , 100), 255(55), 239(14), 192(26), 163(37), 150(20), 137(90), 120(50))

Compound 35

Protocatechualdehyde (3,4-dihydroxybenzaldehyde; rancinamycin IV)



Physical characteristic: brown crystal from dichloromethane-methanol,

m.p. 153-154°C (lit²⁵ 154 °C, lit²⁹ 155-156°C, lit³⁰ 151-153°C)

UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ):

232 (6.81), 278 (6.89), 312 (6.94) (lit³¹ in ethanol : 209 (4.17), 234 (4.15), 281 (4.01), 315 (3.97), 360 (3.05))

FT IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} :

3326, 2875, 2825, 1647, 1595, 1537, 1443, 1168, 1119 (lit²⁹ 3220, 1650, 1595, 1440, 1375, 1296, 1165, lit³⁰ 3240, 1680, 1600)

¹H NMR (CD_3OD , 400 MHz) δ 9.63 (1H, s, CHO), 7.25 (1H, *br s*, H-2), 7.23 (1H, *br d*, H-6), 6.87 (1H, *d*, $J = 8.0$ Hz, H-5)

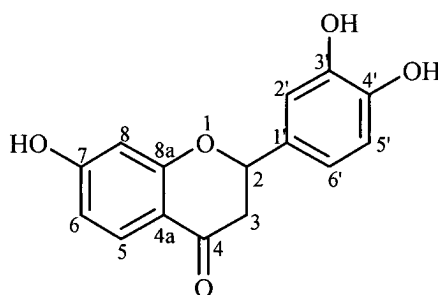
¹³C NMR (CD_3OD , 100 MHz) δ 191.93(CHO), 151.74(C-4), 145.18(C-3), 129.29(C-1), 125.83(CH-6), 114.90(CH-5), 113.97(CH-2)

MS m/z (% relative intensity):

138(M^+ , 70), 137(100), 109(18)

Compound 36

Butin (3',4',7-trihydroxyflavanone; 2-(3,4-dihydroxyphenyl)-2,3-dihydro-7-hydroxy-4*H*-1-benzopyran-4-one)



Physical characteristic: yellow solid from dichloromethane-methanol,

m.p. 204-205°C (lit²⁵ 205°C, lit³² 224-225°C)

UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ):

233 (6.76), 278 (6.84), 311 (6.89) (lit³² in methanol 225 (4.21), 327 (3.99))

FT IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} :

3491, 3371, 3146, 1666, 1607, 1585, 1327, 1287, 1171, 1115

$[\alpha]_{\text{D}}^{29} -87^\circ$ (c 0.03, MeOH) (lit³² (-), lit^{32a} $-18.7^\circ \pm 0.8^\circ$; c 0.5, MeOH)

¹H NMR (CD_3OD , 400 MHz) δ 7.69 (1H, d, $J = 8.7$ Hz, H-5), 6.87 (1H, d, $J = 1.8$ Hz, H-2'), 6.77 (1H, d, $J = 8.2$ Hz, H-5'), 6.74 (1H, dd, $J = 8.2, 1.8$ Hz, H-6'), 6.44 (1H, dd, $J = 8.7, 2.2$ Hz, H-6), 6.32 (1H, d, $J = 2.2$ Hz, H-8), 5.22 (1H, dd, $J = 13.2, 2.8$ Hz, H-2), 2.94 (1H, dd, $J = 13.2, 17.0$ Hz, H-3a), 2.65 (1H, dd, $J = 17.0, 2.8$ Hz, H-3b)

¹³C NMR (CD_3OD , 100 MHz) δ 192.07(CO), 164.86(C-7), 163.77(C-8a), 144.96(C-3'), 144.59(C-4'), 130.31(C-1'), 128.74(CH-5), 118.15(CH-6'), 114.98(CH-5'), 113.64(C-4a), 113.21(CH-2'), 110.65(CH-6), 102.77(CH-8), 79.45(CH-2), 43.62(CH₂-3)

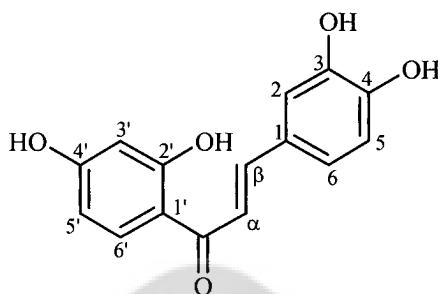
MS m/z (% relative intensity):

272(M^+ , 100), 271(55), 255(24), 163(20), 150(87), 137(52), 136(24)

(lit³² 272(M^+ , 65))

Compound 37

Butein (2',3,4,4'-tetrahydroxychalcone; 1-(2,4-dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)-2-propen-1-one)



Physical characteristic: orange yellow crystal needles (methanol),

m.p. 211-212°C (lit²⁵ 214°C)

UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ):

262 (6.88), 380 (7.04) (lit²⁷ in methanol 239sh, 266, 319sh, 379)

FT IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} :

3546, 3471, 3295, 1638, 1591, 1557, 1513, 1351, 1234, 1123

¹H NMR (CD₃OD, 400 MHz) δ 7.81 (1H, *d*, *J* = 8.9 Hz, H-6'), 7.70 (1H, *d*, *J* = 15.3 Hz, H- β), 7.40 (1H, *d*, *J* = 15.3 Hz, H- α), 7.15 (1H, *d*, *J* = 1.8 Hz, H-2), 7.05 (1H, *dd*, *J* = 8.2, 1.8 Hz, H-6), 6.82 (1H, *d*, *J* = 8.2 Hz, H-5), 6.40 (1H, *dd*, *J* = 8.9, 2.4 Hz, H-5'), 6.33 (1H, *d*, *J* = 2.4 Hz, H-3')

¹³C NMR (CD₃OD, 100 MHz) δ 191.87(CO), 165.49(C-2'), 164.38(C-4'), 147.87(C-3), 144.83(C-4), 144.61(CH- β), 131.56(CH-6'), 126.78(C-1), 122.19(CH-6), 116.86(CH- α), 115.10(CH-5), 114.28(CH-2), 113.21(C-1'), 107.86(CH-5'), 102.66 (CH-3')

MS *m/z* (%relative intensity):

272 (*M*⁺, 60), 255 (9), 163 (30), 150 (30), 137 (100), 123 (12), 110(18)

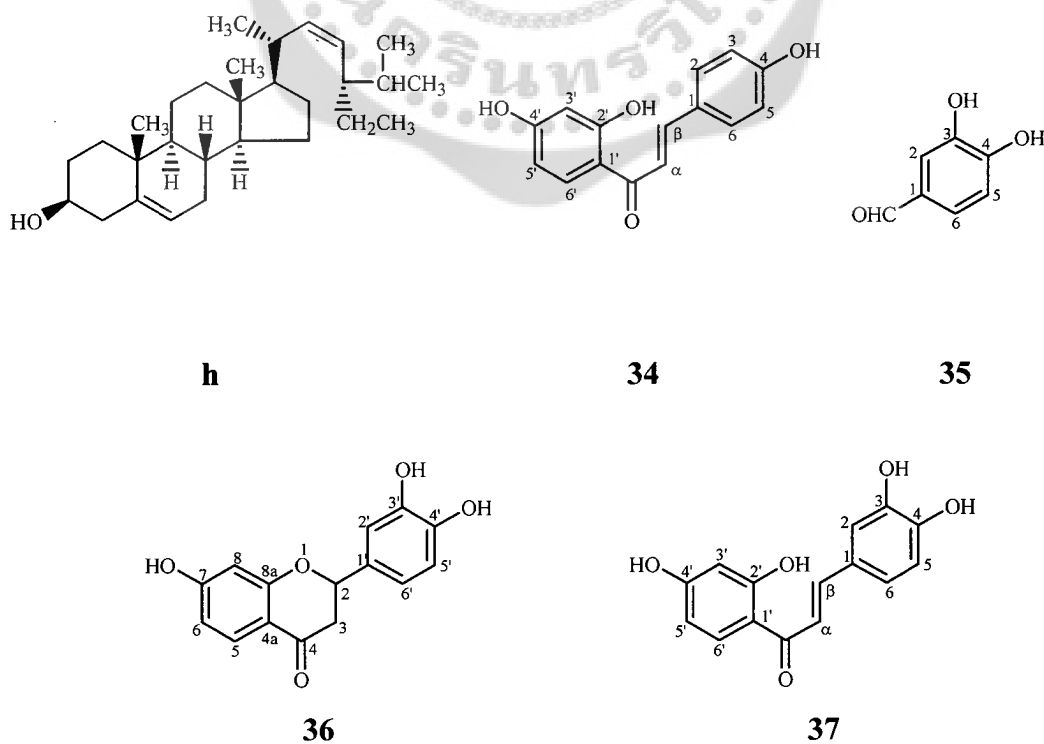
Chapter 4

Results and Discussion

The crude hexane extract (15 g) of dried-tubers of *Hydnophytum formicarum* Jack. was chromatographed on silica gel column using 3-5% acetone-hexane as an eluent to give green gum white solid which after recrystallization providing compound **g** of m.p. 152-153°C. Upon comparison of ^1H and ^{13}C NMR spectral data of compound **g** with authentic sample, compound **g** was determined to be stigmasterol. It is well known that stigmasterol is a sterol isolated from soy beans¹.

Similarly, the crude dichloromethane extract (20 g) was isolated and purified as above to give compound **h**. Comparison of ^1H NMR (60 MHz) of compounds **g** and **h**, thus compound **h** was concluded to be stigmasterol.

The ethyl acetate extract of dried-tubers of *H. formicarum* Jack. was concentrated to give a dark red gum (59 g) which was separated by silica gel column using gradient elution of hexane-acetone with increasing polarity. Several crude fractions were collected and examined by ^1H NMR spectroscopy. Thus selected fractions were further purified by silica gel column and preparative TLC to obtain 4 compounds, **34-37**.



In addition, compound **i** (8 mg) was found from such ethyl acetate extract as outlined in **Fig 3.2**. The structure of compound **i** was determined by 1D and 2D NMR spectral data as shown in appendix. Upon comparison of ^1H and ^{13}C NMR spectral data of compound **i** and **ketone** compound⁶, $\text{C}_{18}\text{H}_{16}\text{O}_2$, which was previously isolated from crude chloroform extract (mentioned in the introduction), now, it is cleared that such **ketone** compound was cyclic polymer of tri-ethylene terephthalate.

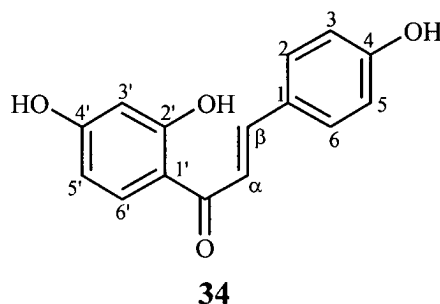
Compounds **35-37** were tested for cytotoxic activities against a panel of cell lines : KB (Human oral cancer) and HuCCA-1 (Human cholangiocarcinoma). All tested compounds were inactive in both HuCCA-1 and KB cell lines showing ED_{50} greater than 10 $\mu\text{g/mL}$. The results are shown in **Table 1**.

Table 1 Cytotoxic activities of compounds **35-37**

Compounds	ED_{50} ($\mu\text{g/mL}$) [†]	
	HuCCA-1 cell lines	KB cell lines
35	>10	>10
36	>11	>11
37	>11	>11

[†] ED_{50} ">" indicate that compounds are inactive at that concentration or inhibit cell growth less than 50 %

Identification of compound 34



Compound **34** was isolated as yellow solid, m.p. 182-183°C. The structure of **34** was elucidated by spectroscopic method. Its molecular formula $C_{15}H_{12}O_4$ was established by EIMS showed the molecular ion at m/z 256, suggesting tenth degree of unsaturations. Carbon-carbon bond cleavage at carbonyl and α -carbons gave two fragments of vinylbenzene m/z 120 and oxonium ion m/z 137. Further loss of $H^\bullet$ then CO from the latter ion m/z 137 provided ketene accounting for m/z 108 (see **appendix p-82**). The IR spectrum of **34** exhibited characteristic absorption bands for hydroxy (3514 cm^{-1}), carbonyl (1634 cm^{-1}) functionalities. While the UV spectrum showed a maximum absorption at 369 nm.

The 100 MHz ^{13}C NMR of compound **34** exhibited 13 signals. The DEPT spectra in **Table 4** (DEPT-90 and DEPT-135) established the existence of nine methine carbons δ 144.40(CH- β), 131.72(CH-6'), 130.48(CH-2,6), 117.20(CH- α), 115.90(CH-3,5), 108.18(CH-5'), 103.08(CH-3'), six quaternary carbons δ 191.92(CO), 165.71(C-4'), 164.36(C-2'), 159.50(C-4), 126.58(C-1), 113.61(C-1').

The 400 MHz 1H NMR of compound **34** showed three protons belonging to 1,2,4-trisubstituted benzene ring system (ABX system) at δ 7.74 (1H, *d*, $J = 8.8$ Hz, H-6'), 6.36 (1H, *dd*, $J = 8.8, 2.4$ Hz, H-5'), 6.31 (1H, *d*, $J = 2.4$ Hz, H-3'). Two doublets at δ 7.36 ($J = 15.4$ Hz) and 7.75 ($J = 15.4$ Hz) were observed suggesting the presence of double bond between C- α and C- β . In addition, four protons belonging to 1,4-disubstituted benzene ring system (AA'BB' system) at δ 7.47 (2H, *d*, $J = 8.6$ Hz, H-2,6), 6.80 (2H, *d*, $J = 8.6$ Hz, H-3,5) were noted. Correlations of these protons were observed in COSY spectral data **Table 5**. Connectivity of C-H bond was shown in HMQC NMR spectral data **Table 6**. HMBC spectral data of **34** in **Table 7** showed the connectivity of C-H through two or three bond correlation.

Based on the spectral data of compound **34** and comparison of its ^1H and ^{13}C NMR with isoliquiritigenin shown in **Table 2** and **Table 3**, structure of **34** was identified to be isoliquiritigenin. Previously, m.p. of isoliquiritigenin was reported^{25,26} to be 202°C and 193.5-195°C. Isoliquiritigenin was naturally first found in 1953 from *Dahlia variabilis* (Compositae)³³. However, we are the first to isolate isoliquiritigenin from *Hydnophytum formicarum* Jack.. Recently in 1997, isoliquiritigenin, in dandelion (*Taraxacum officinale*), has been biosynthetically produced from 4-coumaroyl-CoA and three malonyl CoA catalyzed by pET-PKR and NADPH in *E. coli*³⁴. Similarly, the production of such compound was also catalyzed by 6'-deoxychalcone synthase (DOCS) in yeast³⁵. Recently in 1998, isoliquiritigenin and flavone (liquiritigenin) have been reported to be common characteristic flavonoidal constituents of the Leguminosae family³⁶. In 1996, it was reported the isolation of isoliquiritigenin glycoside from woody part of the thickening root of *Glycyrrhiza glabra* L.³⁷.

In leguminous plants, e.g. licorice have produced isoliquiritigenin using CHS (chalcone synthase) and PKR (polyketide reductase)³⁴. Conversion of isoliquiritigenin to liquiritigenin was catalyzed by chalcone-flavanone isomerase from soya bean seed and was first described by Wong and Moustafa³⁸. Furthermore, isoliquiritigenin was converted to several compounds by using S-adenosyl-L-methionine (SAM) coupled with chalcone 2'-O-methyltransferase (CHMT) to give 4,4'-dihydroxy-2'-methoxychalcone or using SAM with licodione 2'-O-methyltransferase (LMT) to provide 2'-O-methyllicodione³⁹. In addition, isoliquiritigenin was catalyzed by chalcone-3-hydroxylase to afford butein⁴⁰.

In agriculture, isoliquiritigenin was a strong inducer for the transcription of nodulation (*nod*) genes in *Bradyrhizobium japonicum*⁴¹. Furthermore, isoliquiritigenin exhibited an effect on cardiac⁴² and aortic smooth muscle⁴³. Isoliquiritigenin was also shown anti-platelet⁴⁴, vasorelaxant⁴⁵, antioxidant⁴⁶ and antitumour activities⁴⁷.

Table 2 ^1H NMR spectral data of compound **34** in CD_3OD and isoliquiritigenin²⁶

Position	compound 34 δ_{H} , mult, $J(\text{Hz})$	isoliquiritigenin $^*\delta_{\text{H}}$, mult, $J(\text{Hz})$ ²⁶
1		
2	7.47 (2H, <i>d</i> , 8.6)	7.68 (2H, <i>d</i> , 8)
3	6.80 (2H, <i>d</i> , 8.6)	6.87 (2H, <i>d</i> , 8)
4		
5	6.80 (2H, <i>d</i> , 8.6)	6.87 (2H, <i>d</i> , 8)
6	7.47 (2H, <i>d</i> , 8.6)	7.68 (2H, <i>d</i> , 8)
α	7.36 (1H, <i>d</i> , 15.4)	7.66 (1H, <i>d</i> , 16)
β	7.75 (1H, <i>d</i> , 15.4)	7.82 (1H, <i>d</i> , 16)
1'		
2'		
3'	6.31 (1H, <i>d</i> , 2.4)	6.33 (1H, <i>d</i> , 2.5)
4'		
5'	6.36 (1H, <i>dd</i> , 8.8, 2.4)	6.42 (1H, <i>dd</i> , 8, 2.5)
6'	7.74 (1H, <i>d</i> , 8.8)	8.04 (1H, <i>d</i> , 8)
CO		

^1H NMR (100 MHz) in DMSO-d_6

Table 3 ^{13}C NMR spectral data of compound **34** in CD_3OD and isoliquiritigenin⁴⁸

Position	compound 34 δ_{C}	Isoliquiritigenin $^*\delta_{\text{C}}$, mult, $J(\text{Hz})$ ⁴⁸
1	126.58 (C)	125.8 (C)
2	130.48 (CH)	130.6 (CH)
3	115.90 (CH)	115.8 (CH)
4	159.50 (C)	159.9 (C)
5	115.90 (CH)	115.8 (CH)
6	130.48 (CH)	130.6 (CH)
α	117.20 (CH)	117.8 (CH)
β	144.40 (CH)	143.8 (CH)
1'	113.61 (C)	113.2 (C)
2'	164.36 (C)	164.6 (C)
3'	103.08 (CH)	102.6 (CH)
4'	165.71 (C)	165.4 (C)
5'	108.18 (CH)	107.9 (CH)
6'	131.72 (CH)	132.3 (CH)
CO	191.92 (CO)	191.4 (CO)

*in DMSO-d_6

Table 4 ^{13}C and DEPT NMR spectral data of compound **34**

δ_{C}	DEPT-90 (CH)	DEPT-135
191.92		
165.71		
164.36		
159.50		
144.40	144.40	144.40 (CH)
131.72	131.72	131.72 (CH)
130.48	130.48	130.48 (CH)
126.58		
117.20	117.20	117.20 (CH)
115.90	115.90	115.90 (CH)
113.61		
108.18	108.18	108.18 (CH)
103.08	103.08	103.08 (CH)

Table 5 COSY correlation among some protons of compound **34**

δ_{H} (ppm)		Proton correlated with δ_{H} (ppm)
7.47 (H-2)	$\leftrightarrow$	6.80 (H-3)
7.47 (H-6)	$\leftrightarrow$	6.80 (H-5)
6.80 (H-3)	$\leftrightarrow$	7.47 (H-2)
6.80 (H-5)	$\leftrightarrow$	7.47 (H-6)
7.36 (H- α)	$\leftrightarrow$	7.75 (H- β)
7.75 (H- β)	$\leftrightarrow$	7.36 (H- α)
6.31 (H-3')	$\leftrightarrow$	6.36 (H-5')
6.36 (H-5')	$\leftrightarrow$	6.31 (H-3'), 7.74 (H-6')
7.74 (H-6')	$\leftrightarrow$	6.36 (H-5')

Table 6 ^1H -detected Heteronuclear Multiple Quantum Coherence (HMQC) NMR spectral data of compound **34**

δ_{C} (ppm)	δ_{H} (ppm)	assignment
144.40	7.75	CH- β
131.72	7.74	CH-6'
130.48	7.47	CH-2, 6
117.20	7.36	CH- α
115.90	6.80	CH-3, 5
108.18	6.36	CH-5'
103.08	6.31	CH-3'

Table 7 Heteronuclear Multiple Bond Connectivity (HMBC) NMR spectral data of compound **34**

δ_C (ppm)		Proton correlated with δ_H (ppm)*
191.92 (CO)	$\leftrightarrow$	7.36 (H- α), 7.75 (H- β)
165.71 (C-4')	$\leftrightarrow$	7.74 (H-6')
164.36 (C-2')	$\leftrightarrow$	7.74 (H-6')
159.50 (C-4)	$\leftrightarrow$	6.80 (H-3, 5), 7.47 (H-2, 6)
144.40 (C- β)	$\leftrightarrow$	7.47 (H-2, 6)
131.72 (C-6')		
130.48 (C-2, 6)	$\leftrightarrow$	7.47 (H-2, 6), 7.75 (H- β)
126.58 (C-1)	$\leftrightarrow$	6.80 (H-3, 5), 7.36 (H- α)
117.20 (C- α)	$\leftrightarrow$	7.75 (H- β)
115.90 (C-3, 5)	$\leftrightarrow$	6.80 (H-3, 5)
113.61 (C-1')	$\leftrightarrow$	6.31 (H-3'), 6.36 (H-5')
108.18 (C-5')	$\leftrightarrow$	6.31 (H-3')
103.08 (C-3')	$\leftrightarrow$	6.36 (H-5')

* Shown only two or three bond correlation

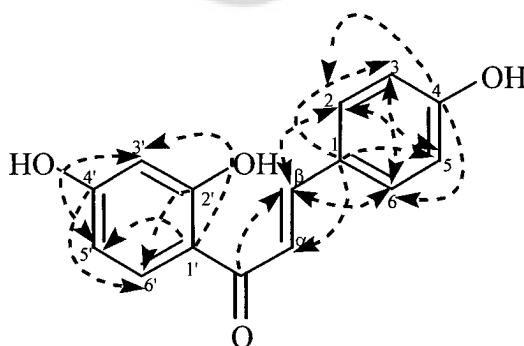
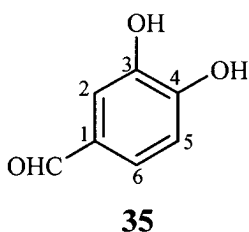


Fig 4.1 HMBC of compound **34**

Identification of compound 35



Compound **35** was isolated as brown crystal, m.p. 153-154°C. The structure of **35** was elucidated by spectroscopic method. Its molecular formula $C_7H_6O_3$ was established by EIMS at m/z 137 suggesting fifth degree of unsaturations. The IR spectrum of **35** exhibited characteristic absorption bands at 3326 cm^{-1} (OH), 1647 cm^{-1} (C=O), 1595 cm^{-1} (aromatic), 1168 cm^{-1} (C-O). While the UV spectrum showed maximum absorption at 232, 278, 312 nm.

The 100 MHz ^{13}C NMR of compound **35** exhibited 7 signals. The DEPT spectra **Table 10** (DEPT-90 and DEPT-135) established the existence of four methine carbons δ 191.93(CHO), 125.83(CH-6), 114.90(CH-5), 113.97(CH-2), three quaternary carbons δ 151.74(C-4), 145.18(C-3), 129.29(C-1).

The 400 MHz ^1H NMR of compound **35** showed one proton low field singlet at δ 9.63 and was assigned to be aldehyde (CHO). In addition, three protons belonging to 1,2,4-trisubstituted benzene ring system (ABX system) was observed at δ 7.25 (1H, *br s*, H-2), 7.23 (1H, *br d*, H-6), 6.87 (1H, *d*, $J = 8.0\text{ Hz}$, H-5). This assignment was confirmed by ^1H - ^1H correlation in COSY spectral data (**Table 11**) including the correlation between H-2 (δ 7.25) with C-2 (δ 113.97) and H-6 (δ 7.23) with C-6 (δ 125.83) in HMQC experiment as shown in **Table 12**. In addition, the connectivity of C-H through two or three bond was observed in HMBC spectra as shown in **Table 13**.

By careful analysis of the obtained spectral data of **35** and comparison of ^1H and ^{13}C NMR (**Table 8** and **Table 9**) including the melting point, thus, compound **35** was determined to be protocatechualdehyde (lit²⁵ m.p. 154°C, lit²⁹ m.p. 155-156°C and lit³⁰ m.p. 151-153°C). This compound **35** has been first isolated from *Hydnophytum formicarum* Jack..

Protocatechualdehyde exhibited various bioactivity such as antitumour and antioxidant activities⁴⁹.

Recently in 2000, protocatechualdehyde has been reported to transform to vanillin and capsaicin by immobilized cell culture of *Capsicum frutescens*⁵⁰.



Table 8 ^1H NMR spectral data of compound **35** in CD_3OD and protocatechualdehyde³⁰

Position	compound 35 δ_{H} , mult, $J(\text{Hz})$	Protocatechualdehyde $^*\delta_{\text{H}}$, mult, $J(\text{Hz})$ ³⁰
2	7.25 (1H, <i>br s</i>)	7.35 (1H, <i>d</i> , 2)
3		
4		
5	6.87 (1H, <i>d</i> , 8.0)	6.93 (1H, <i>d</i> , 8.5)
6	7.23 (1H, <i>br d</i>)	7.29 (1H, <i>dd</i> , 8.5, 2)
CHO	9.63 (1H, <i>s</i>)	9.70 (1H, <i>s</i>)

^{*}in ^1H NMR (100 MHz) DMSO-d_6

Table 9 ^{13}C NMR spectral data of compound **35** in CD_3OD and protocatechualdehyde⁵¹

Position	compound 35 δ_{C}	protocatechualdehyde $^*\delta_{\text{C}}$ ⁵¹
1	129.29 (C)	129.1 (C)
2	113.97 (CH)	114.6 (CH)
3	145.18 (C)	146.1 (C)
4	151.74 (C)	152.3 (C)
5	114.90 (CH)	115.8 (CH)
6	125.83 (CH)	124.7 (CH)
CHO	191.93 (CO)	191.2 (CO)

^{*}in DMSO-d_6

Table 10 ^{13}C and DEPT NMR spectral data of compound **35**

δ_{C}	DEPT-90 (CH)	DEPT-135
191.93	191.93	191.93 (CHO)
151.74		
145.18		
129.29		
125.83	125.83	125.83 (CH)
114.90	114.90	114.90 (CH)
113.97	113.97	113.97 (CH)

Table 11 COSY correlation among some protons of compound **35**

δ_{H} (ppm)		Proton correlated with δ_{H} (ppm)
7.25 (H-2)	$\leftrightarrow$	7.23 (H-6)
6.87 (H-5)	$\leftrightarrow$	7.23 (H-6)
7.23 (H-6)	$\leftrightarrow$	7.25 (H-2), 6.87 (H-5)
9.63 (CHO)		

Table 12 ^1H -detected Heteronuclear Multiple Quantum Coherence (HMQC) NMR spectral data of compound **35**

δ_{C} (ppm)	δ_{H} (ppm)	assignment
191.93	9.63	CHO
125.83	7.23	CH-6
114.90	6.87	CH-5
113.97	7.25	CH-2

Table 13 Heteronuclear Multiple Bond Connectivity (HMBC) NMR spectral data of compound **35**

δ_C (ppm)		Proton correlated with δ_H (ppm)*
191.93 (CHO)	$\leftrightarrow$	7.23 (H-6)
151.74 (C-4)	$\leftrightarrow$	6.87 (H-5), 7.23 (H-6), 7.25 (H-2)
145.18 (C-3)	$\leftrightarrow$	6.87 (H-5), 7.25 (H-2)
129.29 (C-1)	$\leftrightarrow$	6.87 (H-5), 9.63 (CHO)
125.83 (C-6)	$\leftrightarrow$	7.25 (H-2), 9.63 (CHO)
114.90 (C-5)		
113.97 (C-2)	$\leftrightarrow$	7.23 (H-6), 9.63 (CHO)

*Shown only two or three bond correlation

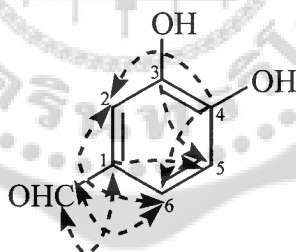
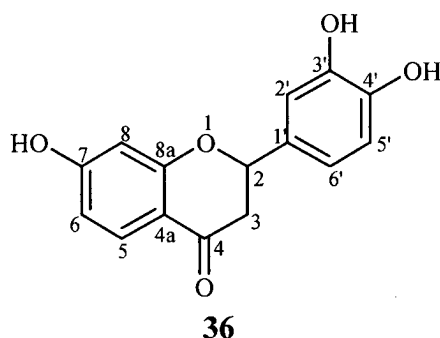


Fig 4.2 HMBC of compound **35**

Identification of compound 36



Compound **36** was isolated as yellow solid, m.p. 204-205°C. The structure of **36** was determined by spectroscopic method. Its molecular formula $C_{15}H_{12}O_5$ was established by EIMS showing molecular ion at m/z 272. Mass fragmentation of **36** took place at the pyrone ring to give two fragments of vinylbenzene m/z 136 and oxonium ion m/z 137. Similar to compound **34**, the oxonium ion was transformed to ketene m/z 108 as depicted in **appendix p-98**. According to the results, it is suggested that compound **36** containing tenth degree of unsaturations. The IR spectrum of **36** show characteristic absorption bands for hydroxy ($3491, 3371\text{ cm}^{-1}$), carbonyl (1666 cm^{-1}) and C-O ($1327, 1287, 1171\text{ cm}^{-1}$) moieties. The UV spectrum showed maximum absorption at 233, 278, 311 nm.

The 100 MHz ^{13}C NMR of compound **36** exhibited 15 signals. The DEPT spectra (DEPT-90 and DEPT-135) in **Table 15** established the existence of one methylene carbon δ 43.62(CH_2 -3), seven methine carbons δ 128.74 (CH-5), 118.15(CH-6'), 114.98(CH-5'), 113.21(CH-2'), 110.65(CH-6), 102.77(CH-8), 79.45(CH-2), seven quaternary carbons δ 192.07(CO), 164.86(C-7), 163.77(C-8a), 144.96(C-3'), 144.59(C-4'), 130.31(C-1'), 113.64(C-4a).

The 400 MHz ^1H NMR of compound **36** showed six protons belonging to two sets of 1,2,4-trisubstitued benzene ring systems (ABX system) at δ 7.69 (1H, *d*, $J = 8.7\text{ Hz}$, H-5), 6.44 (1H, *dd*, $J = 8.7, 2.2\text{ Hz}$, H-6), 6.32 (1H, *d*, $J = 2.2\text{ Hz}$, H-8), and δ 6.87 (1H, *d*, $J = 1.8\text{ Hz}$, H-2'), 6.77 (1H, *d*, $J = 8.2\text{ Hz}$, H-5'), 6.74 (1H, *dd*, $J = 8.2, 1.8\text{ Hz}$, H-6'). In addition, one methine proton at 5.22 (1H, *dd*, $J = 13.2, 2.8\text{ Hz}$, H-2) coupled with two methylene protons at 2.94 (1H, *dd*, $J = 13.2, 17.0\text{ Hz}$, H-3a) and 2.65 (1H, *dd*, $J = 17.0, 2.8\text{ Hz}$, H-3b) was observed. The large coupling constant ($J =$

13.2 Hz) was observed for vicinal H-2 and H-3a. Two geminal protons H-3a and H-3b was coupled showing J value of 17.0 Hz. Comparison with the reported value³² in **Table 14**, suggesting that H-3a oriented at equatorial position.

In COSY experiment, H-2 at δ 5.22 correlated with H-3a (δ 2.94) and H-3b (δ 2.65) as shown in **Table 16**. This suggested that H-2 attaching to chiral C-2 atom. Assignment of direct C-H connectivity was well correlated as observed in HMQC spectral data **Table 17**.

HMBC spectral data of **36** in **Table 18** showed C-H connectivity through two or three bond. In case of C-2 at δ 79.45 which connected with H-3a (δ 2.94) through two bond as shown in **Table 18**.

According to the available spectral data of compound **36** and comparison of its ^1H NMR with butin as shown in **Table 14**, thus, we concluded that compound **36** was butin (lit²⁵ m.p. 205°C, lit³² m.p. 224-225°C). The specific rotation of compound **36** was measured and found to be -87° . It was suggested that **36** was (-)-butin produced by natural enzymatic reaction. According to the literature reported^{32a}, butin isolated from heartwood of *Acacia mearnsii* and *A. pycnantha* exhibited specific rotation of -18.7° . In addition, it was reported that (-) butin, resulting from the irradiation of (+)-fustin (2R, 3R) at 350 nm³², possessed absolute configuration at C-2 as 2S. Our isolated (-)-butin (**36**) is first found in *Hydnophytum formicarum* Jack..

Previously, butin was isolated from the seeds of *Butea frondosa* and possessed postcoital anti-implantation and anticonceptive activity in the pregnant rats when administered orally during the implantation period⁵². It was found that the colour change of butin and butein depending on the pH of solution. In the acid solution butein (orange yellowish) was converted to butin (yellow)⁵³.

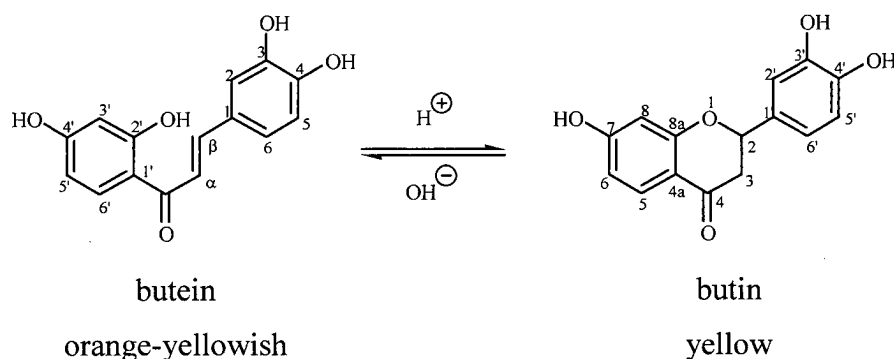


Table 14 ^{13}C , ^1H NMR spectral data of compound **36** in CD_3OD and butin³²

Position	compound 36 δ_{C}	compound 36 δ_{H} , mult, $J(\text{Hz})$	(-)-Butin $^*\delta_{\text{H}}$, mult, $J(\text{Hz})$ ³²
2	79.45 (CH)	5.22 (1H, <i>dd</i> , 13.2, 2.8)	5.31 (1H, <i>dd</i> , 11.8, 4.3)
3a	43.62 (CH ₂)	2.94 (1H, <i>dd</i> , 13.2, 17.0)	2.97 (1H, <i>dd</i> , 11.8, 16.5)-eq
3b	43.62 (CH ₂)	2.65 (1H, <i>dd</i> , 17.0, 2.8)	2.61 (1H, <i>dd</i> , 16.5, 4.3)-ax
4	192.07(CO)		
4a	113.64 (C)		
5	128.74 (CH)	7.69 (1H, <i>d</i> , 8.7)	7.61 (1H, <i>d</i> , 8.5)
6	110.65 (CH)	6.44 (1H, <i>dd</i> , 8.7, 2.2)	6.48 (1H, <i>dd</i> , 8.5, 2.5)
7	164.86 (C)		
8	102.77 (CH)	6.32 (1H, <i>d</i> , 2.2)	6.2 (1H, <i>d</i> , 2.5)
8a	163.77 (C)		
1'	130.31 (C)		
2'	113.21 (CH)	6.87 (1H, <i>d</i> , 1.8)	6.97-6.72 (3H, <i>m</i>)
3'	144.96 (C)		
4'	144.59 (C)		
5'	114.98 (CH)	6.77 (1H, <i>d</i> , 8.2)	6.97-6.72 (3H, <i>m</i>)
6'	118.15 (CH)	6.74 (1H, <i>dd</i> , 8.2, 1.8)	6.97-6.72 (3H, <i>m</i>)

* ^1H NMR (80 MHz) in DMSO-d_6

Table 15 ^{13}C and DEPT NMR spectral data of compound **36**

δ_{C}	DEPT-90 (CH)	DEPT-135
192.07		
164.86		
163.77		
144.96		
144.59		
130.31		
128.74	128.74	128.74 (CH)
118.15	118.15	118.15 (CH)
114.98	114.98	114.98 (CH)
113.64		
113.21	113.21	113.21 (CH)
110.65	110.65	110.65 (CH)
102.77	102.77	102.77 (CH)
79.45	79.45	79.45 (CH)
43.62		43.62 (CH ₂)

Table 16 COSY correlation among some protons of compound **36**

δ_{H} (ppm)		Proton correlated with δ_{H} (ppm)
5.22 (H-2)	$\leftrightarrow$	(H-3b), (H-3a)
2.94 (H-3a)	$\leftrightarrow$	(H-3b), (H-2)
2.65 (H-3b)	$\leftrightarrow$	(H-3a), (H-2)
7.69 (H-5)	$\leftrightarrow$	(H-6)
6.44 (H-6)	$\leftrightarrow$	(H-8), (H-5)
6.32 (H-8)	$\leftrightarrow$	(H-6)
6.87 (H-2')	$\leftrightarrow$	(H-6')
6.77 (H-5')	$\leftrightarrow$	(H-6')
6.74 (H-6')	$\leftrightarrow$	(H-5'), (H-2')

Table 17 ^1H -detected Heteronuclear Multiple Quantum Coherence (HMQC) NMR spectral data of compound **36**

δ_{C} (ppm)	δ_{H} (ppm)	assignment
128.74	7.69	CH-5
118.15	6.74	CH-6'
114.98	6.77	CH-5'
113.21	6.87	CH-2'
110.65	6.44	CH-6
102.77	6.32	CH-8
79.45	5.22	CH-2
43.62	2.94, 2.65	CH ₂ -3

Table 18 Heteronuclear Multiple Bond Connectivity (HMBC) NMR spectral data of compound **36**

δ_C (ppm)		Proton correlated with δ_H (ppm)*
192.07 (CO)	$\leftrightarrow$	2.65 (H-3b), 2.94 (H-3a), 5.22(H-2), 7.69 (H-5)
164.86 (C-7)	$\leftrightarrow$	7.69 (H-5)
163.77 (C-8a)	$\leftrightarrow$	7.69 (H-5)
144.96 (C-3')	$\leftrightarrow$	6.77 (H-5')
144.59 (C-4')	$\leftrightarrow$	6.74 (H-6'), 6.77 (H-5')
130.31 (C-1')	$\leftrightarrow$	2.94 (H-3a), 5.22 (H-2), 6.77 (H-5')
128.74 (C-5)		
118.15 (C-6')	$\leftrightarrow$	5.22 (H-2), 6.87 (H-2')
114.98 (C-5')		
113.64 (C-4a)	$\leftrightarrow$	6.44 (H-6), 6.32 (H-8)
113.21 (C-2')	$\leftrightarrow$	5.22 (H-2), 6.74 (H-6')
110.65 (C-6)	$\leftrightarrow$	6.32 (H-8)
102.77 (C-8)	$\leftrightarrow$	6.44 (H-6)
79.45 (C-2)	$\leftrightarrow$	2.94 (H-3a), 6.74 (H-6'), 6.87 (H-2')
43.62 (C-3)		

*Shown only two or three bond correlation

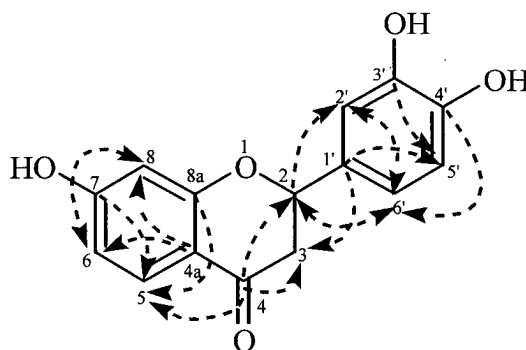
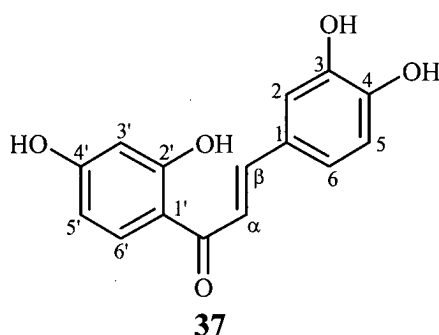


Fig 4.3 HMBC of compound **36**

Identification of compound 37



Compound **37** was isolated as orange-yellowish needles, m.p. 211-212°C. The structure of **37** was elucidated by spectroscopic method. EIMS showed molecular ion at m/z 272 for its molecular formula $C_{15}H_{12}O_5$, suggesting tenth degree of unsaturations. Similarly, the mass fragmentation of compound **37** (see **appendix p-107**) showed three fragments of vinylbenzene 136, oxanium ion m/z 137 and ketene m/z 108 as described for compounds **34** and **36**. The IR spectrum of **37** exhibited characteristic absorption bands for hydroxy ($3546, 3471\text{ cm}^{-1}$), carbonyl (1638 cm^{-1}) and C-O (1234 cm^{-1}) functionalities. While the UV spectrum showed maximum absorption at 262, 380 nm.

The 100 MHz ^{13}C NMR of compound **37** exhibited 15 signals. In **Table 20**, DEPT spectra (DEPT-90 and DEPT-135) established the existence of eight methine carbons [δ 102.66(C-3'), 107.86(C-5'), 114.28(C-2), 115.10(C-5), 116.86(C- α), 122.19(C-6), 131.56(C-6'), 144.61(C- β)], seven quaternary carbons [δ 113.21(C-1'), 126.78(C-1), 144.83(C-4), 147.87(C-3), 164.38(C-4'), 165.49(C-2'), 191.87(CO)].

The 400 MHz ^1H NMR of compound **37** showed six protons belonging to the two sets of 1,2,4-trisubstituted benzene ring system (ABX system) at δ 7.15 (1H, *d*, J = 1.8 Hz, H-2), 7.05 (1H, *dd*, J = 8.2, 1.8 Hz, H-6) 6.82 (1H, *d*, J = 8.2 Hz, H-5) and δ 7.81 (1H, *d*, J = 8.9 Hz, H-6') 6.40 (1H, *dd*, J = 8.9, 2.4 Hz, H-5') 6.33 (1H, *d*, J = 2.4 Hz). Correlation of these ring protons was noted as shown in COSY spectral data **Table 21**. Two doublets at δ 7.40 and 7.70 (J = 15.3 Hz) were observed suggesting the presence of trans-double bond between C- α and C- β . This assignment was confirmed by the three bond correlation between C-6 (δ 122.19), H- β and C-2 (δ

114.28), H- β in HMBC experiment as shown in **Table 23**. The direct C-H connectivity of compound **37** was observed as depicted in **Table 22**.

Based on the spectral data of compound **37** and comparison of its ^1H NMR with butein shown in **Table 19**, therefore, structure of **37** was concluded to be butein which its m.p. was reported²⁵ to be 214°C.

Previously butein was found from *Butea frondosa*⁵⁴, *Viguiera multiflora* (Nutt.)⁵⁵, *Baeria chrysostoma*⁵⁵ (F. and M.), *Cosmos sulphureus*⁵⁶, *Dahlia variabilis*⁵⁵, *Coreopsis maritima*⁵⁷, *Coreopsis gigantea*⁵⁷. However, this is our first isolation of butein from *Hydnophytum formicarum* Jack..

It was reported that butein served as a powerful antioxidant against lipid and LDL peroxidation and protected free radical-induced cell damage⁵⁸.

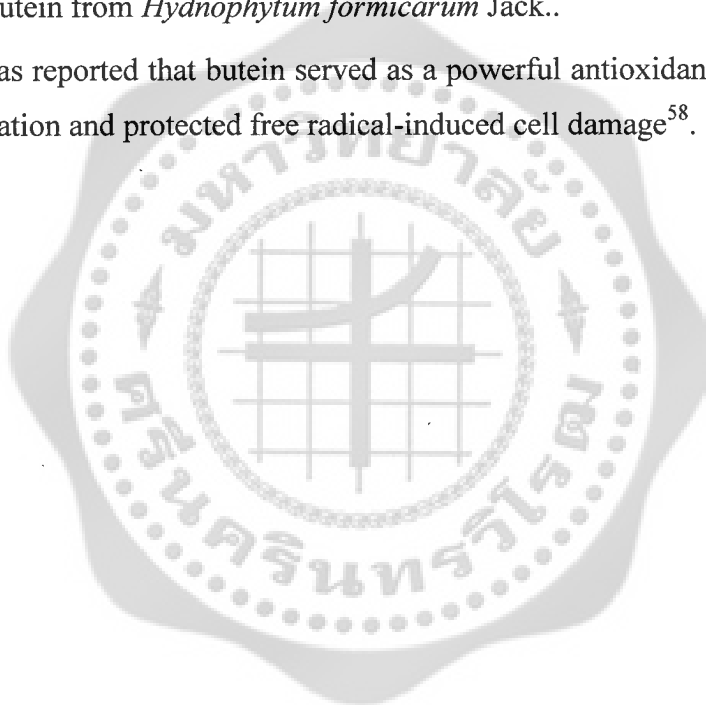


Table 19 ^{13}C , ^1H NMR spectral data of compound **37** in CD_3OD and butein²⁷

Position	compound 37 δ_{C}	compound 37 δ_{H} , mult, $J(\text{Hz})$	Butein $^*\delta_{\text{H}}$, mult, $J(\text{Hz})$ ²⁷
1	126.78 (C)		
2	114.28 (CH)	7.15 (1H, <i>d</i> , 1.8)	7.22 (1H, <i>d</i> , 2)
3	147.87 (C)		
4	144.83 (C)		
5	115.10 (CH)	6.82 (1H, <i>d</i> , 8.2)	6.89 (1H, <i>d</i> , 8)
6	122.19 (CH)	7.05 (1H, <i>dd</i> , 8.2, 1.8)	7.04-7.09 (1H, m)
α	116.86 (CH)	7.40 (1H, <i>d</i> , 15.3)	7.40 (1H, <i>d</i> , 15)
β	144.61 (CH)	7.70 (1H, <i>d</i> , 15.3)	7.75 (1H, <i>d</i> , 15)
1'	113.21 (C)		
2'	165.49 (C)		
3'	102.66 (CH)	6.33 (1H, <i>d</i> , 2.4)	6.41-6.47 (1H, m)
4'	164.38 (C)		
5'	107.86 (CH)	6.40 (1H, <i>dd</i> , 8.9, 2.4)	6.41-6.47 (1H, m)
6'	131.56 (CH)	7.81 (1H, <i>d</i> , 8.9)	7.80 (1H, <i>d</i> , 9)
CO	191.87		

* ^1H NMR (60 MHz) in DMSO-d_6

Table 20 ^{13}C and DEPT NMR spectral data of compound **37**

δ_{C}	DEPT-90 (CH)	DEPT-135
191.87		
165.49		
164.38		
147.87		
144.83		
144.61	144.61	144.61
131.56	131.56	131.56
126.78		
122.19	122.19	122.19
116.86	116.86	116.86
116.80	116.80	116.80
114.28	114.28	114.28
113.21		
107.86	107.86	107.86
102.66	102.68	102.68

Table 21 COSY correlation among some protons of compound **37**

δ_{H} (ppm)		Proton correlated with δ_{H} (ppm)
7.15 (H-2)	$\Leftrightarrow$	7.05 (H-6)
6.82 (H-5)	$\Leftrightarrow$	7.05 (H-6)
7.05 (H-6)	$\Leftrightarrow$	7.15 (H-2), 6.82 (H-5)
7.40 (H- α)	$\Leftrightarrow$	7.70 (H- β)
7.70 (H- β)	$\Leftrightarrow$	7.40 (H- α)
6.33 (H-3')	$\Leftrightarrow$	6.40 (H-5')
6.40 (H-5')	$\Leftrightarrow$	7.81 (H-6'), 6.33 (H-3')
7.81 (H-6')	$\Leftrightarrow$	6.40 (H-5')

Table 22 ¹H-detected Heteronuclear Multiple Quantum Coherence (HMQC) NMR spectral data of compound **37**

δ_{C} (ppm)	δ_{H} (ppm)	assignment
144.61	7.70	CH- β
131.56	7.81	CH-6'
122.19	7.05	CH-6
116.86	7.40	CH- α
116.80	6.82	CH-5
114.28	7.15	CH-2
107.86	6.40	CH-5'
102.66	6.33	CH-3'

Table 23 Heteronuclear Multiple Bond Connectivity (HMBC) NMR spectral data of compound **37**

δ_C (ppm)		Proton correlated with δ_H (ppm)*
191.87 (CO)	$\leftrightarrow$	7.40 (H- α), 7.70 (H- β), 7.81 (H-6')
165.49 (C-2')	$\leftrightarrow$	7.81 (H-6'), 6.33 (H-3')
164.38 (C-4')	$\leftrightarrow$	7.81 (H-6'), 6.33 (H-3')
147.87 (C-3)	$\leftrightarrow$	6.82 (H-5), 7.15 (H-2)
144.83 (C-4)	$\leftrightarrow$	6.82 (H-5), 7.05 (H-6), 7.15 (H-2)
144.61 (C- β)	$\leftrightarrow$	7.05 (H-6), 7.15 (H-2)
126.78 (C-1)	$\leftrightarrow$	6.82 (H-5), 7.40 (H- α)
122.19 (C-6)	$\leftrightarrow$	7.15 (H-2), 7.70 (H- β)
116.86 (C- α)		
116.80 (C-5)		
114.28 (C-2)	$\leftrightarrow$	7.05 (H-6), 7.70 (H- β)
113.21 (C-1')	$\leftrightarrow$	6.33 (H-3'), 6.40 (H-5')
107.86 (C-5')	$\leftrightarrow$	6.33 (H-3')
102.66 (C-3')	$\leftrightarrow$	6.40 (H-5')

*Shown only two or three bond correlation

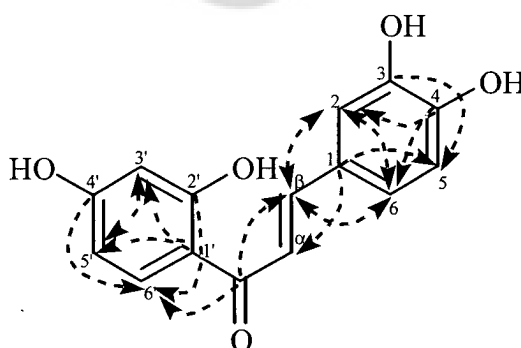


Fig 4.4 HMBC of compound **37**

In conclusion, in this study we have isolated four compounds (**34-37**) from crude ethyl acetate. Although these compounds were previously isolated from various plants, but they have been first isolated from *Hydnophytum formicarum* Jack. in our laboratory. In addition, 1D and 2D-NMR spectral data of these compounds have been established.





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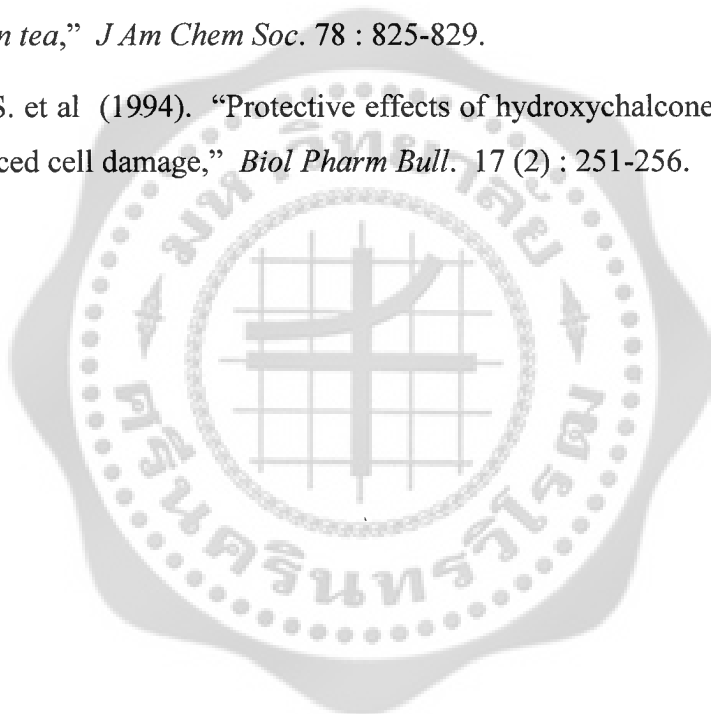
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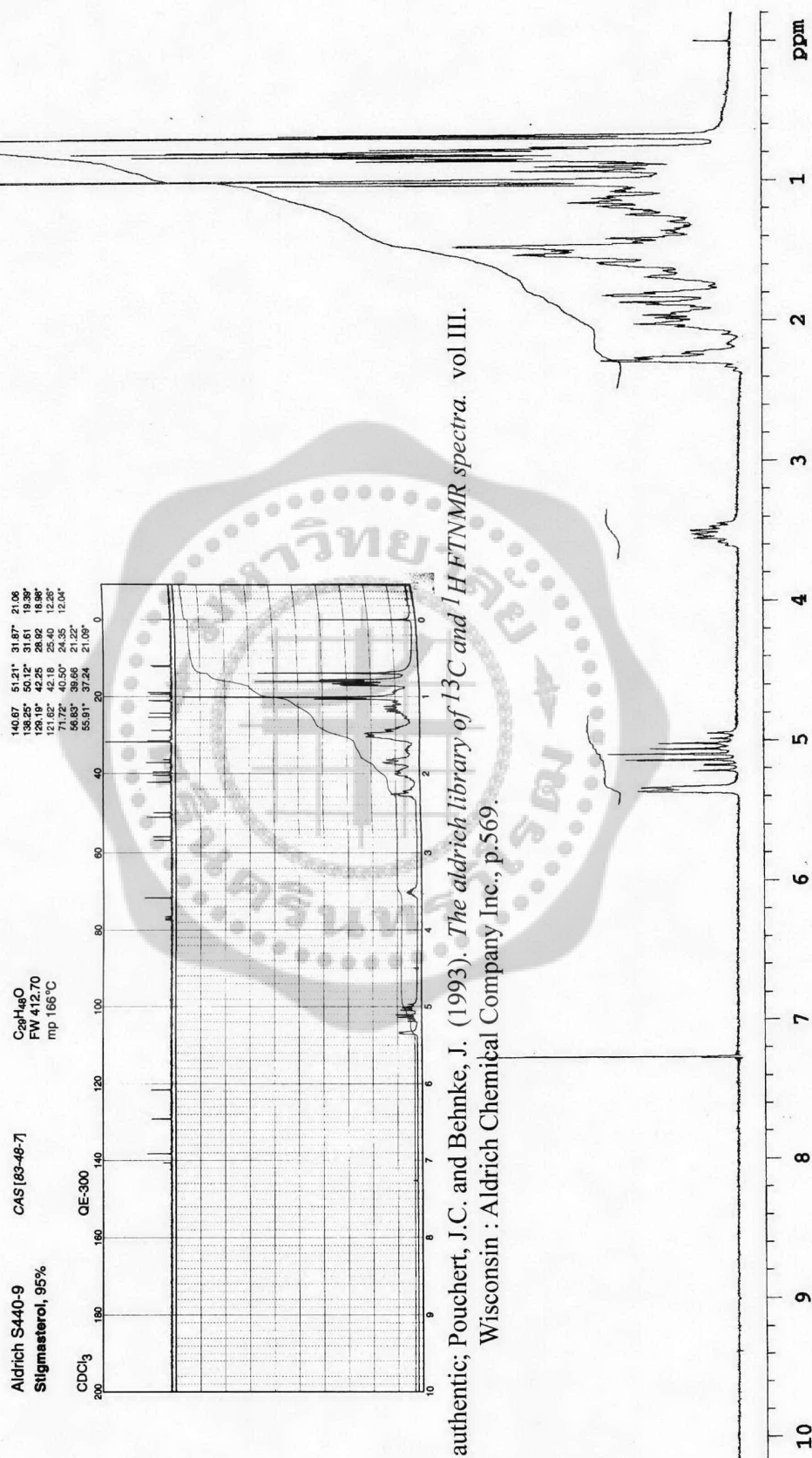
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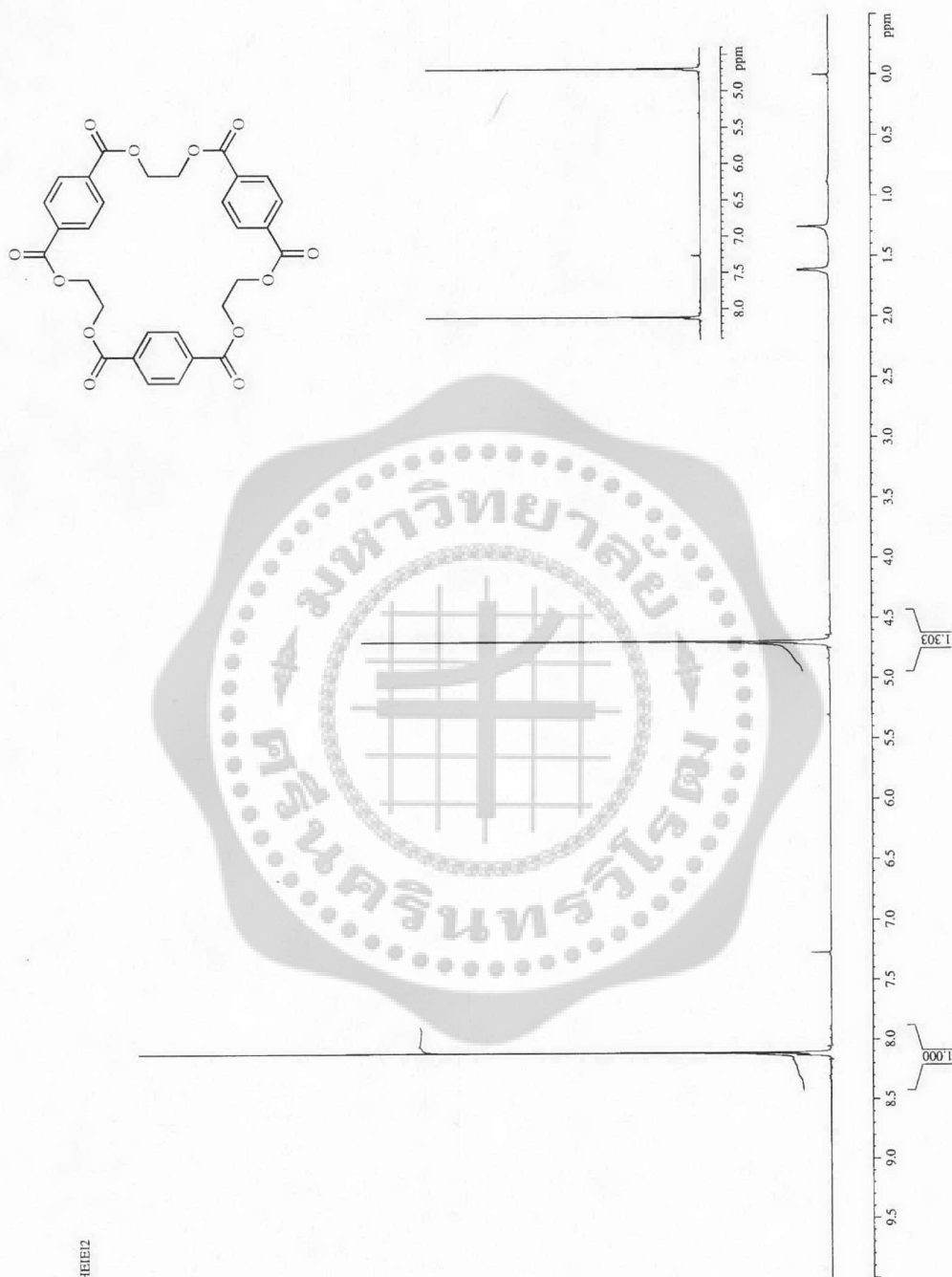




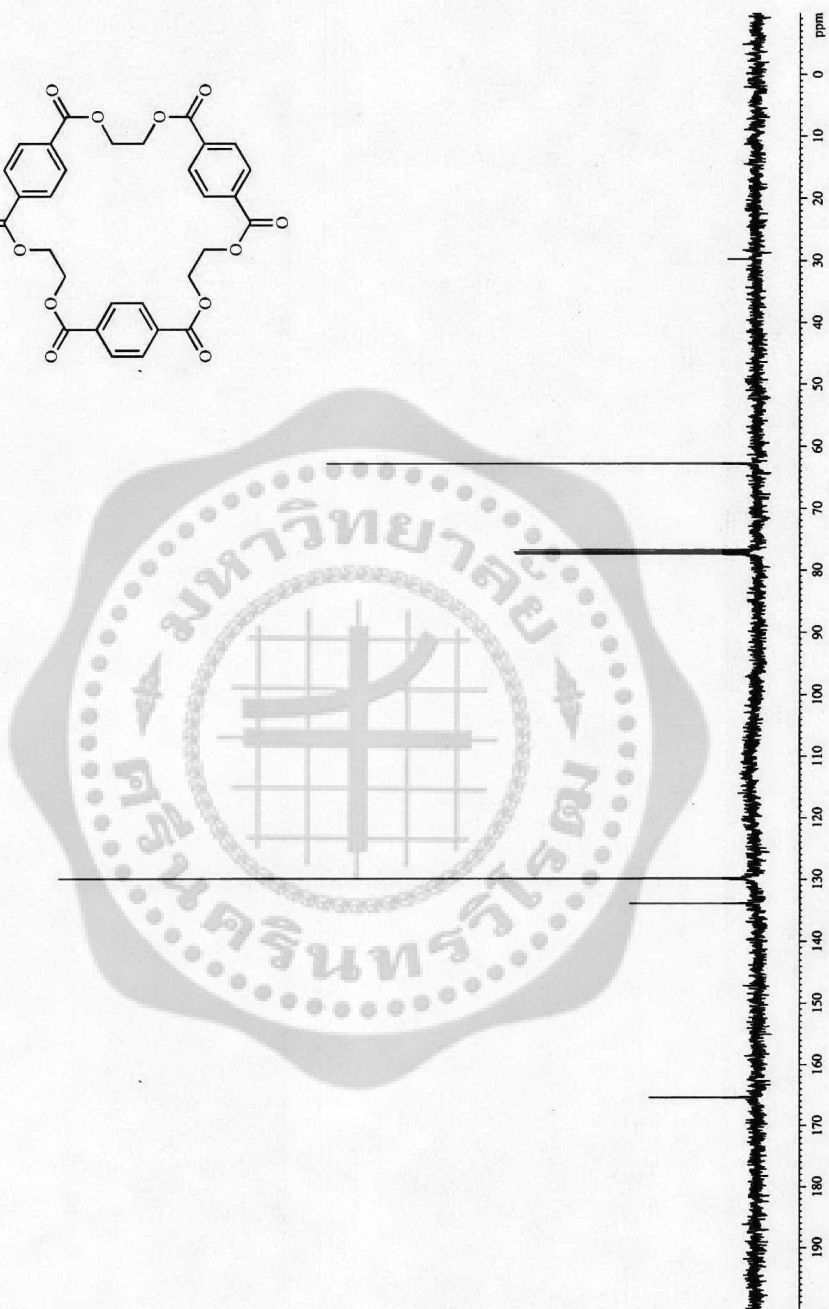
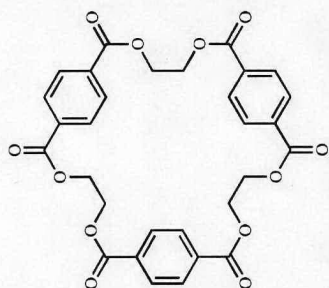


authentic; Pouchert, J.C. and Behnke, J. (1993). *The aldrich library of ¹³C and ¹H FTNMR spectra*. vol III.
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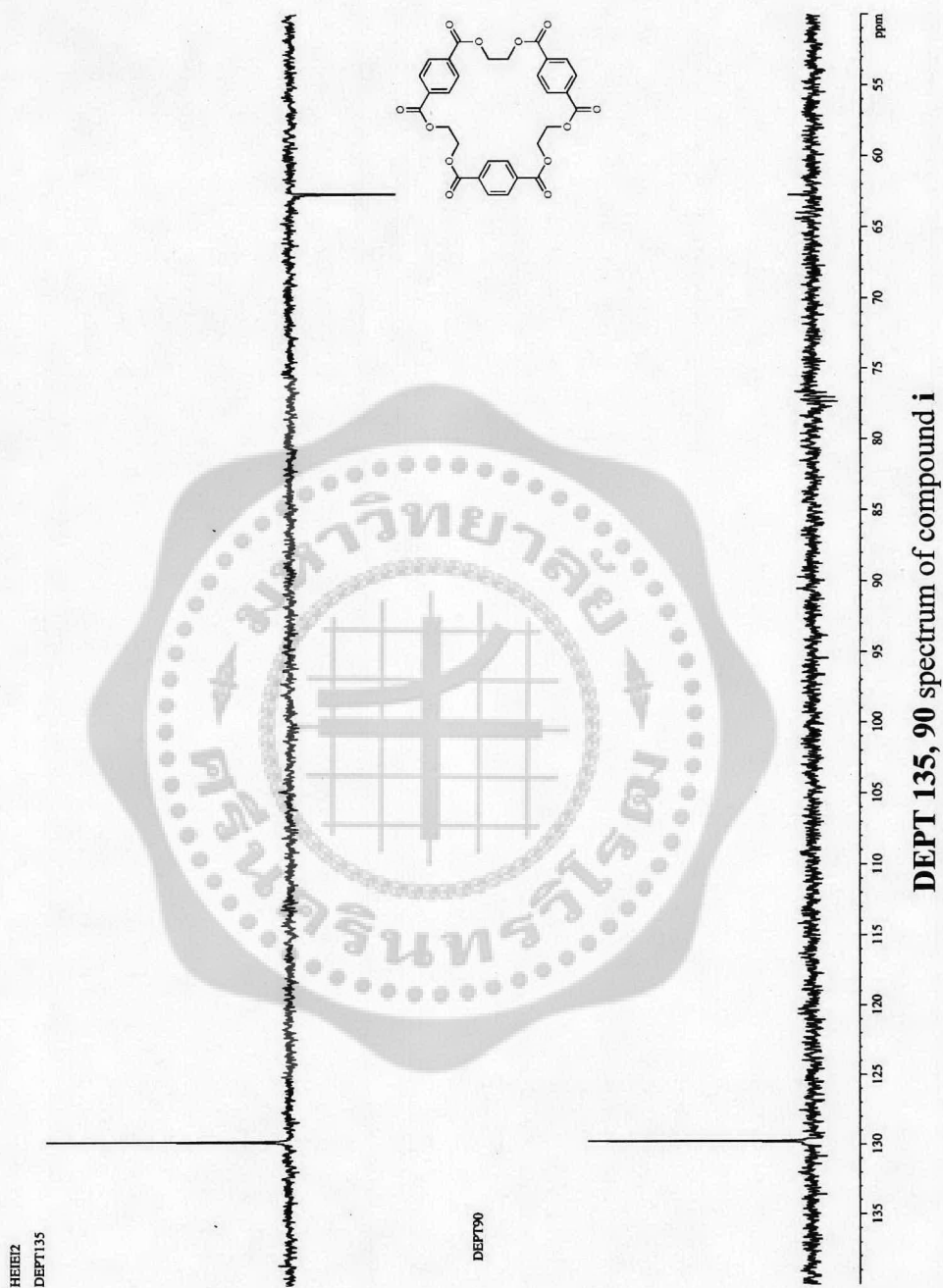
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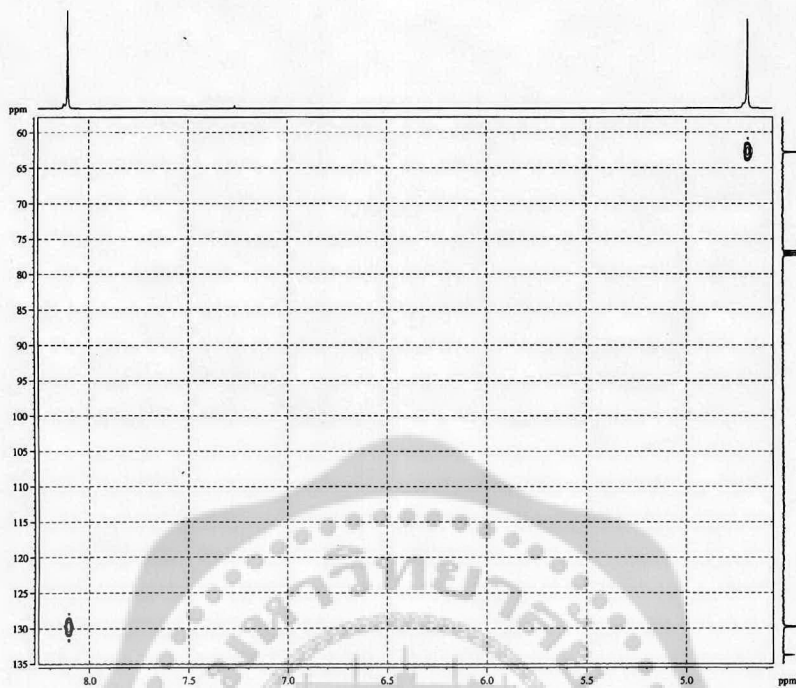


^1H NMR spectrum (400 MHz, CDCl_3) of compound **i**

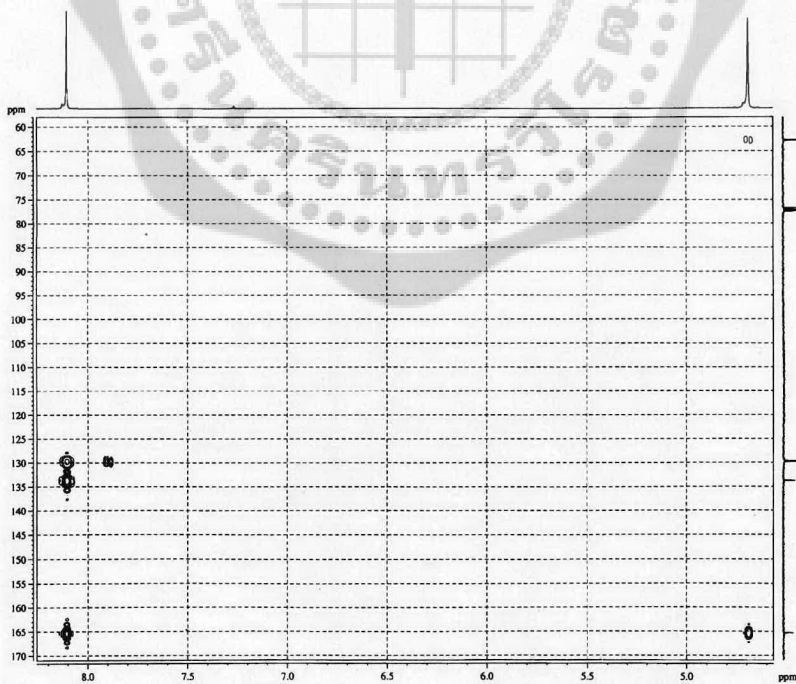


¹³C NMR spectrum (100 MHz, CDCl₃) of compound **1**

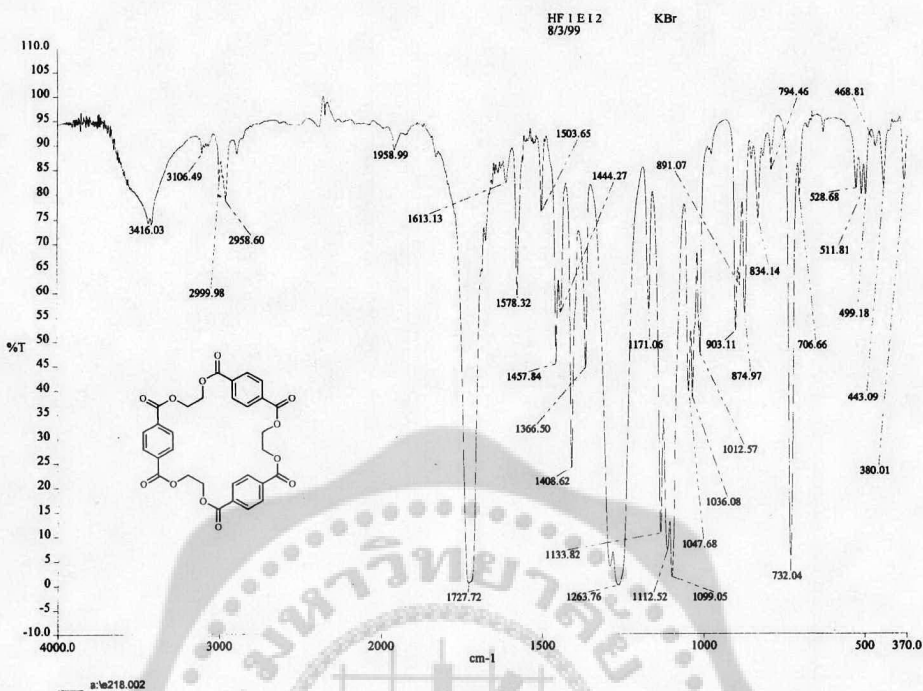


HEIE12
HMQC

HMQC spectrum of compound i

HEIE12
HMBC

HMBC spectrum of compound i

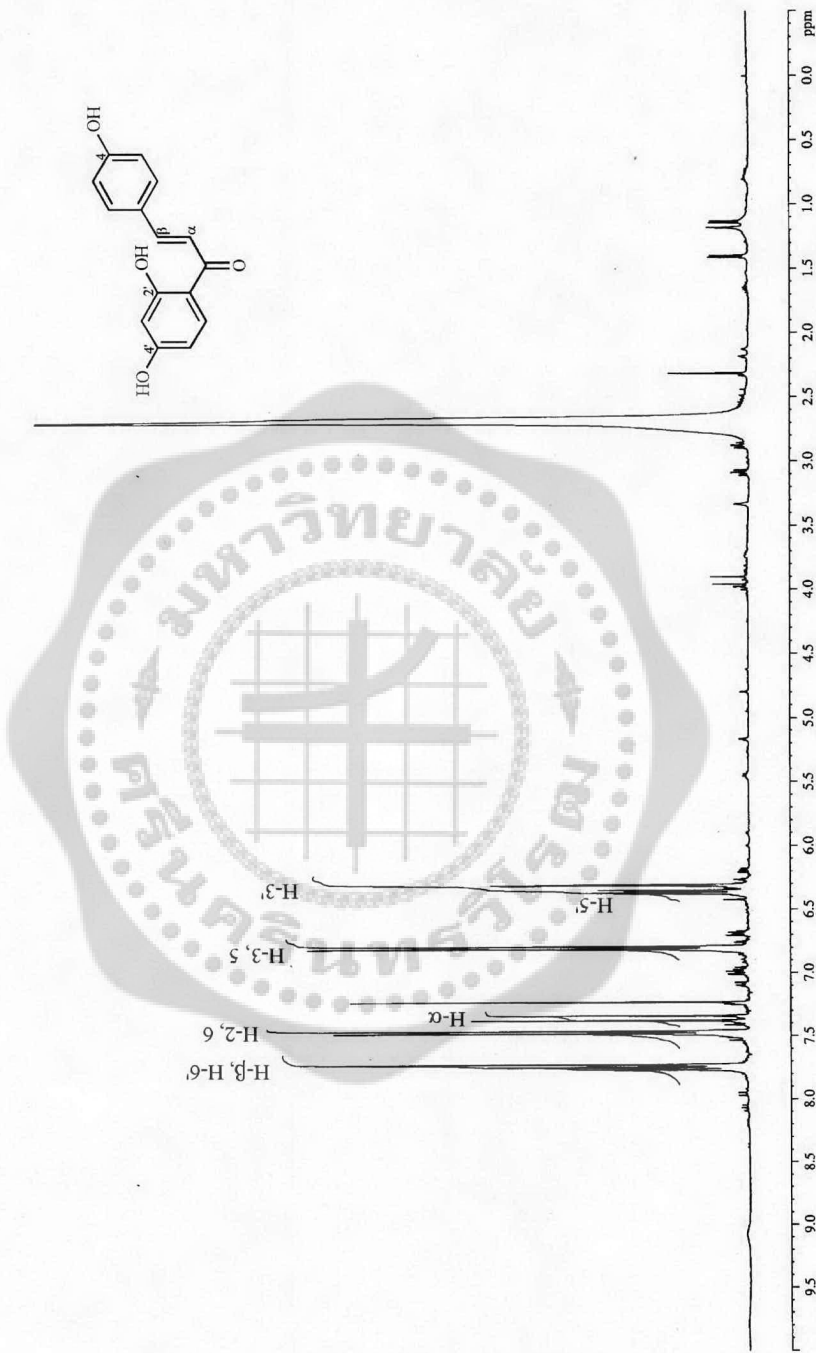


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 Sample: HFIEI2
 Conds.: DIP 40C/300C @60C/MIN

31	0.05	0.	Minima	Min Inten:	19.			
536			Maxima	# 300				
Mass	% RA	Inten.	Mass	% RA	Inten.	Mass	% RA	Inten.
31	1.32	500.	120	1.87	708.	267	0.98	372.
32	8.39	3184.	121	4.29	1628.	268	0.82	312.
39	1.55	588.	122	0.83	316.	269	0.96	364.
40	1.32	500.	126	1.10	416.	283	1.01	384.
41	2.30	872.	129	1.03	392.	284	9.81	3724.
42	1.13	428.	131	1.29	488.	285	2.75	1044.
43	3.91	1484.	132	7.97	3024.	296	7.69	2920.
44	3.33	1264.	133	1.98	752.	297	3.97	1508.
45	1.02	388.	140	1.11	420.	298	1.58	600.
50	3.78	1436.	145	1.05	400.	300	0.84	320.
51	1.44	548.	146	1.84	700.	312	2.47	936.
52	0.90	340.	147	3.49	1324.	313	1.06	404.
53	1.05	400.	148	9.07	3444.	325	1.04	396.
55	3.57	1356.	149	86.85	32960.	328	1.09	412.
56	0.95	360.	150	9.00	3416.	329	2.95	1120.
57	1.89	716.	151	1.97	748.	330	0.98	372.
61	0.83	316.	152	1.88	712.	339	0.93	352.
63	0.87	332.	153	0.81	308.	341	5.05	1916.
65	4.46	1692.	159	1.10	416.	342	1.32	500.
66	2.47	936.	161	1.37	520.	357	1.19	452.
67	0.93	352.	162	12.18	4624.	371	1.47	556.
69	1.74	660.	163	1.34	508.	372	2.79	1060.
71	1.06	404.	164	1.11	420.	373	1.36	516.
73	1.43	544.	169	0.79	300.	382	1.10	416.
74	1.59	604.	175	1.39	528.	385	2.01	764.
75	4.22	1600.	179	1.20	456.	394	2.58	980.
76	15.49	5880.	180	1.87	708.	395	1.33	504.
77	3.73	1416.	184	6.03	2288.	396	2.02	768.
78	0.83	316.	189	1.01	384.	397	1.67	632.
79	1.11	420.	191	5.21	1976.	399	2.54	964.
81	1.37	520.	192	1.16	440.	400	8.18	3104.
83	1.67	632.	193	7.27	2760.	401	2.41	916.
84	0.97	368.	194	1.84	700.	417	1.19	452.
85	1.17	444.	196	2.62	996.	428	2.02	768.
88	1.29	488.	206	1.18	448.	429	0.90	340.
91	1.95	740.	207	2.10	796.	441	0.91	344.
92	0.89	336.	208	1.70	644.	445	29.51	11200.
93	1.58	600.	219	0.95	360.	446	6.87	2608.
95	1.02	388.	224	1.18	448.	447	1.54	584.
97	1.00	380.	225	0.96	364.	460	0.96	364.
98	1.16	440.	228	1.34	508.	473	4.29	1628.
101	0.81	308.	235	1.44	548.	474	1.24	472.
102	0.81	308.	240	5.66	2148.	488	0.79	300.
103	4.61	1748.	241	1.47	556.	500	0.90	340.
104	29.34	11136.	251	1.05	400.	516	6.97	2644.
105	5.90	2240.	252	1.52	576.	517	2.00	760.
106	1.33	504.	253	1.34	508.	519	1.12	424.
107	0.94	356.	255	1.48	560.	528	1.19	452.
109	1.06	404.	256	2.76	1048.	533	100.00	37952.
118	1.02	388.	257	1.03	392.	534	30.23	11472.
119	2.33	884.	258	3.08	1168.	535	6.04	2292.
						536	1.24	472.

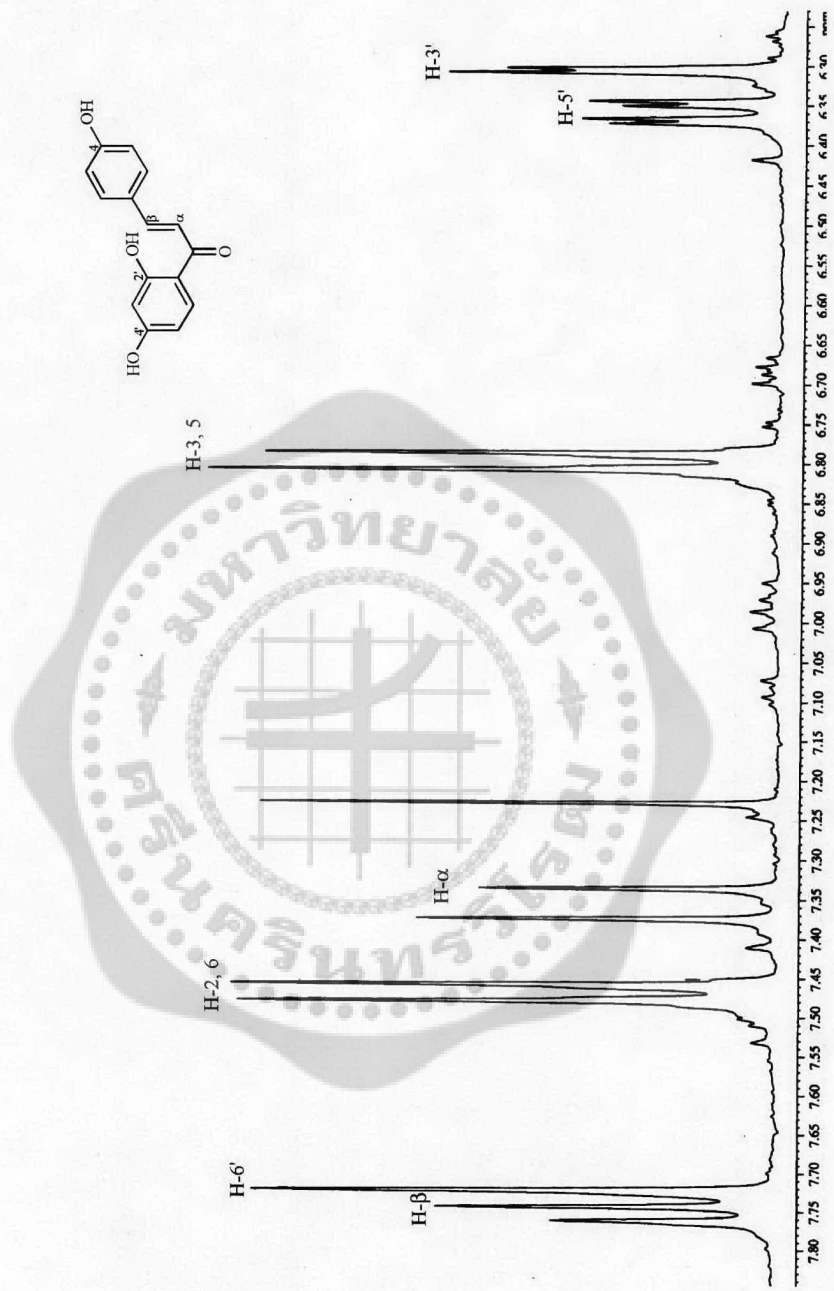
MS data of compound i

HF1E-1411



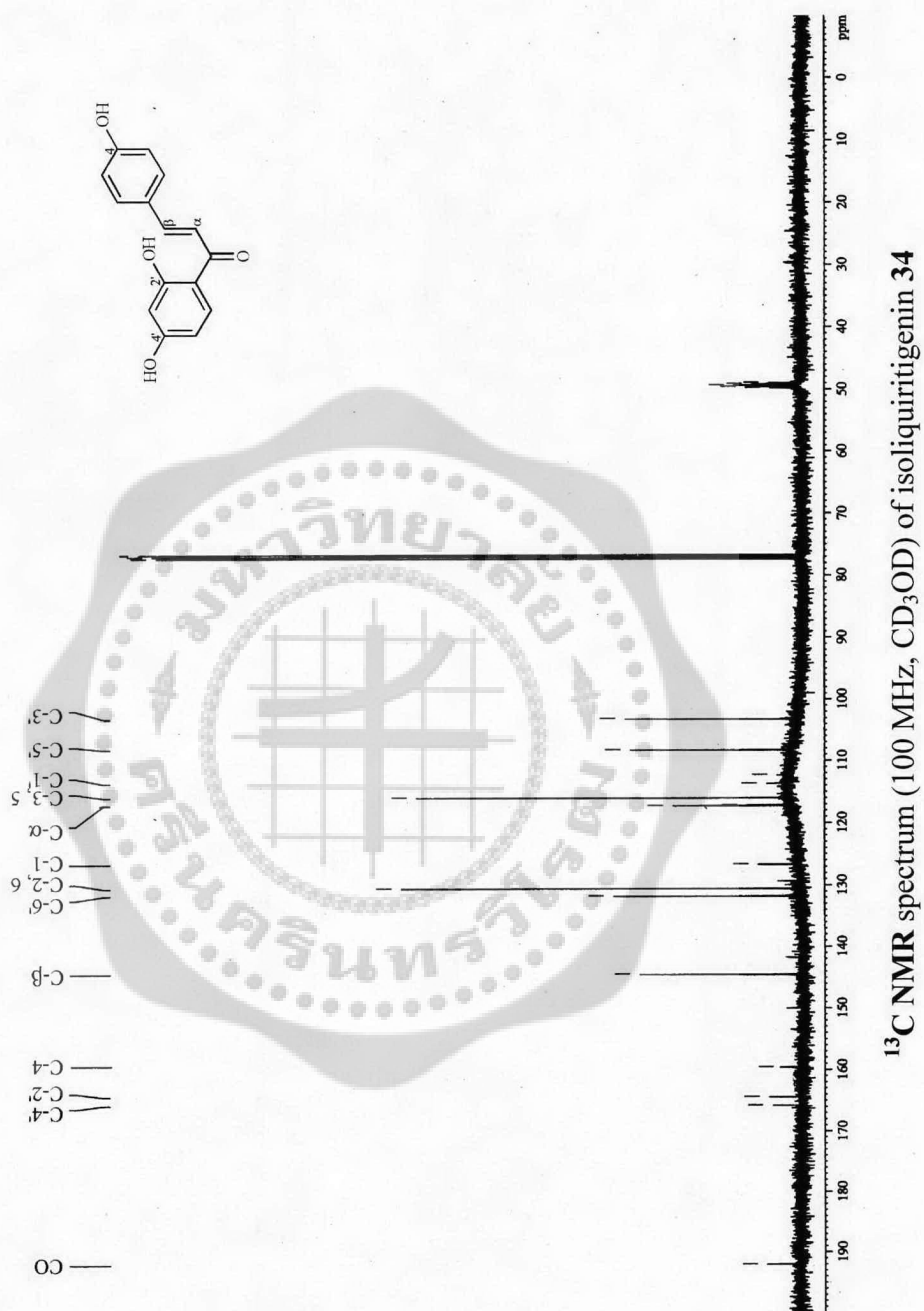
^1H NMR spectrum (400 MHz, CD_3OD) of isoliquiritigenin **34**

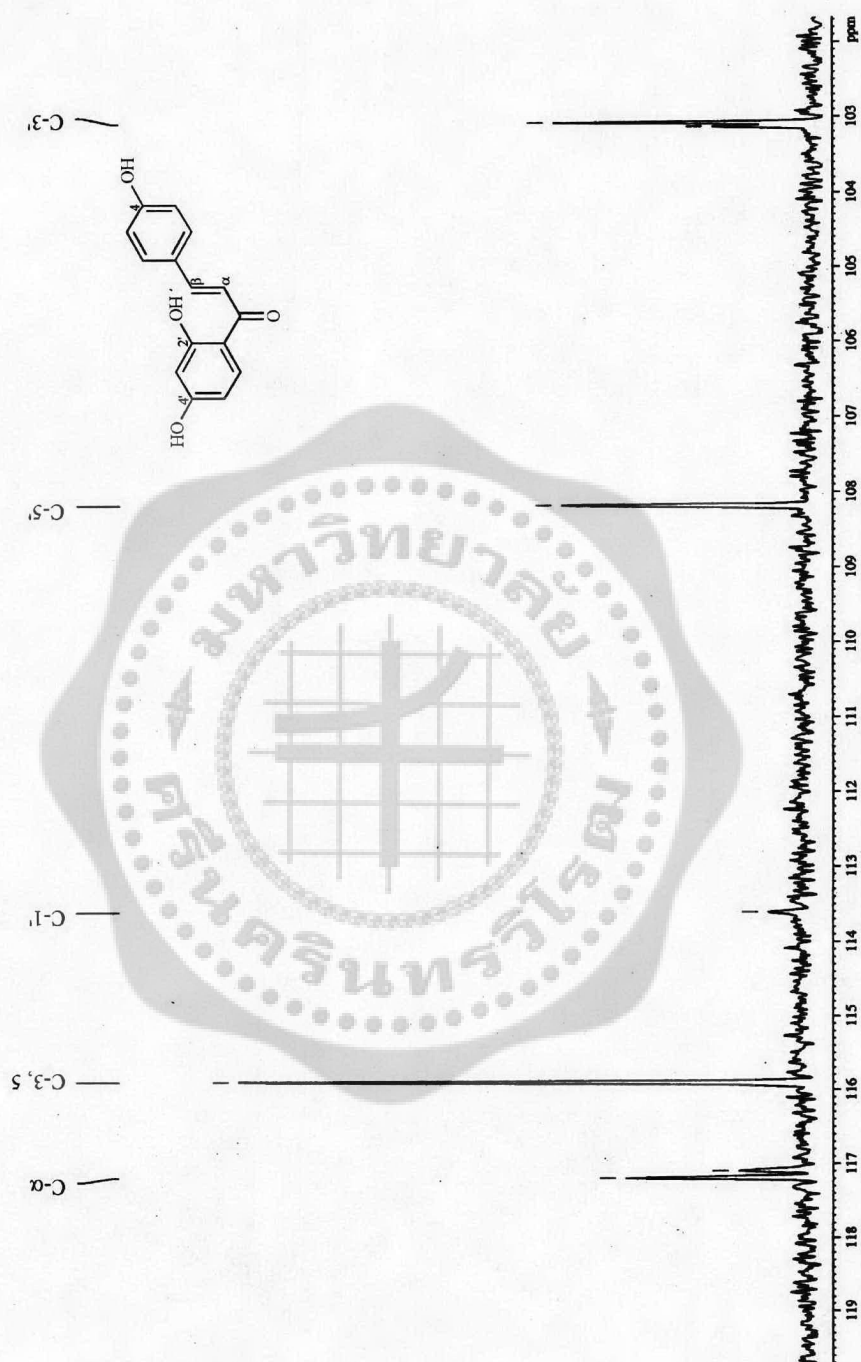
HF1E-1411



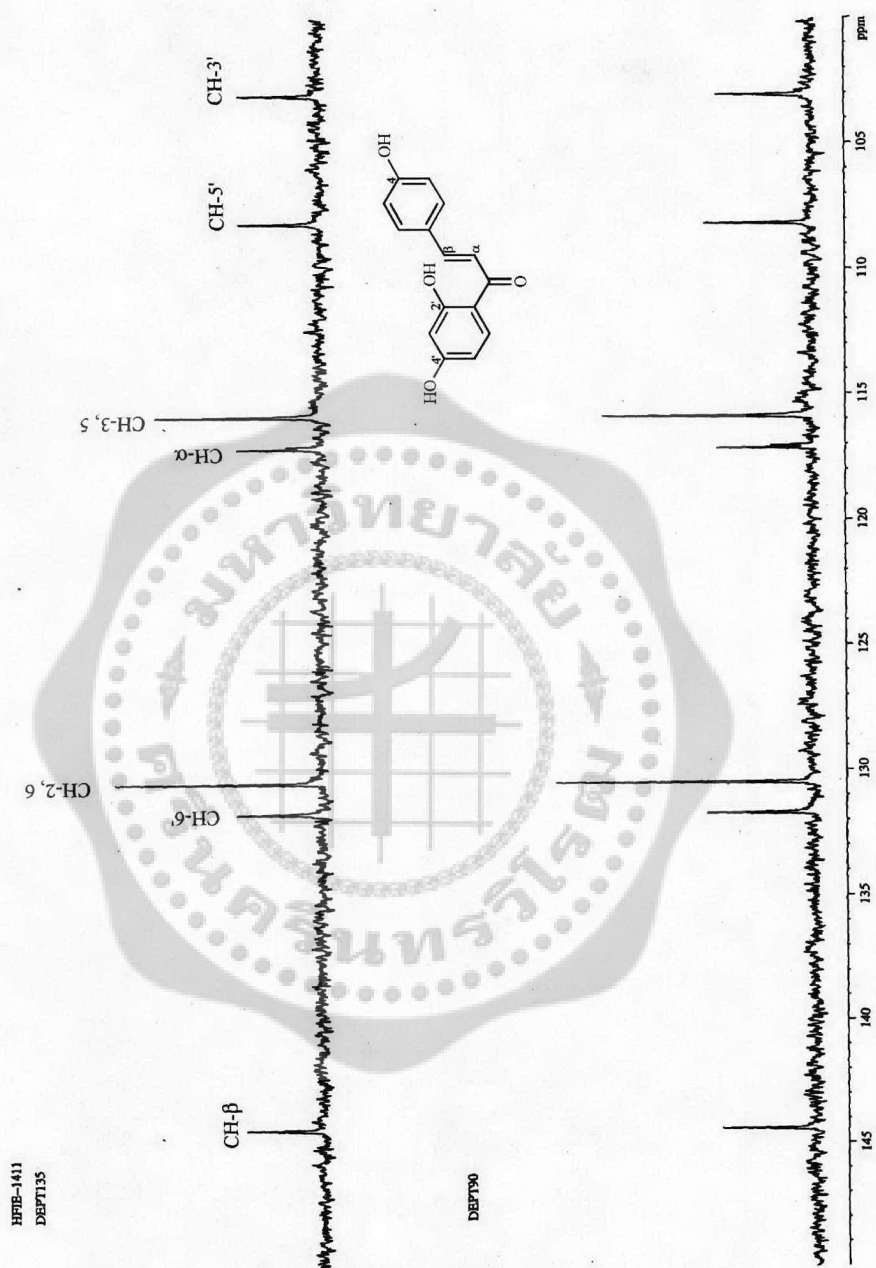
¹H NMR spectrum (400 MHz, CD₃OD) of isoliquiritigenin 34

HF1B-1411

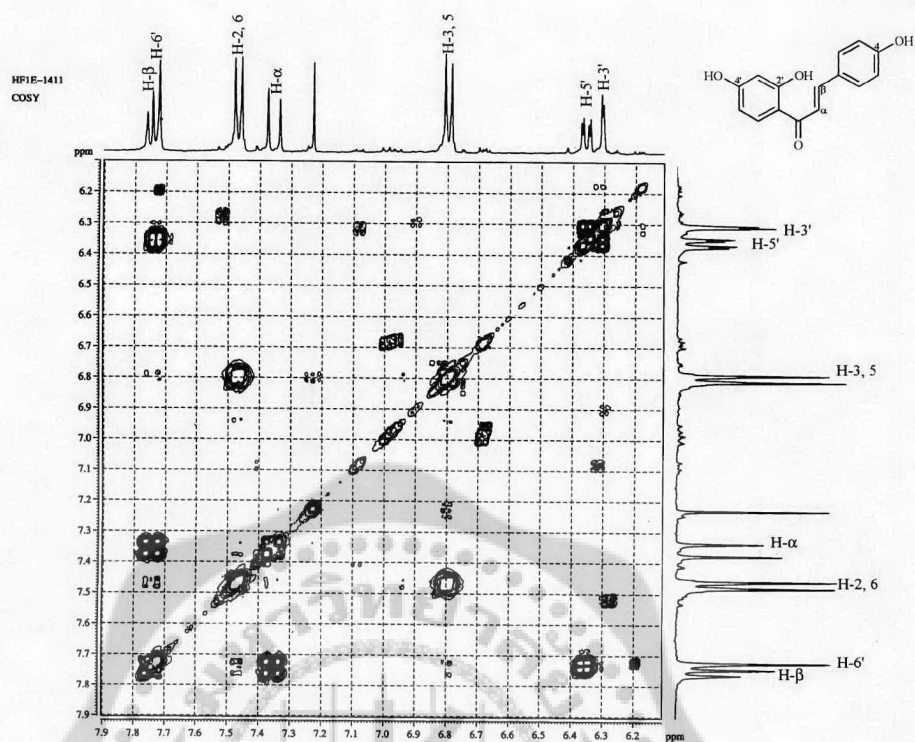




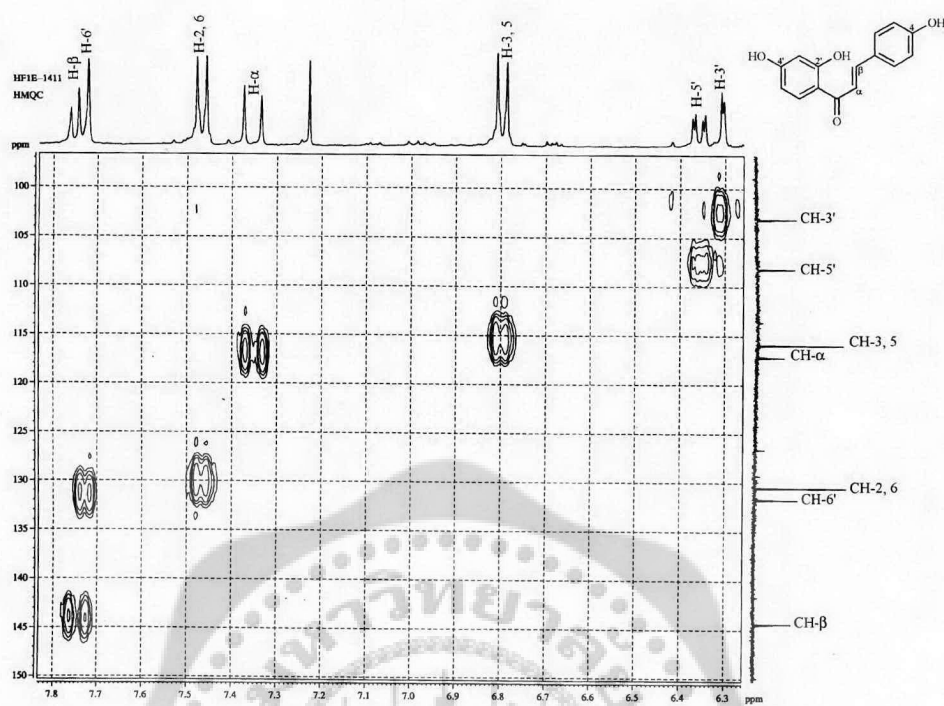
^{13}C NMR spectrum (100 MHz, CD_3OD) of isoliquiritigenin 34



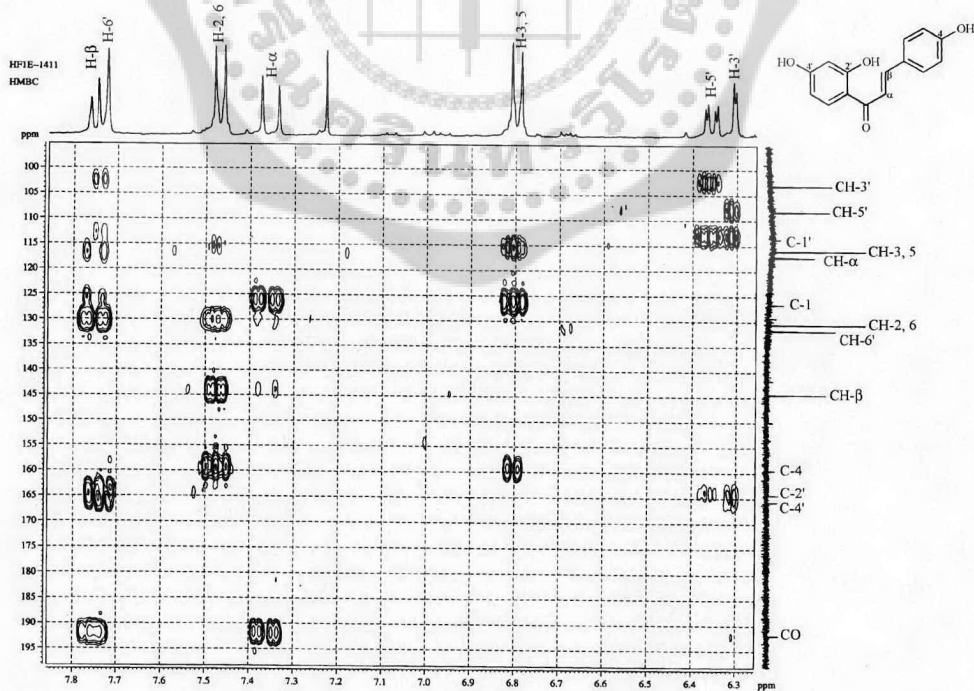
DEPT 135, 90 spectrum of isoliquiritigenin 34



^1H - ^1H COSY spectrum (CD_3OD) of isoliquiritigenin 34

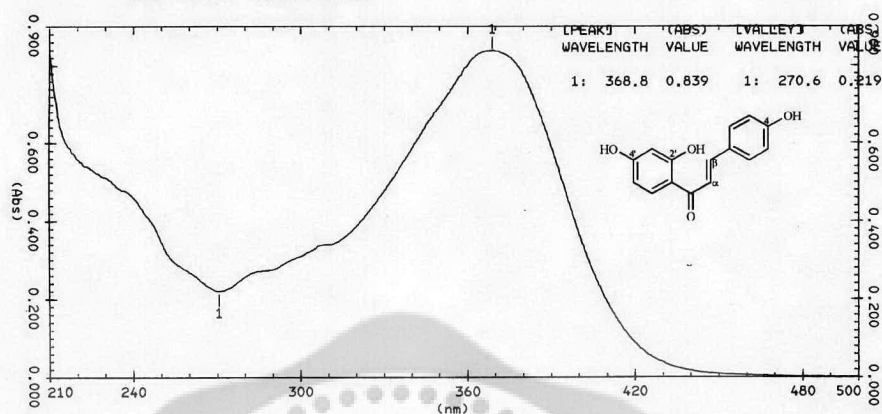


HMQC spectrum of isoliquiritigenin 34



HMBC spectrum of isoliquiritigenin 34

UV-VIS SPECTRA



SAMPLE : HF1E1411
CONC. :

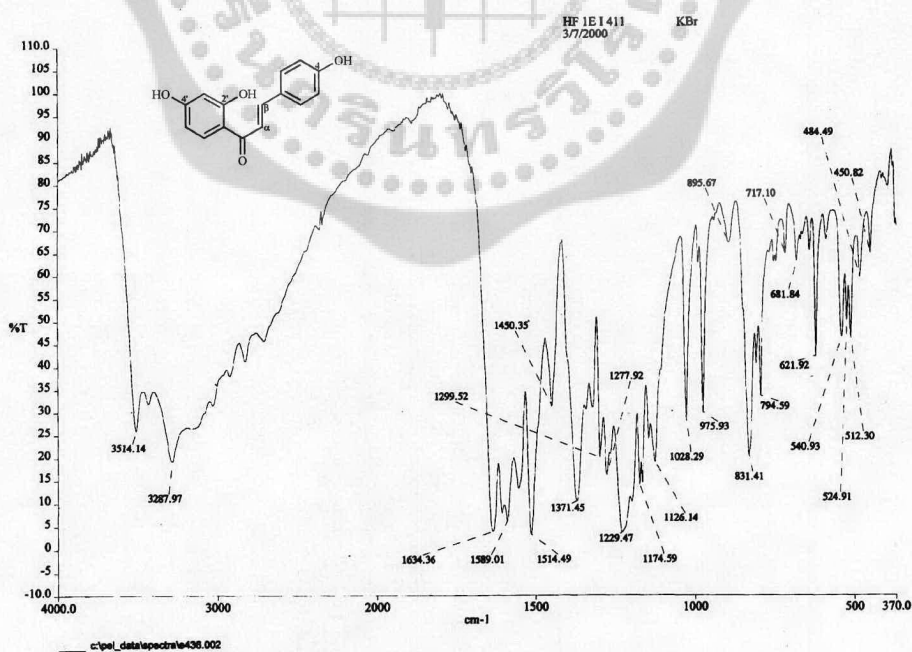
REFERENCE : MeOH
PATHLENGTH : 1 cm.
COMMENT : 0.3 mg. in 4 mL/dil. 7 times/dil. 3 times
threshold 0.005 abs. instrument from envi-tox Lab

SPEED : FAST
DATE : 23-02-00

SLIT : 2.0

ANALYST :

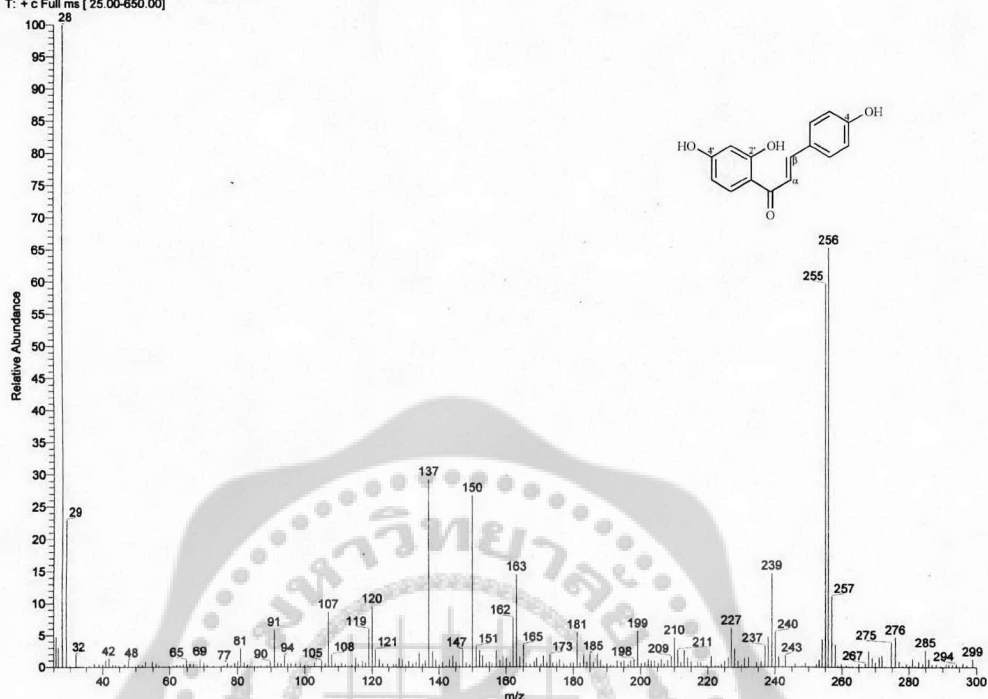
UV spectrum of isoliquiritigenin 34



IR spectrum of isoliquiritigenin 34

C:\Xcalibur\ChemRaw\HF raw\Hf1e1411
mw. 256
Hf1e1411#748 RT: 6.89 AV: 1 NL: 2.01E6
T: + c Full ms [25.00-650.00]

02/18/00 04:09:35



MS spectrum of isoliquiritigenin 34

C:\Xcalibur\ChemRaw\HF raw\Hf1e1411
mw. 256

02/18/00 04:09:35

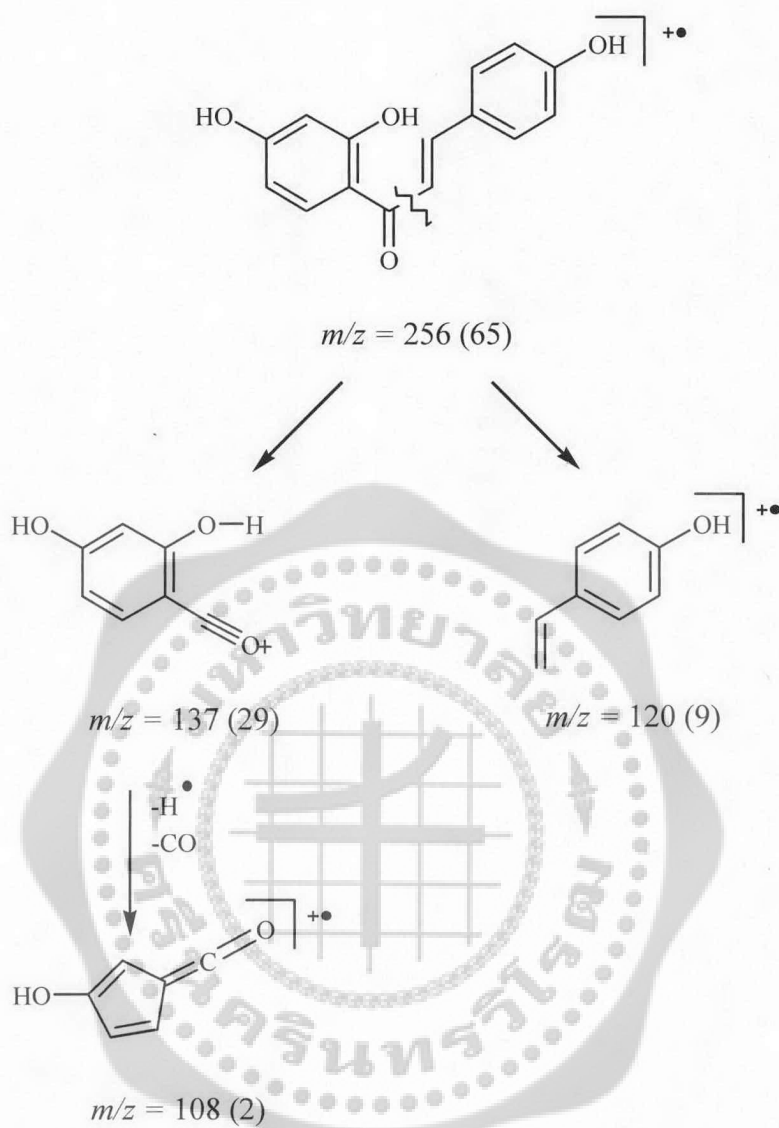
Hf1e1411#748 RT: 6.89

T: + c Full ms [25.00-650.00]

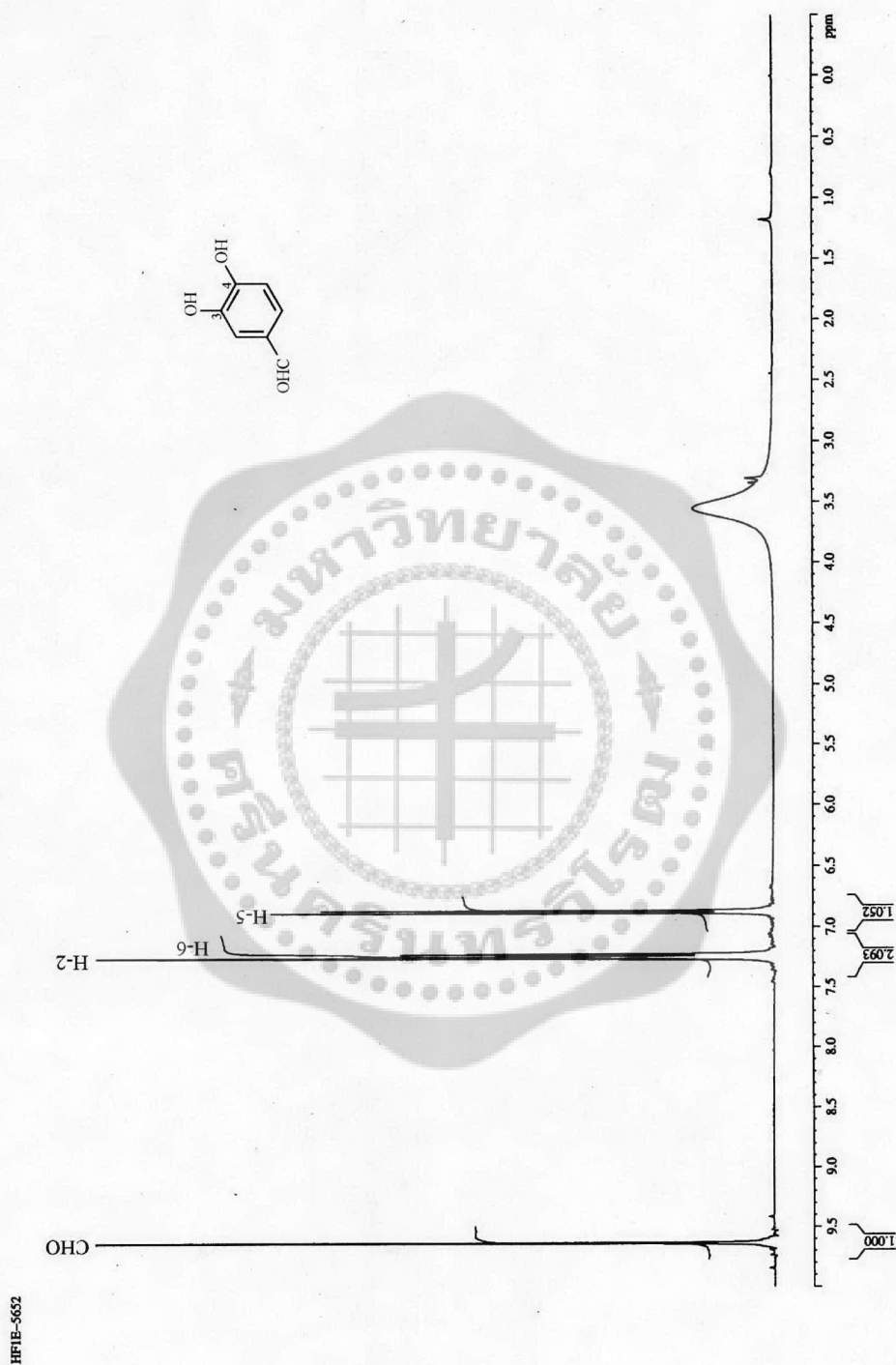
m/z= 25.00-300.00

m/z	Intensity	Relative			
25.66	95216.0	4.73	170.99	35427.0	1.76
26.38	60675.0	3.02	172.99	40235.0	2.00
27.64	2011699.0	100.00	181.05	111002.0	5.52
28.80	462002.0	22.97	183.05	39282.0	1.95
31.74	39015.0	1.94	184.98	42968.0	2.14
80.89	58560.0	2.91	187.09	42244.0	2.10
90.98	118471.0	5.89	199.10	114663.0	5.70
94.01	36436.0	1.81	209.98	93320.0	4.64
107.03	174680.0	8.68	211.03	54976.0	2.73
108.05	38852.0	1.93	212.93	53332.0	2.65
119.01	120706.0	6.00	226.99	124313.0	6.18
119.99	192395.0	9.56	227.99	57118.0	2.84
120.96	55479.0	2.76	237.02	68419.0	3.40
134.10	44602.0	2.22	238.00	95978.0	4.77
136.98	589429.0	29.30	239.02	296856.0	14.76
138.10	49696.0	2.47	240.09	111715.0	5.55
144.11	36130.0	1.80	243.09	37135.0	1.85
147.10	54328.0	2.70	254.10	88027.0	4.38
149.96	539494.0	26.82	255.04	1202361.0	59.77
151.04	66220.0	3.29	255.93	1314875.0	65.36
152.02	53766.0	2.67	256.98	224141.0	11.14
153.02	36943.0	1.84	257.94	72187.0	3.59
157.06	52753.0	2.62	267.96	49600.0	2.47
159.00	35717.0	1.78	274.69	76680.0	3.81
161.98	157340.0	7.82	275.94	93420.0	4.64
163.07	293588.0	14.59	284.90	52848.0	2.63
165.09	71699.0	3.56			

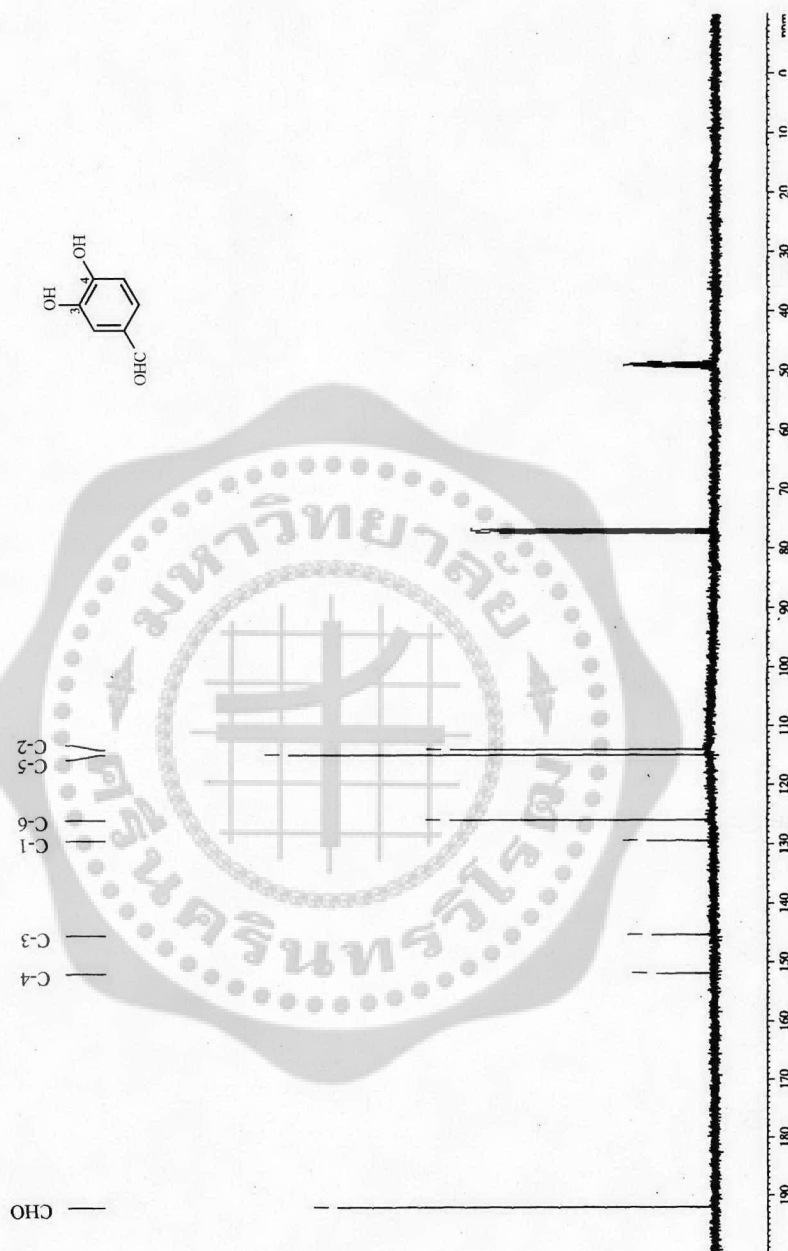
MS data of isoliquiritigenin 34

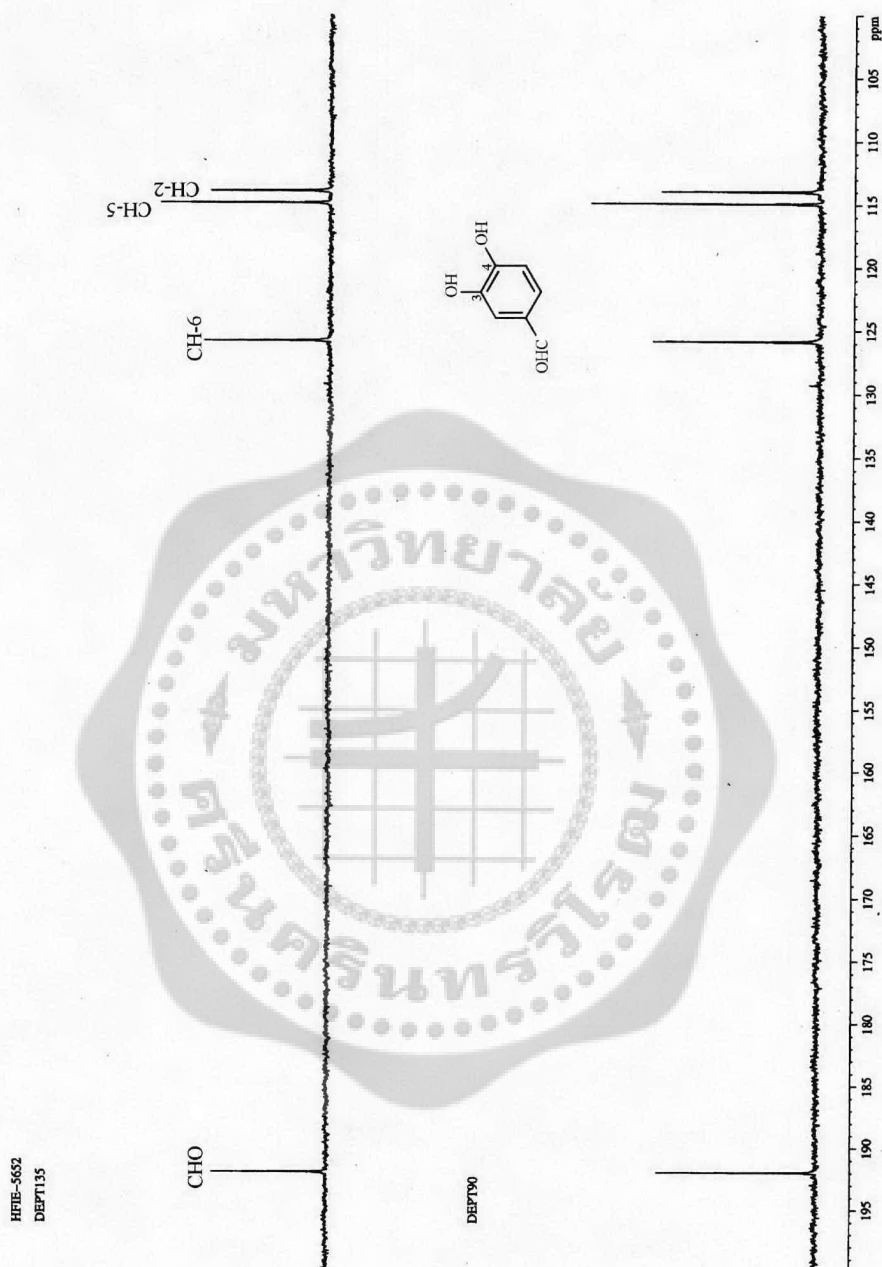


Mass fragmentation of isoliquiritigenin **34**

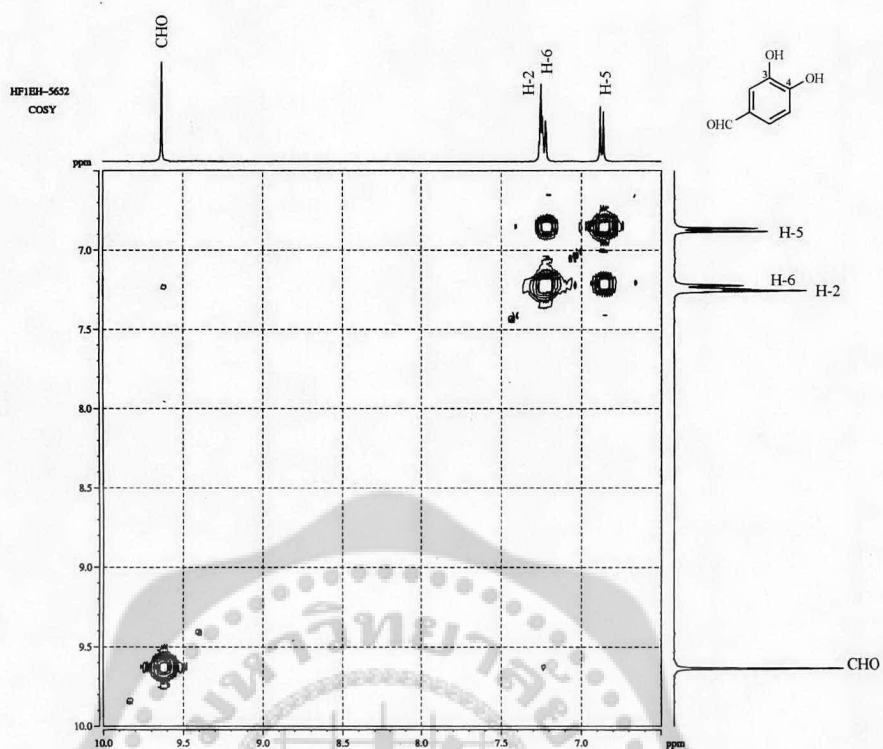


HFIE-5632

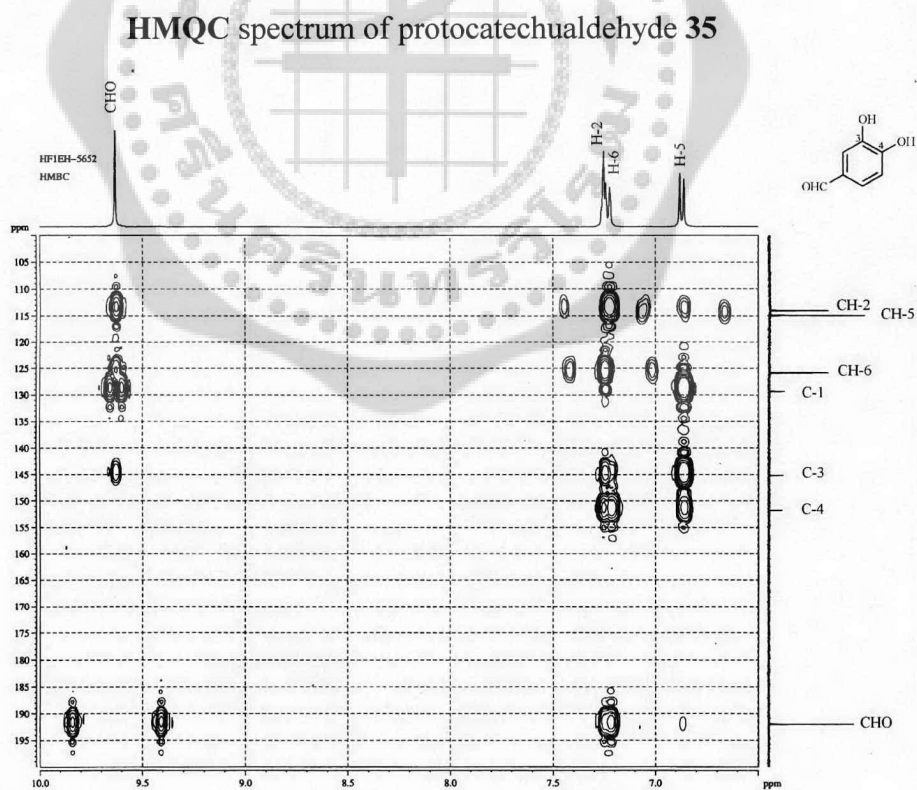
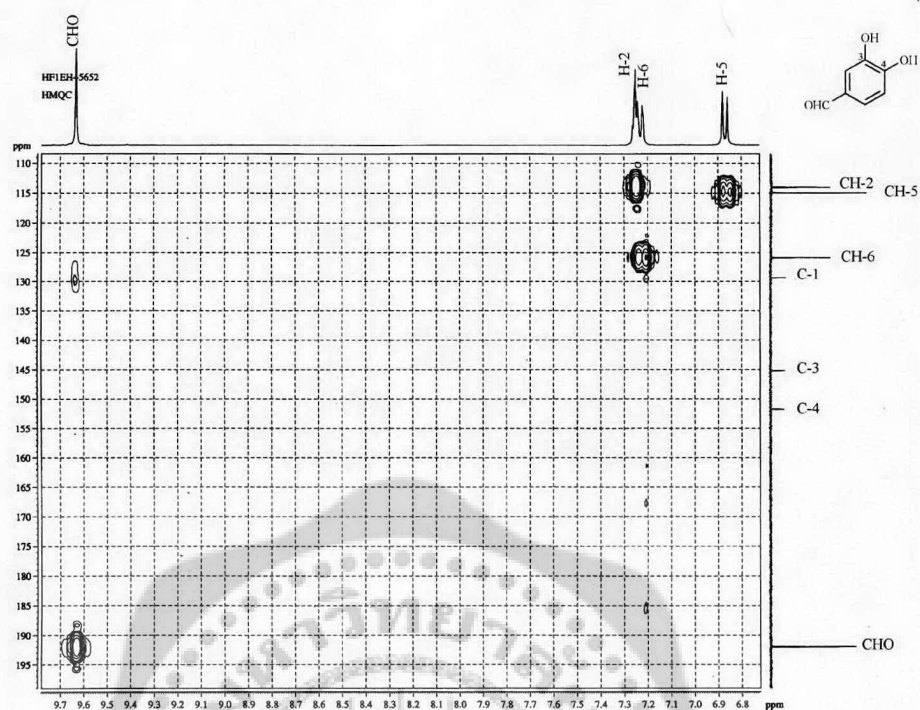
 ^{13}C NMR spectrum (100 MHz, CD_3OD) of protocatechualdehyde 35



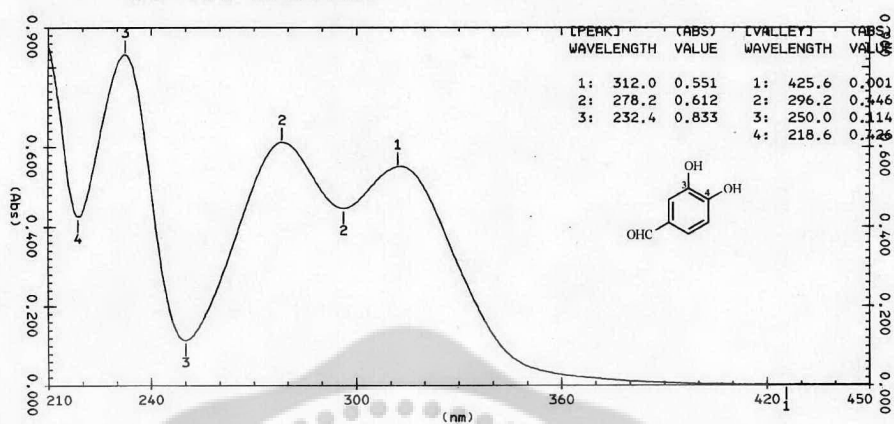
DEPT 135, 90 spectrum of protocatechualdehyde 35



^1H - ^1H COSY spectrum (CD_3OD) of protocatechualdehyde **35**



UV-VIS SPECTRA



SAMPLE : HF1EH5652
CONC. :

REFERENCE : MeOH
PATHLENGTH : 1 cm.

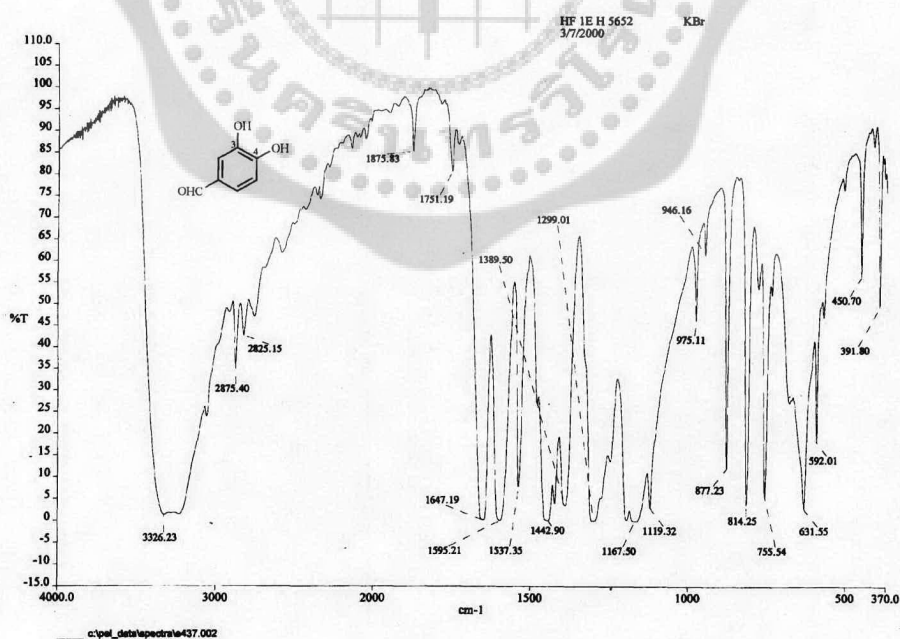
SPEED : FAST
DATE : 23-02-00

SLIT : 2.0

COMMENT : 1.0 mg. in 5 ml./dil. 10 times/dil. 4 times
threshold 0.005 abs. Instrument from envi-tox Lab

ANALYST :

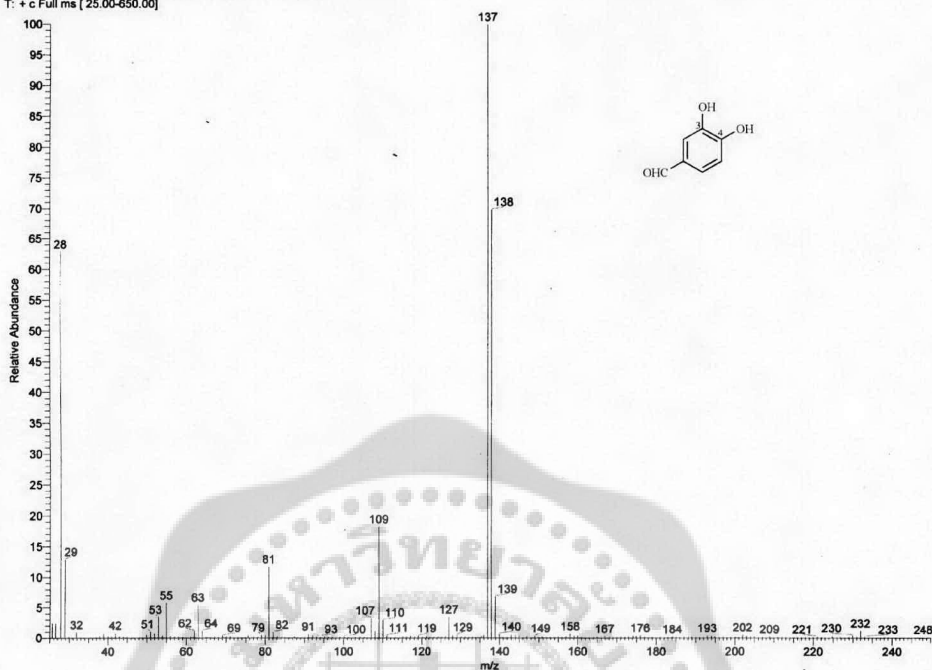
UV spectrum of protocatechualdehyde 35



IR spectrum of protocatechualdehyde 35

C:\Xcalibur\ChemRaw\HF raw\HF1EH5652
 mw. 139
 HF1EH5652#207-241 RT: 1.93-2.25 AV: 35 NL: 3.46E6
 T: + c Full ms [25.00-650.00]

02/18/00 03:51:51



MS spectrum of protocatechualdehyde 35

C:\Xcalibur\ChemRaw\HF raw\HF1eh5652

02/18/00 03:51:51

mw. 139

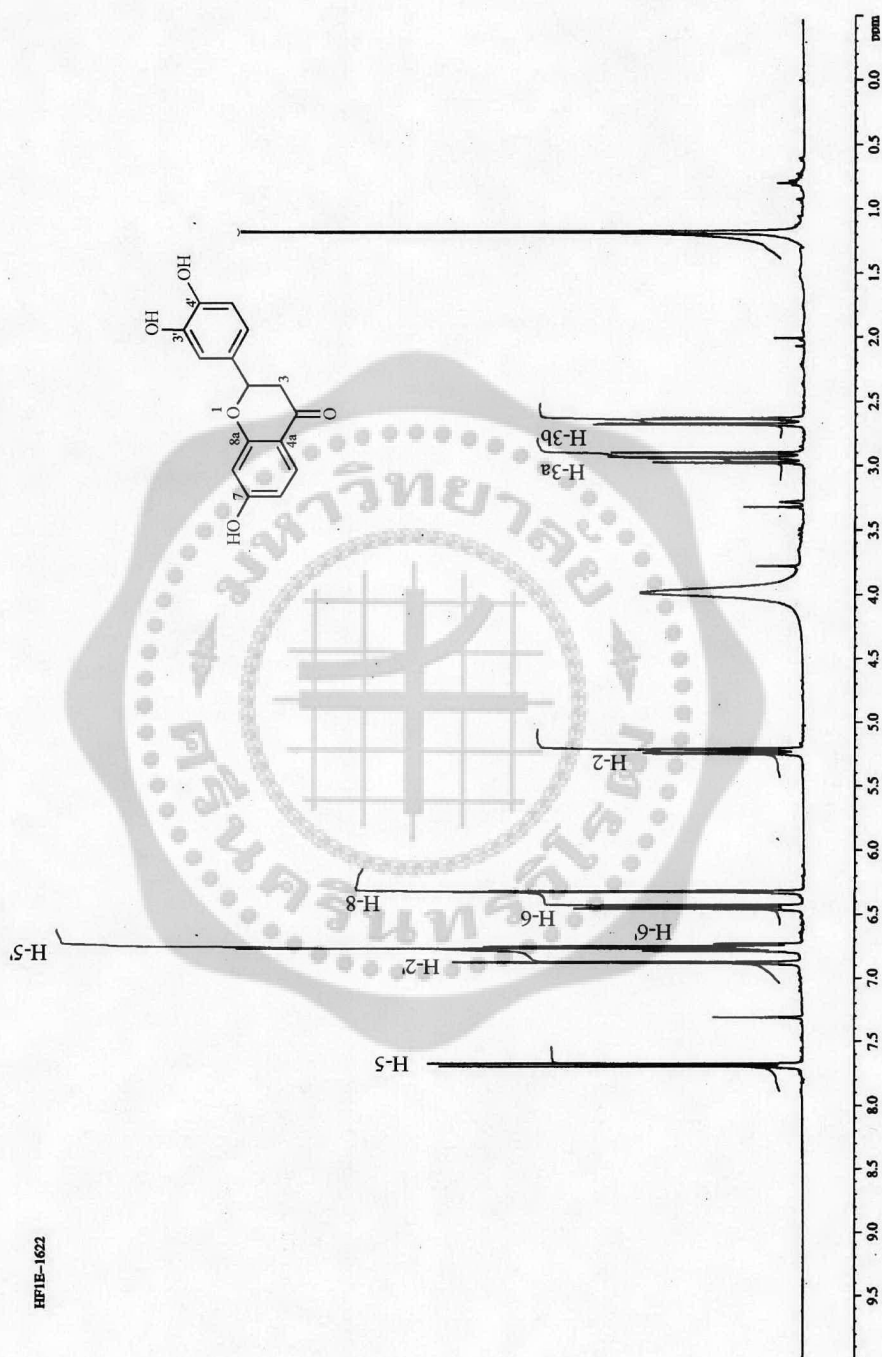
HF1EH5652#207-241 RT: 1.93-2.25 AV: 35

T: + c Full ms [25.00-650.00]

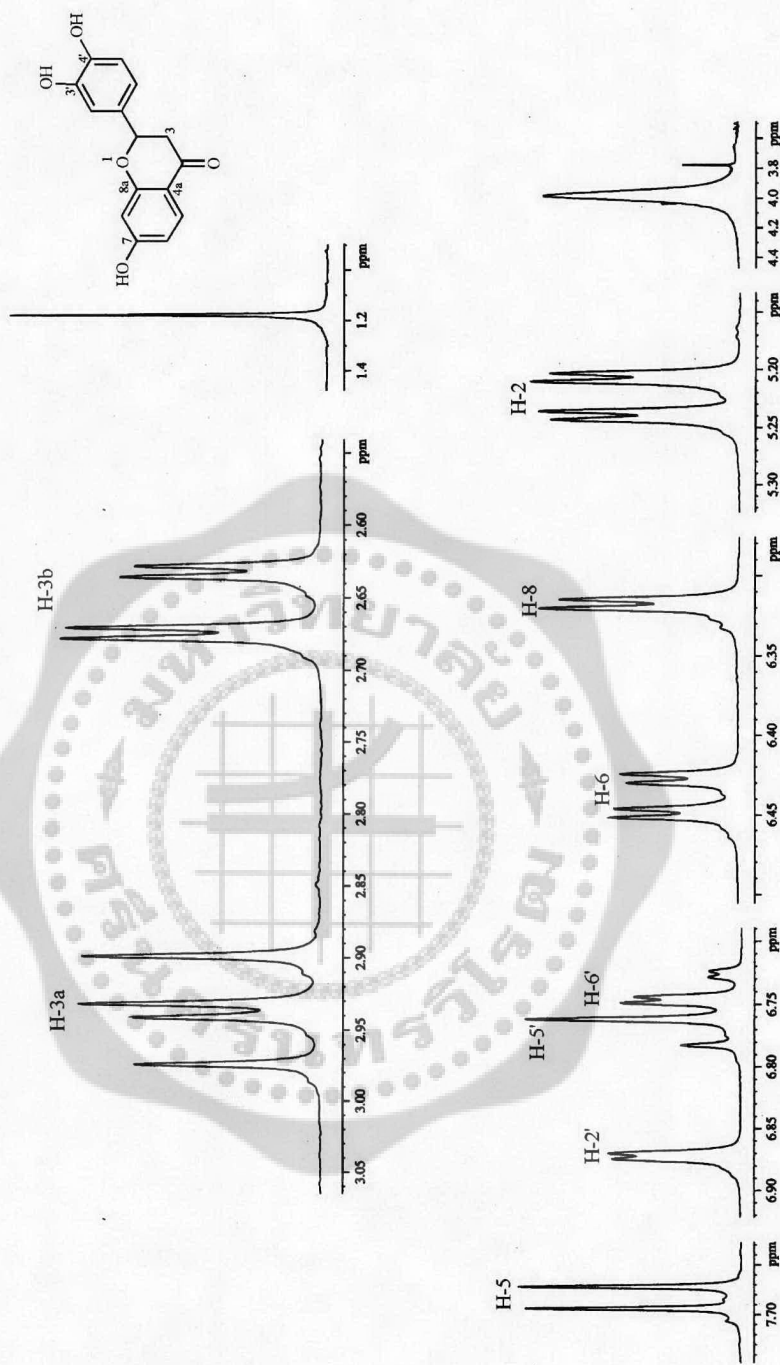
m/z= 25.00-250.00

m/z	Intensity	Relative			
25.60	83139.5	2.40	107.06	113766.4	3.29
26.47	82157.6	2.38	108.00	37584.0	1.09
27.74	2171491.9	62.81	109.04	629648.5	18.21
28.85	445529.8	12.89	110.04	102033.8	2.95
31.86	31355.8	0.91	111.01	14795.9	0.43
38.90	25402.7	0.73	117.10	11409.3	0.33
40.93	11253.1	0.33	119.10	11929.8	0.35
41.92	27540.5	0.80	127.05	119604.0	3.46
49.94	16746.1	0.48	128.04	13177.2	0.38
50.95	35342.9	1.02	129.11	16955.2	0.49
51.95	24912.2	0.72	135.94	16021.1	0.46
52.98	118712.1	3.43	136.96	3457414.3	100.00
53.96	11808.7	0.34	137.92	2416907.6	69.91
54.96	198704.4	5.75	138.96	235827.8	6.82
56.03	10457.0	0.30	139.98	22040.8	0.64
61.97	40315.9	1.17	142.99	17159.5	0.50
62.97	187345.0	5.42	144.02	13247.2	0.38
64.00	39892.5	1.15	145.00	11104.2	0.32
69.03	12581.3	0.36	148.95	11688.6	0.34
75.60	12254.5	0.35	158.03	22756.5	0.66
79.01	15533.1	0.45	160.03	13593.4	0.39
80.05	14460.9	0.42	174.16	11507.7	0.33
81.01	400966.5	11.60	175.98	13363.8	0.39
82.06	31897.7	0.92	192.90	13718.1	0.40
91.05	22221.6	0.64	202.01	16787.7	0.49
92.06	14602.4	0.42	229.97	13312.3	0.39
			232.00	40884.9	1.18

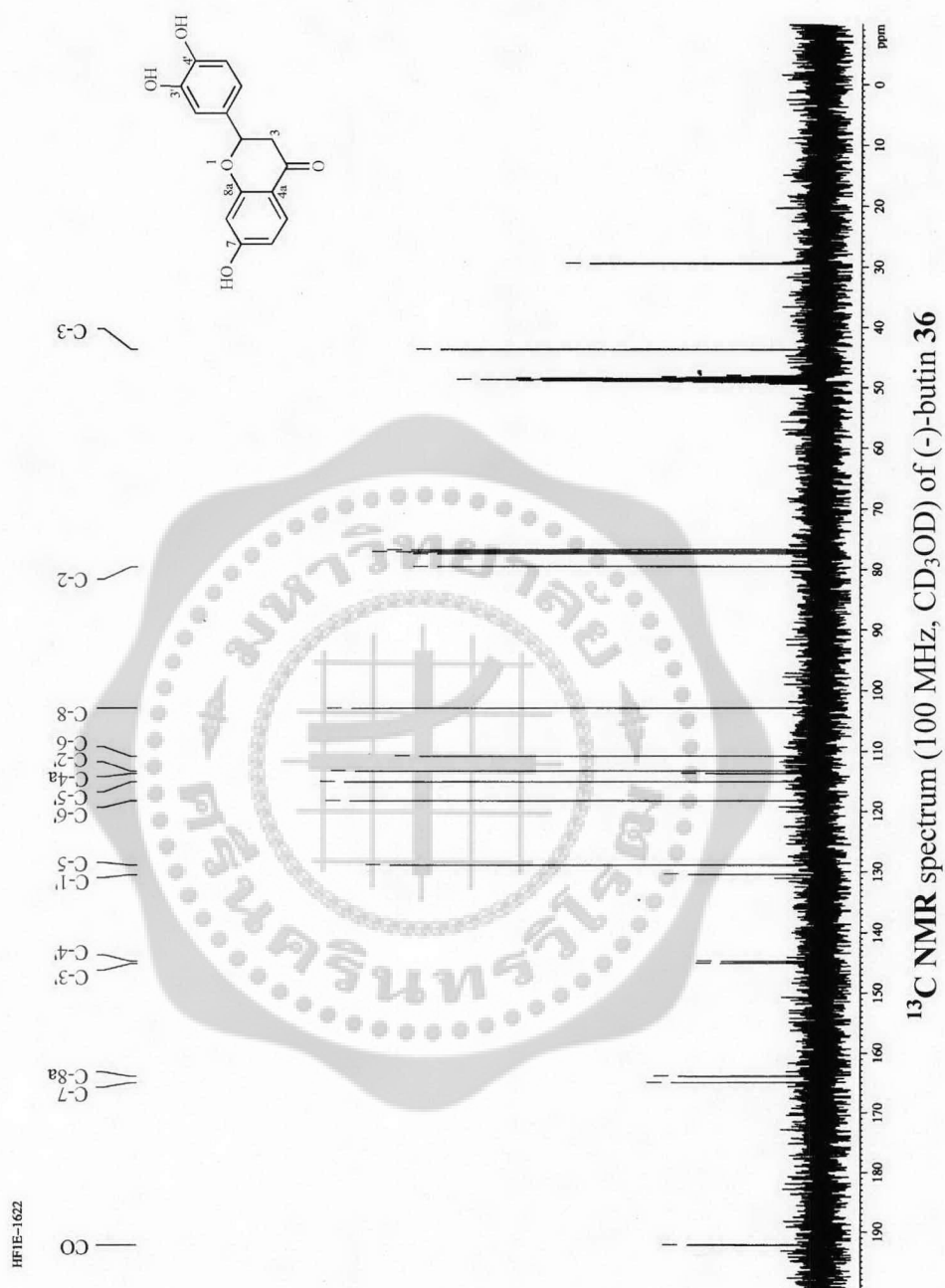
MS data of protocatechualdehyde 35

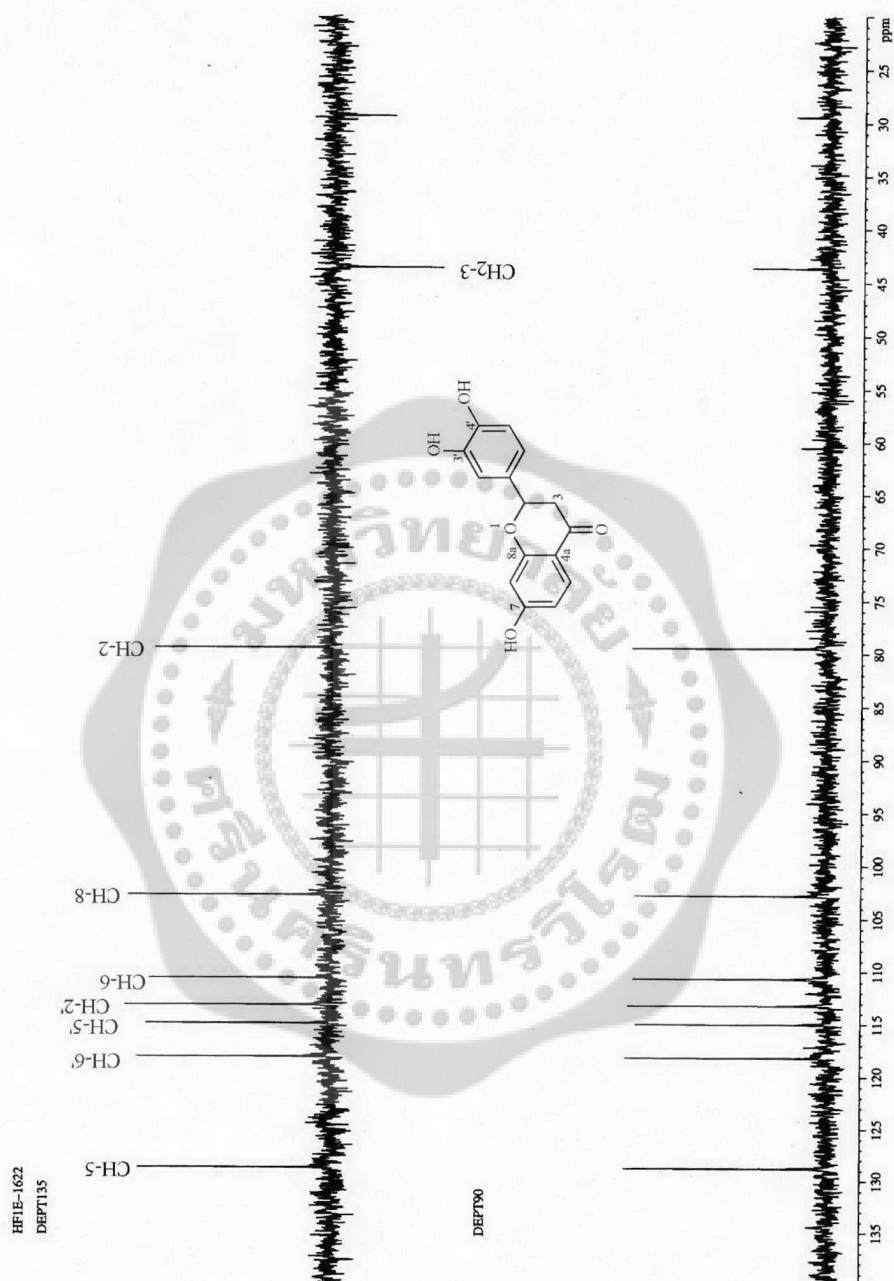


HFIE-1622

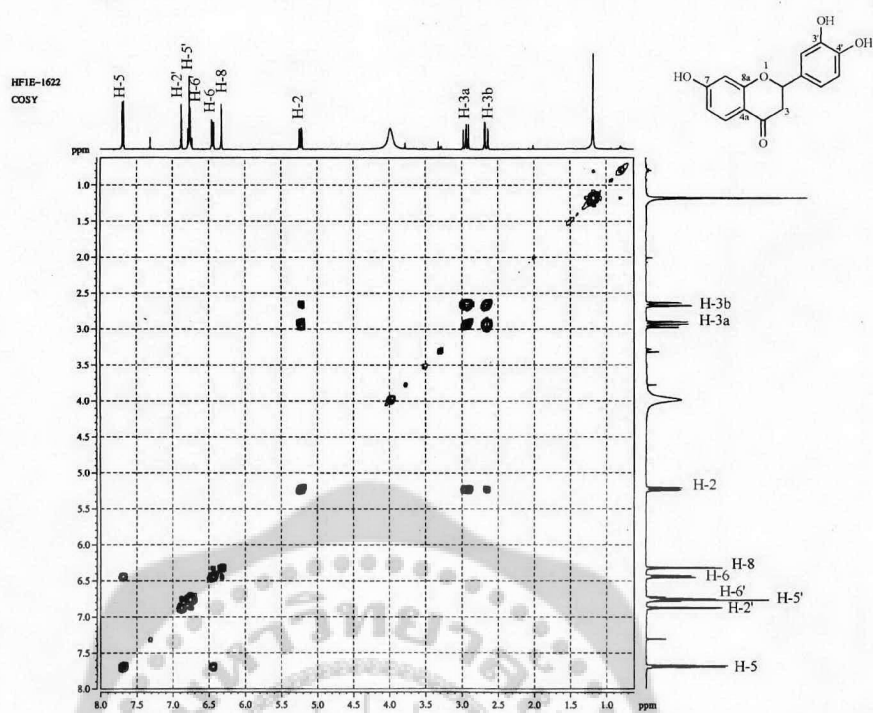


^1H NMR spectrum (400 MHz, CD_3OD) of (-)-butin 36

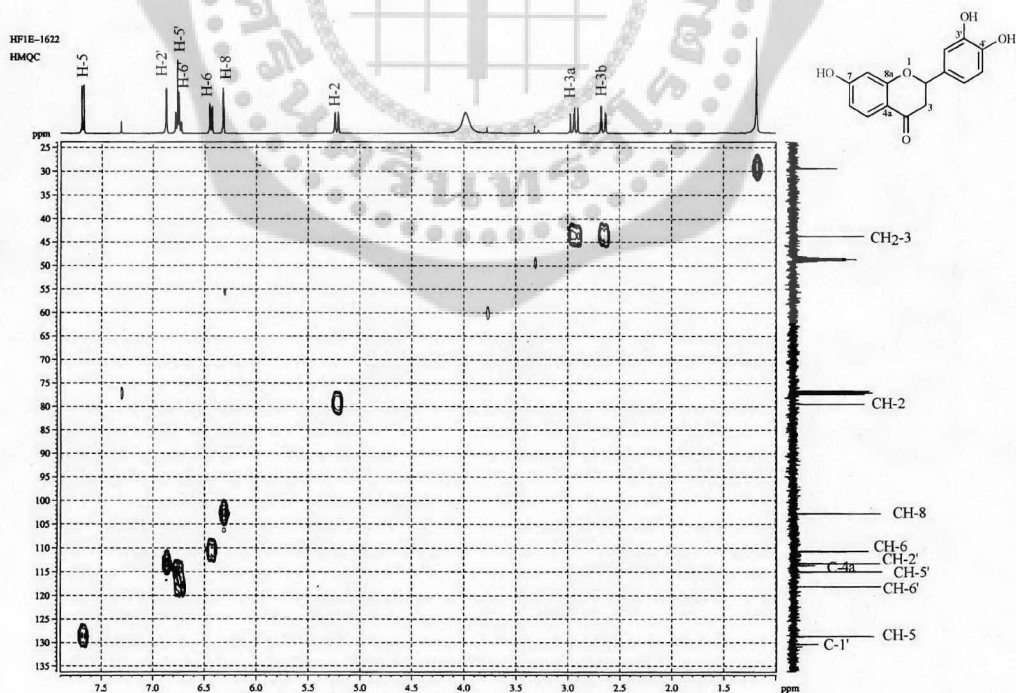




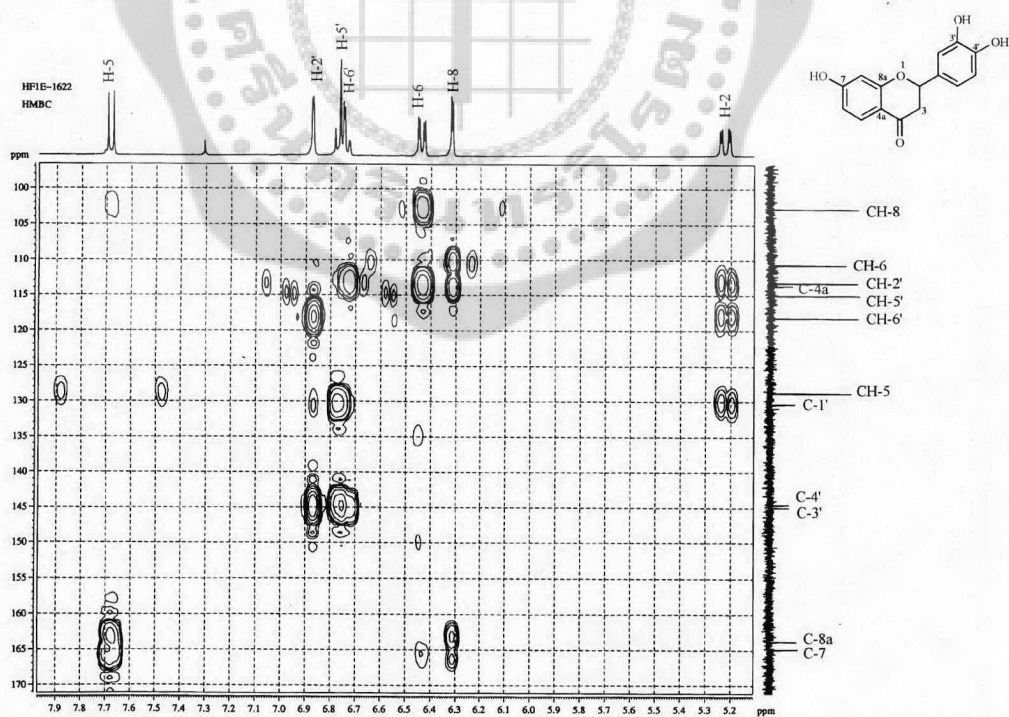
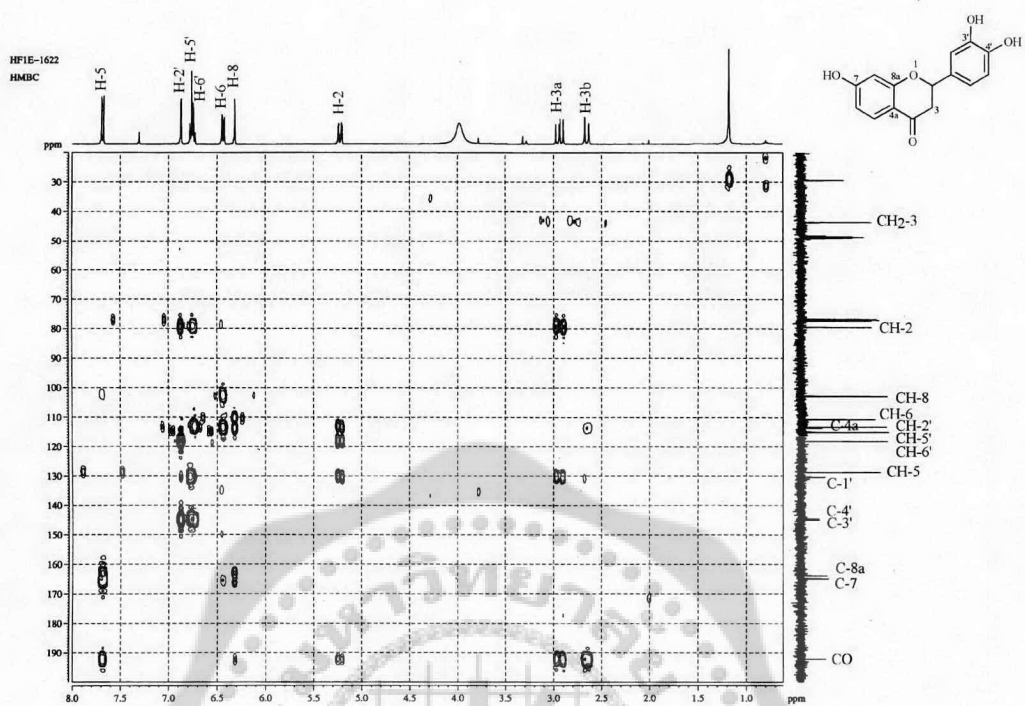
DEPT 135, 90 spectrum of (-)-butin 36



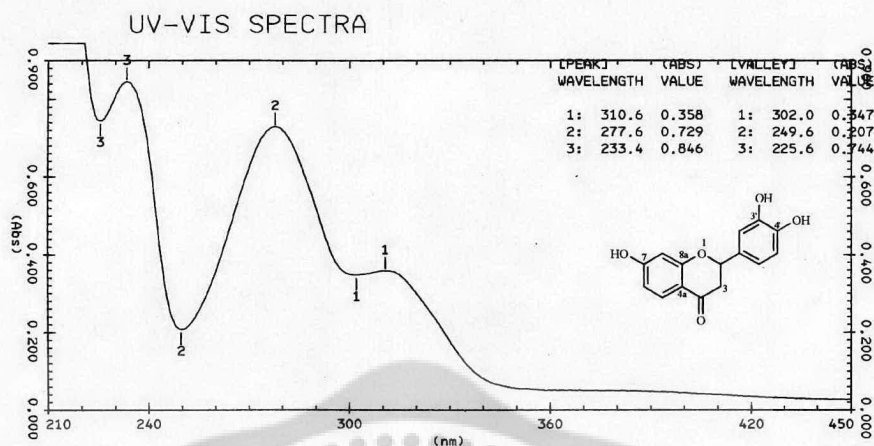
^1H - ^1H COSY spectrum (CD_3OD) of (-)-butin 36



HMQC spectrum of (-)-butin 36



HMBC spectrum of (-)-butin 36



SAMPLE : HF1E1622
CONC. :

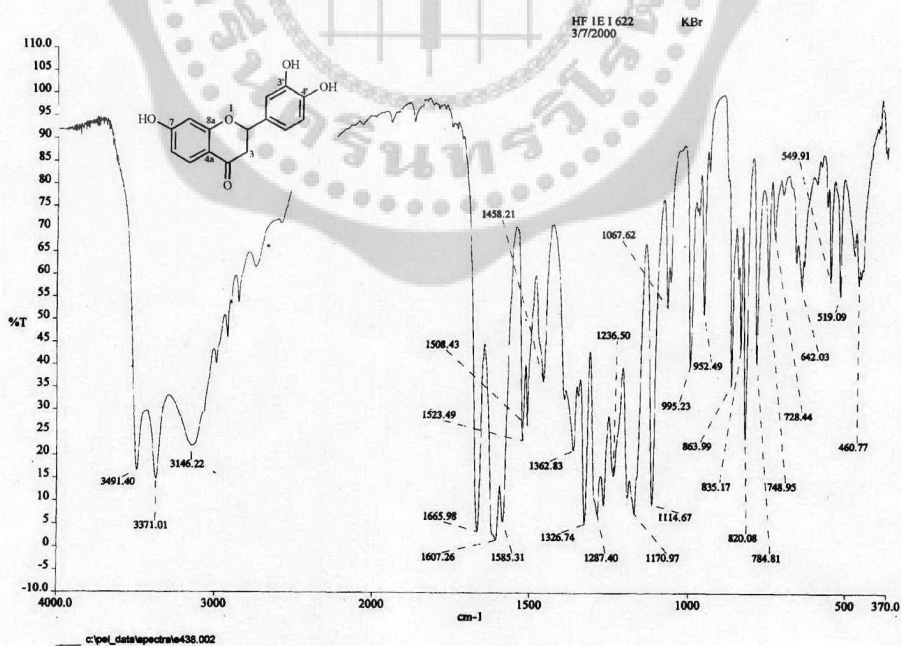
REFERENCE : MeOH
PATHLENGTH : 1 cm.

SPEED : FAST
DATE : 23-02-00

SLIT : 2.0

COMMENT : 1.1 mg in 5 ml/dil. 20 times (prepare 23-02-00)
threshold 0.005 abs. instrument from envi-tox Lab ANALYST :

UV spectrum of (-)-butin 36



IR spectrum of (-)-butin 36

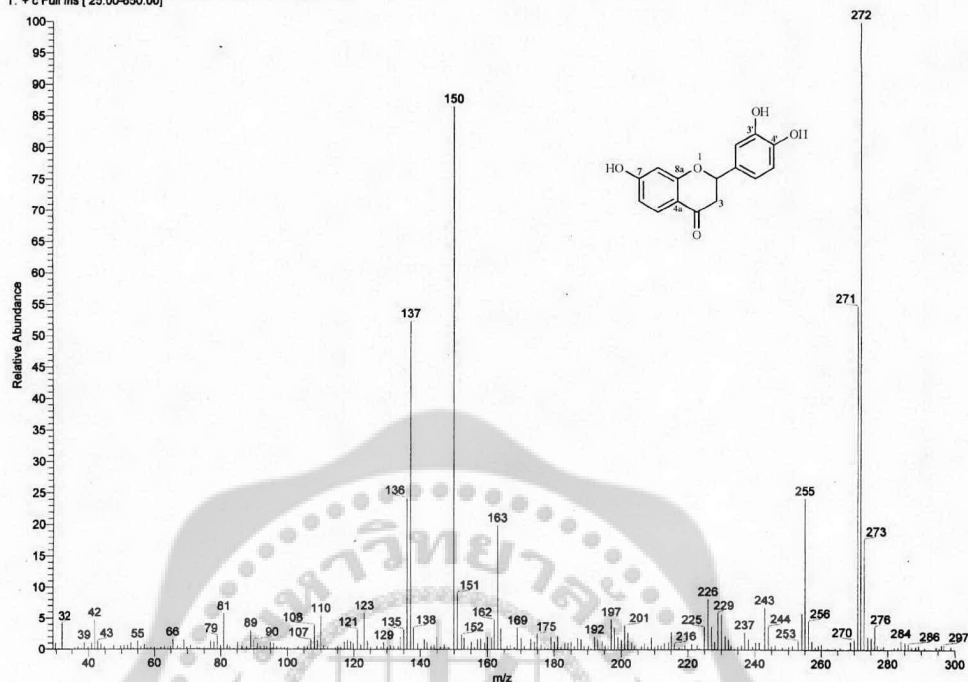
C:\Xcalibur\...HF1E1622_000218165444

02/18/00 04:54:44

mw.272

HF1E1622_000218165444#1233 RT: 11.30 AV: 1 NL: 9.55E5

T: + c Full ms [25.00-650.00]



MS spectrum of (-)-butin 36

C:\Xcalibur\...HF1E1622_000218165444

02/18/00 04:54:44

mw.272

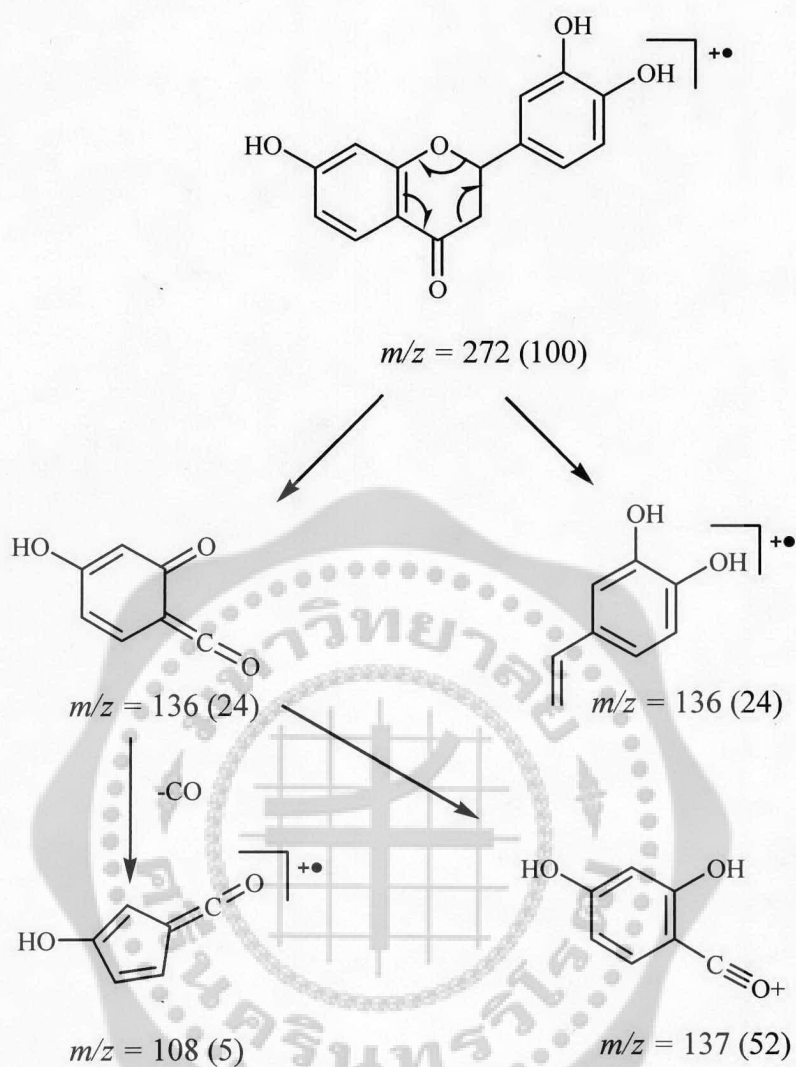
HF1E1622_000218165444#1233 RT: 11.30

T: + c Full ms [25.00-650.00]

m/z= 29.00-300.00

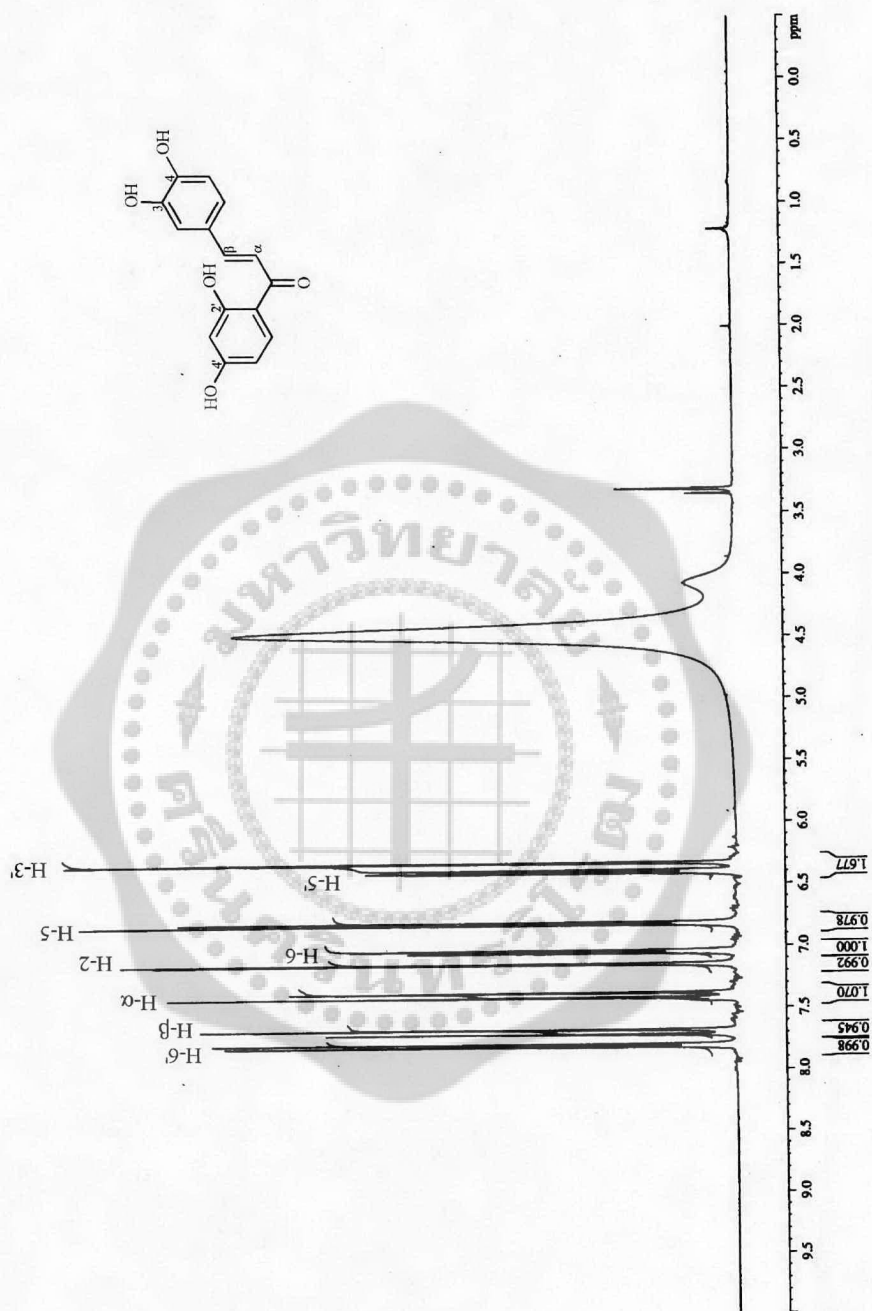
m/z	Intensity	Relative			
31.79	39447.0	4.13	191.98	19706.0	2.06
41.86	45104.0	4.72	196.99	46565.0	4.88
78.99	20286.0	2.12	198.00	33951.0	3.56
80.96	54705.0	5.73	199.02	19150.0	2.01
89.05	28737.0	3.01	200.98	37200.0	3.90
108.01	37339.0	3.91	201.99	26447.0	2.77
110.00	51514.0	5.39	209.09	18905.0	1.98
121.05	30119.0	3.15	215.04	26932.0	2.82
123.10	55625.0	5.83	224.89	34877.0	3.65
134.03	19016.0	1.99	226.06	77812.0	8.15
135.03	30343.0	3.18	227.07	34882.0	3.65
136.02	231315.0	24.22	228.92	56782.0	5.95
137.06	500268.0	52.39	230.03	54611.0	5.72
138.00	33632.0	3.52	231.18	20425.0	2.14
144.89	19584.0	2.05	236.95	26586.0	2.78
149.92	826380.0	86.54	242.92	64872.0	6.79
151.02	87347.0	9.15	244.04	36243.0	3.80
152.26	22720.0	2.38	254.00	55787.0	5.84
160.02	19676.0	2.06	255.02	230923.0	24.18
162.03	45729.0	4.79	256.12	44189.0	4.63
163.05	189867.0	19.88	271.02	524059.0	54.88
163.99	32183.0	3.37	271.90	954882.0	100.00
168.99	33302.0	3.49	272.90	169830.0	17.79
174.91	23502.0	2.46	274.01	19089.0	2.00
179.01	19955.0	2.09	275.02	18233.0	1.91
181.02	20325.0	2.13	276.04	35355.0	3.70
187.02	18194.0	1.91			

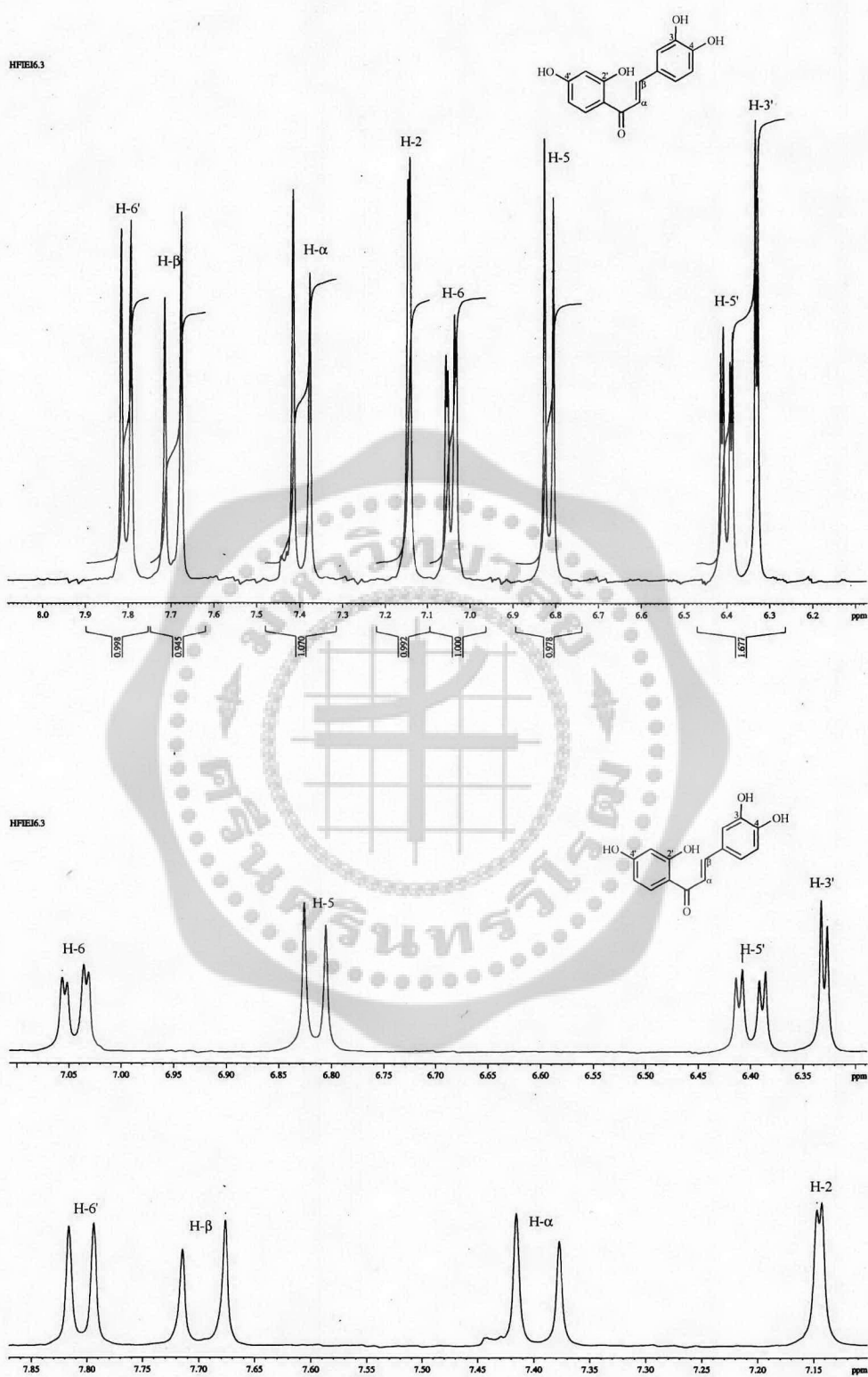
MS data of (-)-butin 36



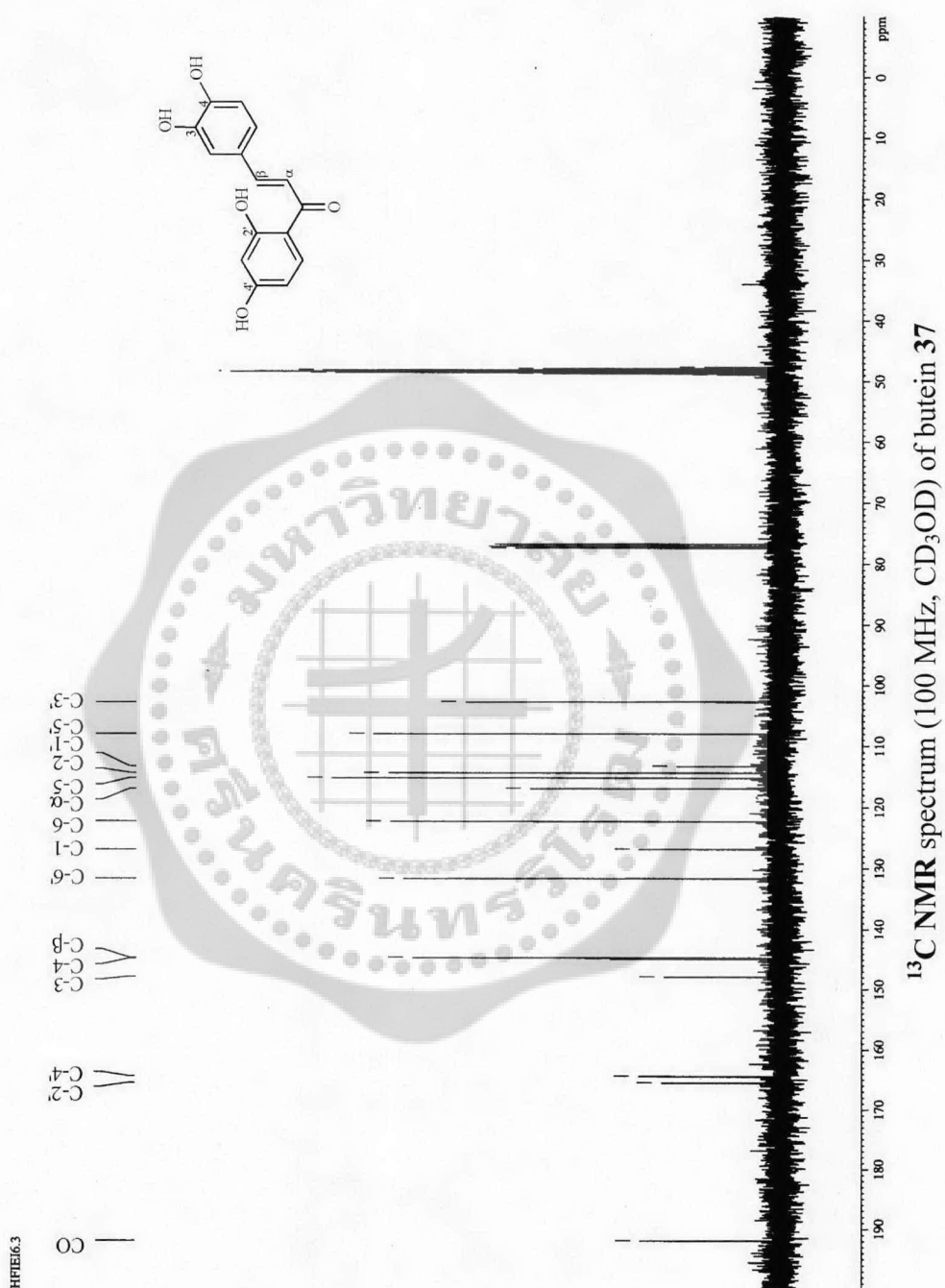
Mass fragmentation of (-)-butin 36

HF16163

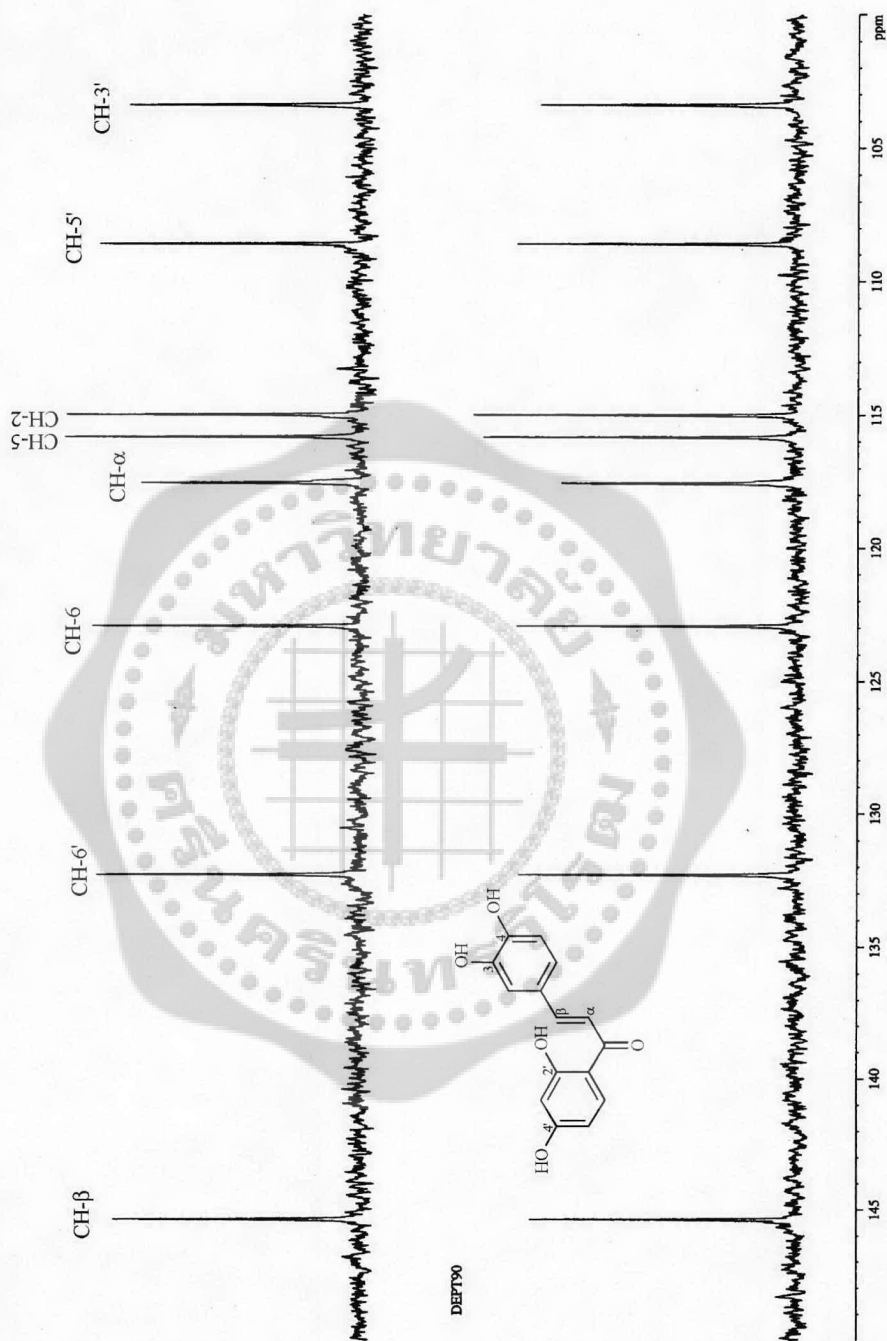
 ^1H NMR spectrum (400 MHz, CD_3OD) of butein 37



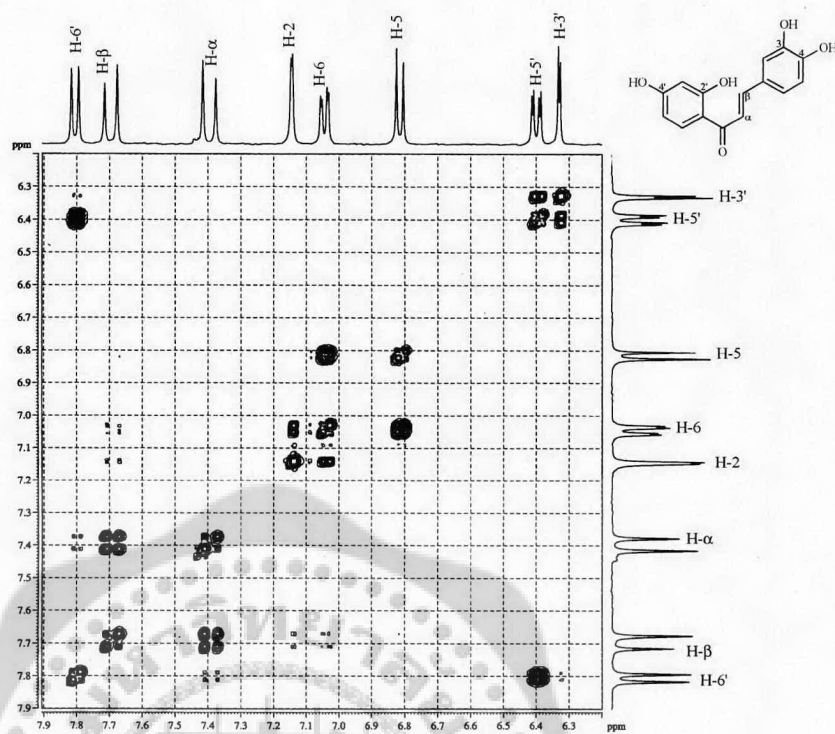
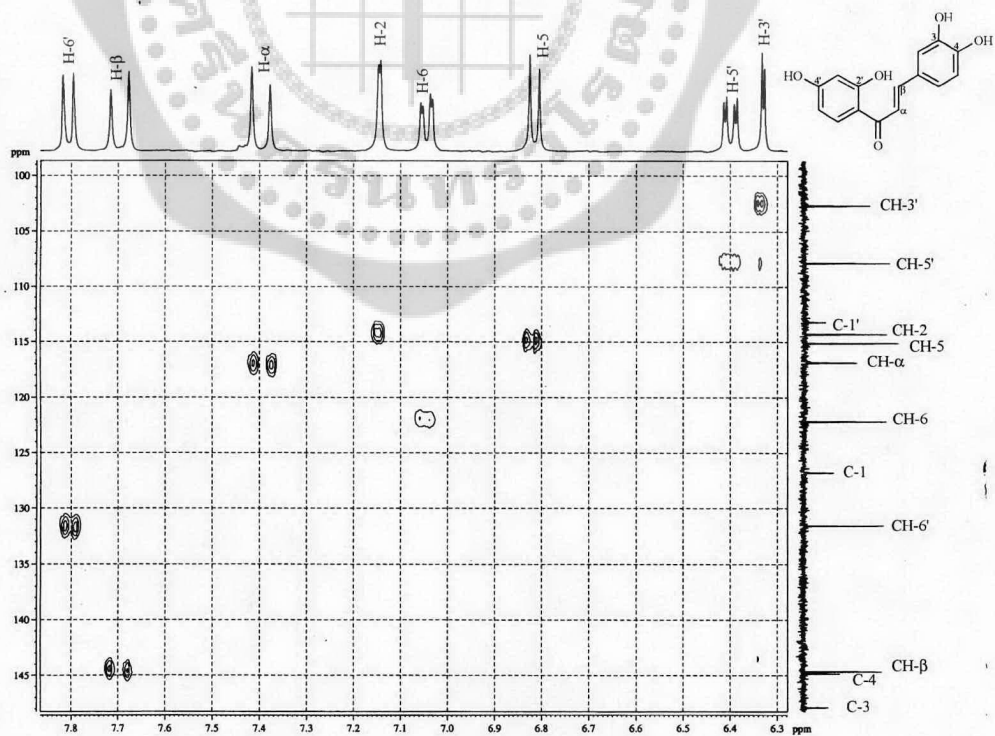
¹H NMR spectrum (400 MHz, CD₃OD) of butein 37

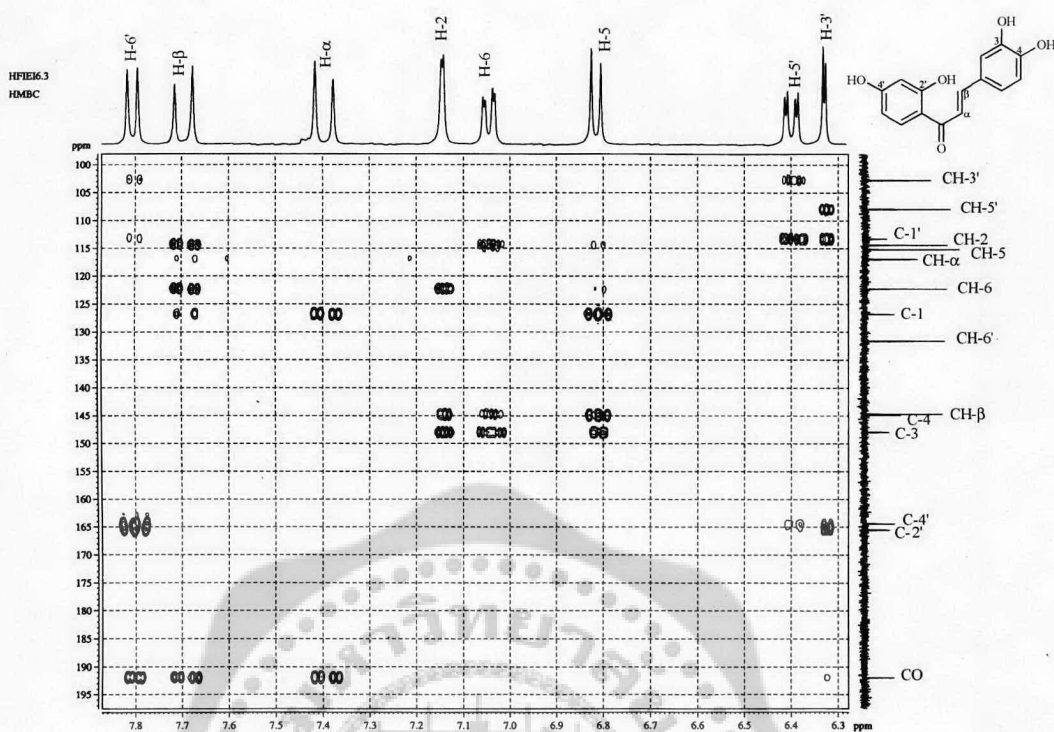


HFIEI6.3 DEPT135



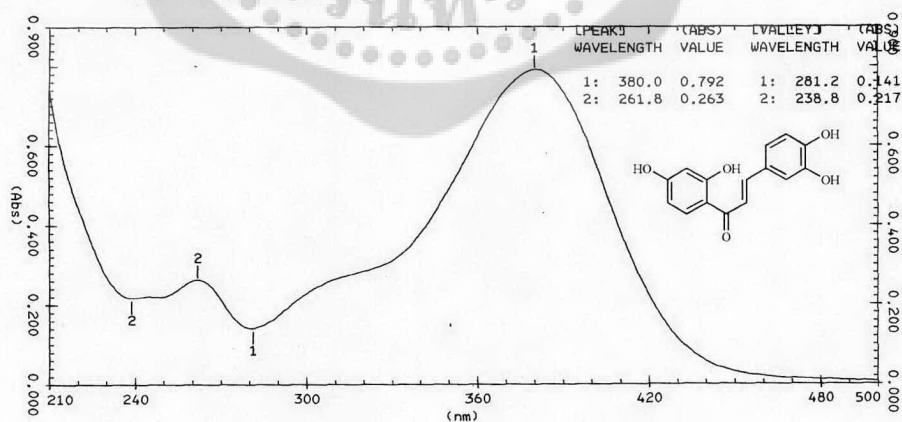
HFIEI6.3 COSY

HFIEI6.3
HMQC



HMBC spectrum of butein 37

UV-VIS SPECTRA



SAMPLE : HFIEI63
CONC. :

REFERENCE : MeOH
PATHLENGTH : 1 cm.

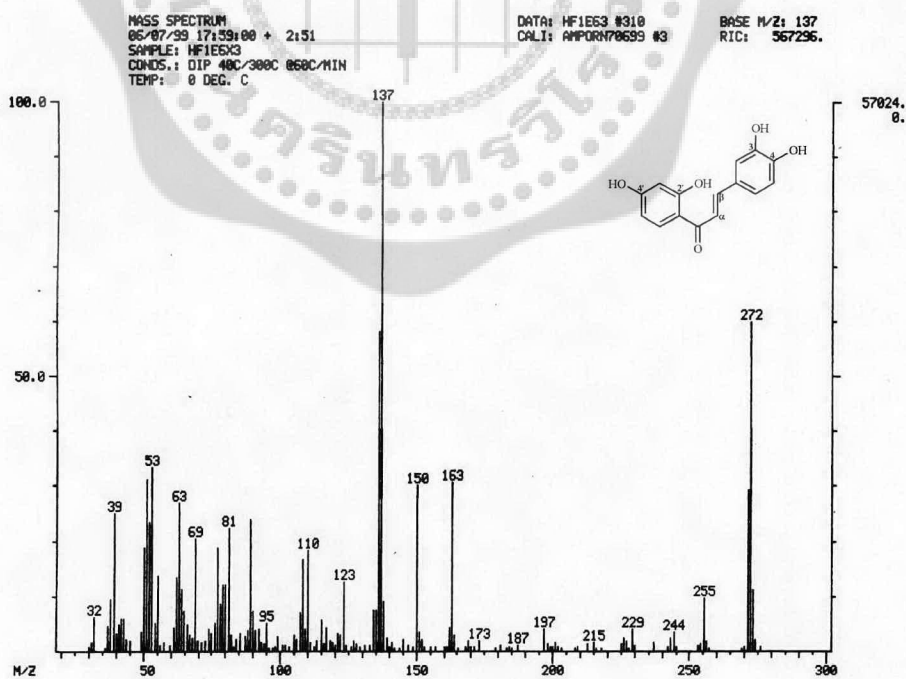
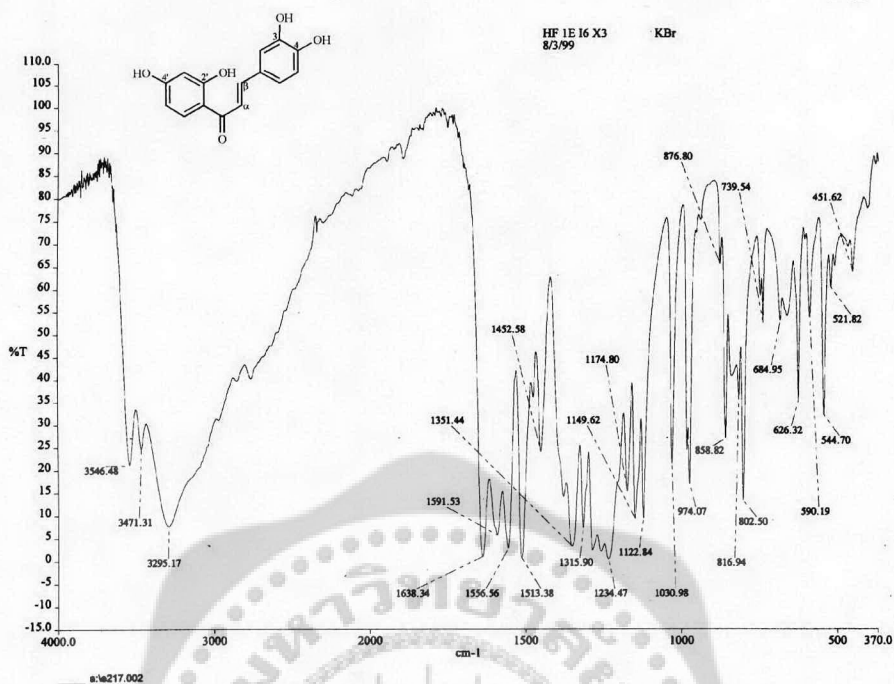
SPEED : FAST
DATE : 24-02-00

SLIT : 2.0

COMMENT : 1.4 mg. in 5 mL./dil. 30 times

threshold 0.005 abs. instrument from envi-tox lab ANALYST :

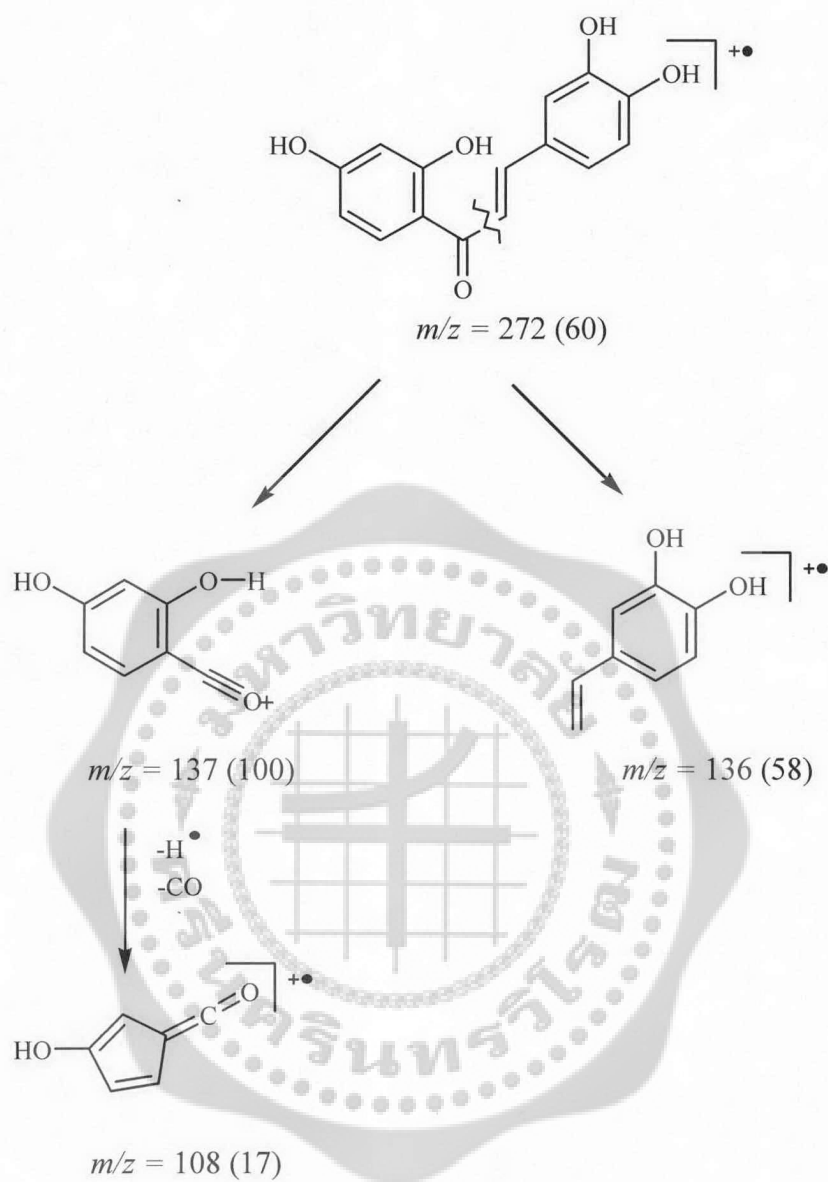
UV spectrum of butein 37



Mass List Data: HF1E63 # 310 Base m/z: 137
 06/07/99 17:59:00 + 2:51 Cali: AMPORN70699 # 3 RIC: 567296.
 Sample: HF1E6X3
 Conds.: DIP 40C/300C @60C/MIN

30	0.05	0.	Minima	Min Inten:	29.			
276			Maxima	# 300				
Mass	% RA	Inten.	Mass	% RA	Inten.	Mass	% RA	Inten.
30	0.79	452.	93	1.54	876.	157	0.84	480.
31	1.49	848.	94	1.28	728.	160	0.78	444.
32	6.12	3488.	95	5.11	2916.	161	0.77	440.
36	0.58	332.	96	0.56	320.	162	4.21	2400.
37	4.70	2680.	97	0.53	304.	163	30.70	17504.
38	9.50	5416.	98	0.77	440.	164	3.02	1720.
39	25.06	14288.	99	2.66	1516.	165	0.62	352.
40	3.14	1792.	101	1.03	588.	168	0.94	536.
41	4.88	2784.	102	1.06	604.	169	1.78	1016.
42	5.83	3324.	103	0.89	508.	170	0.76	436.
43	5.90	3364.	105	3.07	1752.	171	0.74	420.
44	2.14	1220.	106	2.03	1160.	173	1.99	1132.
45	1.87	1064.	107	6.99	3984.	179	0.54	308.
49	3.44	1960.	108	16.55	9440.	181	1.12	640.
50	18.94	10800.	109	4.08	2324.	183	0.54	308.
51	31.09	17728.	110	18.41	10496.	184	0.82	468.
52	23.48	13392.	111	1.53	872.	185	0.67	380.
53	33.33	19008.	112	0.73	416.	187	1.00	572.
54	5.20	2964.	113	1.79	1020.	197	3.95	2252.
55	13.75	7840.	115	5.72	3264.	198	1.22	696.
56	1.07	608.	116	1.29	736.	199	0.88	500.
57	1.70	972.	117	4.28	2440.	200	0.79	448.
60	1.08	616.	118	1.78	1016.	201	1.54	876.
61	4.22	2408.	119	1.52	864.	202	0.86	492.
62	13.43	7656.	120	1.00	572.	203	0.58	332.
63	26.96	15376.	121	3.26	1860.	208	0.60	344.
64	11.41	6504.	122	3.02	1720.	209	0.67	384.
65	7.18	4096.	123	12.77	7280.	213	1.22	696.
66	4.95	2824.	124	1.07	612.	215	1.74	992.
67	2.96	1688.	126	0.82	468.	216	0.62	356.
68	2.30	1312.	127	1.83	1044.	218	0.57	324.
69	20.51	11696.	128	1.38	788.	225	1.26	720.
70	1.83	1044.	129	0.93	528.	226	2.29	1304.
71	1.52	864.	131	1.15	656.	227	1.82	1036.
73	1.94	1104.	133	1.12	636.	228	0.57	324.
74	4.05	2312.	134	7.45	4248.	229	3.95	2252.
75	3.30	1884.	135	7.59	4328.	230	1.02	584.
76	5.10	2908.	136	58.14	33152.	237	1.62	924.
77	18.69	10656.	137	100.00	57024.	242	0.69	392.
78	8.64	4928.	138	9.22	5256.	243	2.43	1388.
79	11.99	6840.	139	2.47	1408.	244	3.60	2052.
80	12.09	6896.	140	0.72	412.	245	0.90	516.
81	22.22	12672.	141	1.69	964.	253	1.08	616.
82	2.94	1676.	142	0.54	308.	254	1.41	804.
83	0.87	496.	144	0.60	340.	255	9.65	5504.
84	2.22	1264.	145	2.04	1164.	256	1.89	1080.
85	3.29	1876.	147	1.16	660.	257	0.64	364.
87	2.74	1560.	149	0.72	412.	269	0.55	316.
88	3.76	2144.	150	30.19	17216.	270	0.70	400.
89	23.79	13568.	151	3.58	2040.	271	29.24	16672.
90	7.34	4184.	152	2.03	1160.	272	59.93	34176.
91	3.73	2128.	153	0.86	488.	273	11.18	6376.
92	4.01	2284.	155	0.88	500.	274	2.14	1220.
						276	0.69	392.

MS data of butein 37



Mass fragmentation of butein 37



GLOSSARY

<i>s</i>	singlet
<i>d</i>	doublet
<i>dd</i>	doublet of doublet
<i>m</i>	multiplet
<i>br s</i>	broad singlet
<i>br d</i>	broad doublet
<i>J</i>	coupling constant
EIMS	electron impact mass spectra
IR	infrared spectrum
<i>m/z</i>	a value of mass divided by charge
°C	degree celcius
g	gram
mL	millilitre
h	hour
%	percent
m.p.	melting point
cm ⁻¹	wave number
TMS	tetramethylsilane
Hz	hertz
TLC	thin layer chromatography
lit	literature
$\nu_{\max}$	maximum absorption frequencies
CDCl ₃	deuteriochloroform
CD ₃ OD	deuteromethanol
DMSO-d ₆	deuterodimethylsulfoxide
¹ H NMR	proton nuclear magnetic resonance
¹³ C NMR	carbon nuclear magnetic resonance
¹ H- ¹ H COSY	H-relayed correlated spectroscopy
DEPT	distortionless enhancement by polarization transfer

HMBC

heteronuclear multiple bond connectivity

HMQC

^1H -detected heteronuclear multiple quantum
coherence





Biography

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