องค์ประกอบทางเคมีจากรากกระทิง *Calophyllum inophyllum* L.

นางสาวศศ พงศ์อัจฉริยกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมี ภาควิชาเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

CHEMICAL CONSTITUENTS FROM THE ROOTS OF *Calophyllum inophyllum* L.

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
Department of Chemistry
Faculty of Science
Chulalongkorn University
Academic Year 2016

Thesis Title	CHEMICAL CONSTITUENTS FROM THE ROOTS OF *Calophyllum inophyllum* L.
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ศศ พงศ์อัจฉริยกุล : องค์ประกอบทางเคมีจากรากกระทิง *Calophyllum inophyllum* L. (CHEMICAL CONSTITUENTS FROM THE ROOTS OF *Calophyllum inophyllum* L.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สันติ ทิพยางค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร. จีราพัสฐ์ สีแจ่ม, 72 หน้า.

จากการศึกษาองค์ประกอบทางเคมีของสิ่งสกัดไดคอลโรมีเทนจากรากกระทิง สามารถแยกสารใหม่ในกลุ่มโครมาโนน (chromanone) ได้ 5 ชนิด คือ caloinophyllins A-E (1-5) นอกจากนี้ยังพบสารที่มีรายงานมาก่อนหน้านี้อีก 9 ชนิด คือ nobiletin (6), pentamethylquercetin (7), 3,5,7,4'-tetramethoxyflavone (8), 5,7,4'- trimethoxyflavone (9), 1,5- dihydroxyxanthone (10), 1,8-dimethoxyxanthone (11), caloxanthone B (12), 4-methoxycaffeic acid (13) และ 6-dihydroxy-7-methoxyxanthone (14) โดยโครงสร้างของสารใหม่ทั้งหมดนี้ (1-5) ได้พิสูจน์ทราบด้วยวิธีทาง 1D และ 2D เอ็นเอ็มอาร์ สเปกโทรสโกปี ส่วนโครงสร้างของสารที่มีรายงานก่อนหน้านี้ (6-14) ได้พิสูจน์ด้วยวิธีทาง ^{1}H และ ^{13}C เอ็นเอ็มอาร์ สเปกโทรสโกปี ร่วมกับการเปรียบเทียบข้อมูลของสารที่มีรายงานก่อนหน้านี้

จากการทดสอบฤทธิ์การยับยั้งเซลล์มะเร็งชนิด KB, HeLa S-3, MCF-7, HT-29 และ Hep G2 ของสารทั้งหมดที่แยกได้ พบว่า สาร 5 มีฤทธิ์ในการยับยั้งเซลล์มะเร็งทั้ง 5 ชนิด อยู่ในระดับที่ดี เนื่องจากมีค่า IC_{50} อยู่ในช่วง 0.78-10.35 µM ส่วนสาร 14 มีฤทธิ์การยับยั้งเซลล์มะเร็งชนิด KB, HeLa S-3 และ MCF-7 ได้ในระดับปานกลางโดยมีค่า IC_{50} อยู่ในช่วง 18.19-25.84 µM นอกจากนี้สาร 1, 2, 4 และ 12 มีฤทธิ์ในการยับยั้งได้ต่ำ เนื่องจากมีค่า IC_{50} อยู่ในช่วง 55.77-95.47 µM และสารชนิดอื่นที่เหลือพบว่าไม่มีฤทธิ์ยับยั้งเซลล์มะเร็ง เนื่องจากมีค่า IC_{50} มากกว่า 100 µM สำหรับการทดสอบฤทธิ์ยับยั้งเอ็นไซม์แอลฟ่ากลูโคซิเดสจากยีสต์ และลำไส้เล็กของหนูของสารทั้งหมดที่แยกได้ พบว่าสารทั้งหมดไม่มีฤทธิ์ในการยับยั้งเอ็นไซม์แอลฟ่ากลูโคซิเดสจากยีสต์ (IC_{50} > 600 µM) และลำไส้เล็กของหนู (IC_{50} > 3,000 µM)

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5872060623 : MAJOR CHEMISTRY

KEYWORDS: CALOPHYLLUM INOPHYLLUM / GUTTIFERAE, CALOINOPHYLLIN / CHROMANONE DERIVATIVE / ALPHA-GLUCOSIDASE INHIBITORY ACTIVITY

SASA PONGUSCHARIYAGUL: CHEMICAL CONSTITUENTS FROM THE ROOTS OF *Calophyllum inophyllum* L.. ADVISOR: ASSOC. PROF. SANTI TIP-PYANG, Ph.D., CO-ADVISOR: JIRAPAST SICHAEM, Ph.D., 72 pp.

Phytochemical investigation of the CH_2Cl_2 extract from the roots of *C. inophyllum* let to the isolation of five new chromanone derivatives, namely caloinophyllins A-E (1-5), along with nine known compounds (6-14), nobiletin (6), pentamethylquercetin (7), 3,5,7,4'-tetramethoxyflavone (8), 5,7,4'-trimethoxyflavone (9), 1,5-dihydroxyxanthone (10), 1,8-dimethoxyxanthone (11), caloxanthone B (12), 4-methoxycaffeic acid (13) and 1,6-dihydroxy-7-methoxyxanthone (14). The structures of five new compounds (1-5) were elucidated by using 1D and 2D-NMR spectroscopy. The structures of known compounds (6–14) were determined and confirmed by comparison of their ^{1}H and ^{13}C NMR spectroscopy data with the previous published data. Moreover, all isolated compounds were assessed for their *in vitro* cytotoxicity against the KB, HeLa S-3, MCF-7, HT-29 and Hep G2 human cancer cell lines. The results revealed that compound 5 showed a good cytotoxicity against five cancer cell lines with IC_{50} values in the range of 0.78-10.35 µM. Compound 14 showed a moderate activity against KB, HeLa S-3 and MCF-7 cell lines with IC_{50} values in the range of 18.19-25.84 µM and compounds 1, 2, 4 and 12 showed a weak activity with IC_{50} values in the range of 55.77-95.47 µM. The other compounds were inactive (IC_{50} > 100 µM) against five human cancer cell lines. In addition, all isolated compounds (1-14) were evaluated for their α-glucosidase inhibitory activity (yeast and rat small intestinal). All of them displayed no activity against both yeast (IC_{50} > 600 µM) and rat small intestinal (IC_{50} > 3,000 µM) α-glucosidase enzymes.

Department:	Chemistry	Student's Signature	.....
Field of Study:	Chemistry	Advisor's Signature	.....
Academic Year:	2016	Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor, Assoc. Prof. Dr. Santi Tip-pyang and my co-advisor Dr. Jirapast Sichaem for the golden opportunity to do this wonderful research on the topic of chemical constituents from the roots of Calophyllum inphyllum L.

I would like to express my sincere thanks towards the members of my research committee, Assoc. Prof. Dr. Vudhichai Parasuk, Assistant Professor Dr. Worawan Bhanthumnavin and Assistant Professor Dr. Jongkolnee Jongaramruong for their times and knowledge for this research. The special thank goes to Dr. Suttira Khumkratok, a botanist at the Biodiversity and Conservation Research Unit, Walai Rukhavej Botanical Research Institute, Mahasarakham University for collecting the roots of Calophyllum inphyllum L., identifying the plant species and providing the voucher specimen.

I am also grateful to Dr. Pongpan Siripong and the staff from Natural Product Research Section, Research Division, National Cancer Institute for performing the cytotoxicity assay.

I would like to thank Santi and Preecha's group for helping me a lot on my research. I came to know so many new things from their comments.

Finally, hearfelt thanks to my family for their great sacrifice, unconditional love and care, and continuous spiritual support.

CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

Page

LIST OF SCHEMES

Page

LIST OF ABBREVIATIONS

IC_{50}	the molar concentration of an antagonist that reduces the response to an agonist by 50%
kg	kilogram
μg	microgram
mg	milligram
μM	micromolar
L	liter
mL	milliliter
h	hour(s)
mmol	millimole
U	unit
m/z	mass per charge number of ions (Mass Spectroscopy)
δ	chemical shift (NMR)
δ_H	chemical shift of proton (NMR)
δ_C	chemical shift of carbon (NMR)
J	coupling constant (NMR)
s	singlet (NMR)
d	doublet (NMR)
dd	doublet of doublet (NMR)
t	tirplet (NMR)
brs	broad singlet (NMR)
Hz	hertz
MHz	megahertz
DMSO-d_6	deuterated dimethyl sulfoxide
$CDCl_3$	deuterated chloroform
HRESIMS	high resolution electrospray ionization mass spectroscopy
^{1}H NMR	proton nuclear magnetic resonance
^{13}C NMR	carbon-13 nuclear magnetic resonance
1D-NMR	one dimensional nuclear magnetic resonance
2D-NMR	two dimensional nuclear magnetic resonance
COSY	correlation spectroscopy
HSQC	heteronuclear single quantum correlation
HMBC	heteronuclear multiple bond correlation
calcd.	calculated
TLC	thin layer chromatography

CHAPTER I
INTRODUCTION

Natural products are claimed to be a rich source of the active ingredients of medicines. This is widely accepted to be true when applied to drug discovery in 'olden times' before the advent of high-throughput screening and the post-genomic era in which more than 80% of drug substances were natural products or inspired by natural compounds [1]. The comparison of the information (**Table 1.1**) on sources of new drugs from 1981 to 2007 indicated that almost half of the drugs approved since 1994 were based on natural products. There were several natural compounds related drugs approved as new classes of drugs such as the peptide (exenatide and ziconotide) and small molecules (ixabepilone, retapamulin and trabectedin) [2, 3].

Table 1.1 Drugs based on natural products at different stages of development.

Development stage	Plant	Bacterial	Fungal	Animal	Semi-synthetic	Total[a]
Preclinical	46	12	7	7	27	99
Phase I	14	5	0	3	8	30
Phase II	41	4	0	10	11	66
Phase III	5	4	0	4	13	26
Pre-registration	2	0	0	0	2	4
Total	108	25	7	24	61	225

Source: Pharmaprojects database (1981-2007 March)

[a] This does not include reformulations of products (66 such products were listed.)

These recently approved that natural products based drugs have been described extensively in earlier reviews [2-5]. Some compounds were obtained from plants (e.g. elliptinium, galantamine and huperzine), microbes (daptomycin) and animals (e.g. exenatide and ziconotide), as well as synthetic or semi-synthetic compounds based on natural products (e.g. tigecycline, everolimus, telithromycin, micafungin and caspofungin) (**Figure. 1.1**).

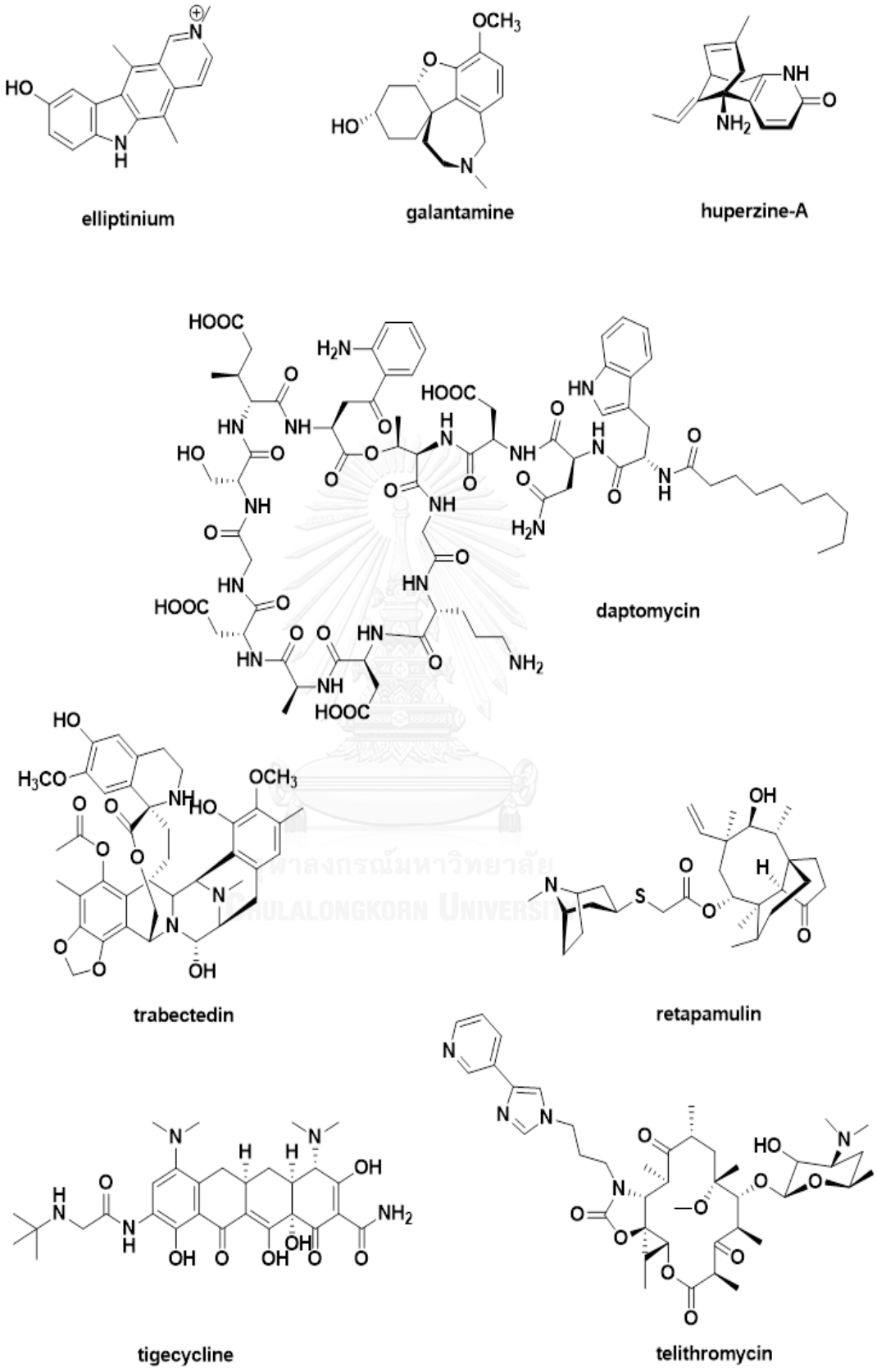


Figure 1.1 Small-molecule natural products contributing in new medicines.

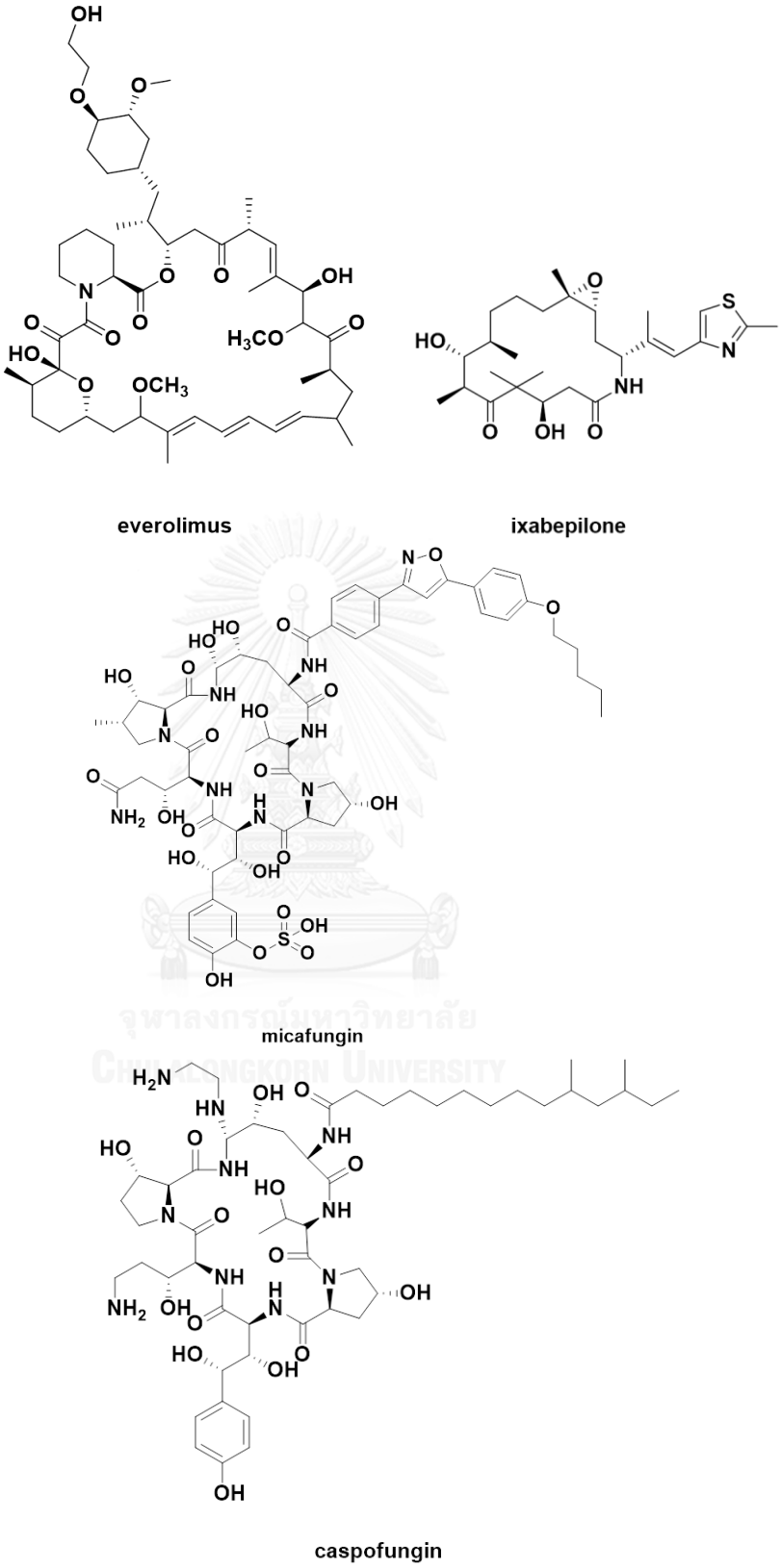


Figure 1.1 (Cont.) Small-molecule natural products contributing in new medicines.

Plant-derived compounds have been used as drugs such as elliptinium for cancer therapy, galantamine and huperzine-A for Alzheimer's disease, the microbial derived compounds (e.g. daptomycin) as antibiotics, the marine anti-cancer compound e.g. trabectedin and natural product derivatives such as retapamulin, tigecycline and telithromycin as an antibiotic, everolimus as an immunosuppressive, ixabepilone as an anti-cancer agent and anti-fungal agent e.g. micafungin and caspofungin.

They covered a range of therapeutic indications as anti-cancer, anti-infective and anti-diabetic agents and they related a great diversity of chemical structures. The chemical properties of the small-molecule natural products that have been recently developed into drugs have been analysed [6]. Half of them were found to be closely compliant with Lipinski's Rule. However, the remaining compounds had a higher molecular weight, more rotable bonds and more stereogenic centers, although they remained relatively low log *P* values. On average, natural products are more readily absorbed than synthetic drugs [7].

An exponential growth has been occurred in the field of herbal medicine since last few years and these drugs are gaining popularity both in developing and developed countries because of their natural origin and less side effects. Many traditional medicines consumed are derived from medicinal plants, minerals and organic matter [8].

1.1 Botanical aspect and distribution of *Calophyllum inophyllum*

Calophyllum is a genus of Guttiferae family, consisting of approximately 187 species and mainly distributed in Asia, East Africa, tropical America, Madagascar, Mascarenes and Australasia [9]. It is a multipurpose plant because it is harvested from the wild for local use as food, medicine and source of materials. The oil from its seed is traded internationally as tamanu oil or foraha oil, but its quantity involved are not known. The tree is planted in reforestation schemes to provide a shelter from the wind and as an ornamental and roadside tree for its deliciously scented white flowers that are reminiscent of orange blossom [10-12].

This genus is a rich source of secondary metabolites such as coumarins, xanthones and terpenoids. Previous reports shown that some compounds from the *Calophyllum* species exhibited a wide range of biological and pharmacological activities such as cytotoxic and antioxidant abilities [13]. Recently, various bioactivities of xanthones such as cytotoxic and antitumor, anti-inflammatory, anti-fungal, enhancement of choline acetyltransferase and inhibition of lipid peroxidase have been reported.

Calophyllum coumarins, represented by calanolides A and B were reported to be the most active in the cell-based anti-HIV-1 assay. Calanolide A fully protected human T-lymphoblastic cells from the cytopathogenic effects of HIV-1 and had no cytotoxic effects. The calanolides isomers were also found to be effective against a wide spectrum of drug-resistant HIV strains isolated from patients' T-cells. Due to the novelty of bioactivity, calanolide A was chosen as a candidate for multicenter Phase II clinical trials on patients with HIV infection in the United States and Malaysia [14]. In addition, inophyllums B and P are currently undergoing preclinical development for activity against AIDS [15]. Some chromanones have been reported to show anti-acetylcholine esterase, anti-bacterial, anti-fungal and HIV-1 inhibitory activity [16].

Calophyllum inophyllum Linn. (**Figure 1.2**) is a slow-growing, medium-sized evergreen tree with a spreading crown. It usually grows up to 25 meters tall, occasionally to 35 meters. The bole is usually short and twisted or leaning and it can be up to 150 cm in diameter. All parts of the plant contain a sticky yellowish latex [10-12]. The balsam from the bark of *C. inophyllum* is called an 'oleoresin' and used as a cicatrisant, whereas an infusion or dew and on of the leaves has been traditionally used as an eye remedy in Asian medicine.

Figure 1.2 *Calophyllum inophyllum* tree

The flowers (**Figure 1.3**) are 25 mm (0.98 in) wide and occurs in racemose or paniculate inflorescences consisting of 4 to 15 flowers. Flowering can occur year-round, but usually two distinct flowering periods are observed, in late spring and in late autumn.

Figure 1.3 The flowers of *C. inophyllum*

The fruits (**Figure 1.4**) are a round, green drupe reaching 2 to 4 cm (0.79 to 1.57) in diameter and having a single large seed. When ripe, the fruit is wrinkled and its color varies from yellow to brownish-red. Besides being a popular ornamental plant, its wood is hard and strong and has been used in construction or boat building. Traditional pacific islanders used wood to construct the keel of their canoes while the boat sides were made from breadfruit (*Artocarpus altilis*) wood.

Figure 1.4 The fruits of *C. inophyllum*

The previous studies have found that substances isolated from the bark, leaves and fruit of *C. inophyllum* had interesting biological activities such as antibacterial, anticancer, antiplatelet, antiinflammatory, antiviral and antioxidant. In addition, the antioxidant was also good for wound healing and UV protection.

1.2 Traditional medicine of *C. inophyllum*

C. inophyllum which have been recorded from latex and bark are applied externally on ulcers, phthisis, orchitis, lung affections and internally as a purgative after childbirth and to treat gonorrhea. The leaf infusion is used to treat sore eyes, haemorrhoids and dysentery. Moreover, heated leaves are applied to cuts, sores, ulcers, boils and skin rash. Seed oil is applied externally as an analgesic against rheumatism, sciatica and as a medication against swellings, ulcers, scabies, ringworm, boils and itch. The seeds are used as a fish poison. The flowers are used as a heart tonic [17].

1.3 Previous investigations of *Calophyllum* species

Iinuma et al.[18, 19] have investigated the hexane, acetone and methanol extracts of *C. inophyllum* roots and four new xanthones were identified as caloxanthones A, B, D and E. The chromatographic isolation of the hexane, chloroform and methanol extracts of *C. inophyllum* roots gave a new prenylated pyranoxanthone, inophyllin A [20], (**Figure 1.5**) which inhibited *Dengue* virus. Two triterpenoids, friedelin and stigmasterol were also reported from this part.

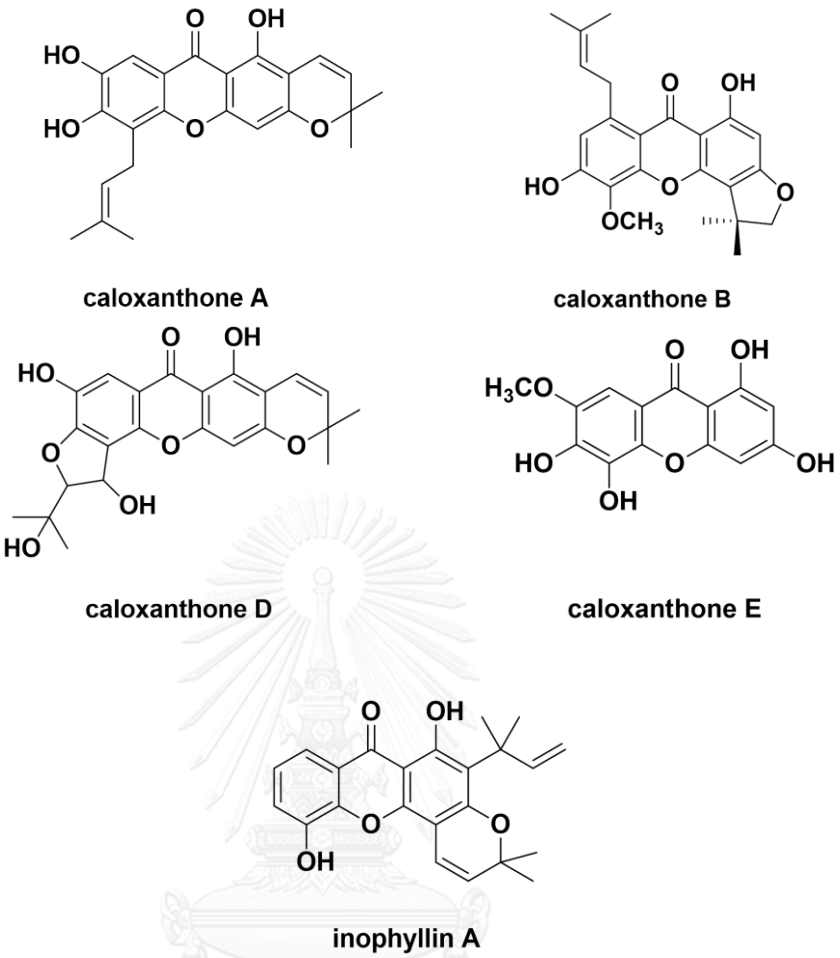


Figure 1.5 Isolated compounds from the roots of *C. inophyllum*

Three triterpenoids have been isolated from the hexane extract of *C. inophyllum* leaves and identified as canophyllal, canophyllol and canophyllic acid [21]. Friedelane-type triterpenoids have been extracted as 3,4-secofriedelan-3,28-dioic acid, 27-hydroxyacetate canophyllic acid and 3-oxo-27-hydroxyacetate friedelan-28-oic acid [22]. Then, four coumarins and two triterpenoids, including 12-*O*-butylinophyllum D, 12-*O*-ethylinophyllum D, inophyllum H, inophyllum I, 27-[(*E*)-*p*-coumaroyloxy]friedelin-28-carboxylic acid and 27-[(*Z*)-*p*-coumaroyloxy]friedelin-28-carboxylic acid were achieved from the chloroform and methanol extracts of *C. inophyllum* leaves [23]. Moreover, four coumarins as (-)-12-methoxyinophyllum A, (+)-12-methoxyinophyllum H-1, (-)-12-methoxyinophyllum H-2 and inophyllum J have been isolated from the ethanol extract of *C. inophyllum* leaves (**Figure 1.6**) [24].

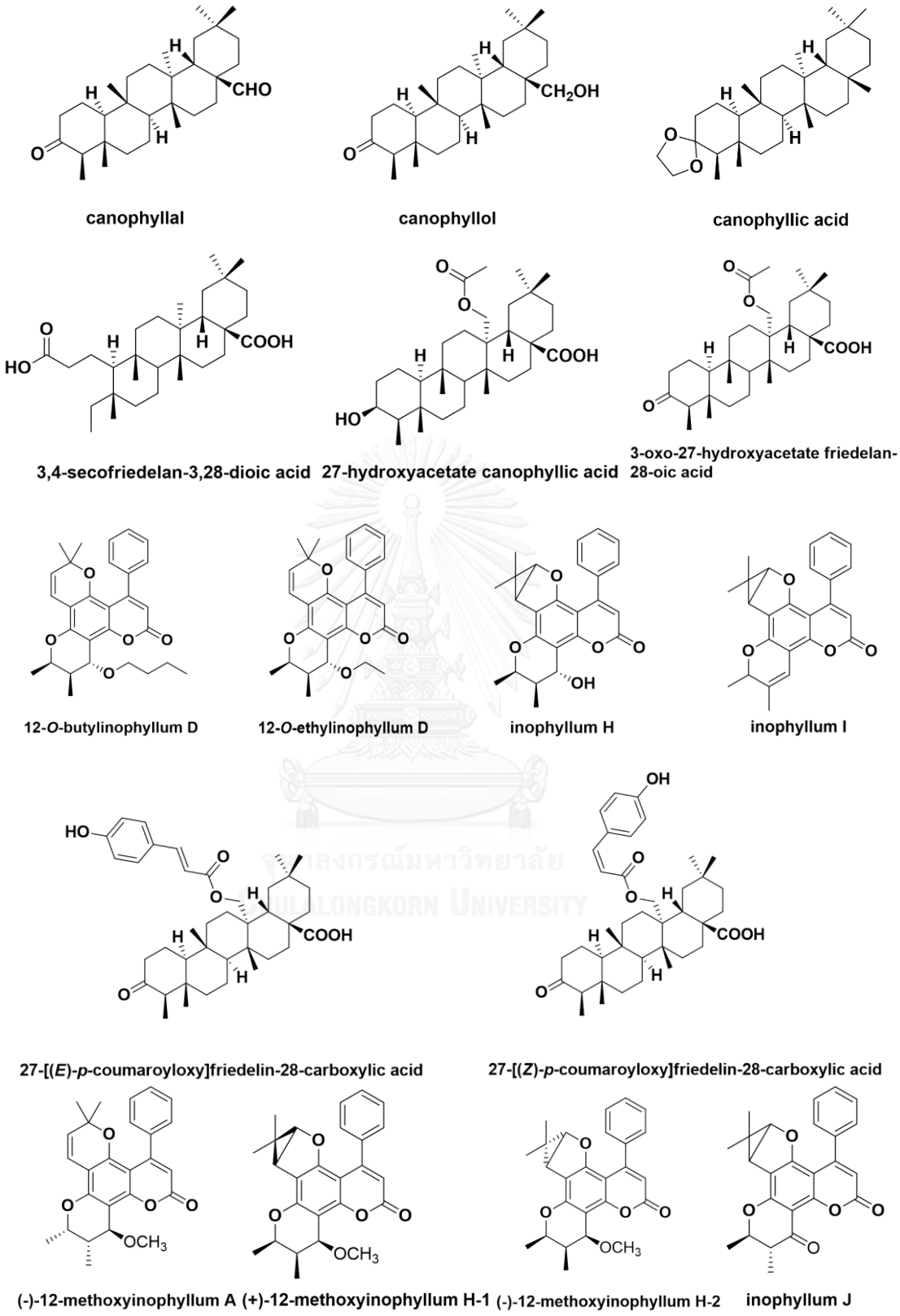
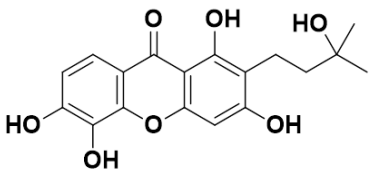


Figure 1.6 Isolated compounds from the leaves of *C. inophyllum*

The chromatographic isolation of the hexane, ethyl acetate and methanol extracts of *C. inophyllum* wood gave one xanthone, 2-(3-hydroxy-3-methylbutyl)-1,3,5,6-tetrahydroxyxanthone (**Figure 1.7**) [25].



2-(3-hydroxy-3-methylbutyl)-1,3,5,6-tetrahydroxyxanthone

Figure 1.7 Isolated compound from the wood of *C. inophyllum*

Joshi et al. [26] have investigated the methanol extract of *C. inophyllum* seeds and obtained one coumarin, 12-methoxyinophyllum-D. Inophyllums A, B, C, D, P and calophyllolide were also reported. In 2003, inocalophyllins A and B were isolated [14]. After that, Leu et al. [27] have extracted the seeds of *C. inophyllum* (hexane extract) followed by several separation methods to obtain three pyranocoumarins, tamanolide, tamanolide D and tamanolide P. In 2010, two prenylated xanthones, caloxanthone *O* and caloxanthone *P* [28] were isolated, which inhibited gastric cancer cells. Five xanthones and three terpenoids, including inophyllin A, macluraxanthone, pyranojacareubin, 4-hydroxyxanthone, friedelin, stigmasterol and betulinic acid were reported in the hexane, dichloromethane, ethyl acetate and methanol extracts of *C. inophyllum* almond seeds. Among them, inophinnin performed anti-inflammatory activity [29] (**Figure 1.8**).

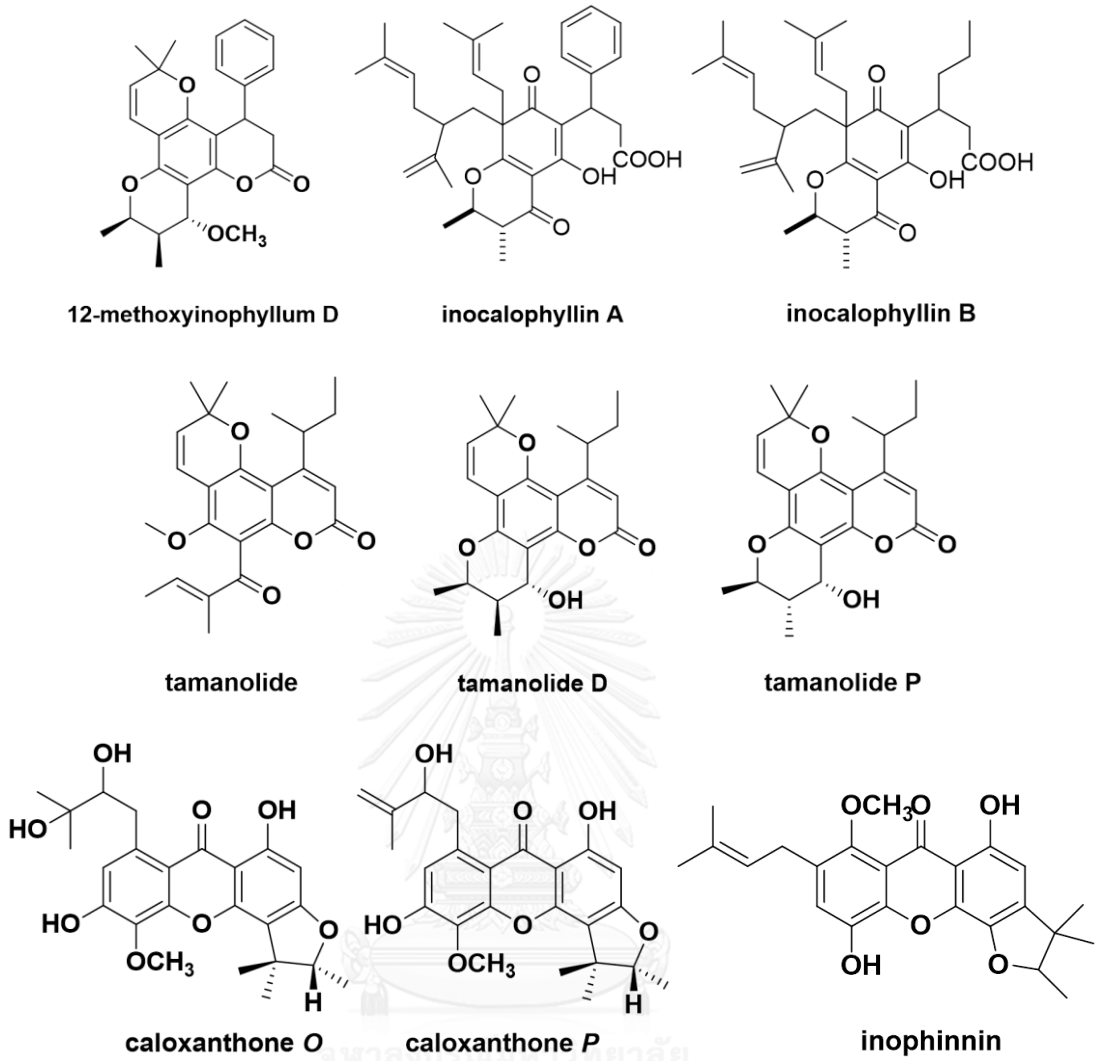
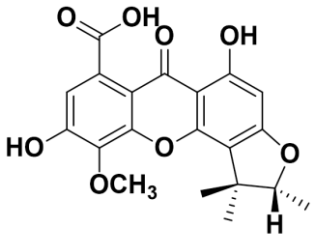


Figure 1.8 Isolated compounds from the seeds of *C. inophyllum*

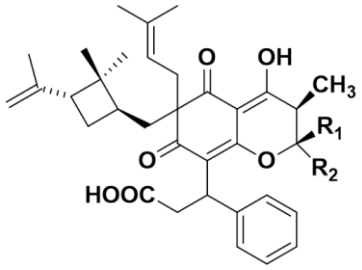
One prenylated xanthone, caloxanthone Q (**Figure 1.9**), was isolated from the ethanol extract of *C. inophyllum* branch, along with three prenylated xanthones such as jacareubin, 2-deprenylrheediaxanthone B and 6-deoxyjacareubin [30].



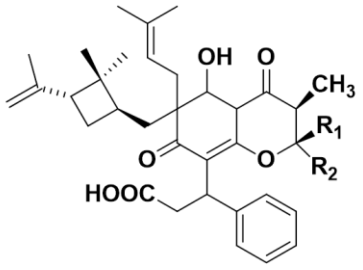
caloxanthone Q

Figure 1.9 Isolated compound from the branch of *C. inophyllum*

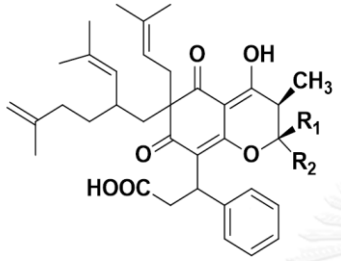
Chromanone acids, brasiliensophyllic acid A, isobrasiliensophyllic acid A, brasiliensophyllic acid B, isobrasiliensophyllic acid B, brasiliensophyllic acid C and isobrasiliensophyllic acid C were isolated from the bark of *C. brasiliense* Cambess and showed moderate to strong antibacterial activity against *Bacillus cereus* and *Staphylococcus epidermidis*, with brasiliensophyllic acid A and isobrasiliensophyllic acid A being the most active. None of the compounds were cytotoxic against KB, Jurkat T, and myosarcoma cancer cells [31]. In 2008, 6-*O*-methyl inophylloidic acid methyl ester has been isolated from *C. brasiliense* stem bark in stable form [32]. In 2010, three new chromanone derivatives, calopolyanic acid isocalopolyanic acid and isorece-densic acid, were isolated from the pericarps of *C. polyanthum* (**Figure 1.10**) [33]. Chromatographic isolation of the acetone extract of the seeds of *C. blancoi* gave one new dihydrocoumarin derivative, isorecedensolide. In addition, it showed mild activity against KB (human oral epidermoid carcinoma) and Hela (human cervical epitheloid carcinoma) tumor cell lines (**Figure 1.11**) [14].



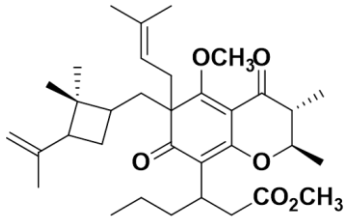
brasiliensophyllic acid A : R_1 = H, R_2 = CH_3
isobrasiliensophyllic acid A : R_1 = CH_3, R_2 = H



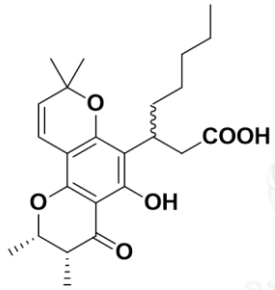
brasiliensophyllic acid B : R_1 = H, R_2 = CH_3
isobrasiliensophyllic acid B : R_1 = CH_3, R_2 = H



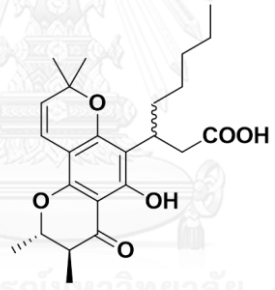
brasiliensophyllic acid C : R_1 = H, R_2 = CH_3
isobrasiliensophyllic acid C: R_1 = CH_3, R_2 = H



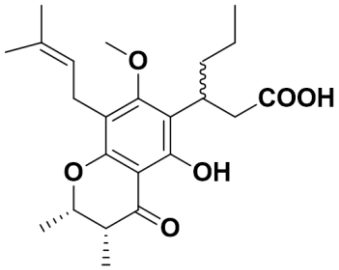
6-*O*-methyl inophylloidic acid methyl ester



calopolyanic acid

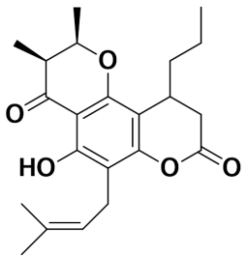


isocalopolyanic acid



isorecedensic acid

Figure 1.10 Isolated compounds from the stem and bark of *C. brasiliense* Cambess, *C. brasiliense* and *C. polyanthum*



isorecedensolide

Figure 1.11 Isolated compound from the seeds of *C. blancoi*

1.4 Biological activities

1.4.1 α-Glucosidase inhibitory activity

Blood sugar levels are mainly determined by absorption of glucose from gut, uptake of glucose by peripheral tissues (muscle, adipose tissue) and the insulin secretion from the pancreas. To enable glucose uptake and absorption by the body and availability as an energy source, intestinal cleavage of starch and oligosaccharides is required. Oligosaccharides are commonly cleaved into monosaccharides by enzyme complexes called α-glucosidases, which are present in the brush border membrane of the small intestine (**Figure 1.12**) [34], [35].

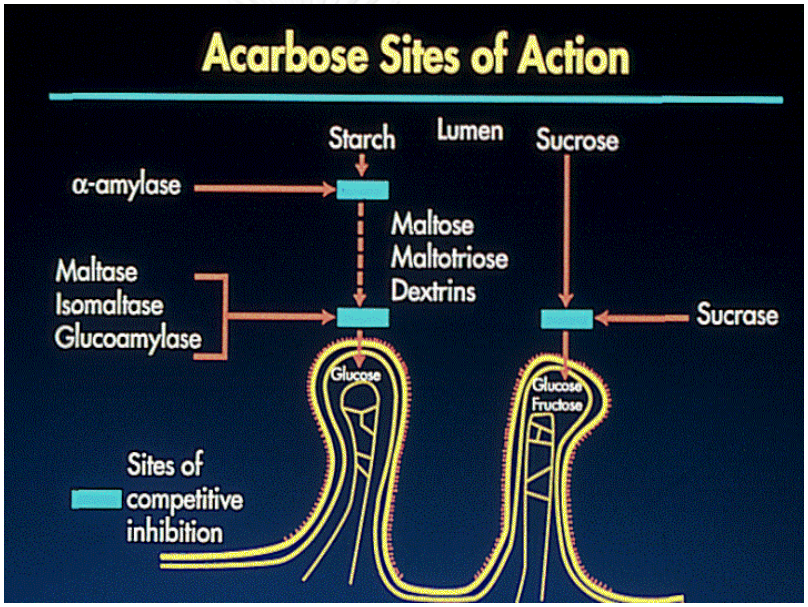


Figure 1.12 In normal digestion, oligosaccharides are hydrolyzed by α-glucosidase located in the intestinal brush border to monosaccharides, which are then absorbed.

Various oral anti-diabetic agents act by modifying the factors aiding in the control of hypoglycemia. These anti-diabetic agents include sulfonylureas (increase insulin secretion), biguannides (increase in glucose uptake) and alpha-glucosidase inhibitors [36].

To date, one potential therapy for diabetes is inhibiting α-glucosidase, thereby providing an alternative to reduce postprandial hyperglycemia. Oral antihyperglycemic

drugs (**Figure 1.13**) currently used for alpha-glucosidase inhibitors include acarbose (Precose® or Glucobay®), miglitol (Glyset®) and voglibose (Basen®) [34], [36].

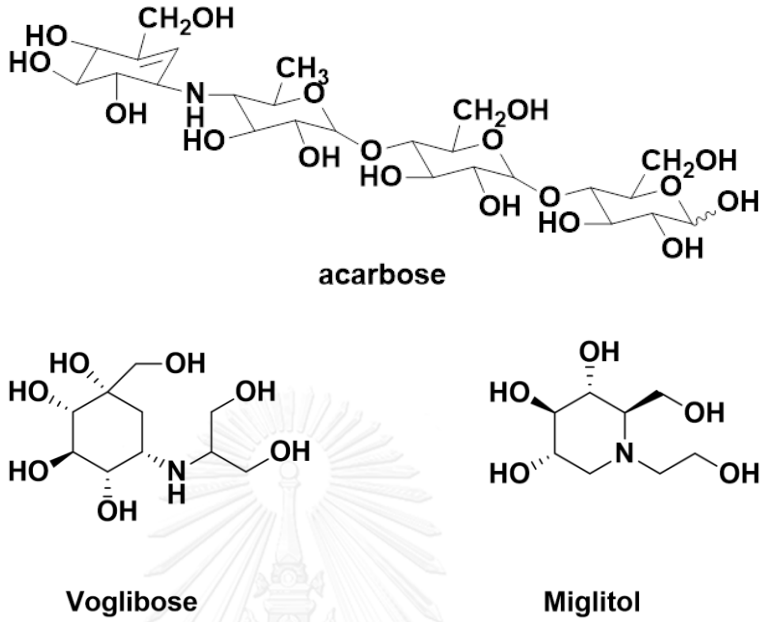


Figure 1.13 Structures of α-glucosidases inhibitors currently used to reduce hyperglycemia.

Acarbose (**Figure 1.13**) is a pseudomaltotetraose produced by strains of genus *Actinoplanes* sp. The compounds is an inhibitor of α-glucosidases and is used in type II diabetes therapy [37]. Acarbose competitively inhibits the function of α-glucosidase in the brush border of enterocytes lining the intestinal villi (**Figure 1.14**). However, continuous use of that acarbose should be limited because long-term administration and may cause side effects such as flatulence, abdominal cramp, vomiting and diarrhea.

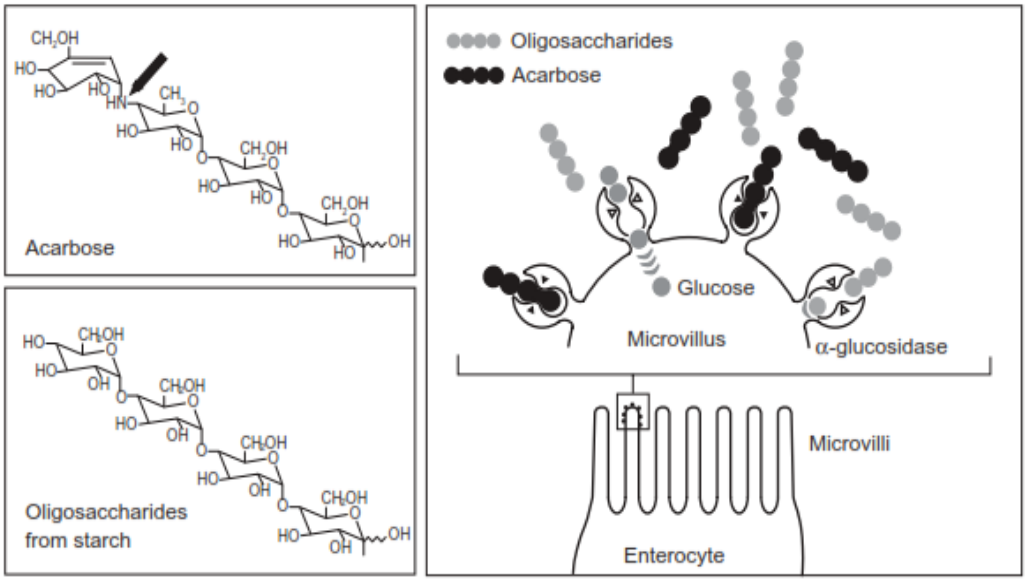
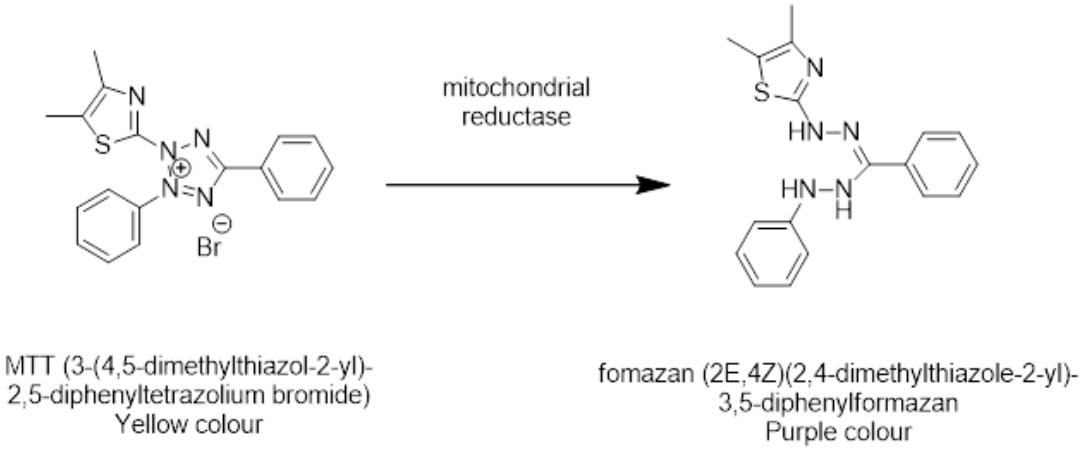


Figure 1.14 Competitive inhibition of acarbose toward intestinal enzymatic hydrolysis of oligosaccharides [35].

1.4.2 Cytotoxic activity against human cancer cell lines

Cancer is the second leading cause of death globally, and was responsible for 8.8 million deaths in 2015. Globally, nearly 1 in 6 deaths is due to cancer and approximately 70% of deaths from cancer occur in low- and middle-income countries. Around one third of deaths from cancer are due to the 5-leading behavioral and dietary risks such as high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, and alcohol use [38]. The discovery of an anticancer agent from natural products has been developed initially through a preliminary screening of drug candidates. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is one of initial methods to screen the cytotoxicity of a substance indicated by viability of the cells. The number of viable cells are determined through the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent by mitochondrial dehydrogenase enzyme inside living cells forming a formazan dye (**Figure 1.15**) which is measured then using colorimetric method. The result of cytotoxic activity can be used for further investigation through *in vivo* test using an animal model to assess the metabolism properties of a drug candidate in a living organism [39].



MTT (3-(4,5-dimethylthiazol-2-yl)-
2,5-diphenyltetrazolium bromide)
Yellow colour

fomazan (2E,4Z)(2,4-dimethylthiazole-2-yl)-
3,5-diphenylformazan
Purple colour

Figure 1.15 MTT reduction in live cells by mitochondrial reductase resulting a formation of insoluble formazan

1.5 The objectives of the research

The objectives in this investigation are as follows:

1. To isolate and purify the compounds from the roots of *C. inophyllum*.
2. To identify the chemical structures of all isolated compounds.
3. To evaluate the α-glucosidase inhibitory activity of the isolated compounds.
4. To evaluate the cytotoxic activity of the isolated compounds against human cancer cell lines.

CHAPTER II
EXPERIMENTAL

2.1 General experimental procedures

NMR spectra were recorded on a Bruker 400 AVANCE spectrometer operated at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. The chemical shifts were reported in ppm and using TMS as the internal standard. Melting points were determined on a Fisher-Johns Melting Point apparatus. Absorbents such as Dianion HP-20 (Sigma-Aldrich), silica gel (60 Merck cat. No. 7730, 7734 and 7749) were used for CC and Sephadex LH-20, preparative TLC and radial chromatography (chromatotron model 7924T, Harrison Research). Merck silica gel $60F_{254}$ plates were used for TLC. HRESIMS were obtained using a Bruker MICROTOF model mass spectrometer. HPLC was conducted on Waters® 600 controller equipped with a Waters® photodiode array detector (USA). Apollo C18 5µm column (10×250 mm) was used for separation purposes. UV-visible absorption spectra were taken on a UV-2550 (SHIMADZU) UV-Vis spectrometer (Shimadzu, Kyoto, Japan).

2.2 Plant material

The roots of *C. inophyllum* was collected from Maha Sarakham province of Thailand in May 2016 and identified by Dr. Suttira Khumkratok, a botanist at the Walairukhavej Botanical Research Institute, Mahasarakham University and the specimen (khumkratok no.2-15) was retained as a reference.

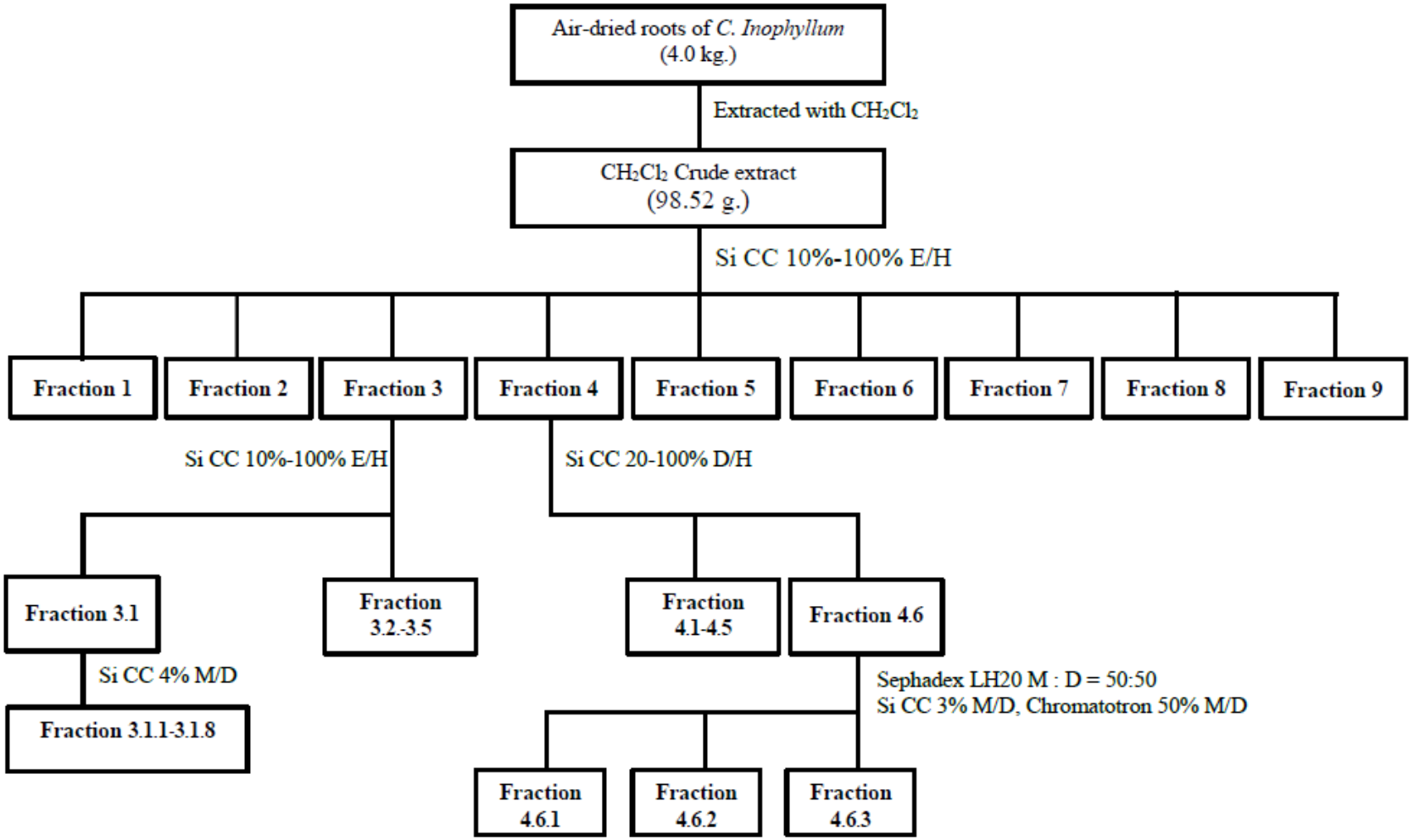
2.3 Extraction and isolation

The air-dried roots of *C. inophyllum* (4.0 kg) were successively extracted for six days with CH_2Cl_2 at room temperature. The solvent was evaporated to give CH_2Cl_2 crude extract (98.52 g). The CH_2Cl_2 extract was subjected to column chromatography on silica gel (Merck Art 7730), using successive elution of hexane, EtOAc and MeOH with increasing polarity to provide nine major fractions (1–9). Fraction 3 was subjected to silica gel column chromatography (Si CC) eluted by a 1:9 (v/v) EtOAc : hexane to pure EtOAc gradient to give five sub-fractions (3.1–3.5). Sub-fraction 3.1 was subjected to Si CC eluted by using 4% MeOH : CH_2Cl_2 to give eight sub-fractions (3.1.1-3.1.8). Sub-fraction 3.1.4 was purified by Sephadex LH 20 column chromatography (50% MeOH : CH_2Cl_2) and radial chromatography (chromatotron; 5% EtOAc : hexane) afforded caloinophyllin A (**1**) (5.7 mg). Sub-fraction 3.1.3 was purified by Sephadex LH 20 column

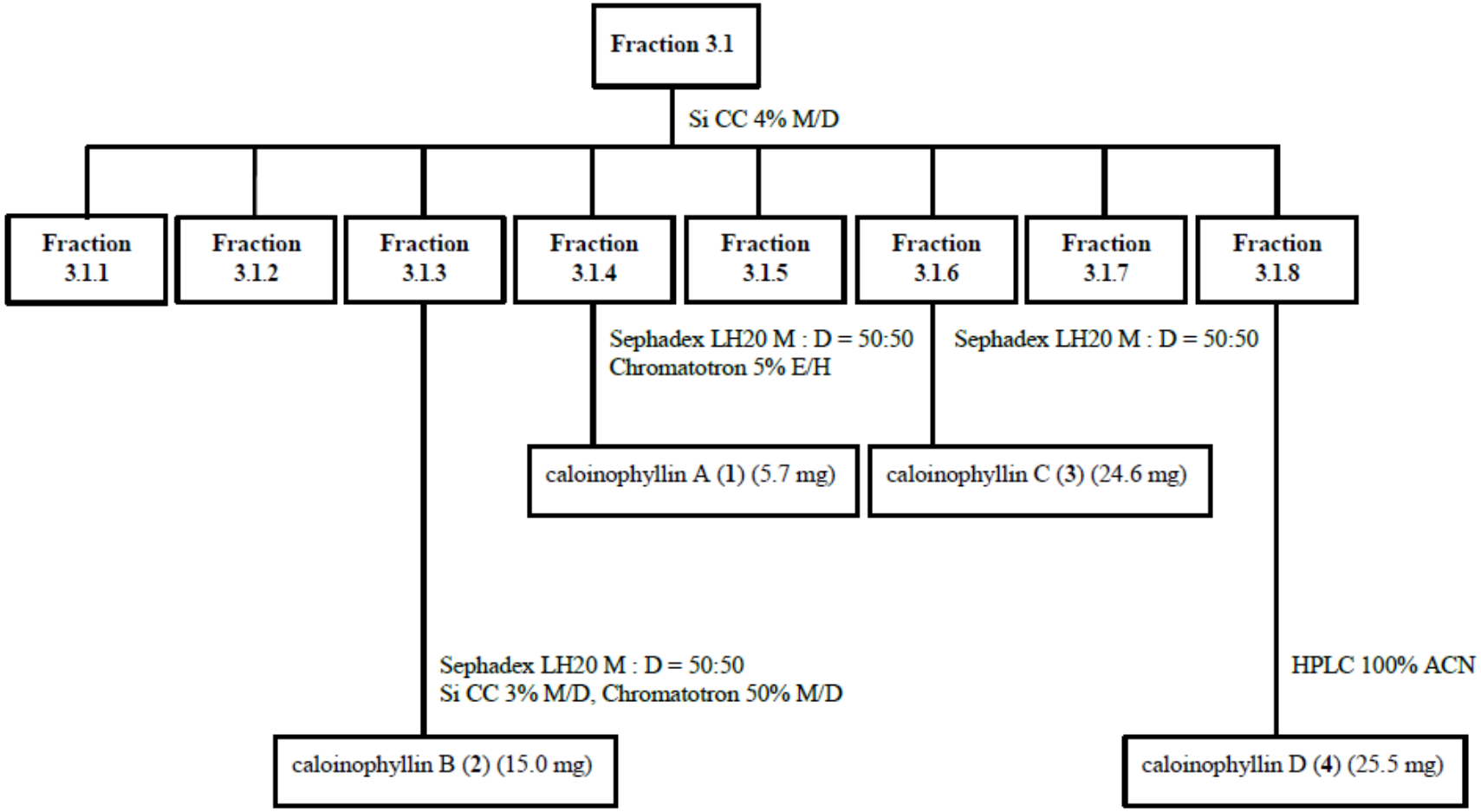
chromatography (50% MeOH : CH_2Cl_2) and Si CC eluted by using 5% MeOH : CH_2Cl_2 to afford caloinophyllin B (**2**) (15.0 mg). Sub-fraction 3.1.6 was purified by Sephadex LH 20 column chromatography (50% MeOH : CH_2Cl_2) to give caloinophyllin C (**3**) (24.6 mg). Sub-fraction 3.1.8 was purified by RP HPLC (ODS, 100% ACN, UV 254 nm) caloinophyllin D (**4**) (25.5 mg, t_R 14 min). Sub-fraction 3.3 was subjected to Si CC eluted by using 4% MeOH : CH_2Cl_2 to give caloinophyllin E (**5**) (23.2 mg). Fraction 7 was subjected to Si CC eluted by using 50% EtOAc : hexane and preparative TLC purification (1% MeOH : CH_2Cl_2) to give nobiletin (**6**) (19.5 mg). Sub-fraction 3.2 was subjected to radial chromatography (chromatotron; 50% CH_2Cl_2 : hexane) to obtain pentamethylquercetin (PMQ) (**7**) (19.5 mg) and 1,6-dihydroxy-7-methoxyxanthone (**14**) (8.5 mg). Sub-fraction 3.5 was purified by Sephadex LH 20 column chromatography (50% MeOH : CH_2Cl_2) to afford 3,5,7,4'-tetramethoxyflavone (**8**) (2.7 mg). Fraction 4 was subjected to Si CC eluted by a 2:8–10:0 (v/v) CH_2Cl_2-hexane gradient to obtain six sub-fractions (4.1–4.6). Sub-fraction 4.6 was subjected to Si CC eluted by using 3% MeOH : CH_2Cl_2 and Sephadex LH 20 column chromatography (50% MeOH : CH_2Cl_2) to obtain three sub-fractions (4.6.1–4.6.3). Sub-fraction 4.6.1 was subjected to Si CC eluted by a 4:6–10:0 (v/v) EtOAc and hexane gradient to afford 5,7,4'-trimethoxyflavone (**9**) (7.5 mg) and 1,5-dihydroxyxanthone (**10**) (5.3 mg). Preparative TLC purification (1% MeOH : CH_2Cl_2) of sub-fraction 4.6.2 gave 1,8-methoxyxanthone (**11**) (11.7 mg). Finally, sub-fraction 4.6.3 was purified by preparative TLC (1% MeOH : CH_2Cl_2) to obtain caloxanthone B (**12**) (20.7 mg) and 4-methoxycaffeic acid (**13**) (2.7 mg).

The isolated compounds (**Figure 2.1**) were identified by means of various spectroscopic methods including MS, 1D and 2D NMR techniques together with comparison with the previous literature data.

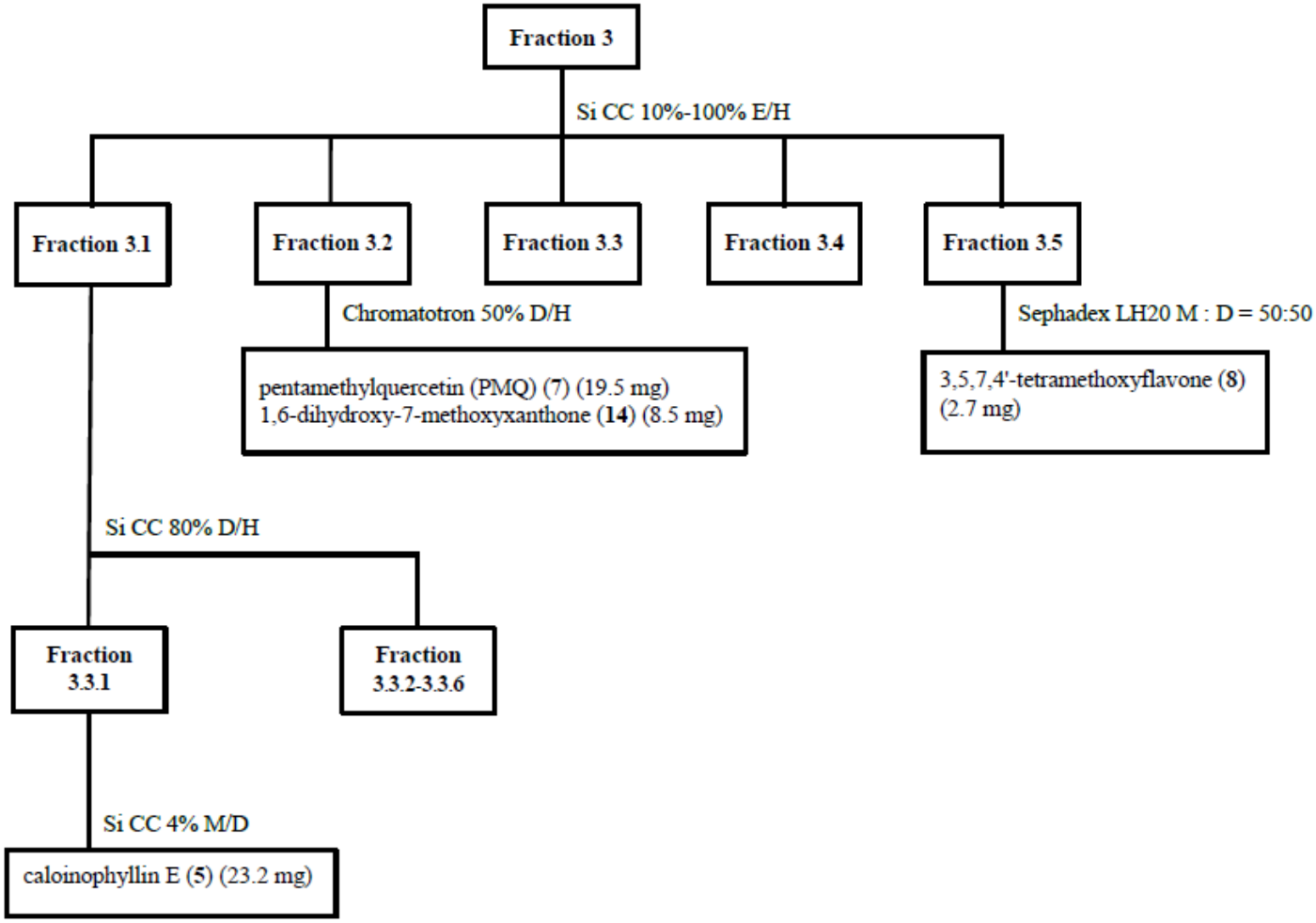
The isolation and purification of all isolated compounds from the roots of *C. inophyllum* were briefly summarized in **Schemes 2.1**, **2.2**, **2.3** and **2.4**



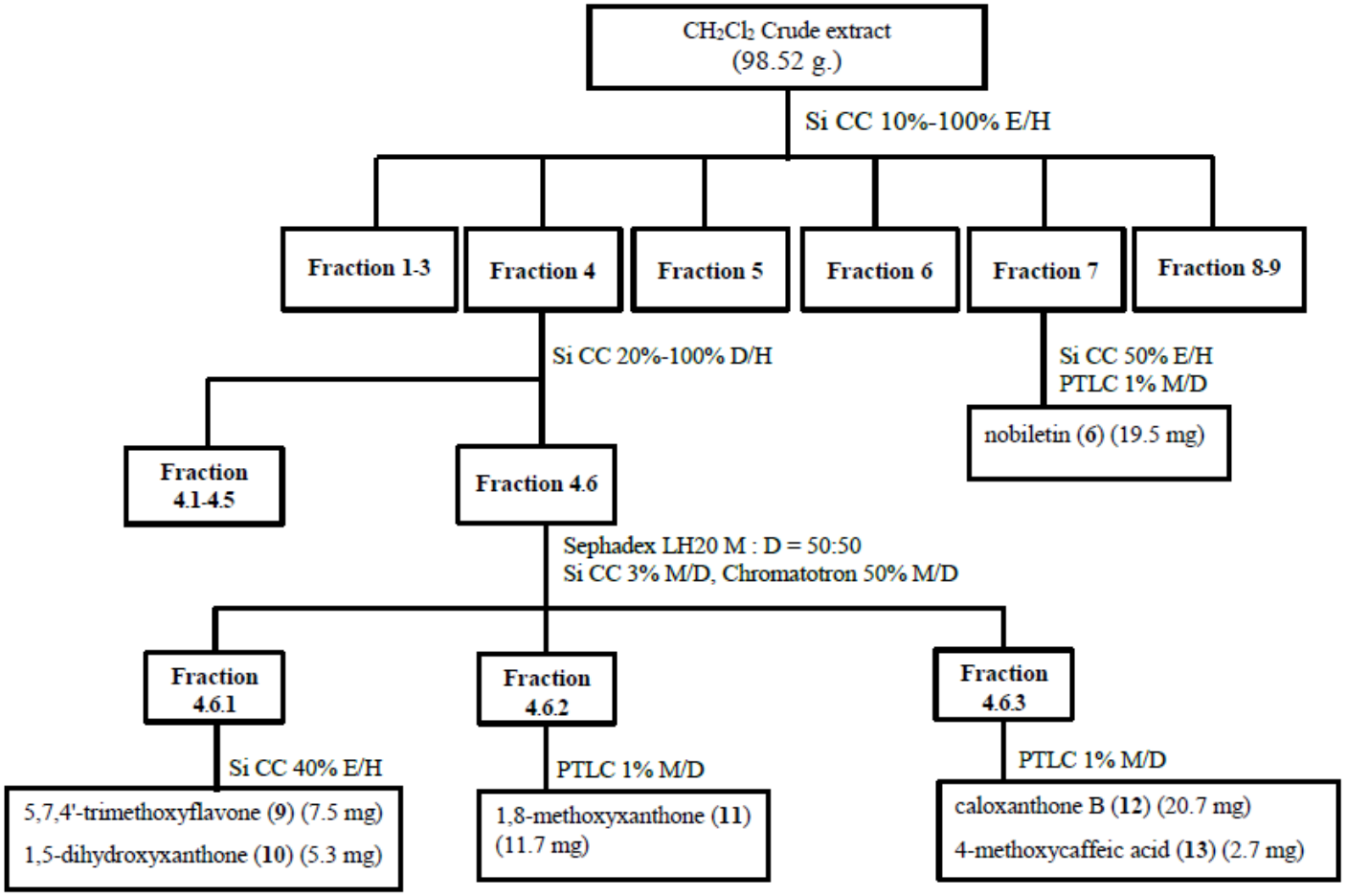
Scheme 2.1 Extraction procedure of *C. inophyllum* roots and fractionation procedure of the CH_2Cl_2 crude extract



Scheme 2.2 Isolation procedure of subfractions 3.1.3, 3.1.4, 3.1.6 and 3.1.8 from the CH_2Cl_2 crude extract of *C. inophyllum* roots



Scheme 2.3 Isolation procedure of subfractions 3.2, 3.3 and 3.5 from the CH_2Cl_2 crude extract of *C. inophyllum* roots



Scheme 2.4 Isolation procedure of subfractions 4.6.1, 4.6.2, 4.6.3 and fraction 7 from the CH_2Cl_2 crude extract of *C. inophyllum* roots

2.4 Bioassay procedures

2.4.1 α -Glucosidase inhibitory activity from Baker's Yeast

The α-glucosidase inhibition assay was performed according to our previous protocols. The α-glucosidase (0.4 U/mL) and substrate (1 mM *p*-nitrophenyl-α-D-glucopyranoside) were dissolved in 0.1 M phosphate buffer (pH 6.9). A 10 µL test sample was pre-incubated with yeast α-glucosidase (40 µL) at 37°C for 10 min. A substrate solution (50 µL) was then added to the reaction mixture and incubated at 37°C for an additional 20 min, and terminated by adding 1 M Na_2CO_3 solution (100 µL). The enzymatic hydrolysis of the *p*-NPG was monitored based on the amount of *p*-nitrophenol released into the reaction mixture (**Figure 2.1**). Enzymatic activity was quantified by measuring the absorbance at 405 nm (Sunrise microplate reader). The percentage inhibition of activity was calculated as follows: % Inhibition = $[(A_0-A_1)/A_0] \times 100$, where: A_0 is the absorbance without the sample; A_1 is the absorbance with the sample. The IC_{50} value was deduced from the plot of % inhibition versus concentration of test sample. Acarbose was used as standard control and the experiment was performed in duplicate [40].

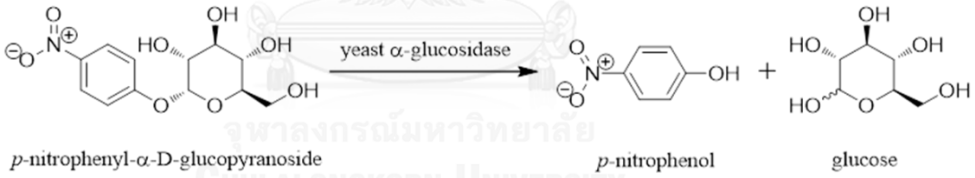


Figure 2.1 Hydrolysis by yeast α-glucosidase.

2.4.2 α-Glucosidase inhibitory activity from rat small intestine

The inhibition against rat small intestinal α-glucosidase (maltase and sucrase) was carried out using a similar protocol with baker's yeast. Briefly, 10 µL of the test sample was added to 0.1 M phosphate buffer (pH 6.9, 30 µL), 20 µL of the substrate solution (maltose: 10 mM; sucrose: 100 mM) in 0.1 M phosphate buffer, glucose kit (80 µL) and the crude enzyme solution (20 µL). The reaction mixture (**Figure 2.2**) was then incubated at 37°C for either 10 min (for maltose) or 40 min (for sucrose). The concentration of glucose released from the reaction mixture was detected by the glucose

oxidase method using a glu-kit (Human, Germany). Enzymatic activity was quantified by measuring absorbance at 503 nm. Experiment was run in duplicate [40].

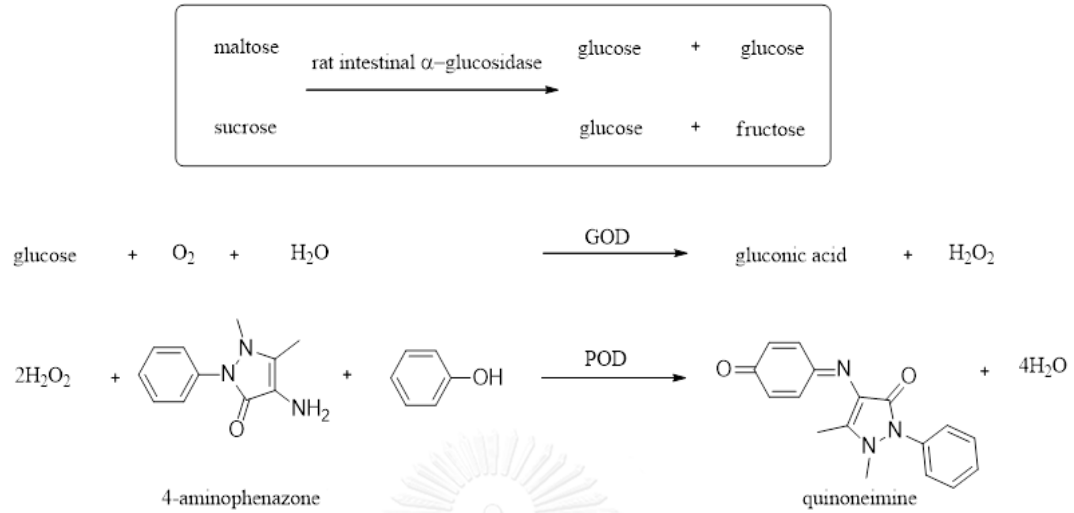


Figure 2.2 Hydrolysis by rat small intestinal α-glucosidase.

2.4.3 Cytotoxic activity against human cancer cell lines

All isolated compounds (**1**-**14**) were evaluated for their *in vitro* cytotoxic activities against the KB (epidermoid carcinoma), HeLa S-3 (cervix adenocarcinoma), HT-29 (colon adenocarcinoma), MCF-7 (breast adenocarcinoma) and Hep G2 (hepatocellular carcinoma) human cancer cell lines using the MTT colorimetric method [41]. Doxorubicin was used as the reference substance.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma Chemical Co., USA) was dissolved in saline to make a 5 mg/mL stock solution. Cancer cells (3 × 103 cells) suspended in 100 µg/wells of MEM medium containing 10% fetal calf serum (FCS, Gibco BRL, Life Technologies, NY, USA) were seeded onto a 96-well culture plate (Costar, Corning Incorporated, NY 14831, USA). After 24 h of pre-incubation at 37 °C in a humidified atmosphere of 5% CO_2/95% air to allow cellular attachment, various concentrations of test solution (10 µL/well) were added and these were then incubated for 48 h under the above conditions. At the end of the incubation, 10 µL of tetrazolium reagent was added into each well followed by further incubation at 37 °C for 4 h. The supernatant was decanted, and DMSO (100 µL/well) was added to allow formosan solubilization. The optical density (OD) of each well was detected using a Microplate reader at 550 nm and for correction at 595 nm. Each determination represented the average mean of six replicates. The 50% inhibition concentration (IC_{50} value) was determined by curve fitting.

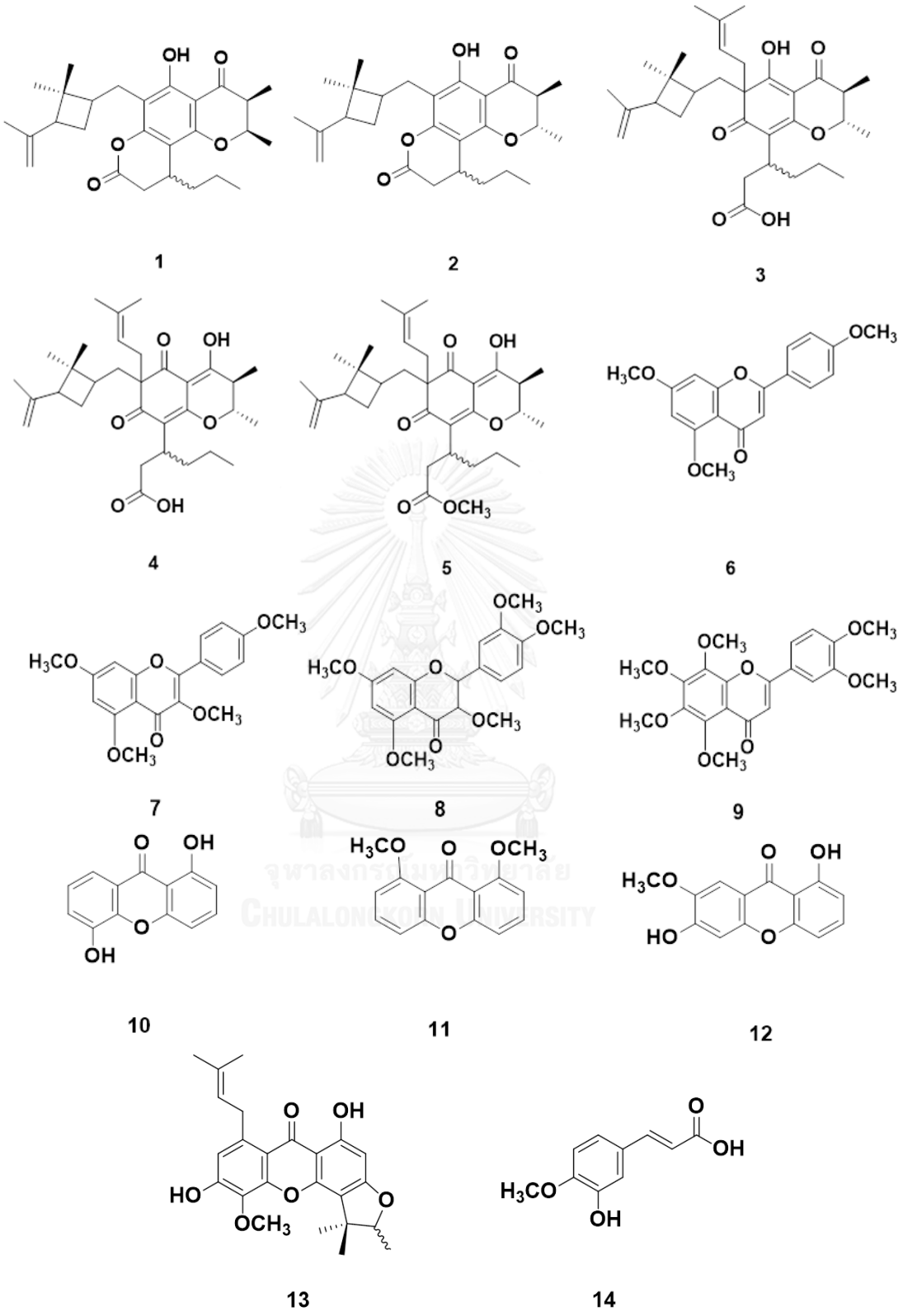


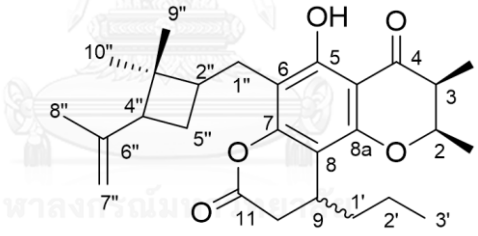
Figure 2.3 Chemical constituents (**1-14**) from the CH_2Cl_2 crude extract of *C. inophyllum* roots

CHAPTER III
RESULTS AND DISCUSSION

3.1 Properties and structural elucidation of isolated compounds

The roots of *C. inophyllum* were grounded and extracted with CH_2Cl_2 at room temperature for six days. The CH_2Cl_2 crude extract was further subjected by various chromatographic techniques to afford fourteen compounds (**1**–**14**), including five new compounds, caloinophyllins A-E (**1**–**5**) and nine known compounds (**6**–**14**), nobiletin (**6**), pentamethylquercetin (**7**), 3,5,7,4'-tetramethoxyflavone (**8**), 5,7,4'-trimethoxyflavone (**9**), 1,5- dihydroxyxanthone (**10**), 1- methoxyxanthone (**11**), caloxanthone B (**12**), 4-methoxycaffeic acid (**13**) and 1,6-dihydroxy-7-methoxyxanthone (**14**). The structures of all isolated compounds were identified by the interpretation of their spectroscopic data and comparison with those reported in the literature.

3.1.1 Caloinophyllin A (**1**)



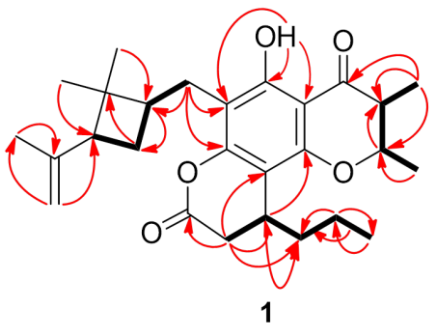
1

Figure 3.1 The chemical structure of **1**.

Caloinophyllin A (**1**) was isolated as yellow gum, and the molecular formula was measured by HRESIMS as $C_{27}H_{35}O_5$ (m/z 441.2640 $[M+H]^+$, calcd. for $C_{27}H_{36}O_5$, 441.2641) (**Figure 3.1**). The ^1H NMR experiment revealed a singlet signal at δ_H 12.22 indicating a chelated hydroxyl moiety in **1**. This hydroxyl proton was assigned to be at C-5 (160.8) where it showed correlations with δ_C 103.5 (C-4a), 110.4 (C-6) and 160.8 (C-5) in the HMBC spectrum. The HMBC correlations between H-1″ [δ_H 2.86 (1H, m[a]) and δ_H 2.67(1H, m[a])] to C-5″ (δ_C 24.6), C-2″ (δ_C 40.5), C-6 (δ_C 110.4), C-7 (δ_C 156.6), C-5 (δ_C 160.8); H-2″ [δ_H 2.00 (1H, m)] and C-5″ (δ_C 24.6), C-4″ (δ_C 48.3); H-8″ [(δ_H 1.66 (3H, s)] and C-4″ (δ_C 48.3), C-7″ (δ_C 109.0) and C-6″ (δ_C 146.6) supported the presence of a 3-

isopropenyl-2,2-dimethylcyclobutylmethyl chain at C-6 (δ_C 110.4).[42] Meanwhile, the signals at δ_H 1.43 (2H, m, H-1′), 1.40 (2H, m, H-2′) and δ_H 0.90 (3H, t, J = 7.2 Hz, H-3′) were attributed to the propyl chain in **1** [33]. This chain was suggested to be at C-9 (δ_C 28.6) from the observation of a HMBC correlation between H-9 [δ_H 3.32 (1H, m)] and C-1′ (δ_C 40.5) and couplings of H-9-H-1′-H-2′-CH_3-3′ in the COSY experiment.

The proton signals at δ_H 2.60 (1H, dq, J = 7.2, 3.2 Hz, H-3), 4.57 (1H, m, H-2), 1.40 (3H, d, J = 6.4 Hz, CH_3-2) and 1.18 (3H, d, J = 7.2 Hz, 3-CH_3), suggested the presence of the highly substituted 2,3-dimethylchromanone ring. The coupling constant of H-2 and H-3 (J = 3.2 Hz) and the carbon signals at 2-CH_3 (δ_C 16.6) and 3-CH_3 (δ_C 9.5) suggested that they were in a *cis* configuration, as in calopolyanic acid and calofolic acid D [16, 33, 43]. In the ^{13}C spectrum, all the carbons were assigned to their respective protons by their correlations in the HSQC spectrum. The ^{13}C NMR spectrum indicated this compound to consist of five methines, six methylenes, six methyls and ten quaternary carbons. The two very downfield signals at δ_C 201.8 (C-4) and 167.5 (C-11) were assigned to carbonyl carbons in this compound. The carbonyl signal at δ_C 167.5 gave cross-peaks with the H-10 [δ_H 2.78 (1H, m[a]) and 2.66 (1H, m[a])] and H-9 [δ_H 3.32 (1H, br m)] signals, indicating it to be at C-11 [44]. The full analysis of both 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR spectra of **1** (**Figure 3.2**) suggested that its structure was close to those of previously semisynthesized compound **2d** [45], except for the presence of *cis*-configuration of H-2 and H-3 consistent with previously reported analogues [16, 33]. Therefore, the chemical structure of **1** was concluded to be a new chromanone derivative, which we name as caloinophyllin A.



1

Figure 3.2 Selected HMBC (single headed arrow curves) and COSY (bold lines) correlations of **1**.

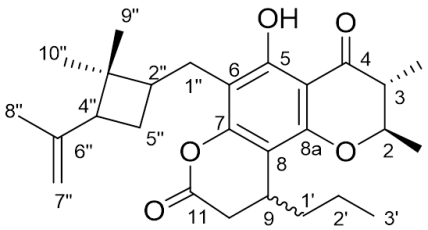
Table 3.1 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of **1** in $CDCl_3$.

Position	**1**		
	δ_H (*J* in Hz)	**δ_C**	**HMBC**
2	4.57 m[a]	76.4	2-CH_3, C-3, 3-CH_3
3	2.60 (dq, 7.2, 3.2)	44.8	3-CH_3, C-4
4		201.8	-
4a		103.5	-
5		160.8	-
6		110.4	-
7		156.6	-
8		105.7	-
8a		155.9	-
9	3.32 br m	28.6	C-8, C-8a, C-10, C-11, C-1′, C-2′
10	2.78 m[a]	34.5	C-8, C-9, C-11, C-1′
	2.66 m[a]		C-8, C-9, C-11, C-1′
11		167.5	-
1′	1.43 m	40.5	C-8, C-9, C-10, C-2′, C-3′
2′	1.40 m	20.0	C-1′, C-3′
3′	0.90 (t, 7.2)	14.1	C-1′, C-2′
1″	2.86 m[a]	23.0	C-5, C-6, C-7, C-2″, C-5″
	2.67 m[a]		C-5, C-6, C-7, C-2″, C-5″
2″	2.00 m	40.5	C-4″, C-5″
3″		36.9	-

4″	2.68 m[a]	48.3	C-2″, C-6″, C-7″, C-10″
5″	1.89 m[a]	24.6	C-1″, C-2″, C-4″
	1.58 m[a]		C-2″, C-4″
6″		146.6	-
7″	4.78 s	109.0	C-4″, C-8″
	4.54 s		C-4″, C-8″
8″	1.66 s	23.5	C-4″, C-6″, C-7″
9″	0.92 s	24.1	C-2″, C-3″, C-4″, C-10″
10″	1.18 s	25.1	C-2″, C-4″
2-CH_3	1.40 (d, 6.4)	16.6	C-2, C-3
3-CH_3	1.18 (d, 7.2)	9.5	C-2, C-3, C-4
5-OH	12.22 s		C-4a, C-5, C-6

[a]Signals overlapped.

3.1.2 Caloinophyllin B (2)

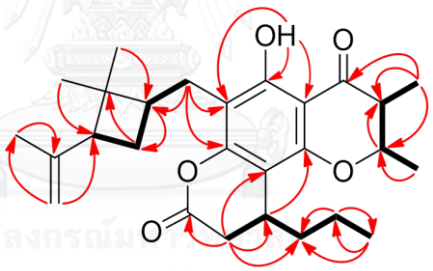


2

Figure 3.3 The chemical structure of **2**.

Caloinophyllin B (**2**), yellow gum, was obtained as a pair of compounds, **2a** and **2b**, with a molecular formula $C_{27}H_{35}O_5$, deduced from the HR-ESI-MS data (m/z 441.2605 $[M+H]^+$, $C_{27}H_{36}O_5$; calcd. 441.2641) (**Figure 3.3**). In the ^1H and ^{13}C NMR

spectra, some signals were found in double, by which **2** was speculated as a mixture of two isomers (**2a** and **2b**) as occurs in isoclopolyanic acid. Unfortunately, several attempts to separate these compounds by HPLC using various eluent systems have been failed. The ratio **2a**/**2b** was ca. 2 : 1 by the proton integration in the ^{1}H NMR spectrum of **2**. The ^{1}H and ^{13}C NMR data of **2a** and **2b** showed similar signals to those of **1**, except the appearance of *trans*-orientation of two protons at C-2 and C-3 (δ_H 2.60: 1H, dq, 7.2, 12.0 Hz, H-3) of the 2,3-dimethylchromanone ring. The 2-CH_3 (δ_C 19.8) and 3-CH_3 (δ_C 10.3) suggested that they were in a *trans* configuration [43]. Based on the 1D and 2D NMR analyses, the planar structure of **2b** were very similar to those of **2a** (**Figure 3.4**). From the values of the coupling constant (H-2 and H-3) of both **2a** and **2b** were determined as *trans*-configuration, which implied that **2a** and **2b** might be a pair of C-9-epimers [33]. Thus, the structure of **2** was established and named caloinophyllin B.



2

Figure 3.4 Selected HMBC (single headed arrow curves) and COSY (bold lines) correlations of **2**.

Table 3.2 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of **2** in $CDCl_3$.

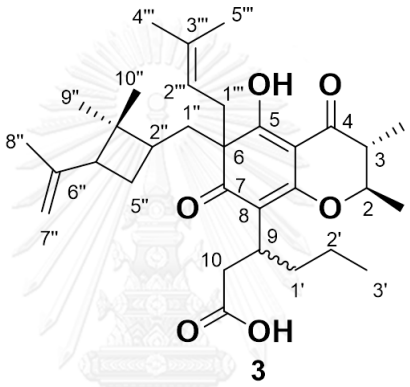
Position	**2a**		**2b**		**HMBC**
	δ_H (*J* in Hz)	**δ_C**	**δ_H (*J* in Hz)**	**δ_C**	
2	4.20 m[a]	79.3	4.20 m[a]	79.1	-
3	2.60 (dq, 7.2, 12.0)	46.2	2.60 (dq, 7.2, 12.00)	46.2	C-2, 3-CH_3, C-4

4		200.0		199.8	-
4a		104.1		104.1	-
5		160.5		160.5	-
6		110.3		110.2	-
7		155.6		155.6	-
8		105.6		105.6	-
8a		155.9		155.9	-
9	3.31 br m	28.6	3.31 br m	28.6	C-8, C-11, C-1′, C-2′
10	2.79 m[a]	34.4	2.79 m[a]	34.4	C-8, C-11, C-1′
	2.64 m[a]		2.64 m[a]		C-9, C-11, C-1′
11		167.6		167.5	-
1′	1.45 m	35.7	1.45 m	35.7	C-2′
2′	1.38 m	19.9	1.38 m	19.9	C-3′
3′	0.91 (t, 7.2)	14.0	0.91 (t, 7.2)	14.0	C-1′, C-2′
1″	2.87 m	23.0	2.87 m	22.7	C-6, C-7, C-2″
	2.68 m		2.68 m		C-6, C-7, C-2″
2″	2.01 m	40.5	2.01 m	40.4	C-4″
3″		36.9		36.9	-
4″	2.70 m[a]	48.3	2.70 m[a]	48.3	C-6″
5″	1.89 m[a]	24.5	1.89 m[a]	24.5	C-1″, C-2″
	1.57 m[a]		1.57 m[a]		C-4″
6″		146.6		146.6	-
7″	4.79 s	109.5	4.79 s	109.5	C-4″, C-8″
	4.56 s		4.56 s		C-4″, C-8″
8″	1.67 s	23.5	1.67 s	23.5	C-4″, C-6″, C-7″

9″	0.93 s	24.1	0.93 s	24.1	C-2″, C-4″, C-10″
10″	1.18 s	25.1	1.18 s	25.1	C-3″, C-4″, C-9″
2-CH_3	1.52 (d, 6.4)	19.8	1.52 (d, 6.4)	19.7	C-2, C-3
3-CH_3	1.23 (d, 7.2)	10.3	1.22 (d, 7.2)	10.1	C-2, C-3, C-4
5-OH	12.29 s		12.27 s		C-4a, C-5, C-6

[a]Signals overlapped.

3.1.3 Caloinophyllin C (3)



3

Figure 3.5 The chemical structure of **3**.

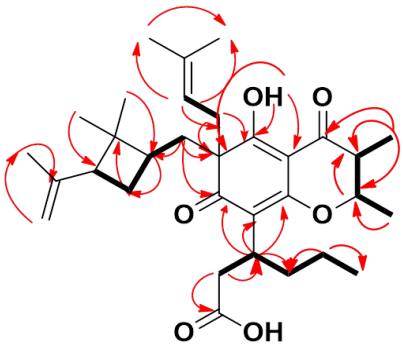
Caloinophyllin C (**3**) was obtained as pale yellow gum, and the molecular formula was determined to be $C_{32}H_{46}O_6$ by HRESIMS, giving a molecular ion at m/z 527.3343 $[M+H]^+$ (calcd. for $C_{32}H_{47}O_6$, 527.3373) (**Figure 3.5**) and by the ^1H and ^{13}C NMR data (**Table 3.3**).

Some signals in the ^1H and ^{13}C NMR spectra of **3** were presented in duplicates; we proposed that **3** was a mixture of two isomers (**3a** and **3b**) that similarly occurs in **2**. The separation of these compounds by HPLC using various eluent systems has been failed. The careful analysis of the ^1H NMR data of **3** were very similar to those of inophylloidic acid [46] except the stereochemistry of methyl groups between C-2 and C-3 (**Figure 3.6**).

^1H and ^{13}C NMR data of **3** (**Table 3.3**) pointed to the presence of a prenylated chromanone acid substituted with a 2-carboxyl-1-propyl group. The proton signals corresponding to two methane at δ_H 2.46 (1H, dq, 6.8, 11.5 Hz, H-3) and 4.24 (1H, m[a], H-2)] and two secondary methyl groups at δ_H 1.19 (3H, d, J = 6.8 Hz, 3-CH_3) and 1.53 (3H,

d, J = 6.0 Hz, 2-CH_3) confirmed the presence of the highly substituted 2,3-dimethylchromanone ring. A downfield shifted proton signal resonating at δ 12.16 (br s) revealed the presence of a hydroxyl proton participating in a strong hydrogen bond. Two signals resonating at δ_C 197.0 (C-4) and 199.0 (C-7) indicated two unsaturated ketone carbonyl groups, and a carbon signal at δ_C 185.5 (C-5) pointed to the presence of a carboxylic group. The ^{1}H NMR and COSY spectra showed the presence of a vinyl proton δ_H 4.76 (m, H-2′′′) coupled to two vinylic methyl groups at δ_H 1.53 (3H, s, H-5′′′) and 1.63 (3H, s, H-4′′′), indicating an isopent-2-enyl group. Two signals of terminal methylenes at δ_H 4.50 (1H, s, H-7′′) and 4.76 (1H, s, H-7′′) together with a methyl group δ_H 1.59 (3H, s, H-8′′) pointed to the presence of an isopropenyl group. The HMBC correlations (**Figure 3.6**) between H-1′′′ and C-5, C-6 and C-7 established that the isopent-2-enyl group was substituted at C-6. HMBC correlations of the hydroxyl proton (δ_H 12.16, br s) to C4a, C-5 and C-6 determined the position of the hydroxyl group at C-5. The HMBC cross-peaks between H-4′′ and C-3′′, C-6′′, C-9′′; H-5′′ and C-4′′, C-6′′; H-8′′and C4′′, C6′′, C7′′; H-9′′ and C-2′′, C-4′′, C-10′′; H-10′′ and C-4′′, C-9′′ supported the presence of a 3-isopropenyl-2,2-dimethylcyclobutylmethyl chain at C-6. Finally, HMBC cross-peaks between H-9 and C-7, C-8, C-8a, C-10, C-11, C-1′; H-10 and C-9, C-11, C-1′clearly showed that the 2-carboxyl-1-propyl moiety must be substituted at C-8 of the chromanone. The ^{13}C NMR spectrum of **3** showed signals corresponding to 32 carbons, eight methyls, seven methylenes, six methines and eleven quaternary carbons. The comparison of the ^{13}C NMR of 2-CH_3 (δ_C 18.2) and 3-CH_3 (δ_C 10.7) with the literature data of 2,3-dimethylchromanone [33] clearly indicated that compounds **3a** and **3b** were the *trans* isomer.

All of the above arguments allowed the complete assignment of all signals and the identification of compound **3** was caloinophyllin C.



3

Figure 3. 6 Selected HMBC (single headed arrow curves) and COSY (bold lines) correlations of **3**.

Table 3.3 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of **3** in $CDCl_3$.

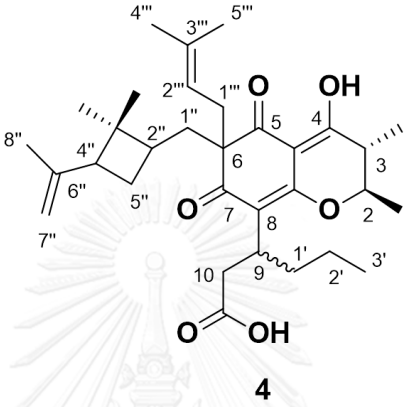
Position	**3a**		**3b**		**HMBC**
	δ_H (*J* in Hz)	**δ_C**	**δ_H (*J* in Hz)**	**δ_C**	
2	4.24 m[a]	81.3	4.24 m[a]	81.2	C-4
3	2.46 (dq, 6.8, 11.5)	44.6	2.45 m	46.6	C-2
4		197.0		197.0	-
4a		105.3		105.0	-
5		185.5		185.3	-
6		57.2		57.1	-
7		199.0		199.0	-
8		112.8		112.7	-
8a		166.9		166.9	-
9	3.42 m	30.1	3.42 m	29.8	C-7, C-8, C-8a, C-10, C-11, C-1′
10	2.71 m[a]	37.9	2.71 m[a]	37.9	C-9, C-11, C-1′
	1.46 m[a]		1.46 m[a]		C-9, C-11, C-1′

11		177.5		177.5	-
1′	1.76 m	34.8	1.76 m	34.6	C-2′, C-3′
	1.48 m		1.48 m		C-9, C-3′
2′	1.19 m	21.2	1.19 m	21.2	C-3′
3′	0.85 (t, 7.2)	14.4	0.85 (t, 7.2)	14.4	C-1′, C-2′
1″	2.72 m	40.7	2.72 m	40.2	C-5, C-6
	2.50 m		2.50 m		C-5, C-7, C-2″
2″	1.37 m	38.0	1.37 m	38.0	-
3″		41.0		40.9	-
4″	2.39 (t, 6.8)	48.6	2.39 (t, 6.8)	48.6	C-3″, C-6″, C-9″
5″	1.84 m[a]	26.7	1.84 m[a]	26.5	C-4″, C-6″
6″		145.8		145.7	-
7″	4.76 s	109.2	4.76 s	109.2	C-4″, C-8″
	4.50 s		4.50 s		C-4″, C-8″
8″	1.59 s	23.8	1.59 s	23.8	C-4″, C-6″, C-7″
9″	0.78 s	25.0	0.78 s	24.9	C-2″, C-4″, C-10″
10″	1.03 s	24.5	1.03 s	24.4	C-4″, C-9″
1‴	2.19 m	40.7	2.19 m	40.7	C-5, C-6, C-7
	1.91 m		1.91 m		C-5, C-6, C-7
2‴	4.76 m	117.6	4.76 m	117.6	C-6, C-1″, C-4‴, C-5‴
3‴		135.3		134.9	-
4‴	1.63 s	25.9	1.63 s	25.9	C-2‴, C-3‴, C-5‴
5‴	1.53 s	18.0	1.53 s	18.0	C-2‴, C-3‴, C-4‴
2-CH_3	1.53 (d, 6.0)	18.2	1.53 (d, 6.0)	18.2	C-2, C-3

3-CH_3	1.19 (d, 6.8)	10.7	1.19 (d, 6.8)	10.7	C-2, C-3, C-4
5-OH	12.16 br s		12.16 br s		C-4a, C-5, C-6

[a]Signals overlapped.

3.1.4 Caloinophyllin D (**4**)

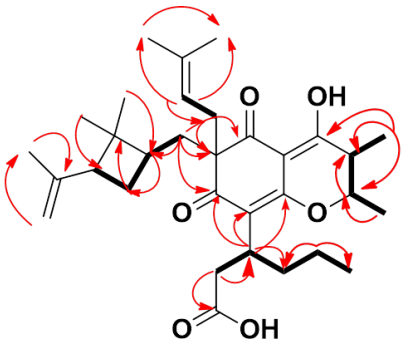


4

Figure 3.7 The chemical structure of **4**.

Caloinophyllin D (**4**) was isolated as light yellow gum, and its molecular formula was established as $C_{32}H_{46}O_6$ through the protonated molecular ion at m/z 527.3377 ([M + H]$^+$, calcd 527.3373) in the HRESIMS spectrum (**Figure 3.7**). The combination of ^{13}C NMR and HSQC spectra showed the presence of 32 signals, including eight methyls, seven methylenes, six methines and eleven quaternary carbons. The careful consideration of the ^{1}H and ^{13}C NMR spectra (**Table 3.4**) showed some signals being duplicates as a mixture of two isomers (**4a** and **4b**) that occurs in **2** and **3**. The ^{1}H and ^{13}C NMR data obtained for **4** were similar to those of brasiliensophyllic acid C [33], except for the presence of 3-isopropenyl-2,2-dimethylcyclobutylmethyl chain instead of 5-methyl-2-(2-methylpropenyl)hex-5-enyl chain linked to C-6, and 2-carboxyl-1-propyl chain instead of a benzyl ring linked to C-8 (**Figure 3.8**).

The comparison of the ^{13}C NMR of 2-CH_3 (δ_C 19.6) and 3-CH_3 (δ_C 9.9) with the previous literature indicated that compound **4** was also a *trans* isomer.



4

Figure 3.8 Selected HMBC (single headed arrow curves) and COSY (bold lines) correlations of **4**.

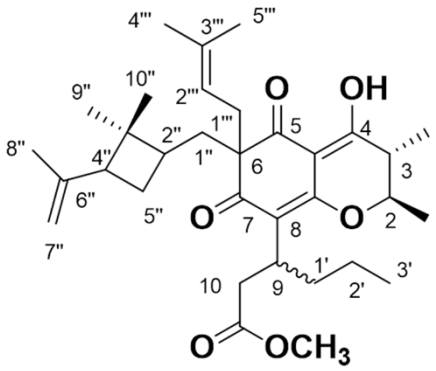
Table 3.4 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of **4** in $CDCl_3$.

Position	**4a**		**4b**		**HMBC**
	δ_H (*J* in Hz)	**δ_C**	**δ_H (*J* in Hz)**	**δ_C**	
2	4.10 m[a]	77.2	4.10 m[a]	77.1	C-4, C-8a, 3-CH_3
3	2.60 (dq, 6.8, 11.8)	42.7	2.60 (dq, 6.8, 11.8)	42.6	C-2, C-4, 2-CH_3, 3-CH_3
4		190.6		190.3	-
4a		103.0		102.8	-
5		196.8		196.7	-
6		59.8		59.8	-
7		199.3		199.2	-
8		113.7		113.7	-
8a		163.7		163.5	-
9	3.46 m	30.7	3.46 m	30.6	C-7, C-8, C-8a, C-11, C-1′
10	2.71 m[a]	38.0	2.71 m[a]	37.9	C-8, C-9, C-11, C1′
11		179.3		179.3	-

1′	1.76 m	34.9	1.76 m	34.9	C-9, C-10, C-2′, C-3′
	1.49 m		1.49 m		C-9, C-10, C-2′, C-3′
2′	1.17 m	21.0	1.17 m	21.0	C-9, C-3′
3′	0.85 (t, 6.2)	14.3	0.85 (t, 6.2)	14.2	C-1′, C-2′
1″	2.58 m	40.6	2.58 m	40.6	C-5, C-6, C-7, C-2″
2″	1.46 m	38.1	1.46 m	38.0	C-5″
3″		39.5		39.5	-
4″	2.36 m[a]	48.5	2.36 m[a]	48.5	C-2″, C-3″, C-5″,C-6″,C-7″
5″	1.79 m[a]	25.7	1.79 m[a]	25.7	C-1″
6″		145.9		145.9	-
7″	4.73 s	108.9	4.73 s	108.8	C-4″, C-6″, C-8″
	4.49 s		4.49 s		C-4″, C-6″, C-8″
8″	1.58 s	23.7	1.58 s	23.7	C-4″, C-6″, C-7″
9″	0.75 s	23.8	0.75 s	23.8	C-2″, C-3″, C-4″, C-10″
10″	1.03 s	24.8	1.03 s	24.8	C-3″, C-4″, C-9″
1‴	2.19 m	41.1	2.16	41.1	C-7, C-1″
	2.03 m		2.00		C-6, C-1″
2‴	4.77 m	117.8	4.77	117.8	C-4‴, C-5‴
3‴		135.2		135.1	-
4‴	1.58 s	25.8	1.58 s	25.7	C-2‴, C-3‴, C-5‴
5‴	1.56 s	17.8	1.56 s	17.8	C-4‴
2-CH_3	1.46 (d, 6.4)	19.6	1.46 m	19.5	C-2, C-3
3-CH_3	1.18 (d, 6.8)	9.9	1.18 m[a]	9.9	C2, C3, C4
4-OH	12.00 s		12.00 s		-

[a]Signals overlapped.

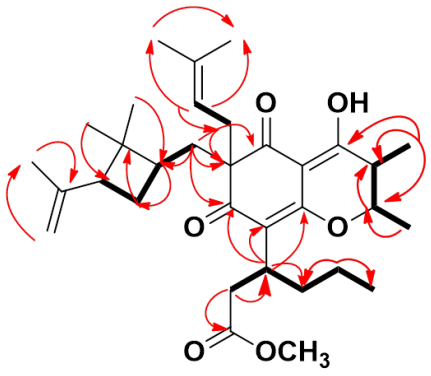
3.1.5 Caloinophyllin E (**5**)



5

Figure 3.9 The chemical structure of **5**.

Caloinophyllin E (**5**) was obtained as a yellow amorphous powder. Its molecular formula was determined as $C_{33}H_{48}O_6$ from the positive-ion HRESIMS data (m/z 563.3266 ($[M+Na]^+$, calcd 563.3349) (**Figure 3.9**). The careful consideration of the ^1H and ^{13}C NMR spectra of **5**, some of signals in were presented in duplicates as a mixture of two isomers (**5a** and **5b**). The ^1H and ^{13}C NMR data in which 33 carbon signals were observed (**Table 3.5**). Compound **5** was similar to those of **4**, except for the presence of methoxy group instead of hydroxyl group at C-11, and application of the same structure elucidation strategy confirmed that **5** as *trans* form. The structure of compound **5** was finally confirmed by COSY, HSQC and HMBC experiments (**Figure 3.10**). We proposed the trivial name as caloinophyllin E (**5**) which was methyl ester of compound **4**.



5

Figure 3.10 Selected HMBC (single headed arrow curves) and COSY (bold lines) correlations of **5**.

Table 3.5 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of **5** in $CDCl_3$.

Position	**5a**		**5b**		**HMBC**
	δ_H (*J* in Hz)	**δ_C**	**δ_H (*J* in Hz)**	**δ_C**	
2	4.51 m[a]	74.7	4.39 m[a]	74.6	C-4, C-8a, 3-CH_3
3	2.59 (dq, 7.2, 11.5)	43.1	2.60 (dq, 7.2, 11.5)	43.0	C-2, C-4, 2-CH_3, 3-CH_3
4		190.7		190.7	-
4a		103.4		103.3	-
5		200.0		200.0	-
6		60.1		60.1	-
7		196.9		196.8	-
8		114.3		114.3	-
8a		163.6		163.6	-
9	3.49 m	31.3	3.49 m	31.3	C-7, C-8, C-8a, C-11, C-1′
10	2.70 m[a]	38.3	2.70 m[a]	38.3	C-8, C-9, C-11, C-1′
11		174.4		174.4	-

1′	1.73 m	35.5	1.73 m	35.5	C-9, C-10, C-2′, C-3′
	1.42 m		1.42 m		C-9, C-10, C-2′, C-3′
2′	1.18 m	21.4	1.18 m	21.4	C-9, C-3′
3′	0.86 (t, 6.4)	14.7	0.86 (t, 6.4)	14.7	C-1′, C-2′
1″	2.63 m	41.0	2.63 m	41.0	C-5, C-6
	2.58 m		2.58 m		C-6, C-2″
2″	1.48 m	38.4	1.48 m	38.4	C-5″
3″		39.9		38.3	-
4″	2.36 m[a]	49.0	2.36 m[a]	49.0	C2″, C3″, C5″,C6″,C7″
5″	1.64 m[a]	26.1	1.64 m[a]	26.1	C1″
6″		146.4		146.4	-
7″	4.76 s	109.2	4.76 s	109.2	C-4″, C-6″, C-8″
	4.51 s		4.51 s		C-4″, C-6″, C-8″
8″	1.65 s	22.9	1.65 s	22.9	C-4″, C-6″, C-7″
9″	0.76 s	24.5	0.76 s	24.5	C-2″, C-3″, C-4″, C-10″
10″	1.03 s	25.2	1.03 s	25.2	C-1″, C-3″, C-4″, C-9″
1‴	2.16 m	40.7	2.16	40.6	C-7, C-1″
	1.90 m		1.90		C-6, C-1″
2‴	4.80 m	118.4	4.80	118.3	C-4‴, C-5″
3‴		135.4		135.4	-
4‴	1.59 s	24.2	1.59	24.1	C-2‴, C-3‴, C-5‴
5‴	1.35 s	16.7	1.35	16.7	C-4‴
2-CH_3	1.50 (d, 6.4)	18.2	1.50 (d, 6.4)	18.1	C-2, C-3
3-CH_3	1.17 (d, 7.2)	10.3	1.17 (d, 7.2)	10.3	C-2, C-3, C-4

OCH_3	3.58 s		51.6	3.58 s	51.6	C11

[a]Signals overlapped.

3.1.6 Nobiletin (**6**)

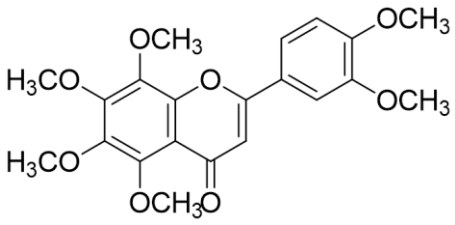


Figure 3.11 The chemical structure of **6**.

Nobiletin (**6**) (**Figure 3.11**): a yellow granule, ^{1}H-NMR (400 MHz, $CDCl_3$); δ_H 7.57 (1H, dd, J = 2.0 and 8.4 Hz), 7.42 (1H, d, J = 2.0 Hz), 7.00 (1H, d, J = 8.4 Hz), 6.63 (1H, s), 4.10, 4.03, 3.98, 3.97, and 3.95 (each s, OCH_3×5). The structure of compound **6** was determined and confirmed by comparison of the physical and spectroscopic data with a previous report [47].

3.1.7 Pentamethylquercetin (**PMQ**) (**7**)

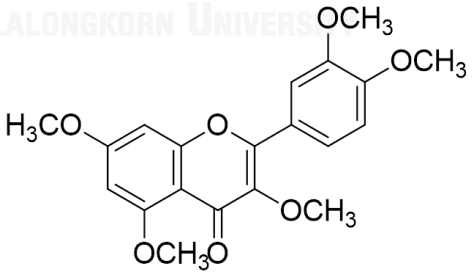


Figure 3.12 The chemical structure of **7**.

Pentahydroxyflavone (**7**) (**Figure 3.12**): a yellow gum, ^{1}H-NMR (400 MHz, $CDCl_3$); δ_H 7.70 (1H, d, J = 2.0 Hz), 6.98 (1H, d, J = 8.8 Hz), 6.51 (1H, d, J = 2.0 Hz), 6.35 (1H, d, J = 1.6 Hz). The structure of compound **7** was determined and confirmed by comparison of the physical and spectroscopic data with a previous report [48].

3.1.8 3,5,7,4'-Tetramethoxyflavone (**8**)

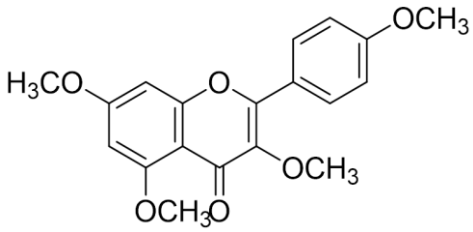


Figure 3.13 The chemical structure of **8**.

3,5,7,4'-Tetramethoxyflavone (**8**) (**Figure 3.13**): a yellow gum, ^{1}H-NMR (400 MHz, $CDCl_3$); δ_H 8.09 (1H, d, J = 8.8 Hz), 7.03 (1H, d, J = 8.8 Hz), 6.53 (1H, d, J = 2.0 Hz), 6.37 (1H, d, J = 2.4 Hz), 3.98 (3H, s), 3.91 (1H, d, J = 3.6 Hz), 3.89 (1H, s). The structure of compound **7** was determined and confirmed by comparison of the physical and spectroscopic data with a previous report [49].

3.1.9 5,7,4'-Trimethoxyflavone (**9**)

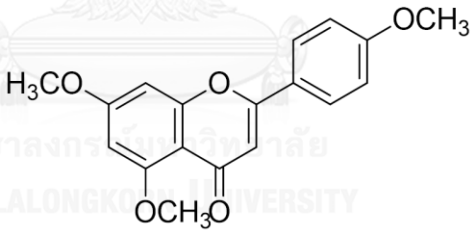


Figure 3.14 The chemical structure of **9**.

5,7,4'-Trimethoxyflavone (**9**) (**Figure 3.9**): a pale of yellow gum, ^{1}H NMR (400 MHz, $CDCl_3$); δ_H 7.83 (1H, d, J = 8.8 Hz),7.00 (1H, d, J = 8.8 Hz), 6.60 (1H, d, J = 2.0 Hz), 6.40 (1H, d, J = 2.0 Hz), 3.96 (3H, s), 3.90 (1H, d, J =12.0 Hz). The structure of compound **9** was determined and confirmed by comparison of the physical and spectroscopic data with a previous report [50].

3.1.10 1,5-Dihydroxyxanthone (**10**)

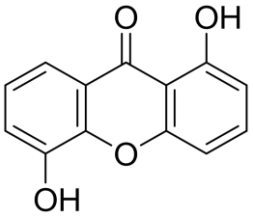


Figure 3.15 The chemical structure of **10**.

1,5-Dihydroxyxanthone (**10**) (**Figure 3.15**): a yellow granule, ^{1}H NMR (400 MHz, DMSO-D_6); 7.74 (lH, t, J = 8.4 Hz), 7.60 (lH, dd, J = 8.0, 1.6 Hz). 7.40 (lH, dd, J = 8.0,1.6 Hz), 7.30 (lH, t, J = 8.0 Hz), 7.10 (lH, d, J = 8.4 Hz), 6.84 (lH, dd = 8.2, 0.8 Hz), 3.89 (3H, s, OCH_3). The structure of compound **10** was determined and confirmed by comparison of the physical and spectroscopic data with a previous report [18].

3.1.11 1,8-Dimethoxyxanthone (**11**)

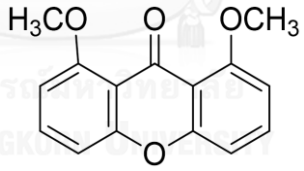


Figure 3. 16 The chemical structure of **11**.

1,8-Dimethoxyxanthone (**11**) (**Figure 3.16**): a yellow granule, ^{1}H NMR (400 MHz, $CDCl_3$); δ_H 7.56 (2H, t, J = 8.4), 6.99 (2H, d, J = 8.4,), 6.77 (2H, d, J = 8.4,) and 4.5,4.01 (6H, s, OCH_3). The structure of compound **11** was determined and confirmed by comparison of the physical and spectroscopic data with a previous report [51].

3.1.12 Caloxanthone B (12)

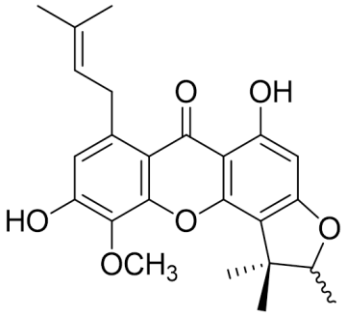


Figure 3.17 The chemical structure of **12**.

Caloxanthone B (**12**) (**Figure 3.17**): a yellow granule, ^{1}H NMR (400 MHz, $CDCl_3$); δ_H 13.70 (lH, s, OH), 6.82 (lH, s), 6.24 (lH, s), 5.37 (1H, t-like m), 4.53 (lH, q, J = 7.8), 4.03 (3H, s, OCH_3), 4.00 (2H, d, J = 6.8 Hz), 1.77 (6H, s, 2 × CH_3), 1.44 (3H, d, J=8.0 Hz), 1.33,1.61 (3H each, s). The structure of compound **12** was determined and confirmed by comparison of the physical and spectroscopic data with a previous report [18].

3.1.13 4-Methoxycaffeic acid (13)

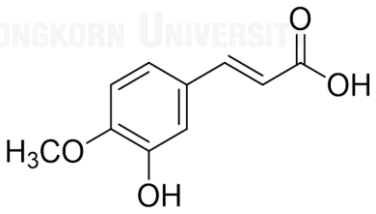


Figure 3.18 The chemical structure of **13**.

4-Methoxycaffeic acid (**13**) (**Figure 3.18**): a pale of yellow gum, ^{1}H NMR (400 MHz, $CDCl_3$); δ_H 7.60 (1H, d, J = 16.0 Hz), 7.07 (1H, dd, J = 8.0, 1.6 Hz), 7.03 (1H, d, J = 1.2 Hz), 6.91 (1H, d, J = 8.4 Hz), 6.29 (1H, d, J = 16.0 Hz), 3.93 (3H, s). The structure of compound **13** was determined and confirmed by comparison of the physical and spectroscopic data with a previous report [52].

3.1.14 1,6-Dihydroxy-7-methoxyxanthone (14)

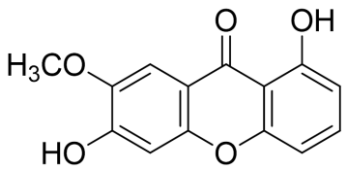


Figure 3.19 The chemical structure of **14**.

1,6-Dihydroxy-7-methoxyxanthone (**14**) (**Figure 3.19**): a pale of yellow gum, ^{1}H NMR (400 MHz, DMSO-d_6); δ_H 12.99 (1H, s, OH), 12.63 (1H, s, OH), 7.65 (1H, t, J = 8.2 Hz), 7.46 (1H, s), 7.02 (1H, dd, J = 8.4, 1.0 Hz), 6.94 (1H, s), 6.77 (1H, dd, J = 8.4, 1.0 Hz), 3.36 (3H, s, OCH_3). The structure of compound **14** was determined and confirmed by comparison of the physical and spectroscopic data with a previous report [53].

3.2 Bioassay activities of isolated compounds

3.2.1 α-Glucosidases inhibitory activities

All the isolated compounds (**1**–**14**) afforded in the present investigation were evaluated for their Baker's yeast and rat intestinal (maltase and sucrase) α-glucosidase inhibitory activities. The results revealed that all isolated compounds (**1**-**14**) displayed no activity against both yeast (IC_{50} > 600 μM) and rat intestinal (IC_{50} > 3,000 μM) α-glucosidases.

3.2.2 Cytotoxic activity of isolated compounds against human cancer cell lines

All isolated compounds (**1**-**14**) were evaluated *in vitro* for their cyctotoxicity potential against KB, HeLa S-3, MCF-7, Hep G2 and HT-29 cancer cell lines using the modified MTT method with doxorubicin used as a positive control [39]. The *in vitro* cytotoxic activities of these compounds were shown in **Table 3.6**. Caloinophyllin E (**5**) exhibited cytotoxicity against five cancer cell lines with an IC_{50} values of 0.79, 0.78, 1.83, 10.35 and 7.58 μM, respectively .1,6-Dihydroxy-7-methoxyxanthone (**14**) exhibited cytotoxicity against KB, HeLa S-3 and MCF-7 cell lines with IC_{50} values of 25.84, 18.19 and 23.46 μM. Moreover, Caloinophyllin A, B, D (**1**, **2**, **4**) and Caloxanthone B (**12**) also

exhibited cytotoxicity against KB, HeLa S-3 and MCF-7 cell lines with an IC_{50} values in the range of 55.77-95.47 µM. The other compounds were inactive (IC_{50} > 100 µM).

Table 3.6 The in vitro cytotoxic activity of compounds **1**-**14** against five human cancer cell lines

Compound	IC_{50} (μM) ± SD				
	KB	Hela S-3	MCF-7	Hep G2	HT-29
Caloinophyllin A (**1**)	83.21 ± 1.71	70.50 ± 2.92	94.54 ± 6.83	>100	>100
Caloinophyllin B (**2**)	58.58 ± 1.60	55.77 ± 0.90	65.26 ± 1.84	95.47 ± 1.48	>100
Caloinophyllin C (**3**)	>100	>100	-	-	-
Caloinophyllin D (**4**)	58.58 ± 0.76	88.13 ± 4.60	92.70 ± 6.43	>100	>100
Caloinophyllin E (**5**)	0.79 ± 0.01	0.78 ± 0.04	1.83 ± 0.05	10.35 ± 0.21	7.58 ± 0.41
Nobiletin (**6**)	>100	>100	-	-	-
Pentahydroxyflavone (**7**)	>100	>100	-	-	-
3,5,7,4'-Tetramethoxyflavone (**8**)	>100	>100	-	-	-
5,7,4'-Trimethoxyflavone (**9**)	>100	>100	-	-	-
1,5-Dihydroxyxanthone (**10**)	>100	>100	-	-	-
1,8-Dimethoxyxanthone (**11**)	>100	>100	-	-	-
Caloxanthone B (**12**)	56.32 ± 1.91	58.58 ± 2.15	95.47 ± 4.62	>100	>100
4-Methoxycaffeic acid (**13**)	>100	>100	-	-	-
1,6-Dihydroxy-7-methoxyxanthone **(14)**	25.84 ± 2.43	18.19 ± 0.19	23.46 ± 0.79	>100	>100
Doxorubicin	0.15 ± 0.03	0.02 ± 0.01	0.67 ± 0.02	1.97 ± 0.05	0.59 ± 0.03

$IC_{50} \leq 10$ μM= active; $10 < IC_{50} \leq 30$ μM= moderate; $30 < IC_{50} \leq 100$ μM= weak; $IC_{50} > 100$ μM= inactive

CHAPTER IV
CONCLUSION

In conclusion, the isolated compounds from the CH_2Cl_2 crude extract from the roots of *C. inophyllum* by various chromatographic techniques, preparative TLC, chromatotron, HPLC, silica gel and sephadex LH-20 column chromatographies were five new chromanone derivatives, caloinophyllins A-E (**1**-**5**) and nine known compounds (**6**-**14**), nobiletin (**6**), pentamethylquercetin (PMQ) (**7**), 3,5,7,4'-tetramethoxyflavone (**8**), 5,7,4'-trimethoxyflavone (**9**), 1,5-dihydroxyxanthone (**10**), 1,8-dimethoxyxanthone (**11**), caloxanthone B (**12**), 4-methoxycaffeic acid (**13**) and 1,6-dihydroxy-7-methoxyxanthone (**14**). The chemical structures of all isolated compounds were characterized by means of NMR and compared with previous reports. Moreover, all isolated compounds were assessed for their in vitro cytotoxicity against the KB, HeLa S-3, MCF-7, HT-29 and Hep G2 human cancer cell lines. The results revealed that compound **5** showed a good cytotoxicity against five cancer cell lines with IC_{50} values in the range of 0.78-10.35 µM. Compound **14** showed a moderate activity against KB, HeLa S-3 and MCF-7 cell lines with IC_{50} values in the range of 18.19-25.84 µM and compounds **1**, **2**, **4** and **12** showed a weak activity with IC_{50} values in the range of 55.77-95.47 µM. The other compounds were inactive (IC_{50} > 100 µM) against five human cancer cell lines. In addition, all isolated compounds (**1**-**14**) were evaluated for their α-glucosidase inhibitory activity (yeast and rat small intestinal). All of them displayed no activity against both yeast (IC_{50} > 600 µM) and rat small intestinal (IC_{50} > 3,000 µM) α-glucosidase enzymes.

The future work may involve the modification and synthesis of natural compounds for increasing a potent new drug and the active compounds will afford to the target. These results might provide the knowledge to better understanding the reaction between active compounds and diseases for the drug improvement.

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APPENDIX

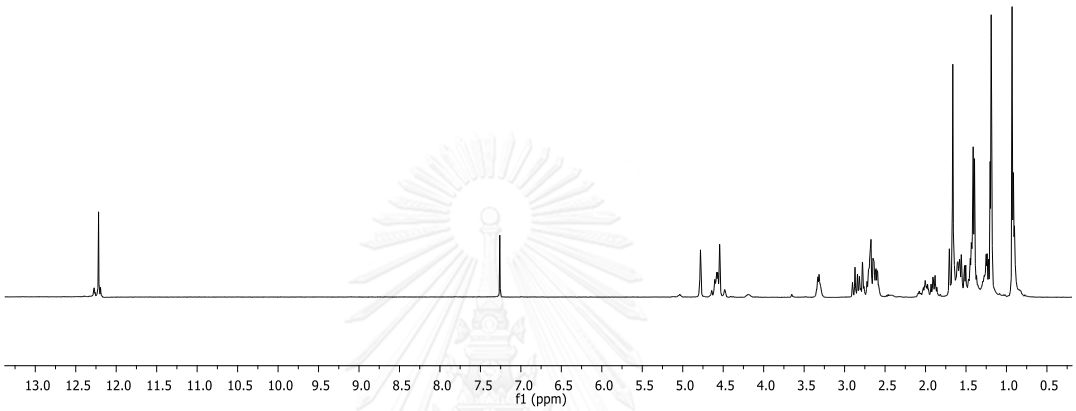

Figure A-1 ^{1}H NMR spectrum of caloinophyllin A (**1**) in $CDCl_3$.

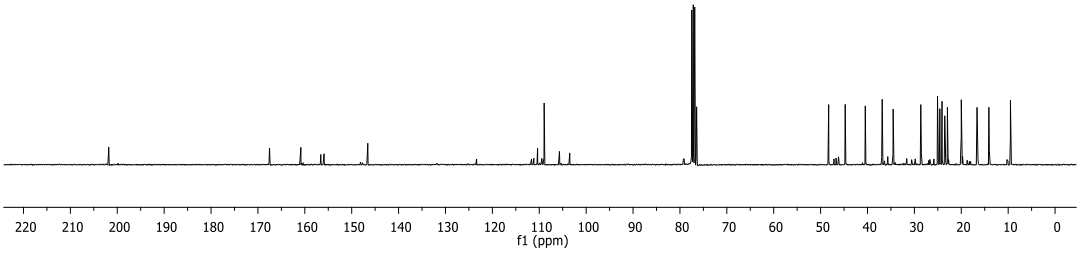

Figure A-2 ^{13}C NMR spectrum of caloinophyllin A (**1**) in $CDCl_3$.

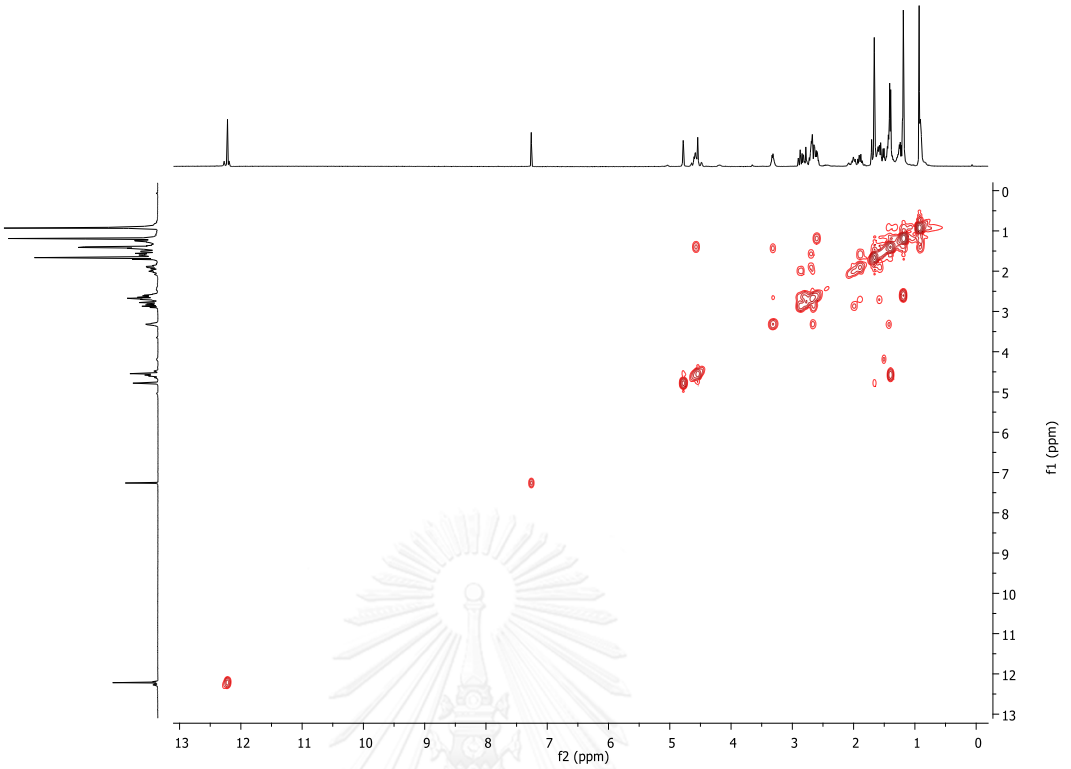

Figure A-3 COSY NMR spectrum of caloinophyllin A (**1**) in $CDCl_3$.

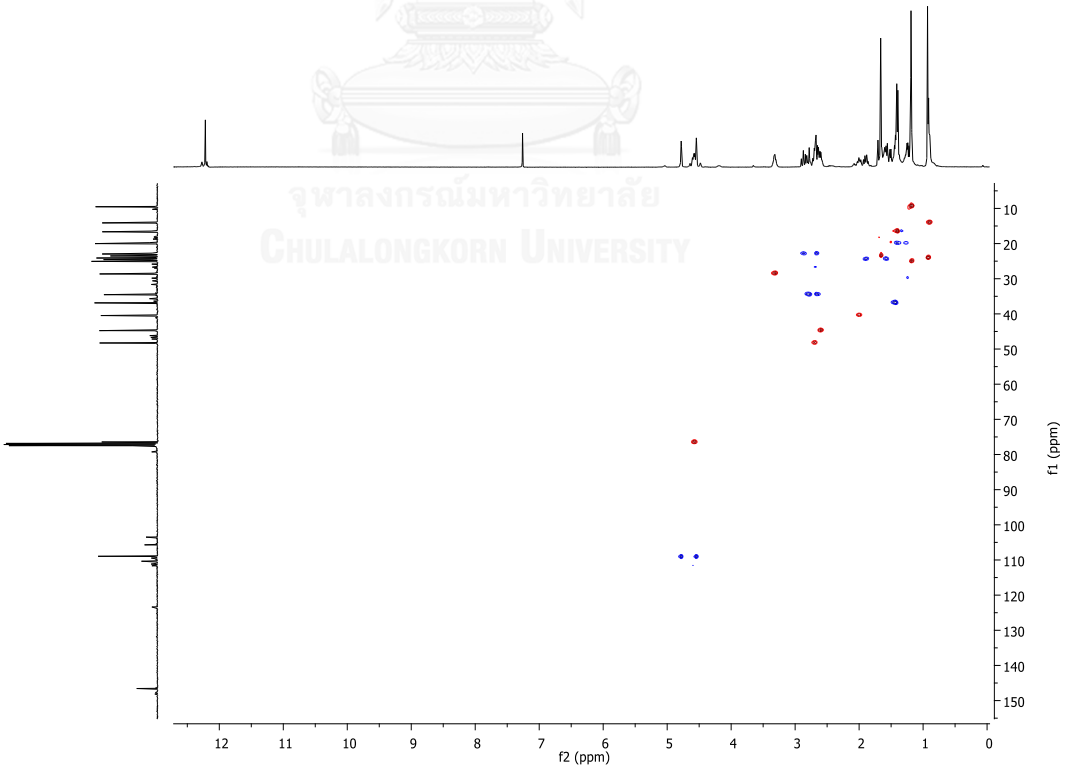

Figure A-4 HSQC NMR spectrum of caloinophyllin A (**1**) in $CDCl_3$.

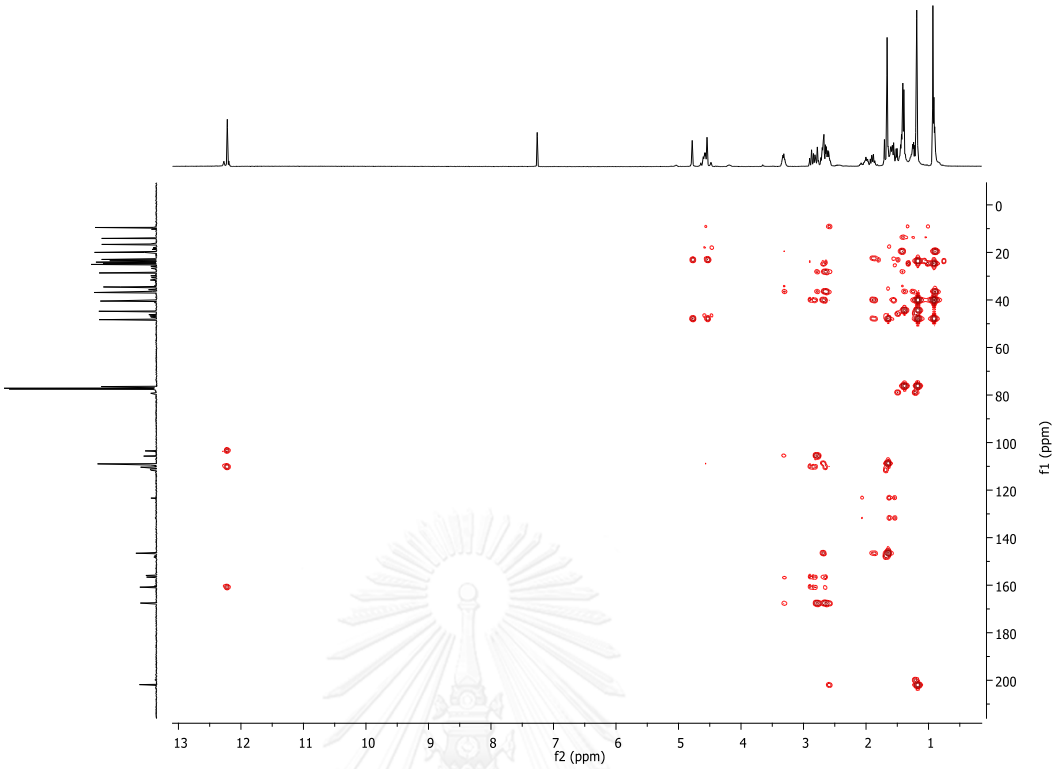

Figure A-5 HMBC NMR spectrum of caloinophyllin A (**1**) in $CDCl_3$.

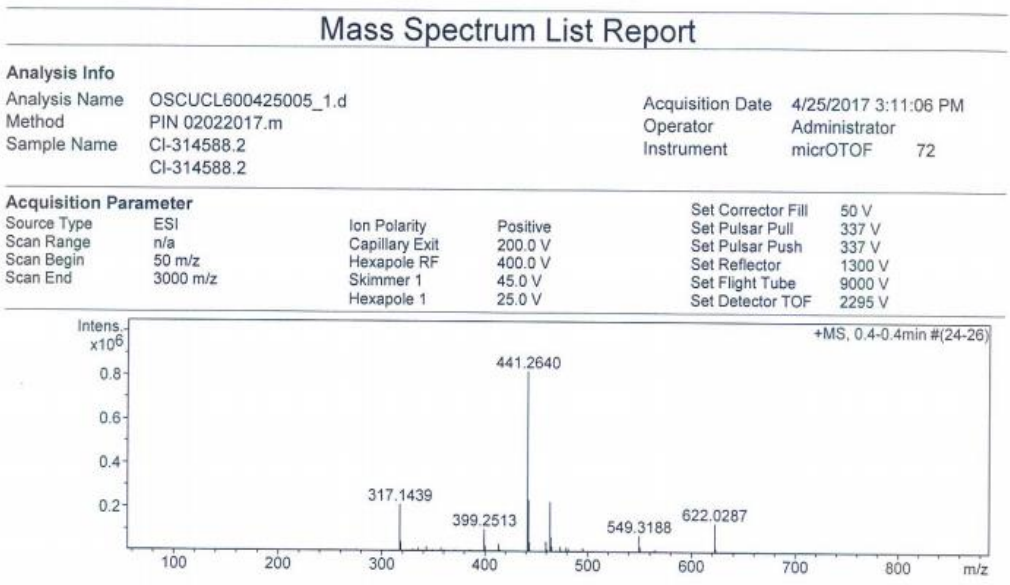

Figure A-6 HRESIMS spectrum of caloinophyllin A (**1**) in $CDCl_3$.

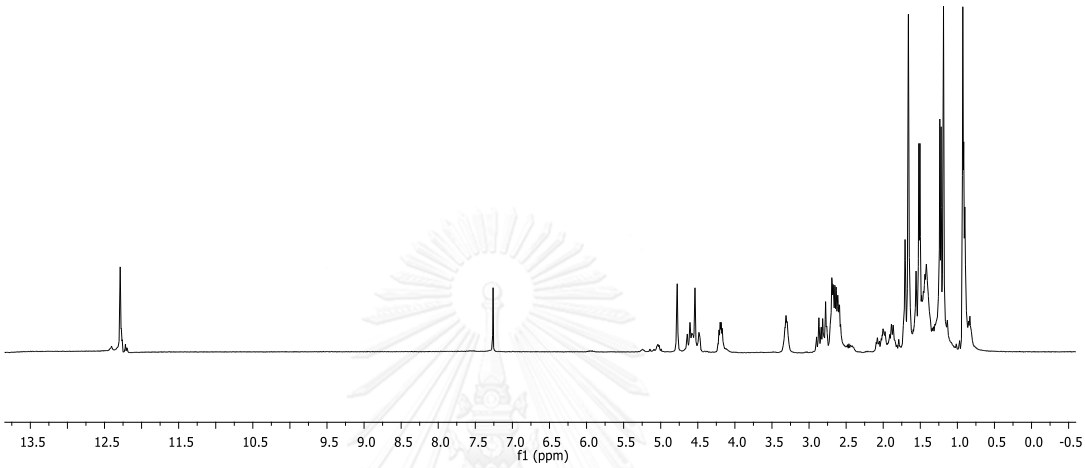

Figure A-7 ^{1}H NMR spectrum of caloinophyllin B (**2**) in $CDCl_3$

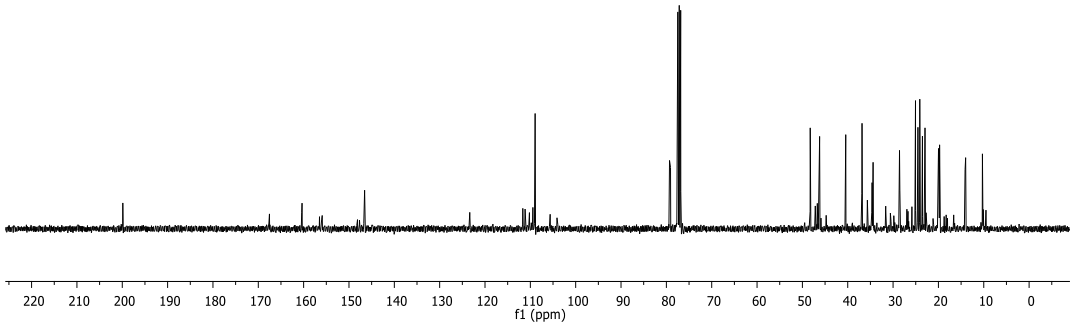

Figure A-8 ^{13}C NMR spectrum of caloinophyllin B (**2**) in $CDCl_3$.

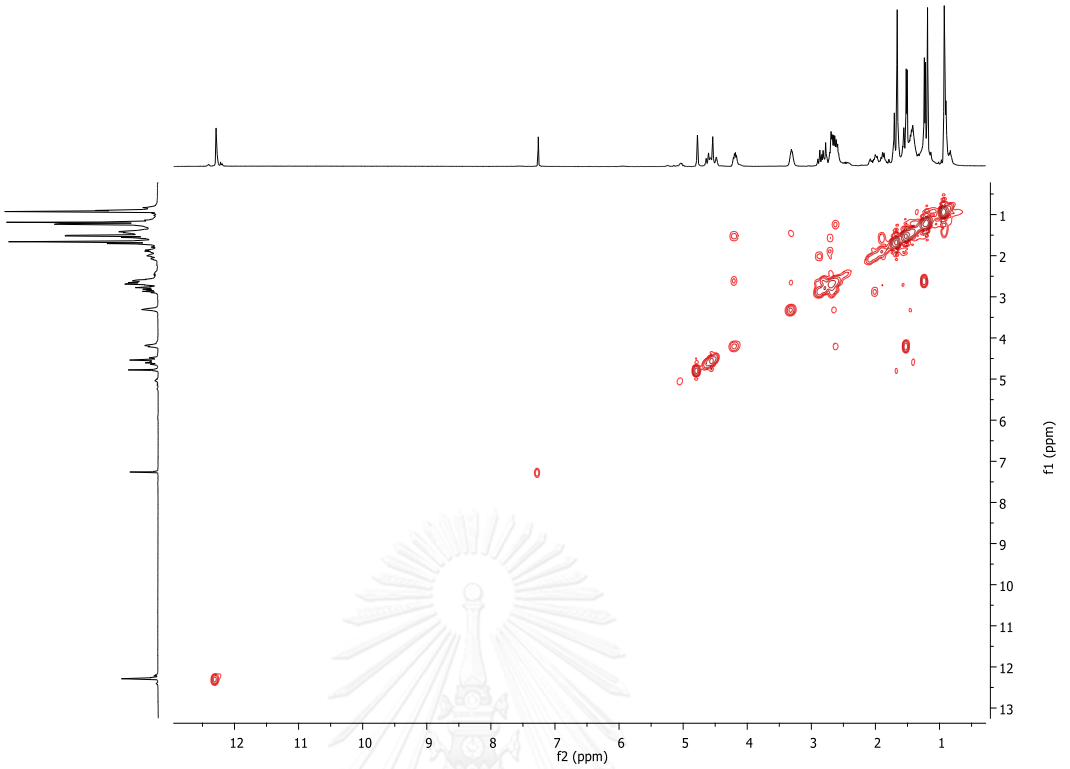

Figure A-9 COSY NMR spectrum of caloinophyllin B (**2**) in $CDCl_3$.

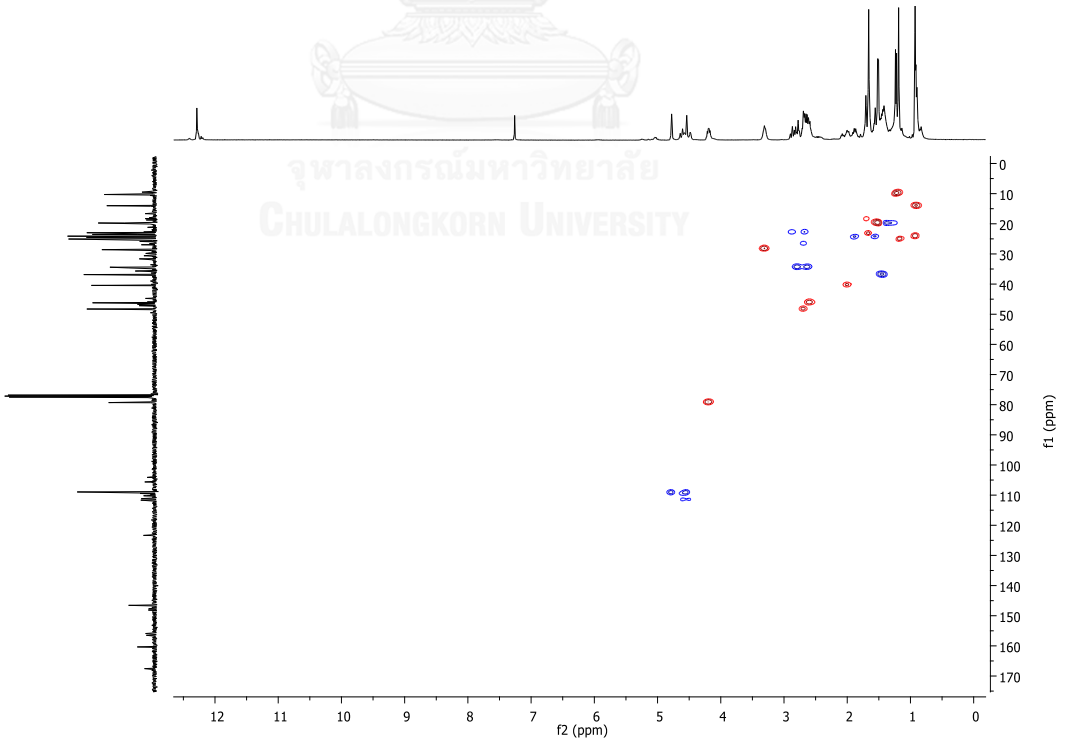

Figure A-10 HSQC NMR spectrum of caloinophyllin B (**2**) in $CDCl_3$.

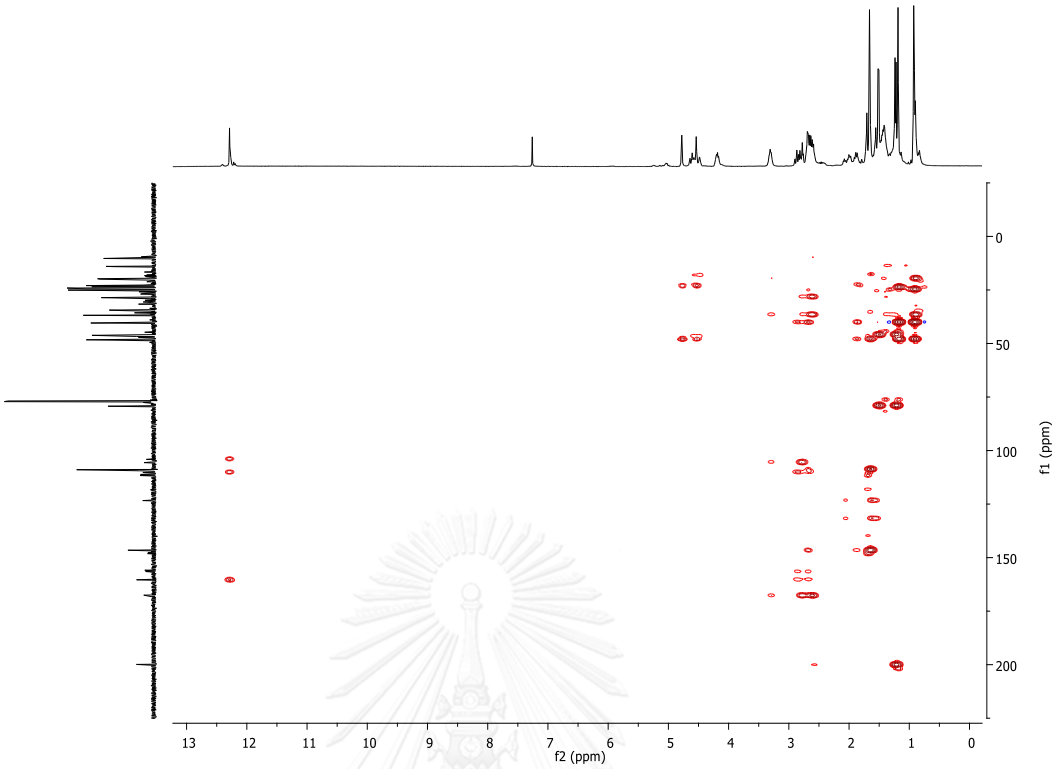

Figure A-11 HMBC NMR spectrum of caloinophyllin B (**2**) in $CDCl_3$.

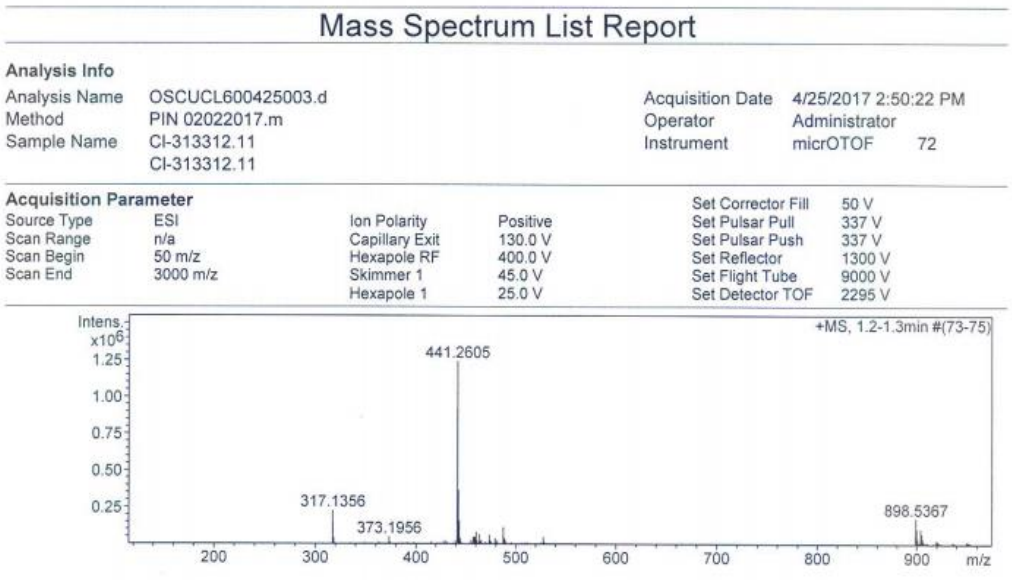

Figure A-12 HRESIMS spectrum of caloinophyllin B (**2**) in $CDCl_3$.

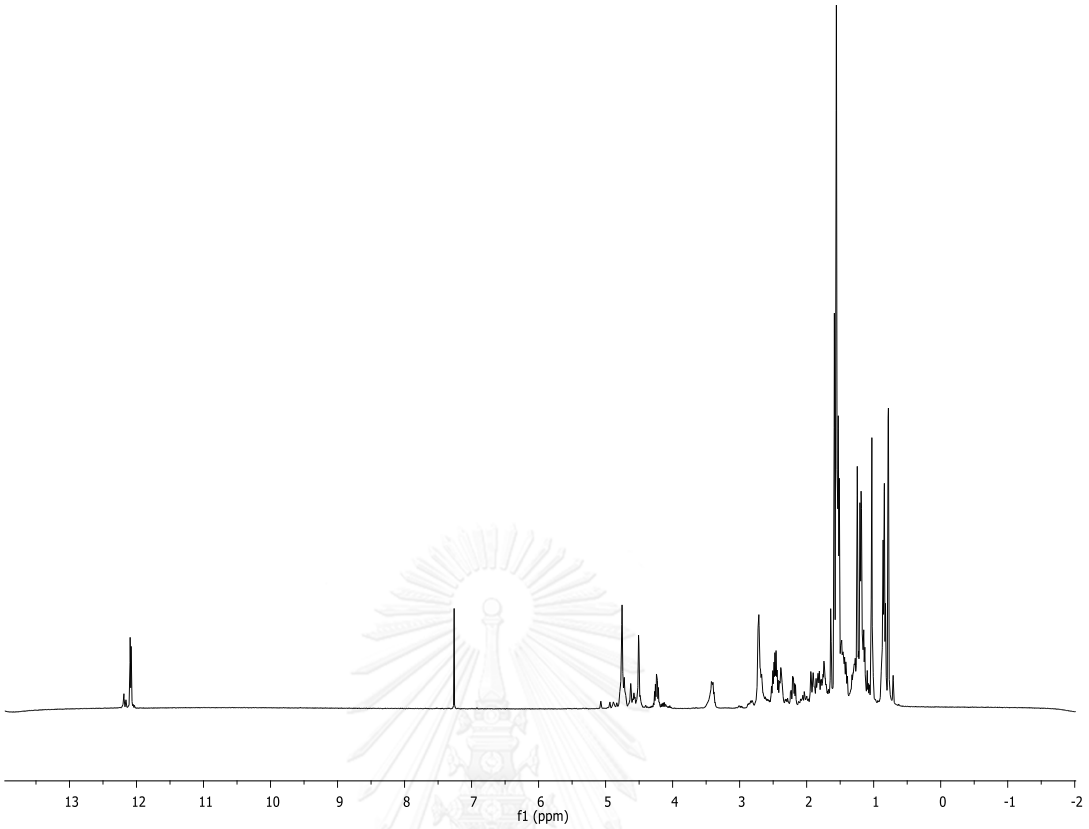

Figure A-13 ^{1}H NMR spectrum of caloinophyllin C (**3**) in $CDCl_3$

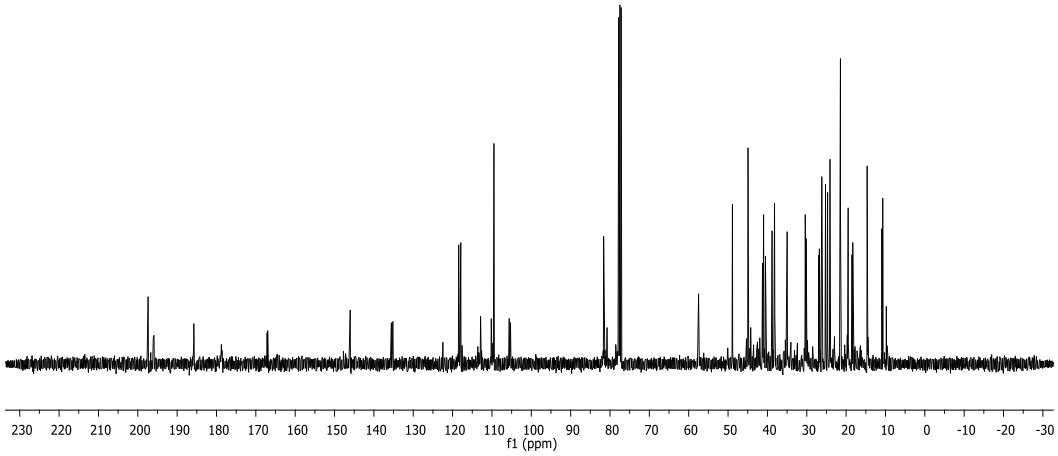

Figure A-14 ^{13}C NMR spectrum of caloinophyllin C (**3**) in $CDCl_3$.

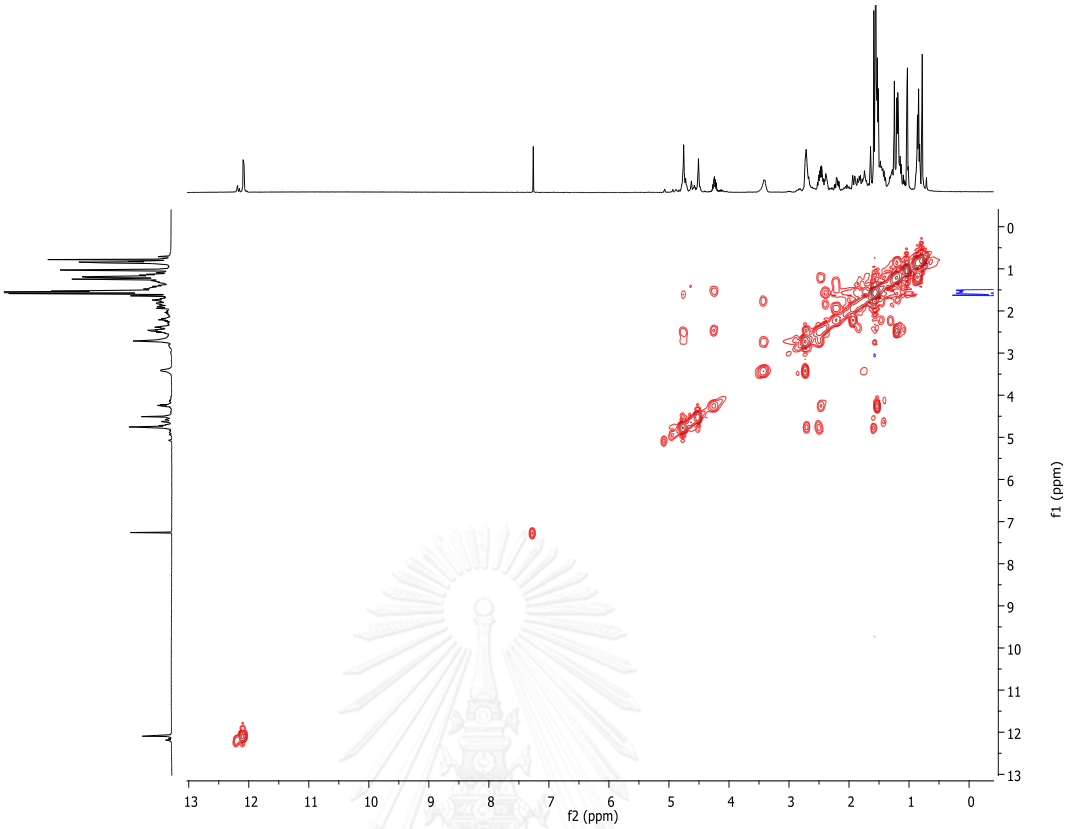

Figure A-15 COSY NMR spectrum of caloinophyllin C (**3**) in $CDCl_3$.

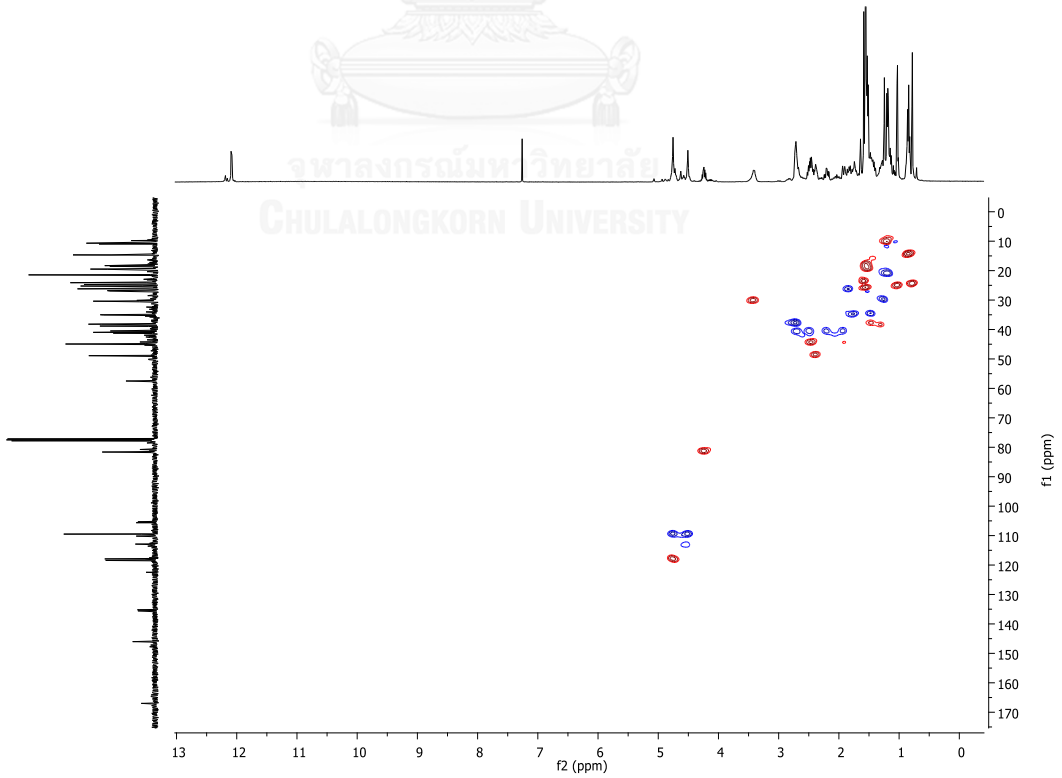

Figure A-16 HSQC NMR spectrum of caloinophyllin C (**3**) in $CDCl_3$

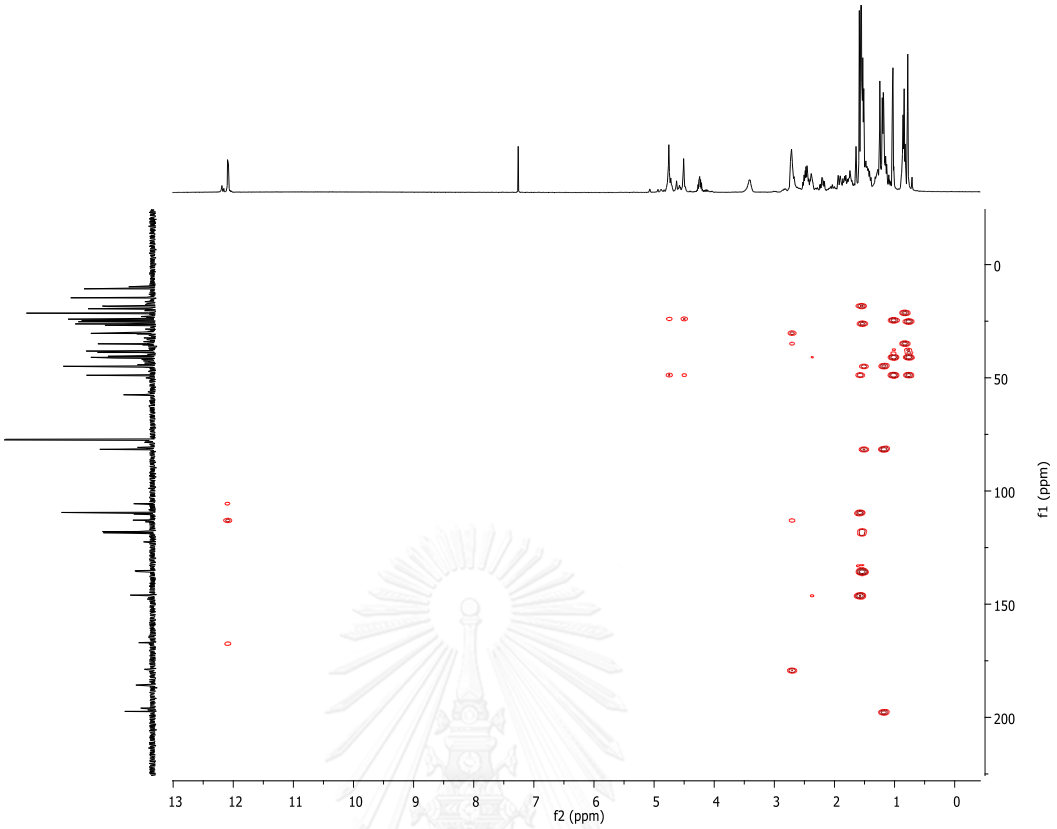

Figure A-17 HMBC NMR spectrum of caloinophyllin C (**3**) in $CDCl_3$

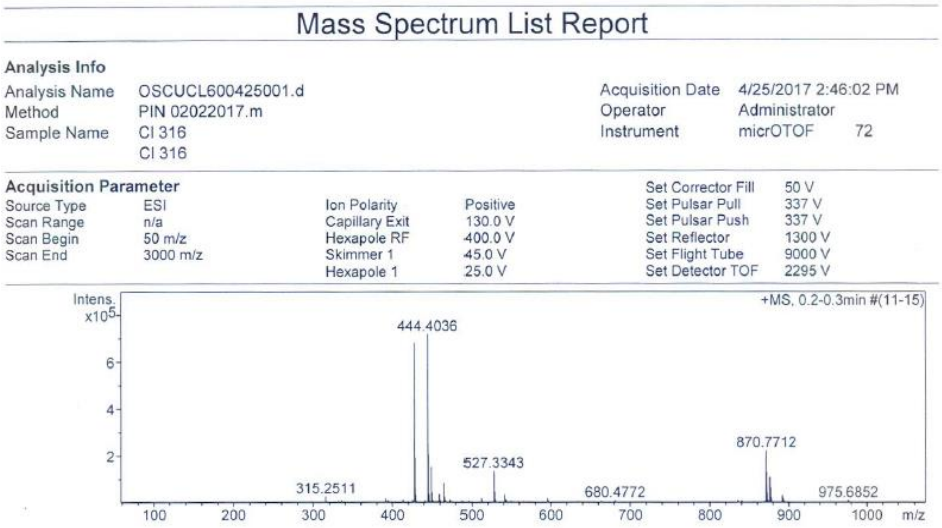

Figure A-18 HRESIMS spectrum of caloinophyllin C (**3**) in $CDCl_3$

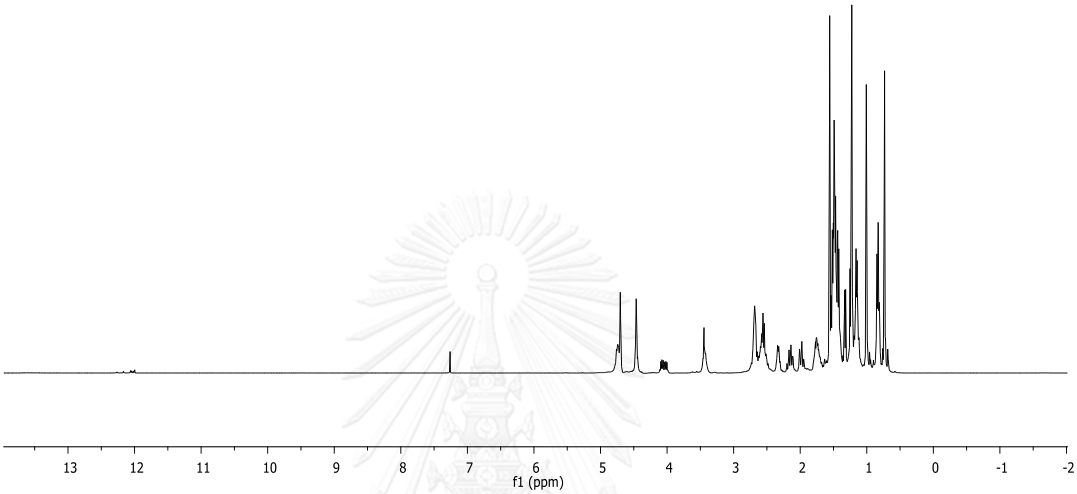

Figure A-19 ^{1}H NMR spectrum of caloinophyllin D (**4**) in $CDCl_3$

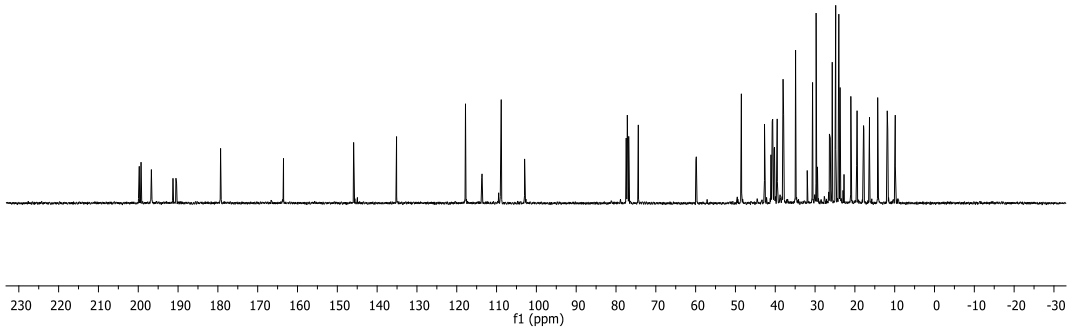

Figure A-20 ^{13}C NMR spectrum of caloinophyllin D (**4**) in $CDCl_3$.

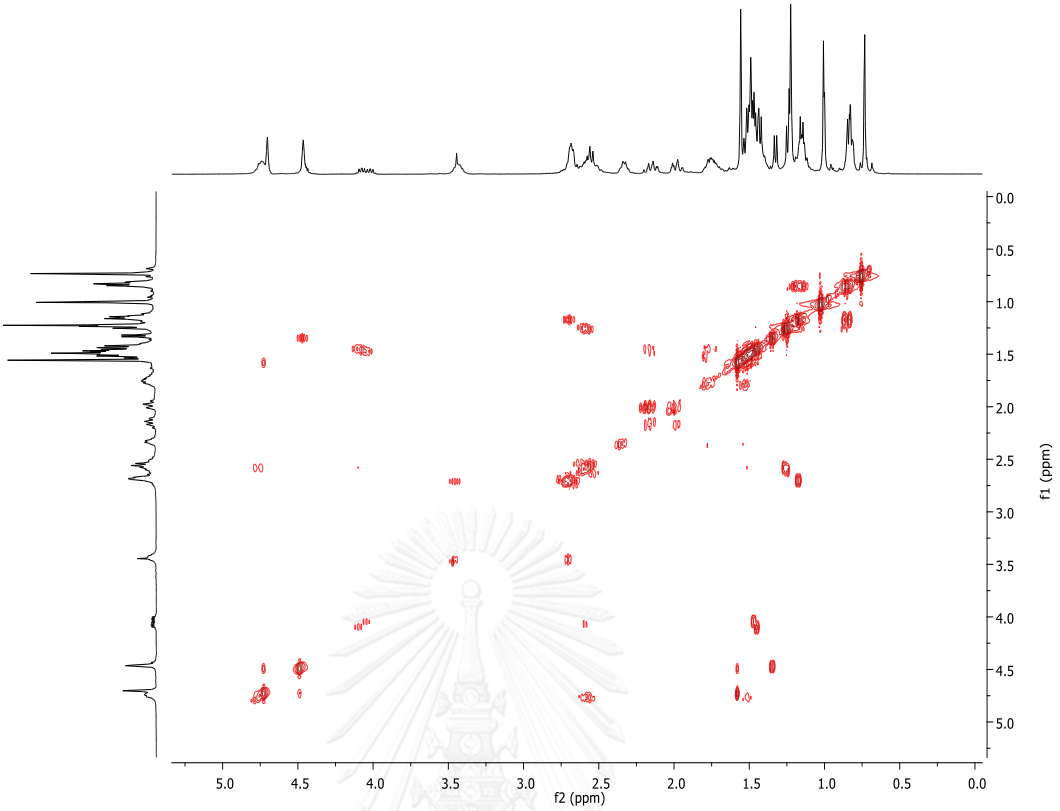

Figure A-21 COSY NMR spectrum of caloinophyllin D (**4**) in $CDCl_3$

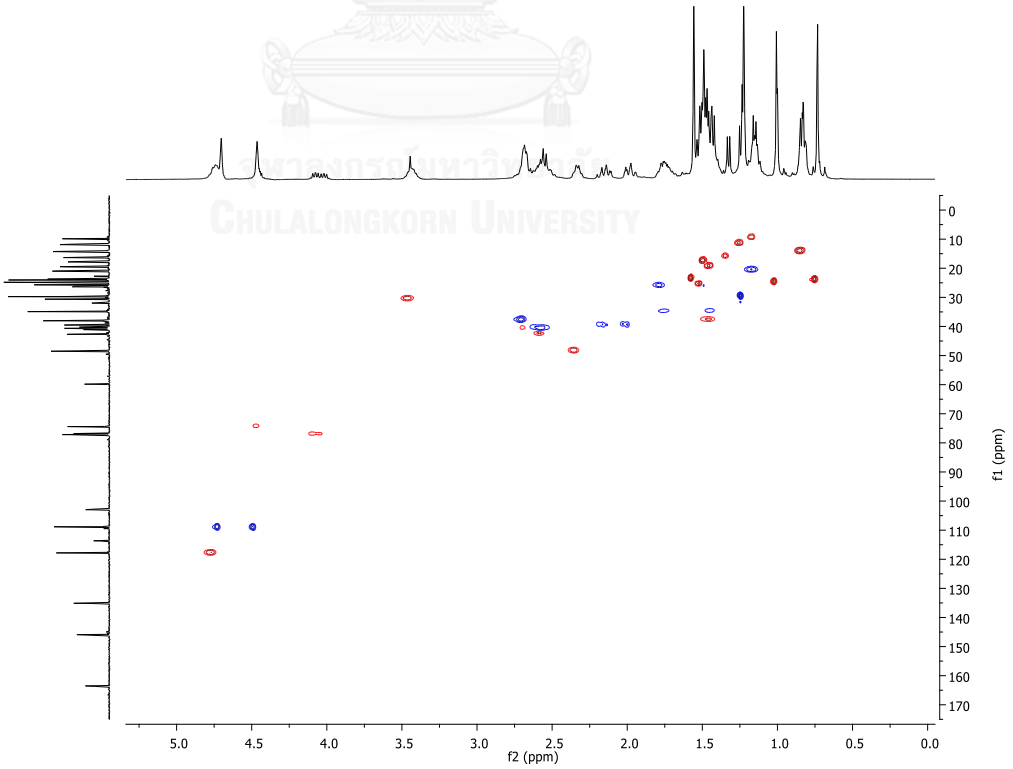

Figure A-22 HSQC NMR spectrum of caloinophyllin D (**4**) in $CDCl_3$

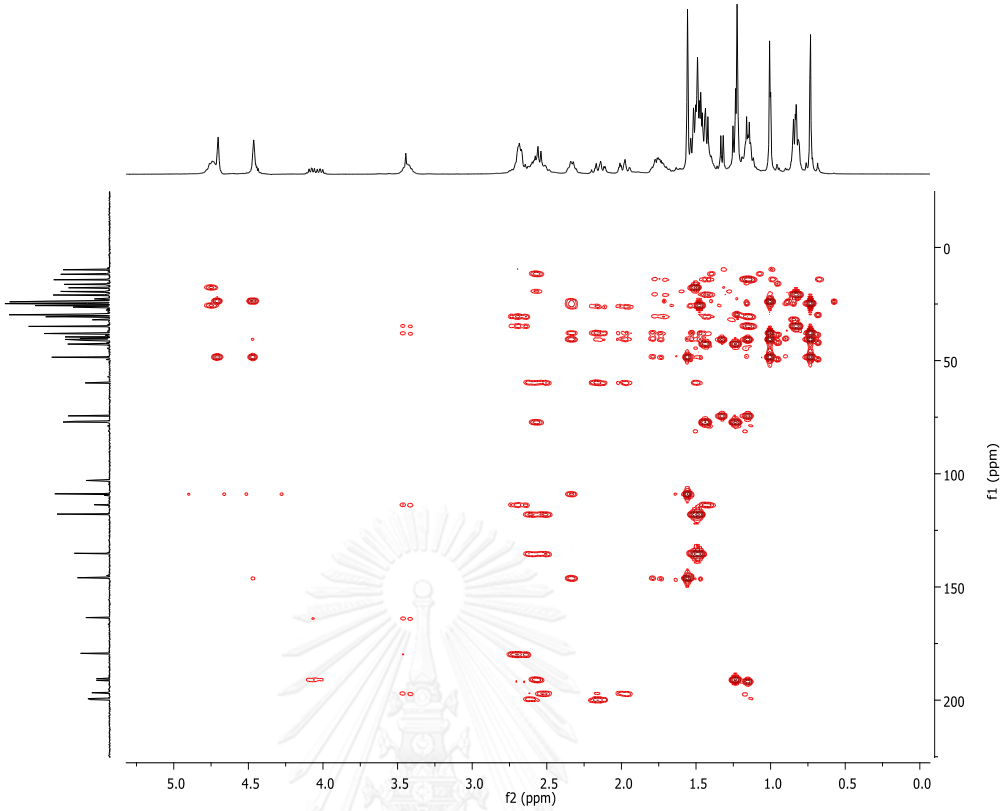

Figure A-23 HMBC NMR spectrum of caloinophyllin D (**4**) in $CDCl_3$

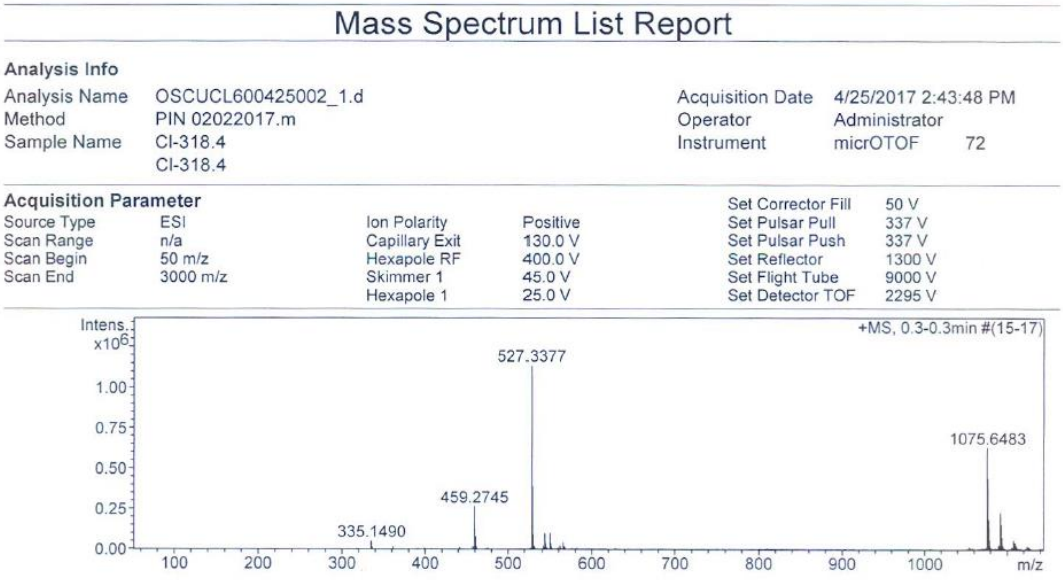

Figure A-24 HRESIMS spectrum of caloinophyllin D (**4**) in $CDCl_3$.

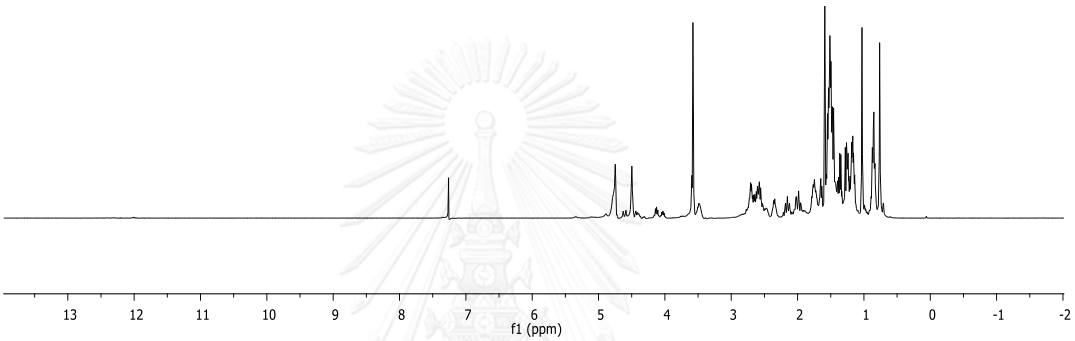

Figure A-25 ^{1}H NMR spectrum of caloinophyllin E (**5**) in $CDCl_3$.

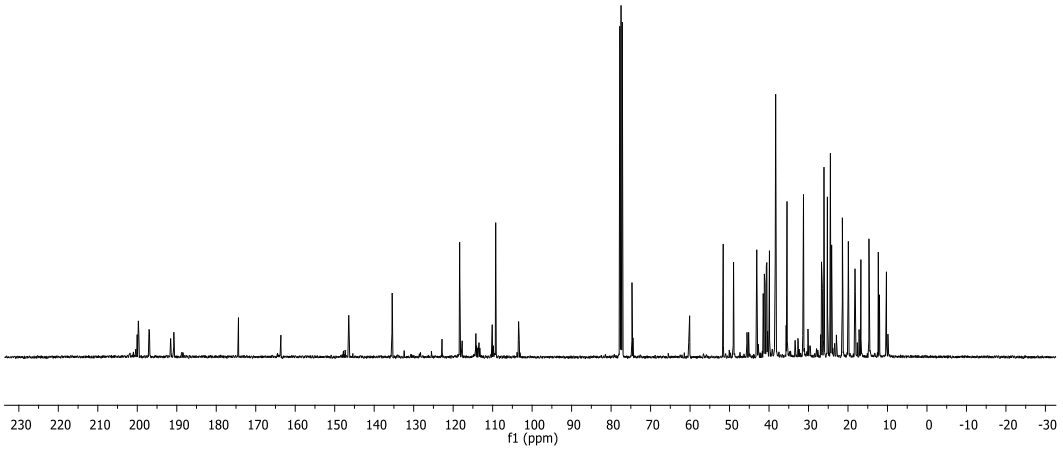

Figure A-26 ^{13}C NMR spectrum of caloinophyllin E (**5**) in $CDCl_3$.

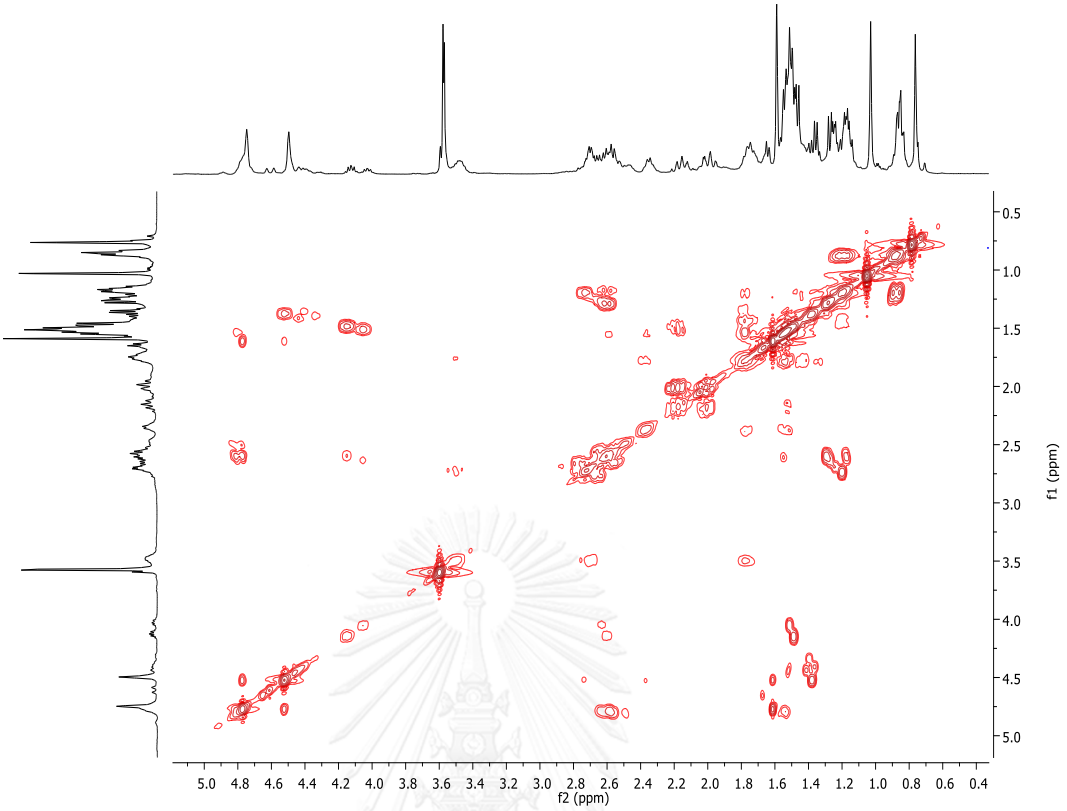

Figure A-27 COSY NMR spectrum of caloinophyllin E (**5**) in $CDCl_3$.

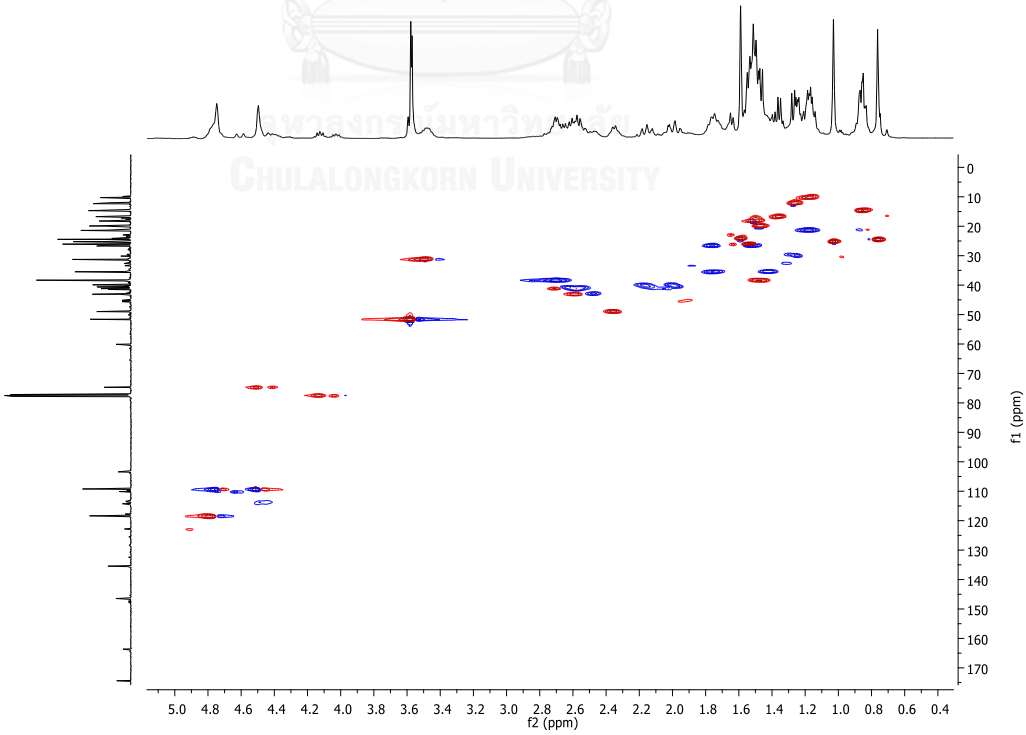

Figure A-28 HSQC NMR spectrum of Caloinophyllin E (**5**) in $CDCl_3$

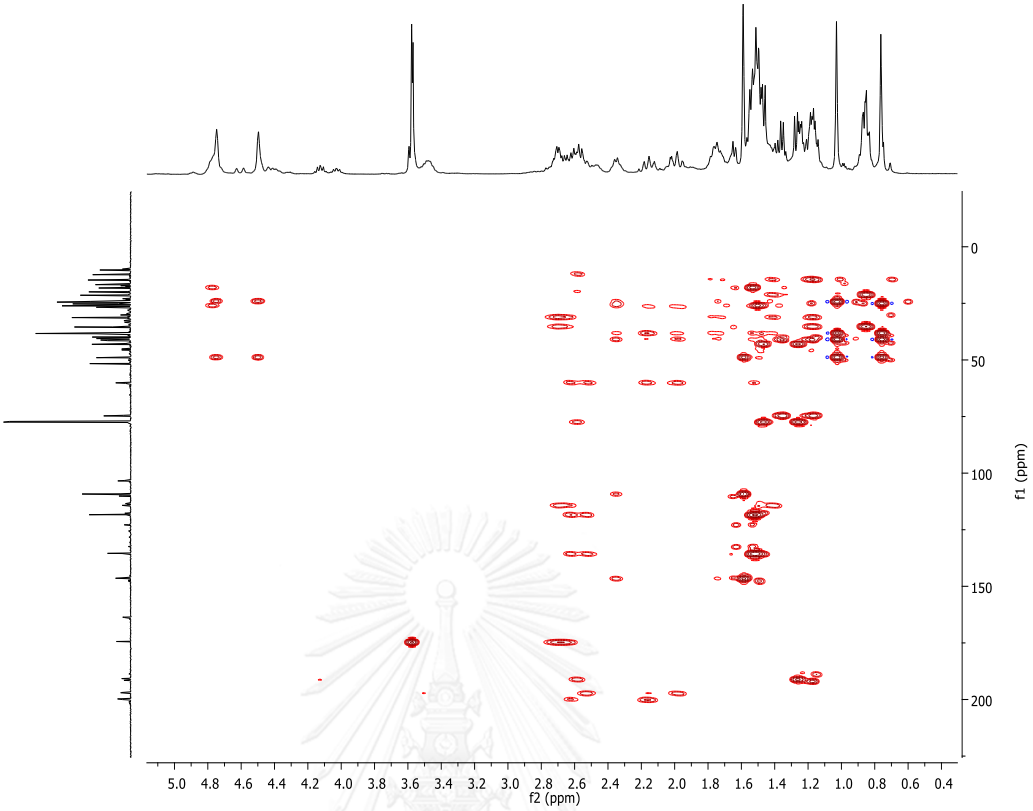

Figure A-29 HMBC NMR spectrum of caloinophyllin E (**5**) in $CDCl_3$

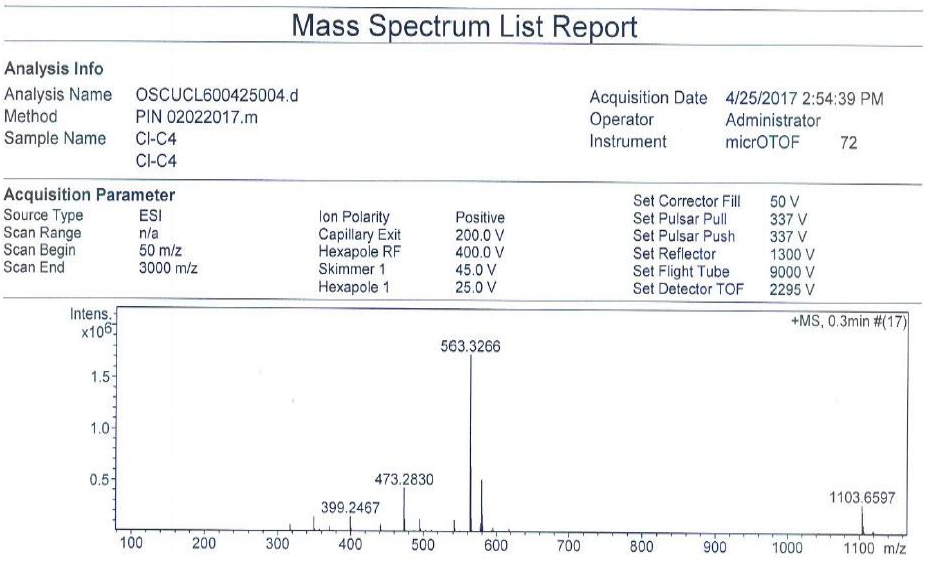

Figure A-30 HRESIMS spectrum of caloinophyllin E (**5**) in $CDCl_3$.

VITA

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