SYNTHESIS OF HALOGENATED FLAVONOIDS AS ANTI-DENGUE AGENTS

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การสังเคราะห์แฮโลจิเนเทตเฟลโวนอยด์เพื่อเป็นสารต้านไข้เลือดออก

น.ส.เถา ฮุน เหวียน ธานห์

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เถา ฮุน เหวียน ธานห์ : การสังเคราะห์แฮโลจิเนเทตเฟลโวนอยด์เพื่อเป็นสารต้าน
ไข้เลือดออก. (SYNTHESIS OF HALOGENATED FLAVONOIDS AS ANTI-DENGUE
AGENTS) อ.ที่ปรึกษาหลัก : ผศ. ดร.วรินทร ชวศิริ

เฟลโวนอยด์แสดงฤทธิ์ทางชีวภาพหลากหลาย อนุพันธ์เฟลโวนอยด์บางชนิดแสดงฤทธิ์
ดีกว่าสารเริ่มต้น เมื่อไม่นานมานี้ มีรายงานว่าแฮโลจิเนเทตเฟลโวนอยด์แสดงฤทธิ์ต้านไข้เลือดออก
ในการศึกษานี้ ได้สังเคราะห์อนุพันธ์โบรมิเนเทตและไอโอดิเนเทตเฟลโวนอยด์ยี่สิบสองตัว และ
ศึกษาฤทธิ์ทางชีวภาพ พบว่าเป็นการรายงานการศึกษาของอนุพันธ์สิบเจ็ดชนิดเป็นครั้งแรก 8-
bromobaicalein, 6,8-dibromopinocembrin, 6,8-dibromopinostrobin และ 6,8-
diiodopinostrobin มีศักยภาพเป็นสารต้านไข้เลือดออกและไม่มีความเป็นพิษ สารเหล่านี้แสดงค่า
EC_{50} ต่ำและค่า CC_{50} สูง ซึ่งสามารถพิจารณาได้ว่าเป็นสารออกฤทธิ์ในการรักษาที่มีศักยภาพ
นอกจากนี้ได้ศึกษาสารในกลุ่ม brominated O^7-ether chrysin เบื้องต้น พบว่า 6,8-dibromo-
O^7-butylchrysin และ 6,8-dibromo-O^7-hexylchrysin แสดงฤทธิ์ที่น่าสนใจ อย่างไรก็ตาม
ภายใต้ภาวะของการทดลองนี้สารบางตัวไม่ละลาย แม้ว่าความสัมพันธ์ระหว่างความยาวของสายโซ่
และฤทธิ์ทางชีวภาพไม่สอดคล้องกัน เปอร์เซ็นต์การยับยั้งพลักซ์เพิ่มขึ้น จากสายโซ่ที่มีหมู่เอทิลถึง
หมู่เฮกซิล

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Flavonoids have been recognized as promising compounds with various bioactivities. Certain flavonoid derivatives exhibited more potent activities than their parent compounds. Recently, halogenated flavonoids have been addressed for their anti-dengue activity. In this research, a series of twenty-two brominated and iodinated flavonoid derivatives were synthesized and explored for dengue infection inhibition. Seventeen derivatives have been investigated for the first time. 8-Bromobaicalein, 6,8-dibromopinocembrin, 6,8-dibromopinostrobin, 6,8-diiodopinostrobin were disclosed as effective and non-toxic anti-dengue agents. These compounds displayed remarkably low EC_{50} as well as high CC_{50}, which could be considered as potent therapeutic agents. Moreover, the preliminary screening on brominated O^7-ether chrysins discovered some noticeable compounds, for example, 6,8-dibromo-O^7-butylchrysin and 6,8-dibromo-O^7-hexylchrysin; nonetheless, some synthesized brominated O^7-ether chrysins were insoluble under tested conditions. Though the relationship between the length of side chain and bioactivity were inconsistent, there was a gradually increasing in percentage of plaque inhibition from ethyl to hexyl in case of 6,8-dibromo O^7-ether chrysins.

Field of Study: Chemistry   Student's Signature ....

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calcd	calculated
CC	Cytotoxic concentration
CH_2Cl_2	dichloromethane
concd	concentrated
d	doublet (NMR)
dd	doublet of doublets (NMR)
DENV	dengue virus
DF	dengue fever
DHF	dengue hemorrhagic fever
DMD	dimethyl dioxirane
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
EC	Effective concentration
equiv.	equivalent
EtOAc	Ethyl acetate
g	gram (s)
HMBC	Heteronuclear multiple bond correlation

HR-ESI-MS	High resolution-electrospray ionization mass
HSQC	Heteronuclear single quantum correlation
m	multiplet (NMR)
MHz	Mega Hertz
mL	milliliter (s)
mm	millimeter (s)
mmol	millimole (s)
NBS	*N*-bromosuccinimide
NMR	Nuclear Magnetic Resonance
ppm	part per million
s	singlet (NMR)
SI	Selective Index
rt	room temperature
TI	Therapeutic Index
TLC	Thin layer chromatography
$[M+Na]^+$	pseudomolecular ion
$[M+ 2Na - H]^+$	pseudomolecular ion
α	alpha
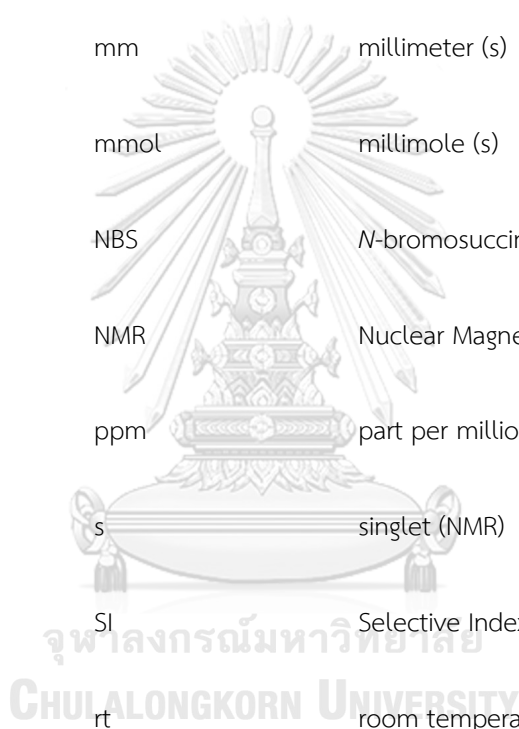
δ	chemical shift
δ_H	chemical shift of proton
μg	microgram (s)
μL	microliter (s)
μM	micromolar (s)
2-D NMR	2-dimensional nuclear magnetic resonance

Chapter 1

Introduction

1.1 Dengue

1.1.1 Introduction

Dengue is mosquito-borne viral disease, caused by the four dengue virus serotypes 1-4. The dengue virus (DENV) belonging to the genus *Flavivirus* (family Flaviviridae), is considered as one of the most widely-spreading and endangered pathogen in the tropics. The virus is transmitted by the vector *Aedes* mosquitoes, commonly *Aedes agypti* species. The infective symptoms can vary from asymptomatic to dengue fever (DF) and dengue hemorrhagic fever (DHF).[1] Typically, dengue fever symptoms include sudden onset of high fever, headaches, muscle and joint paints, which generally misleading with common flu, and can be recovered after two to seven days.[2] However, when the disease develops to DHF, the patient can suffer serve capillary leakage, hemorrhage, thrombocytopenia and fatality.[2]

Nowadays, the affected areas have been spreading out more than hundred countries in Southeast Asia, the Western Pacific, the Americas and Eastern Mediterranean.[3] Noticeably, the most seriously threatened regions are Southeast Asia and the Western Pacific.[3] The first reported case of dengue in Thailand was in 1949; infrequent cases was discovered through 1950s later on and the first epidemic DHF in Bangkok was stated in 1958.[4-7] Moreover, before 2004, Thailand announced the highest number of annual infected patients in Southeast Asia, with an average of

almost 69,000 cases every years in the period 1985-1999.[8] These data warned the emergent situation of this global illness and the urgency of long-term medical treatments.

1.1.2 Clinical therapies

Up to now, diagnosis is principally clinical while medical therapies is supportive and the disease control mainly target on the vector. Briefly, up to now, there are three major elements in dengue analysis[3]:

- Development of ELISAs for dengue-specific IgM detection

- Mosquito cell lines and monoclonal antibody development for viral isolation and identification.

- Reverse transcriptase-polymerase chain reaction (RT-PCR)

These methods considered the serological, virological and molecular diagnosis of dengue.[9]

So far, treatment of dengue infection consists of four methods which can either succeed or fail:

- Vaccination: there are four different serotypes of DENV 1-4, therefore, an effective vaccine must be tetravalent and provides a permanent protection for human. However, the accomplished vaccine has not been disclosed.[3]

- Medication/Treatment: there are no special antiviral therapies currently existing for dengue fever. Other supportive cares can be used to overcome the DF symptoms as well as lessen the serve DHF.[3]

- Natural treatment: Some of herbal cures can be listed such as *Boesenbergia rotunda, Kaempferia parviflora, Carica papaya, etc.* due to their activity against *Aedes agypti*.[3]

- Prevention: The practical and simple protection from dengue is to avoid being bitten by the vector.[3] In addition, the elimination of vectors should be considered properly in order to limit the transmission of dengue virus in community.

The shortage of medicinal treatments for dengue infection has been received a huge attraction of scientists in several years. In the effort to look for the promising agents, thousands of antiviral research on different targets have been conducting from all over the world.

1.2 Flavonoids

1.2.1 Introduction

Flavonoids are well-known secondary metabolites existing in a variety of plants. The basic skeleton of this class consists of fifteen carbons with two fused six-membered rings and one benzene ring. This enormous type of natural products is classified into many sub-divisions by the degree of oxidation of ring C or the positions of ring B (**Figure 1.1**).
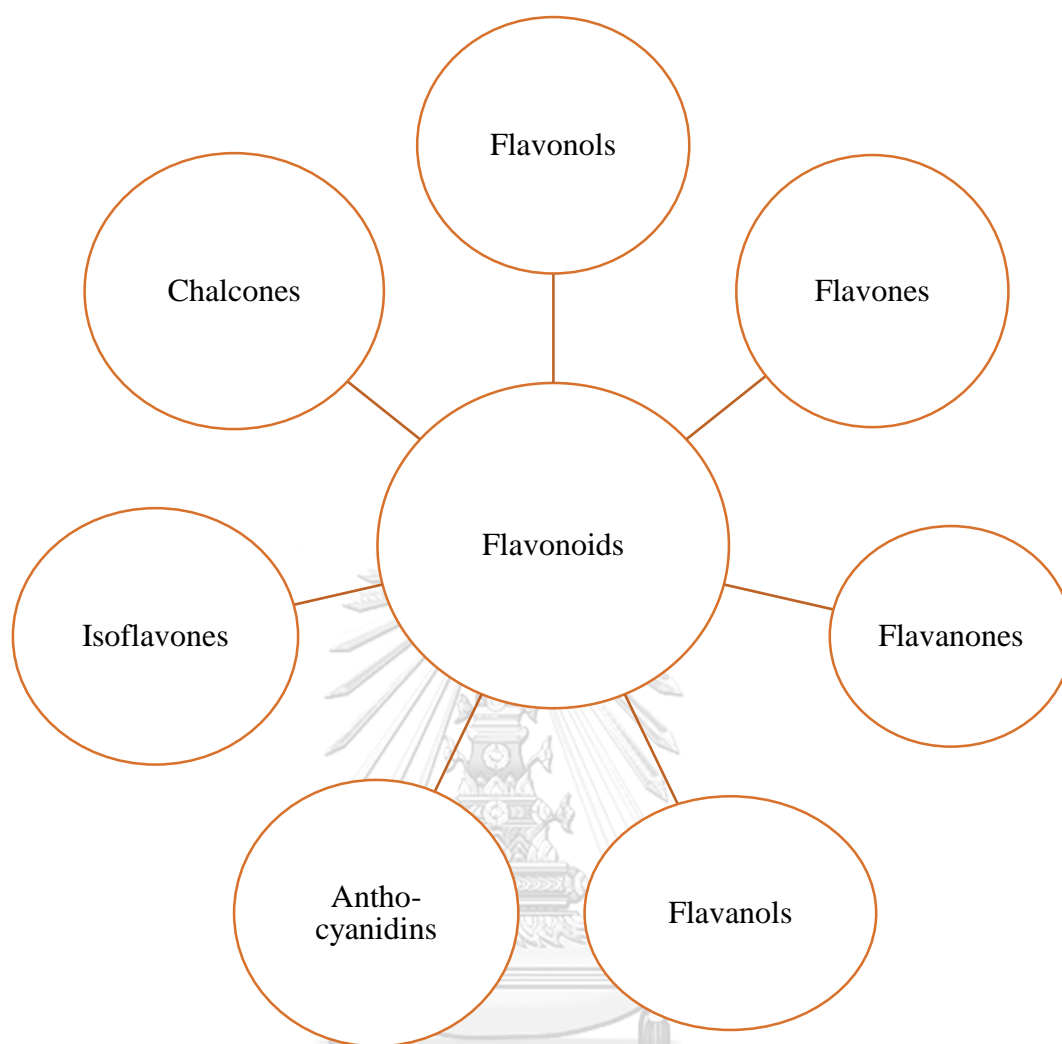
Figure 1.1 Classification of flavonoids.

Up to now, flavonoids were reported to possess a wide range of bioactivities such as anti-oxidant, anti-inflammatory, anti-bacterial, anti-cancer, *etc*.[10] Daidzein, a representative of isoflavone, is dominant in soybean and proved to have anti-cancer, anti-cardiovascular disease, anti-diabetic activity, *etc*.[11] Another example is chrysin, a flavone which is abundantly contributed as one of main ingredients in honey, propolis and blue passion flower (*Pasiflora caerulea*).[12] Chrysin is convinced of its

anti-oxidant, anti-inflammatory, neuroprotective activity in several studies.[12] Because of their diverse bioactivities and natural sources, a hundred of researches have been conducted continuously on different flavonoids.

1.2.2   Anti-dengue activity of flavonoids

Recently, the intensive investigation of flavonoids as anti-dengue agents has been addressed. In 2000, Sanchéz *et al.* evaluated *in vitro* antiviral effect of **1-5**, isolated from Mexican *Tephrosia* species, using LLC-MK2 cells and DENV-2 serotype (**Figure 1.2**).[13] Only glabranine (**1**) and 7-*O*-methylglabranine (**2**) exhibited notable inhibition of 70% virus infection at 25 μM.

R₁ = OMe, R₂ = OH: glabranine (**1**)
R₁ = R₂ = OMe: 7-*O*-methylglabranine (**2**)

3

R₁ = OMe, R₂ = OH: hildgardtol (**4**)
R₁ = R₂ = OMe: methylhildgardtol (**5**)
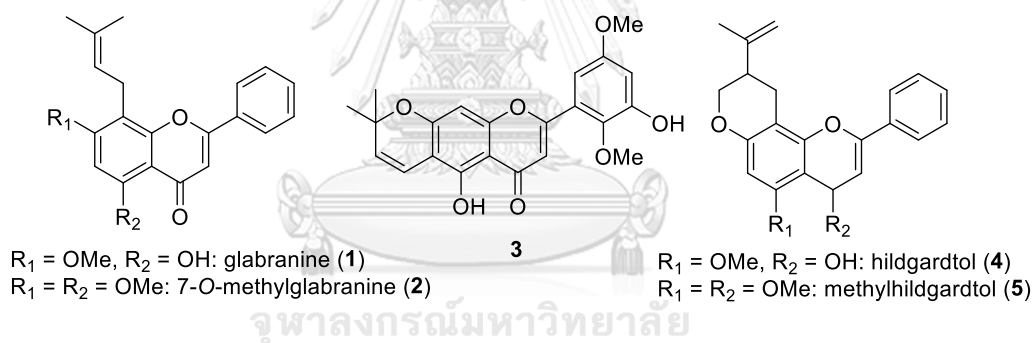
Figure 1.2 Flavonoids from Mexican Tephrosia species.

Tan *et al.* worked on three flavanones **6-8**, a phenylpropanoid **9** and two cyclohexenyl chalcone derivatives **10-11** derived from *Boesenbergia rotunda* (L.), a familiar spice of the ginger family (Zingiberaceae), testing against DENV-2 virus NS2B-NS3 protease by enzymatic assay (**Figure 1.3**).[14] Even though pinocembrin (**7**) and cardamonin (**9**) acted as inactive components, there was an interesting synergetic effect when these compounds were mixed (**Table 1.1**).
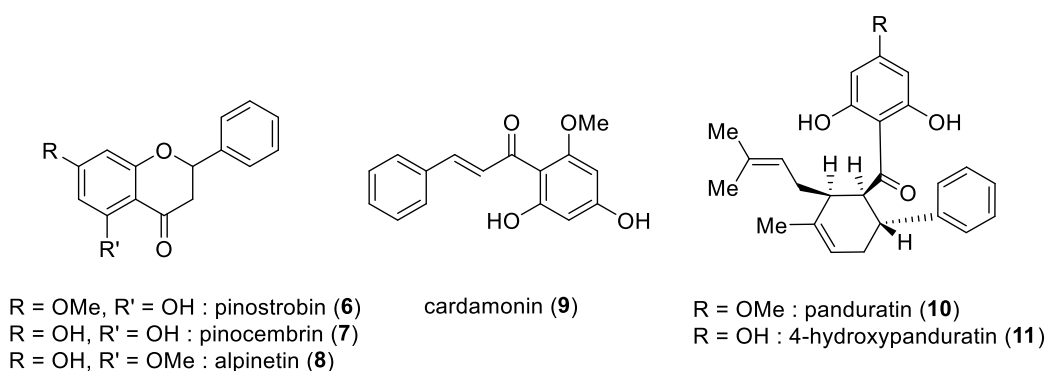
Figure 1.3 Structures of **6-11**.

Table 1.1 Percentage inhibition of DENV-2 NS2B-NS3 virus protease cleavage[a] of compounds **7** and **9**. [14]

Compound(s)	Percentage Inhibition of DENV-2 NS2B-NS3 Protease; Concentration Used (ppm)		
	120	240	400
7	30.1 ± 0.5	47.3 ± 0.5	56.1 ± 0.4
9	39.4 ± 0.6	50.1 ± 0.4	71.3 ± 0.3
7 + 9	52.6 ± 0.4	63.5 ± 0.5	81.8 ± 0.3

[a] The substrate cleaved by NS2B-NS3 protease used in the experiment corresponded to the peptide Boc-Gly-Arg-Arg-MCA. ± corresponds to standard deviation.

Researches developed in 2011 released some noticeable results of anti-dengue activity of flavonoids. In two separately different scope of tested compounds of Zandi and co-workers, naringenin (**12**), quercetin (**13**), daidzein (**14**), and hesperetin (**15**) were evaluated against DENV-2 serotype infection *in vitro* utilizing Vero cells,[15] whilst naringenin (**12**), rutin (**16**) and fisetin (**17**) were examined *in vitro* on antiviral effects at

the different stages of DENV-2 (NGC strain) infection (**Figure 1.4**).[16] **13** presented

significant inhibitory activity (IC$_{50}$ 35.7 μg/mL) against DENV-2 infection, and **17** inhibited

virus replication (IC$_{50}$ 55 μg/mL) after virus adsorption on Vero cells. However, the

mechanism of affecting or interaction of these compounds remained unknown.
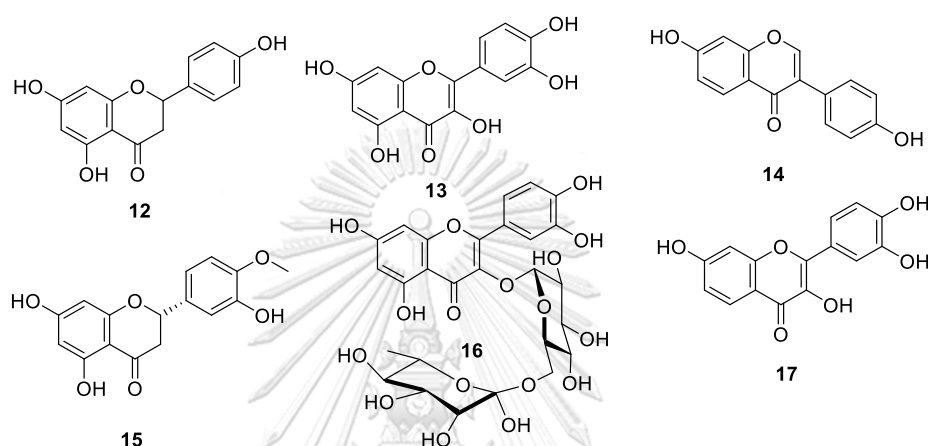
Figure 1.4 Structures of **12** - **17**.

Baicalein (**18**) (**Figure 1.5**) is usually extracted from the root of *Scutellaria*

baicalensis, a traditional Chinese medicinal herb in the Lamiaceae family.[17] Zandi *et*

al. studied on *in vitro* assay using Vero cells and Foci Forming Unit Reduction Assay

(FFURA) to assess antiviral activity of **18** against DENV-2.[17] This flavone displayed IC$_{50}$

value of 6.46 μg/mL affecting on DENV-2 serotype replication in Vero cells when it was

added after adsorption to the cells. Moreover, **18** also possessed direct virucidal (IC$_{50}$

1.55 μg/mL) as well as anti-adsorption (IC$_{50}$ 7.14 μg/mL) effects against DENV-2.
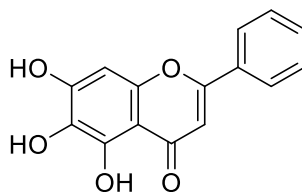
Figure 1.5 Structures of baicalein (**18**).

An investigation on naringenin (**12**) in 2017 by Frabasile and co-workers demonstrated the anti-dengue virus activity as well as its ability to inhibit the replication of four DENV serotypes in Huh7.5 cells (**Table 1.2**).[18] This result supported the potential use of **12** for the development of specific dengue virus treatment.

Table 1.2 Half maximal inhibitory concentration (IC_{50}) and selective index (SI) of naringenin (**12**) for anti – DENV activity in Huh7.5 cells.[18]

DENV	IC_{50}	SI
DENV-1/FGA	35.81	8.60
DENV-2/ICC265	17.97	17.32
DENV-3/5532	117.1	2.66
DENV-4/TVP360	177.5	1.75

SI = CC_{50}/IC_{50}.

1.3 Halogenated flavonoid derivatives

1.3.1 Halogenation of flavonoids

Since the beginning of the 21st century, researchers have been fond of studying on halogenation of flavonoids. At that time, halogenation of aromatic compounds normally used molecular halogens (Cl_2, Br_2, I_2) in chlorinated solvents such as CCl_4,

which caused several drawbacks on environment. Toxic reagents (chlorine gas or bromine liquid) and solvents, pollutant wastes as by-products were considered as limitations of this method. Other methods, including NBS-amberlyst, metal-oxo-catalyzed KBr-H_2O_2, KBr-$NaBO_3$, required strict conditions or complicated work-up steps.[19-21] To overcome these problems, in 2002, Bovicelli *et al.* reported a mild, efficient and regioselective method by DMD/NaX or Oxone®/acetone/water/NaX applied on selected flavanones.[22] The results showed that the mixture of substrate, DMD or Oxone® and NaX in an appropriate proportion achieved high yield of products with regioselectivity depending on the structures of starting materials.

After a decade since Bovicelli's investigation, in 2015, Bernini and partners revealed a new ecofriendly method of halogenation by making use of aqueous solution of hydrogen peroxide (H_2O_2), sodium halides (NaCl or NaBr) and acetic acid on different classes of flavonoids.[23] The regioselectiviy on products was controlled by the effects of ethereal oxygen on ring C endowed with the methoxy groups existing on ring B. Moreover, this method solved the disadvantages of general halogenation, such as non-toxic reagents and by-product, facile procedure and conditions.

These studies opened a new application of halogenation method on flavonoids with environmental friendliness.

1.3.2 Anti-dengue activity of halogenated flavonoids

In a past few years, the examination of halogenated flavonoids as anti-dengue candidate has been attracted interests. The first report of Suroengsit *et al.* in 2017 on 8 selected flavonoid derivatives (**Figure 1.6**) showed that two halogenated chrysins **19-20** strongly inhibited virus production with >99%.[24] These compounds also exhibited broad spectrum activities against all dengue serotypes and Zika virus. Both halogenated chrysins were relatively non-toxic with cell-viability over 80% (**Table 1.3**).

R = Br, R$_1$ = R$_2$ = OH, R$_3$ = H: **19**
R = I, R$_1$ = R$_2$ = OH, R$_3$ = H: **20**
R = H, R$_1$ = R$_3$ = OMe, R$_2$ = OH: **21**
R = H, R$_1$ = R$_2$ = OMe, R$_3$ = H: **22**

6

R = OMe: **23**
R = OH: **24**
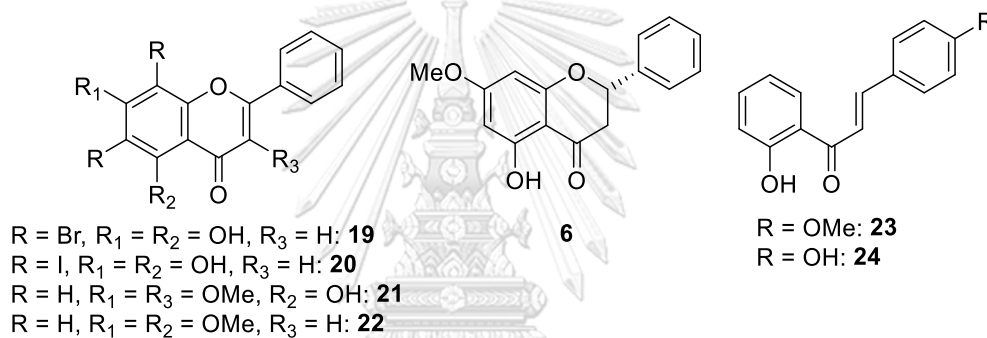
Figure 1.6 Structures of **19** - **24**.

Table 1.3 EC_{50} and SI values of halogenated chrysins (**19**) and (**20**). [24]

| Serotypes | Compounds | | | |
| | 19 | | 20 | |
	EC_{50} $(\mu M)^a$	SI^c	EC_{50} $(\mu M)^a$	SI^c
DENV1 (16007)	2.30 ± 1.04	19.42	2.30 ± 0.92	19.39
DENV2 (NGC)	1.47 ± 0.86	30.43	2.19 ± 0.31	20.37
DENV3 (16562)	2.32 ± 1.46	19.22	1.02 ± 0.31	43.64
DENV4 (c0036)	1.78 ± 0.72	25.12	1.29 ± 0.60	34.50
ZIKV (SV0010/15)[b]	1.65 ± 0.86	27.02	1.39 ± 0.11	32.14

[a]EC_{50} were determined by plaque assay in 96-well plates. [b]EC_{50} was determined by plaque assay in 24-well plates. [c]Selectivity index (CC_{50}/EC_{50}). Mean ± standard error of the means (SEM) of two independent experiments, in which each experiment was performed in triplicate.

1.3.3   Review of other bioactivities of halogenated flavonoids

Beside anti-dengue activity, halogenated derivatives of flavonoids have been evaluated for their bio-potentials in many researches since late 20th century.

In 1996, halogenated flavones were examined as aryl hydrocarbon (Ah) receptor agonists and antagonists by Lu and co-workers.[25] The halogenated flavones **25-27** (**Figure 1.7**) exhibited competitive Ah receptor binding affinities (IC_{50} 0.79 to 2.28 nM) compared with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (1.78 nM). In addition, these compounds also induced transformation of the rat cytosolic Ah receptor and induced CXP1A1 gene expression in MCF-7 human breast cancer cells.

R=Cl: **25**
R=Br: **26**
R=I: **27**
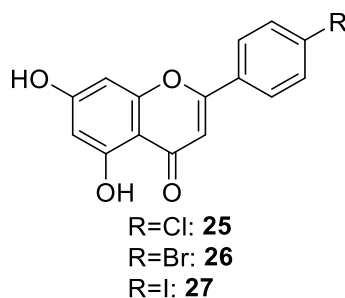
Figure 1.7 Structures of **25** - **27**.

In 2005, Park *et al.* synthesized 6,8-disubstituted chrysin derivatives and tested for inhibition of PGE_2 production.[26] Two halogenated chrysins **19** and **20** expressed strong inhibitory activities with 99.93 and 98.41% of inhibition of COX-2 catalyzed PGE_2 production from LPS-induced RAW 264.7 cells, respectively. Four years later, these two compounds were also investigated for *in vitro* and *in vivo* anti-inflammatory effect in a study of Do and colleagues.[27] Despite of an increasing of six to seven folds comparing with chrysin in *in vitro* anti-inflammatory activity, there were no remarkable difference between the halogenated derivatives and chrysin in *in vivo* test. This result implied an important role of molecular size affecting on the penetration of compounds into body. Lastly, Krongkan and partners examined brominated and iodinated chrysins **19** and **20** on anti-bacterial activity against *Propionibacterium acnes*, *Staphylococcus aureus*, *Streptococcus sobrinus*, *Salmonella typhi* and *Streptococcus mutans*.[28] These compounds exhibited excellent inhibition zone on five tested strains in a range of 18 to 21 mm. Furthermore, **19** and **20** were bacteriostatic agents toward *P. acnes*, *S. aureus*, and bactericidal agents for others. The combination of **19** and streptomycin

displayed the most synergetic effect with sixteen-fold increasing in activity, compared to individual antibiotics, against *P. acnes*, *S. sobrinus* and *S. mutans*, meanwhile the synergetic effect only showed on *P. acnes* when mixing **20** with streptomycin. In conclusion, **19** and **20** have been reported as new potent anti-inflammatory and anti-bacterial agents.

In addition, Bernini and his group reported anti-fungal activity of synthesized chlorinated and brominated flavonoids on *Trichoderma koningii*, *Fusarium oxysporum* and *Cladosporium cladosporioides*.[23] 8-Chloro-5,7,3′,4′-tetramethoxyepicatechin (**28**) (**Figure 1.8**) was the most active compounds against all types of fungi, compared to other tested derivatives.
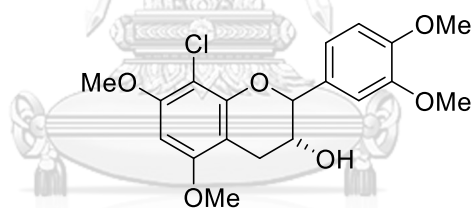
Figure 1.8 Structure of 8-chloro-5,7,3′, 4′-tetramethoxyepicatechin (**28**).

A study published in 2016 of Zhang and co-workers conducted on 5,7-dihydroxyflavanone derivatives including halogenated compounds **29-33** (**Figure 1.9**), evaluating their antimicrobial efficacy on Gram-positive, Gram-negative and yeast.[29] Most of halogenated compounds showed better antimicrobial activity than their natural analogs: **33** exhibited the lowest MIC value of 10 μg/mL towards Gram-positive strains *Bacillus anthracis*, *Bacillus cereus* and 20 μg/mL towards *Bacillus subtilis*,

Staphylococcus aureus; **29** expressed 30 μg/mL of MIC towards all tested Gram-positive strains. Moreover, dicholoro derivative **33** also inhibited the growth of *Saccharomyces cerevisiae* with MIC 10 μg/mL.

$R_1=R_3=H, R_2=F$: **29**
$R_1=R_2=F, R_3=H$: **30**
$R_1=R_2=R_3=F$: **31**
$R_1=F, R_2=Cl, R_3=H$: **32**
$R_1=R_2=Cl, R_3=H$: **33**
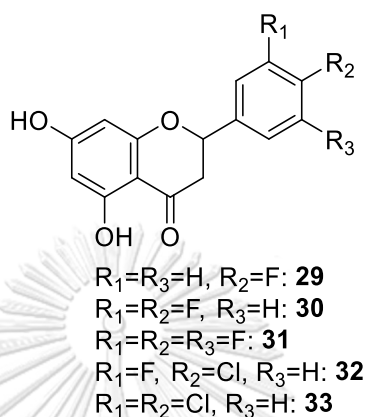
Figure 1.9 Structures of **29** - **33**.

Literature review has given out strong evidences to support the possibility of halogenated flavonoids as new bioactive agents, such as anti-fungal, anti-bacteria, anti-microbial activity. In addition, the promising dengue inhibition of di-halogenated chrysins suggested that halogenated flavonoids could be investigated as new anti-dengue agents.

Thus, the objective of this research was focused on these targets:

1. To synthesize a series of halogenated flavonoid derivatives under mild, effective and ecofriendly conditions.

2. To evaluate and investigate the inhibition of dengue infection of synthesized compounds.

Chapter 2

Experimental

2.1 Instruments and equipment

Thin layer chromatography (TLC) was performed on aluminum sheet precoated with silica gel, Kieselgel 60 F_{254} (Merck, Germany), column chromatography was conducted on silica gel no. 7734 (Merck, Germany). All NMR spectra (^{1}H and ^{13}C NMR) were recorded in deuterated chloroform (CDCl$_3$), acetone-d_6, methanol-d$_4$ (MeOD) or dimethylsulfoxide-d_6 (DMSO-d_6) on a Bruker AV400 and Varain Mercury 400 plus spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. The chemical shifts (δ) are assigned in comparison with residual solvent protons.

2.2 Chemicals

Chrysin (**34**), naringenin (**12**) and quercetin hydrate (**13**) were purchased from Tokyo Chemical Industry company and used without further purification. Pinostrobin (**6**) and pinocembrin (**7**) isolated from *Boesenbergia rotunda* were received as a gift from Mr. Pablo Chourreu Alba. Hesperetin (**15**) and baicalein (**18**) were hydrolyzed from their corresponding *O*-glycosides: hesperidin (**35**) and baicalin (**36**).[30, 31] A series of O^7-ether chrysins **37-41** with variation of chain length was obtained from Ms. Krongkan Kingkaew *et al*.[28] Other synthetic reagents were purchased from Merck chemical company or otherwise stated. All solvents used in this study were purified by standard methods except for those which were reagent grades.

2.3 Synthesis of halogenated flavonoids

2.3.1   Hydrolysis of hesperetin (**15**) and baicalein (**18**) from their corresponding glycosides

<u>Hydrolysis of hesperetin (**15**) from hesperidin (**35**) [30]</u>

To a flask containing 0.5 g of hesperidin (**35**), 500 mL of hydrolysis system concd. H_2SO_4:CH_3COOH:C_2H_5OH (1:1:9) were carefully poured and well-stirred at 80°C for 8.5 h. The reaction mixture was extracted with EtOAc until the organic phase was neutralized. The solvent was evaporated by reduced evaporation and hesperetin (**15**) (206 mg, 83% yield) was isolated by column chromatography with mobile phase hexane:EtOAc 6:4.

<u>Hydrolysis of baicalein (**18**) from baicalin (**36**) [31]</u>

98% H_2SO_4 (2.0 mL, 0.037 mmol) was added dropwise to a round flask (100 mL) containing baicalin (**36**) (50 mg, 0.11 mmol) and stirred on magnetic stirrer at room temperature, following by slowly dropped 2.0 mL of water. After the evolution of heat terminated, one portion of water (15 mL) was poured directly to the mixture and the yellow powder was achieved by suction filtration and washed with water.

Hesperetin (**15**): yellow powder (83%) ^{1}H NMR (100MHz, DMSO-d$_6$) δ 12.13 (s, 1H), 10.8 (b, 1H), 9.10 (s, 1H), 6.94-6.87 (m, 3H), 5.88 (d, J = 3.5 Hz, 2H), 5.42 (dd, J = 12.4, 3.1 Hz, 1H), 3.77 (s, 3H), 3.19 (dd, J = 17.1, 12.4 Hz, 1H), 2.70 (dd, J = 17.1, 3.1 Hz, 1H).

Baicalein (**18**): yellow powder (81%) ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 8.06 (m, 2H), 7.59 (m, 3H), 6.92 (s, 1H), 6.63 (s, 1H).

2.3.2   Brominated flavonoids

<u>General procedure for bromination by NaBr/H$_2$O$_2$/CH$_3$COOH [23]</u>

A 30% aqueous solution of H$_2$O$_2$ (3.0 equiv.) was added to the flask of flavonoid (0.1 mmol) and an appropriate amount of NaBr (2.0-4.0 equiv.) in CH$_3$COOH (2.5 mL); then, the mixture was stirred at room temperature for 24 h. The reaction was monitored by TLC. At the end, the crude was treated with Na$_2$S$_2$O$_3$ and extracted with EtOAc (3✕10 mL). The reunited organic fractions were dried over anhydrous Na$_2$SO$_4$; after filtration, the solvent was evaporated under reduced pressure. Final products were isolated and purified by chromatographic column using hexane: EtOAc: acetone = 60:40:4 as eluent.

<u>General procedure for bromination by NaBr/Oxone® [27]</u>

To the solution of chrysin (**34**) (0.1 mmol) in acetone:water 5:1 was added NaBr (0.3 mmol). After cooling, Oxone® (0.3 mmol) was added and the mixture was stirred at room temperature for 2 h. The final solution was treated with Na$_2$S$_2$O$_3$ and evaporated under reduced pressure.

<u>General procedure for bromination of baicalein (**18**) [32]</u>

A mixture of baicalein (**18**) (35 mg, 0.13 mmol) and NBS (33 mg, 0.19 mmol) in THF (4.0 mL) and concd. H$_2$SO$_4$ (5.0 µL) was stirred at room temperature for 12 h. The reaction mixture was extracted with EtOAc, washed with 10% aqueous NaHSO$_4$ solution and water, dried over anhydrous Na$_2$SO$_4$, and then concentrated under reduced

pressure. The residue was recrystallized from MeOH to give the target compound (35%) as yellow powder.

2.3.3 Iodinated flavonoids

<u>General procedure for iodination by KI/H_2O_2/CH_3COOH</u>

A 30% aqueous solution of H_2O_2 (3.0 equiv.) was added to the flask of flavonoid (0.1 mmol) and an appropriate amount of KI (2.0-4.0 equiv.) in CH_3COOH (2.5 mL); then, the mixture was stirred at room temperature for 24 h. The reaction was monitored by TLC. At the end, the crude was treated with $Na_2S_2O_3$ and extracted with EtOAc (3×10 mL). The separated organic fractions were dried over anhydrous Na_2SO_4; after filtration, the solvent was evaporated under reduced pressure. Final products were isolated and purified by chromatographic column using hexane: EtOAc: acetone = 60:40:4 as eluent.

2.3.4 Brominated O^7-ether chrysin

<u>General procedure for bromination of O^7-ether chrysin [33]</u>

A solution of O^7-ether chrysin **37-41** (0.1 mmol) in CH_2Cl_2 (10 mL) was added NBS (0.1-0.2 mmol) and stirred for 6 h at room temperature. The solvent then was removed by evaporation and the residues were purified by silica gel column chromatography to obtain the derivatives **53-62**.

Twenty-two synthesized flavonoid derivatives are tabulated in **Table 2.1**.

Table 2.1 Structures of flavonoids derivatives **19**, **42** - **62**.
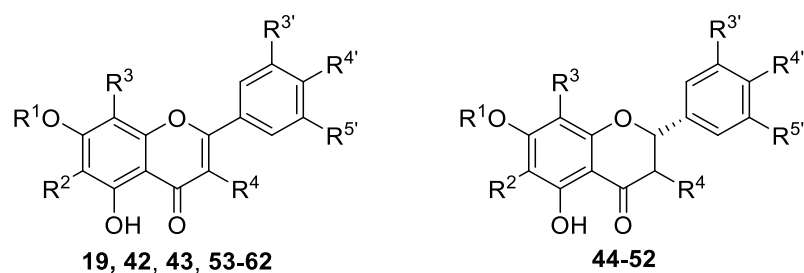
Entry	Cpd	R^1	R^2	R^3	R^4	$R^{3'}$	$R^{4'}$	$R^{5'}$
1	**19**	H	Br	Br	H	H	H	H
2	**42**	H	OH	Br	H	H	H	H
3	**43**	H	Br	Br	OH	H	OH	OH
4	**44**	H	Br	Br	H	H	H	H
5	**45**	H	I	H	H	H	H	H
6	**46**	CH_3	Br	Br	H	H	H	H
7	**47**	CH_3	I	I	H	H	H	H
8	**48**	CH_3	I	H	H	H	H	H
9	**49**	H	Br	Br	H	Br	OH	Br
10	**50**	H	I	I	H	I	OH	I
11	**51**	H	Br	Br	H	H	OH	H
12	**52**	H	Br	Br	H	OH	OCH_3	H
13	**53**	C_2H_5	Br	Br	H	H	H	H
14	**54**	C_2H_5	Br	H	H	H	H	H
15	**55**	C_4H_9	Br	Br	H	H	H	H
16	**56**	C_4H_9	Br	H	H	H	H	H
17	**57**	C_6H_{13}	Br	Br	H	H	H	H

Table 2.1 (Continue)

Entry	Cpd	R^1	R^2	R^3	R^4	R$^{3'}$	R$^{4'}$	R$^{5'}$
18	**58**	C$_6$H$_{13}$	Br	H	H	H	H	H
19	**59**	C$_8$H$_{17}$	Br	Br	H	H	H	H
20	**60**	C$_8$H$_{17}$	Br	H	H	H	H	H
21	**61**	C$_{12}$H$_{25}$	Br	Br	H	H	H	H
22	**62**	C$_{12}$H$_{25}$	Br	H	H	H	H	H

6,8-dibromo-5,7-dihydroxyflavone (**19**): yellow powder (92% yield). ^{1}H NMR (DMSO-d_6) δ 13.74 (s, 1H), 8.14 (dd, J = 7.4, 2.2 Hz, 2H), 7.61 (m, 3H), 7.12 (s, 1H). [28]

8-bromo-5,6,7-trihydroxyflavone (**42**): yellow powder (35% yield). ^{1}H NMR (MeOD) δ 8,04 (m, 2H), 7.56 (m, 3H), 6.77 (s, 1H).[32]

6,8-dibromo-3,5,7,3′,4′-pentahydroxyflavone (**43**): light yellow powder (52% yield). ^{1}H NMR (Acetone-d_6) δ 13.14 (s, 1H), 7.99 (d, J = 2.2 Hz, 1H), 7.87 (dd, J = 8.5, 2.2 Hz, 1H), 7.03 (d, J = 8.5 Hz, 1H).[34]

6,8-dibromo-5,7-dihydroxyflavanone (**44**): light brown powder (93% yield). ^{1}H NMR (CDCl$_3$) δ 12.74 (s, 1H), 7.45 (m, 5H), 5.58 (dd, J = 12.2, 3.5 Hz, 1H), 3.15 (dd, J = 17.3, 12.2 Hz, 1H), 3.00 (dd, J = 17.3, 3.5 Hz, 1H); ^{13}C NMR (CDCl$_3$) δ 195.7, 159.3, 158.0, 157.4, 137.5, 129.1, 126.0, 103.85, 90.6, 89.0, 79.7, 42.6; HR-ESI-MS m/z calcd. for C$_{15}$H$_9$Br$_2$O$_4$Na [M + 2Na - H]$^+$ 456.8663, found 456.8657.

6-iodo-5,7-dihydroxyflavanone (**45**): light brown powder (74% yield). ^{1}H NMR (CDCl$_3$)

δ 13.03 (s, 1H), 7.44 (m, 5H), 6.31 (s, 1H), 5.44 (dd, J = 12.8, 3.2 Hz, 1H), 3.25 (dd, J =

17.3, 12.8 Hz, 1H), 2.89 (dd, J = 17.3, 3.2 Hz, 1H); ^{13}C NMR (CDCl$_3$) δ 195.4, 163.3, 162.8,

162.7, 137.9, 129.0, 128.9, 126.1, 103.2, 95.3, 79.2, 67.3, 42.7.

6,8-dibromo-5-hydroxy-7-methoxyflavanone (**46**): yellow powder (96% yield). ^{1}H

NMR (CDCl$_3$) δ 12.61 (s, 1H), 7.48 (m, 5H), 5.61 (dd, J = 12.3, 3.4 Hz, 1H), 3.98 (s, 3H),

3.18 (dd, J = 17.3, 12.3 Hz, 1H), 3.05 (dd, J = 17.3, 3.4 Hz, 1H); ^{13}C NMR (CDCl$_3$) δ 196.3,

162.1, 159.0, 157.7, 137.1, 128.8, 125.7, 105.9, 98.3, 96.6, 79.2, 60.7, 42.4; HR-ESI-MS

m/z calcd. for C$_{16}$H$_{12}$Br$_2$O$_4$Na [M + Na]$^+$ 448.90000, found 448.89938.

6,8-diiodo-5-hydroxy-7-methoxyflavanone (**47**): light green crystal (73% yield). ^{1}H

NMR (Acetone-d_6) δ 13.02 (s, 1H), 7.47 (m, 5H), 5.85 (dd, J = 12.6, 3.2 Hz, 1H), 3.91 (s,

3H), 3.37 (dd, J = 17.3, 12.6 Hz, 1H), 3.14 (dd, J = 17.3, 3.2 Hz, 1H); ^{13}C NMR (Acetone-

d_6) δ 198.2, 167.6, 164.0, 163.0, 139.2, 129.6, 127.1, 106.4, 95.61, 94.7, 80.5, 61.2, 42.6;

HR-ESI-MS m/z calcd. for C$_{16}$H$_{11}$I$_2$O$_4$Na [M + 2Na - H]$^+$ 566.8542, found 566.8525.

6-iodo-5-hydroxy-7-methoxyflavanone (**48**): yellow oil (10% yield). ^{1}H NMR

(Acetone-d_6) δ 12.95 (s, 1H), 7.53 (m, 5H), 6.33 (s, 1H), 5.68 (dd, J = 13.0, 3.1 Hz, 1H),

3.98 (s, 3H), 3.29 (dd, J = 17.4, 13.0 Hz, 1H), 2.92 (dd, J = 17.4, 3.1 Hz, 1H). ^{13}C NMR

(Acetone-d_6) δ 195.3, 165.6, 164.8, 163.6, 162.2, 137.8, 129.0, 128.9, 126.1, 103.2, 93.2,

91.9, 79.4, 66.0, 42.6.

6,8,3′,5′-tetrabromo-5,7,4′-trihydroxyflavanone (**49**): white powder (42% yield). ^{1}H NMR (Acetone-d_6) δ 12.93 (s, 1H), 7.82 (s, 2H), 5.74 (dd, J = 12.7, 3.1 Hz, 1H), 3.38 (dd, J = 17.2, 12.7 Hz, 1H), 3.07 (dd, J = 17.2, 3.1 Hz, 1H); ^{13}C NMR (Acetone-d_6) δ 196.1, 159.3, 158.4, 157.3, 151.1, 132.2, 130.6, 128.1, 114.0, 111.9, 102.5, 91.1, 90.0, 77.5, 40.9.

6,8,3′,5′-tetraiodo-5,7,4′-trihydroxyflavanone (**50**): yellow powder (48% yield). ^{1}H NMR (DMSO-d_6) δ 12.84 (s, 1H), 7.74 (s, 2H), 5.67 (dd, J = 12.8, 3.0 Hz, 1H), 3.43 (dd, J = 17.2, 12.8 Hz, 1H), 2.95 (dd, J = 17.2, 3.0 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 196.6, 165.9, 165.1, 162.6, 156.3, 138.8, 138.6, 135.9, 135.5, 113.2, 96.7, 84.3, 78.2, 42.6.

6,8-dibromo-5,7,4′-trihydroxyflavanone (**51**): light yellow powder (25% yield). ^{1}H NMR (DMSO-d_6) δ 12.97 (s, 1H), 7.45 (d, J = 8.6 Hz, 2H), 6.92 (d, J = 8.6 Hz, 2H), 5.67 (dd, J = 12.6, 3.2 Hz, 1H), 3.33 (dd, J = 17.2, 12.6 Hz, 1H), 2.95 (dd, J = 17.2, 3.2 Hz, 1H).[35]

6,8-dibromo-5,7,3′-trihydroxy-4′-methoxyflavanone (**52**): yellow powder (61% yield). ^{1}H NMR (Acetone-d_6) δ 12.92 (s, 1H), 7.29 (s, 1H), 7.21 (s, 1H), 7.01 (s, 1H), 5.87 (dd, J = 13.1, 3.0 Hz, 1H), 3.92 (s, 3H), 3.27 (dd, J = 17.3, 13.1 Hz, 1H), 2.94 (dd, J = 17.3, 3.0 Hz, 1H). [35]

6,8-dibromo-7-ethoxy-5-hydroxyflavone or 6,8-dibromo-O^7-ethylchrysin (**53**): yellow powder (86% yield). ^{1}H NMR (CDCl$_3$) δ 13.58 (s, 1H), 8.02 (dd, J = 8.0, 3.5 Hz, 2H), 7.58 (m, 3H), 6.83 (s, 1H), 4.24 (q, J = 7.0 Hz, 2H), 1.57 (t, J = 7.0 Hz, 3H); ^{13}C NMR (100MHz, CDCl$_3$) δ 182.1, 164.8, 159.6, 158.0, 152.5, 132.6, 130.4, 129.3, 126.6, 108.6,

105.5, 101.7, 95.8, 70.4, 10.7; HR-ESI-MS m/z calcd for $C_{17}H_{12}Br_2O_4Na$ [M + Na]$^+$ 460.90000, found 460.90024.

6-bromo-7-ethoxy-5-hydroxyflavone or 6-bromo-O^7-ethylchrysin (**54**): yellow powder (91% yield). ^{1}H NMR (CDCl$_3$) δ 13.46 (s, 1H), 7.89 (m, 2H), 7.41 (m, 3H), 6.73 (s, 1H), 6.56 (s, 1H), 4.22 (q, J = 7.0 Hz, 2H), 1.55 (t, J = 7.0 Hz, 3H); ^{13}C NMR (CDCl$_3$) δ 181.9, 164.2, 161.0, 158.3, 156.7, 132.0, 131.0, 129.1, 126.3, 105.9, 94.8, 91.5, 65.5, 14.4; HR-ESI-MS m/z calcd for $C_{17}H_{13}BrO_4Na$ [M + Na]$^+$ 389.98949, found 389.98970.

6,8-dibromo-7-butoxy-5-hydroxyflavone or 6,8-dibromo-O^7-butylchrysin (**55**): yellow powder (82% yield). ^{1}H NMR (CDCl$_3$) δ 13.57 (s, 1H), 8.01 (m, 2H), 7.58 (m, 3H), 6.82 (s, 1H), 4.15 (q, J= 6.5 Hz, 2H), 1.94 (m, 2H), 1.62 (m, 2H), 1.03 (t, J = 6.5 Hz, 3H); ^{13}C NMR (CDCl$_3$) δ 182.2, 164.8, 160.0, 158.0, 152.5, 132.6, 130.4, 129.3, 126.6, 108.6, 105.5, 101.7, 95.7, 74.2, 32.1, 29.7, 13.9; HR-ESI-MS m/z calcd for $C_{19}H_{16}Br_2O_4Na$ [M + Na]$^+$ 488.93130, found 488.93206.

6-bromo-7-butoxy-5-hydroxyflavone or 6-bromo-O^7-butylchrysin (**56**): yellow powder (90% yield). ^{1}H NMR (CDCl$_3$) δ 13.46 (s, 1H), 7.88 (m, 2H), 7.54 (m, 3H), 6.69 (s, 1H) 6.54 (s, 1H), 4.12 (q, J = 6.4 Hz, 2H), 1.89 (m, 2H), 1.58 (m, 2H), 1.02 (t, J = 6.4 Hz, 3H); ^{13}C NMR (CDCl$_3$) δ 182.0, 164.3, 161.3, 158.5, 156.8, 132.2, 131.2, 129.3, 126.5, 106.0, 95.1, 91.6, 69.7, 31.0, 19.3, 13.9.

6,8-dibromo-7-hexyloxy-5-hydroxyflavone or 6,8-dibromo-O^7-hexylchrysin (**57**): yellow powder (87% yield). ^{1}H NMR (CDCl$_3$) δ 13.57 (s, 1H), 8.00 (d, J = 7.2 Hz, 2H), 7.58

(m, 3H), 6.82 (s, 1H), 4.14 (q, J = 6.6 Hz, 2H), 1.95 (m, 2H), 1.58 (s, 6H), 0.93 (t, J = 6.6 Hz, 3H); ^{13}C NMR (CDCl$_3$) δ 182.1, 164.7, 159.6, 157.9, 152.5, 132.6, 130.4, 129.3, 126.6, 108.5, 105.4, 101.7, 95.7, 74.5, 31.6, 30.0, 2.5, 22.6, 14.1; HR-ESI-MS m/z calcd for C$_{21}$H$_{20}$Br$_2$O$_4$Na [M + Na]$^+$ 516.96260, found 516.96247.

6-bromo-7-hexyloxy-5-hydroxyflavone or 6-bromo-O^7-hexylchrysin (**58**): yellow powder (89% yield). ^{1}H NMR (CDCl$_3$) δ 7.89 (m, 2H), 7.54 (m, 3H), 6.71 (s, 1H), 6.55 (s, 1H), 4.12 (q, J = 6.4 Hz, 2H), 1.91 (m, 2H), 1.53 (m, 2H), 1.38 (m, 4H), 0.93 (t, J = 6.4 Hz, 3H); ^{13}C NMR (CDCl$_3$) δ 182.0, 164.3, 161.3, 158.5, 156.9, 132.2, 131.2, 129.5, 129.3, 126.5, 106.0, 95.1, 91.7, 70.0, 31.6, 28.9, 25.7, 22.7, 14.1.

6,8-dibromo-7-octyloxy-5-hydroxyflavone or 6,8-dibromo-O^7-octylchrysin (**59**): yellow powder (90% yield). ^{1}H NMR (CDCl$_3$) δ 13.53 (s, 1H), 8.00 (m, 2H), 7.56 (m, 3H), 6.82 (s, 1H), 4.12 (q, J = 6.6 Hz, 2H), 1.92 (m, 2H), 1.56 (m, 2H), 1.34 (m, 8H), 0.93 (t, J = 6.5, 3H); ^{13}C NMR (CDCl$_3$) δ 182.3, 177.7, 164.9, 159.8, 158.1, 152.7, 132.7, 130.6, 129.4, 126.8, 108.7, 105.6, 101.8, 95.9, 74.6, 32.0, 30.2, 29.7, 29.5, 29.4, 26.0, 22.8, 14.2.

6-bromo-7-octyloxy-5-hydroxyflavone or 6-bromo-O^7-octylchrysin (**60**): yellow powder (89% yield). ^{1}H NMR (CDCl$_3$) δ 7.88 (d, J = 7.4 Hz, 2H), 7.54 (m, 3H), 6.70 (s, 1H), 6.55 (s, 1H), 4.11 (q, J = 6.4 Hz, 2H), 1.90 (m, 2H), 1.54 (m, 2H), 1.35 (m, 8H), 0.89 (t, J = 6.4, 3H); ^{13}C NMR (CDCl$_3$) δ 181.8, 164.1, 161.2, 158.3, 156.7, 132.0, 131.1, 129.1, 126.3, 105.9, 105.3 94.9, 91.5, 69.9, 31.8, 29.2, 28.8, 25.9, 22.6, 14.1.

6,8-dibromo-7-dodecyloxy-5-hydroxyflavone or 6,8-dibromo-O^7-dodecylchrysin (**61**): yellow powder (88% yield). ^{1}H NMR (CDCl$_3$) δ 13.58 (s, 1H), 8.02 (m, 2H), 7.58 (m, 3H), 6.82 (s, 1H), 4.14 (q, J = 6.6 Hz, 2H), 1.95 (m, 2H), 1.27 (s, 18H), 0.88 (t, J = 6.5 Hz, 3H); ^{13}C NMR (CDCl$_3$) δ 182.3, 165.0, 159.9, 158.2, 152.8, 132.7, 130.7, 129.5, 126.8, 108.8, 105.7, 101.9, 95.9, 74.7, 30.2-14.3 (11C); HR-ESI-MS m/z calcd for C$_{27}$H$_{32}$Br$_2$O$_4$Na [M + Na]$^+$ 601.0564, found 601.0573.

6-bromo-7-dodecyloxy-5-hydroxyflavone or 6-bromo-O^7-dodecylchrysin (**62**): yellow powder (90% yield). ^{1}H NMR (CDCl$_3$) δ 13.48 (s, 1H), 7.89 (m, 2H), 7.55 (m, 3H), 6.73 (s, 1H), 6.57 (s, 1H), 4.13 (q, J = 6.0 Hz, 2H), 1.89 (m, 2H), 1.27 (s, 18H), 0.88 (t, J = 6.0 Hz, 3H); ^{13}C NMR (CDCl$_3$) δ 181.8, 164.1, 161.1, 158.3, 156.7, 132.0, 131.0, 129.1, 126.3, 105.9, 96.8, 94.9, 91.5, 69.8, 31.9-14.1 (11C); HR-ESI-MS m/z calcd for C$_{27}$H$_{34}$BrO$_4$ [M + H]$^+$ 501.16405, found 501.16529.

2.4 Anti-dengue activity study

The experiments were conducted under the collaboration with Department of Microbiology, Faculty of Medicine, Chulalongkorn University.

2.4.1. Preliminary screening

LLC/MK2 cells (ATCC® CCL-7™) and DENV2 NGC were maintained as previously described.[24] LLC-MK2 (5x10^4 cells) were seeded into each well of 24-well plate and incubated overnight at 37°C under 5% CO$_2$. DENV2 was added to the cells at a multiplicity of infection (M.O.I.) of 0.1 and the plate was incubated at 37°C under 5% CO$_2$ with gentle rocking every 15 min. The plate was washed with phosphate buffer

saline and incubated in maintenance media.[24] Each compound was prepared in DMSO and was introduced into each well to the final concentration of 10 μM during and after infection. One percent DMSO was used as a no-inhibition control and the maintenance media alone was used as a no-virus control. Supernatants were collected at day 3 of incubation and the virus titers were determined by plaque titration. The active compounds were determined by the ability of the compound to inhibit greater than 1-log or 90% of viral titer (plaque forming unit/mL).

2.4.2. Cytotoxicity of compounds with cell lines

The compound toxicity was determined at 10 μM final concentration. LLC/MK2 $(1 \times 10^4 \text{cells/well})$ were seeded into each well of 96-well plates and incubated overnight at 37°C 5% CO_2. Compounds were prepared and added to the cells to the final concentration of 10 μM. 1% DMSO was used as a 100% cell viability control. Cells were incubated for 48 h before analyzing their viability by CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega) according to manufacturer's protocol. The plate was read by spectrophotometry at A_{450} and data were calculated to percent cell viability proportioned to DMSO control.

2.4.3. Effective concentration (EC_{50})

Effective concentration (EC_{50}) was also studied using LLC/MK2 5×10^4 cells, which were seeded and infected with DENV2 (M.O.I. of 0.1). Flavonoids were serially diluted in DMSO to the final concentrations as follows; 0, 0.1, 0.25, 0.5, 1, 2, 2.5, 5, 7.5, 10, and 25 μM. DMSO at the concentration of 1% was used as a mock treatment referring to

100% infection. Infected cells were treated with the designated compounds during and post infection. Supernatants were collected at 3 days after infection for analysis of virion production by plaque titration. Non-linear regression analysis was used to determine the effective concentrations. Three independent experiments were performed and results were as means and standard error of the means (SEM). The therapeutic index (TI) was a ratio of CC_{50}/EC_{50} of the compound.

2.4.4. Cytotoxic concentration (CC_{50})

Cytotoxic concentrations (CC_{50}) were also studied using LLC/MK2 cells. Briefly, the 10^4 cells were seeded into each well of 96-well plates. After an overnight incubation, flavonoids were serially diluted in DMSO to the final concentrations as follows: 1, 5, 7.5, 10, 25, 50, 100, and 250 μM. Each concentration was performed in triplicates. DMSO at 1% was used as a mock treatment referring to 100% cell viability. Cells were incubated for 48 h before cell viability was accessed using CellTiler 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Wisconsin, USA). Non-linear regression analysis was used to determine cytotoxic concentrations (CC_{50}) of each experiment. Results were derived from three independent experiments and reported as means and standard error of the means (SEM).

Chapter 3

Results and discussion

Structural modification of flavonoids has been investigated for improvement in biological activity along with penetration ability.[36-38] Bioactive halogenated derivatives were proved as promising agents for further study. In this research, halogenation of selected flavonoids was conducted to obtain a range of brominated and iodinated derivatives as new candidates for anti-dengue infection. Moreover, a series of O^7-ether chrysin was brominated for structure-activity relationship (SAR) evaluation. The inhibition of infection DENV2 was examined on LLC-MK2 cells.

3.1 Halogenation of flavonoids

3.1.1 Preliminary study on the halogenation of chyrsin (**34**)

To find out a suitable method for halogenation of flavonoids, chrysin (**34**) was chosen as a model (**Figure 3.1**). Following the reported reaction conditions with suitable modifications, the results are summarized in **Table 3.1**.[23, 27]
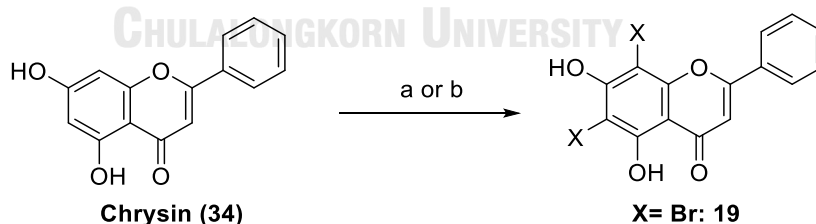
Figure 3.1 Preliminary screening of halogenation of chrysin (**34**).

Reagents and conditions: a) NaBr/KI (3.0 equiv.), H_2O_2 (3.0 equiv.), CH_3COOH (2.5 mL), rt, 24 h; b) NaBr/KI (3.0 equiv.), Oxone® (3.0 equiv.), acetone:H_2O, rt, 2 h.

Table 3.1 Screening of halogenation of chrysin (**34**).

Entry	Reagent	Oxidant (3.0 equiv.)	Solvent	Time (h)	Note
1	NaBr	H_2O_2	CH_3COOH	24	% yield of **19**: 92
2	NaBr	Oxone®	acetone:H_2O	2	% yield of **19**: 92
3	KI	H_2O_2	CH_3COOH	24	Insoluble product
4	KI	Oxone®	acetone:H_2O	2	Insoluble product

All the reactions were conducted at rt.

In previous reports, the common electrophilic halides were derived from hydrogen halides or halide salts and the amount was limited to 1.0 equiv. to obtain the monosubstitution as a major component.[22, 23] The proposed mechanism using chrysin as an instance were illustrated below (**Figure 3.2**)
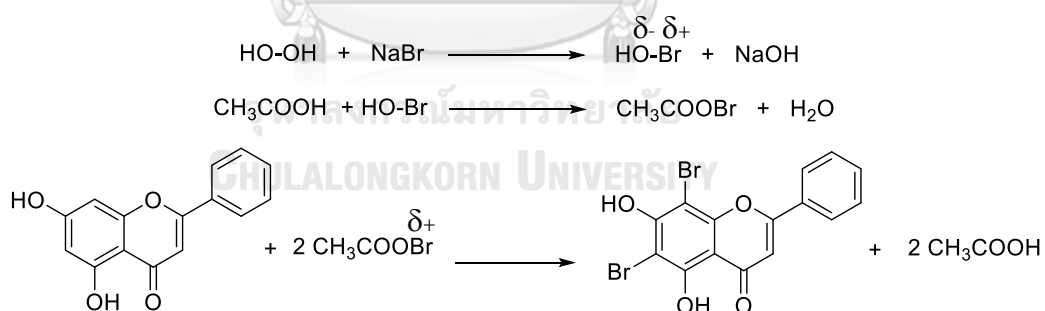
Figure 3.2 Proposed mechanism of bromination of chrysin (**34**).

In this research, the desired targets are a series of disubstituted flavonoids (**19, 42-52**) because of their promising bioactive value.[24] The reaction was taken place at rt and checked by TLC every hour. Using an excess of NaBr (3.0 equiv.), 6,8-

dibromochrysin (**19**) was generated as only product in excellent yield (>90%). The expected product was characterized by the absence of signals of 6- and 8- protons at δ_H 6.50 and 6.21 ppm, and the significant shift of the chelated hydroxyl proton at position 5 from 12.82 ppm of chrysin (**34**) to 13.74 ppm of **42,** due to the effect of bromine atom. The ^{1}H NMR spectral data of **19** was also confirmed with those of the previous report.[26] In addition, NaBr/H_2O_2/CH_3COOH or NaBr/Oxone®/acetone:H_2O 5:1 changed from halogenated solvents to other low toxic solvents and had non-toxic by-product (H_2O), which could consider as ecofriendly conditions.

6,8-dibromo-5,7-dihydroxyflavone (**19**): yellow powder. ^{1}H NMR (DMSO-d_6, Figure A3.) δ_H 13.74 (s, 1H), 8.14 (dd, J = 7.4, 2.2 Hz, 2H), 7.61 (m, 3H), 7.12 (s, 1H).

On the other hand, there was a failure when changing from NaBr to KI, for both oxidants. 6,8-Diiodochrysin (**20**) was synthesized before using I_2 in CH_2Cl_2 and HNO_3, and could be dissolved in DMSO.[28] However, in repeated experiments, the insoluble product was received without identifying since it was not able to dissolve in any kind of solvents, leading to the limit of applicability for iodination. Nonetheless, the developed conditions 1.0 equiv. of precursor – 3.0. of NaBr/KI – 3.0 equiv. of H_2O_2 were applied for other selected flavonoids in this study.

3.1.2 Halogenation of selected flavonoids

After applying on chrysin (**34**), the bromination and iodination of selected flavonoids, including flavone: baicalein (**18**); flavanone: pinostrobin (**6**), pinocembrin

(**7**), naringenin (**12**), hesperetin (**15**); flavonol: quercetin (**13**), were carried out (**Figure 3.3**). Isolation yields were reported in **Table 3.2**.
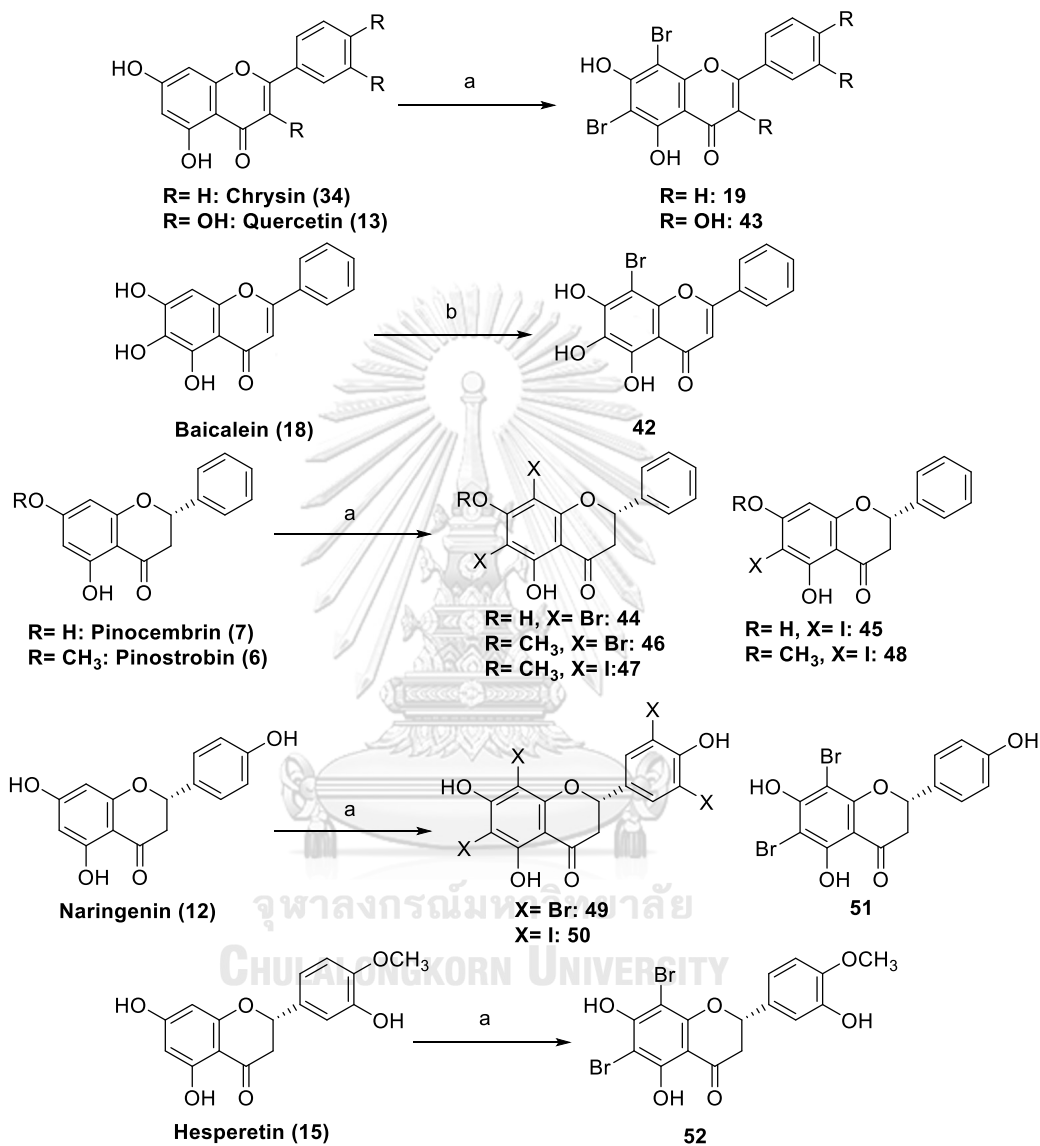
Figure 3.3 Halogenation of selected flavonoids.

Reagents and conditions: a) NaBr/KI (3.0 equiv.), H_2O_2 (3.0 equiv.), CH_3COOH (2.5 mL), rt, 24 h; b) NBS (1.5 equiv.), concd. H_2SO_4, THF (4.0 mL), rt, 12 h.

Table 3.2 Halogenation of selected flavonoids.

Entry	Halogenation	Starting material	% Isolated yield		
			Mono-	Di-	Tetra-
1		Baicalein (**18**)			
2		Baicalein (**18**)[a]	35		
3		Pinocembrin (**7**)		93	
4		Pinostrobin (**6**)		96	
5	Bromination	Naringenin (**12**)		25	42
6		Naringenin (**12**)[b]		10	30
7		Naringenin (**12**)[c]		12	21
8		Quercetin (**13**)		52	
9		Hesperetin (**15**)		61	
10		Pinocembrin (**7**)	74		
11		Pinocembrin (**7**)[d]	31		
12		Pinostrobin (**6**)	10	73	
13	Iodination	Naringenin (**12**)			48
14		Naringenin (**12**)[d]			40
15		Naringenin (**12**)[e]			23

Reaction condition: Precursor (1.0 equiv.), NaBr/KI (3.0 equiv.), H_2O_2 (3.0 equiv.), rt, 24 h.

[a] NBS (1.5 equiv.), concd. H_2SO_4, THF (4.0 mL), rt, 12 h.

[b] NaBr (4.0 equiv.) with H_2O_2 (3.0 equiv.) in CH_3COOH (2.5 mL), rt, 24 h.

[c] NaBr (2.0 equiv.) with H_2O_2 (3.0 equiv.) in CH_3COOH (2.5 mL), rt, 24 h.

[d] KI (4.0 equiv.) with H_2O_2 (3.0 equiv.) in CH_3COOH (2.5 mL), rt, 24 h.

[e] KI (2.0 equiv.) with H_2O_2 (3.0 equiv.) in CH_3COOH (2.5 mL), rt, 24 h.

6-Bromobaicalein (**42**) was unsuccessfully generated by using NaBr/H_2O_2/CH_3COOH, but it could be obtained by following previously reported method (entries 1, 2).[32] Compound **42** was confirmed by comparing ^{1}H NMR spectrum (Figure A4, Appendix) with the reference. In contrast, the bromination of pinocembrin (**7**) and pinostrobin (**6**) was accomplished by using NaBr/H_2O_2/CH_3COOH to gain 6,8-dibromo derivatives in high yield (more than 90%, entries 3, 4). It should be noted here that 6,8-dibromopinocembrin (**44**) and 6,8-dibromopinostrobin (**46**) were synthesized for the first time. These two new compounds were identified from the missing of signals of two protons of 6- and 8-positions at δ_H 6.07 ppm of pinocembrin (**7**) and δ_H 5.94 ppm of pinostrobin (**6**). Furthermore, the large shift of chelated hydroxyl proton at position 5 due to the addition of bromine atom was also the characteristic signal of the spectra (Figures A7, A12, Appendix). The bromination of naringenin (**12**) was complicated as the existence of two products with moderate yield (entries 5-7). 6,8,3′,5′-Tetrabromonaringenin (**49**) and 6,8-dibromonaringenin (**51**) were similar in the disappearance of signal of two protons at positions 6 and 8 at δ_H 5.88 ppm and the switch of specific hydroxyl proton at position 5 from δ_H 12.14 ppm of naringenin (**12**) to 12.93 and 12.98 ppm of **49** and **51**, respectively. Moreover, in the range of aromatic signals of **49**, there is only one signal with integration equal to 2.0 representing for two protons at positions 2′ and 6′ of ring B. This evidence strongly confirmed for the substitution at 3′- and 5′-positions on phenyl ring B of naringenin (**12**) (Figure A19,

Appendix). In the circumstances of quercetin (**13**) and hesperetin (**15**), the replacement of bromine atom only occurred at activated phenyl ring A, which was proved by the loss of the proton signals at positions 6 and 8 at δ_H 6.41 and 6.18 ppm of quercetin (**13**) and at δ_H 5.88 ppm of hesperetin (**15**) (entries 8, 9, Figures A23, A24, Appendix). Compounds **43** and **52** are known compounds and were compared with the reference.[34, 35]

Similarly, the iodination of selected flavonoids gave the same characteristics on the spectra. The obtained products were recognized by the missing of the signals of protons at positions 6 and 8 when collating with their precursors (Figures A9, A15, A17, A21). Besides, these iodinated compounds **45**, **47**, **48** and **50** are firstly reported.

For bromination, there was a noticeable difference in components of the reaction mixture between substituted and unsubstituted phenyl ring B. The parents with plain phenyl ring B gave out dibromo derivatives in excellent yield (>90%). This circumstance could be explained by the inactive reactivity of the unsubstituted benzene ring. 6,8-Dibromochrysin (**19**), 6,8-dibromopinocembrin (**44**) and 6,8-dibromopinostrobin (**46**) were selected as examples for illustration (entries 3, 4). On the contrary, the presence of hydroxyl groups activated the benzene ring, especially at 3′ and 5′ positions, resulted in tetrabromonaringenin (**49**). In spite of using only 2.0 equiv. of NaBr, the multiple substitution still occurred (entry 7), indicating the crucial role of the hydroxyl group on ring B affecting on its reactivity. Increasing to 4.0 equiv. of NaBr only affected

to the yield, since the percentage yield of each product slightly increased without changing the reaction components (entry 6). Furthermore, there was a regioselectivity occurred on electron-deficient ring A only when applying on quercetin (**13**) and hesperetin (**15**), despite the similarity of ring B structure with naringenin (**12**). The formation of dibromo product in the circumstance of quercetin could be explained by the deprotonation of acidic proton of hydroxyl group at postion 7 and the canonical resonance structures of dibromoquercetin quinonemethide,[34] meanwhile hesperetin was still ambiguous. To find out the reasonable explanation, more experiments on similar structures such as apigenin (**63**), luteolin (**64**) are necessitated (**Figure 3.4**).
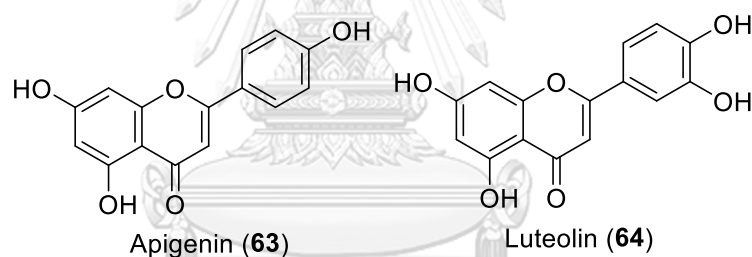
Figure 3.4 Apigenin (**63**) and luteolin (**64**).

Despite of the failure of using KI in iodination of chrysin (**34**), the iodinaion of pinostrobin (**6**), pinocembrin (**7**) and naringenin (**12**) derivatives were achieved in moderate yields (entries 10-15). This success suggested that the iodination using halide salts as iodinating reagents can be applied on some selected class of flavonoids, in this case is flavanone. Similarly, the iodination of naringenin (**12**) generated tetraiodo derivative (**50**) as a major compound while diiodo one was not achieved (entries 13-

15), which reaffirmed the important role of hydroxyl group on phenyl ring B on activating positions 3′ and 5′. In addition, varying the amount of KI did not change the reaction components, but there was a remarkable difference in the yield of reaction (entries 13-15). Interestingly, the regioselectivity on 6-position of ring A could be observed for pinostrobin (**6**) and pinocembrin (**7**) (entries 10-12). There is the difference between two flavanones, *i.e.* the substituent at the 7-position of ring A: pinostrobin (**6**) has a methoxyl group while pinocembrin (**7**) bears a hydroxyl. This observation indicated the role of hydroxyl group at 5-position on the selectivity, which was mentioned in some previous reports.[33, 39]

It should be notified here that this is the first report on the halogenation of pinostrobin (**6**) and pinocembrin (**7**). In addition, tetrahalo-substituted naringenins (**49-50**) have not been addressed before. These new synthesized derivatives were characterized by ^{1}H and ^{13}C NMR and their exact mass were confirmed by HR-ESI-MS (**Table 3.3**).

Table 3.3 Tentative ^{1}H NMR spectral assignment and exact mass of compounds **44-50**.

Cpds	H-2	H-3 α	H-3 β	5-OH	H-6	H-8	H-2',6'	H-3',5'	H-4'	-OCH$_3$	Exact mass Calcd.	Found
44	5.58 (dd, J = 12.2, 3.5 Hz)	3.15 (dd, J = 17.3, 12.2 Hz)	3.00 (dd, J = 17.3, 3.5 Hz)	12.74 (s)	-	-		7.45 (m)		-	456.8663	456.8657
45	5.44 (dd, J = 12.8, 3.2 Hz)	3.25 (dd, J = 17.3, 12.8 Hz)	2.89 (dd, J = 17.3, 3.2 Hz)	13.03 (s)	-	6.31 (s)		7.44 (m)		-		
46	5.61 (dd, J = 12.3, 3.4 Hz)	3.18 (dd, J = 17.3, 12.3 Hz)	3.05 (dd, J = 17.3, 3.4 Hz)	12.61 (s)	-	-		7.48 (m)		3.98 (s)	448.90000	448.89938
47	5.85 (dd, J = 12.6, 3.2 Hz)	3.37 (dd, J = 17.3, 12.6 Hz)	3.14 (dd, J = 17.3, 3.2 Hz)	13.02 (s)	-	-		7.47 (m)		3.91 (s)	566.8542	566.8525

Table 3.3 (Continue)

Cpds	H-2	H-3 α	H-3 β	5-OH	H-8	H-2′, 6′	H-3′, 5′	H-4′	-OCH$_3$	Exact mass Calcd.	Found
48	5.68 (dd, J = 13.0, 3.1 Hz)	3.29 (dd, J = 17.4, 13.0 Hz)	2.92 (dd, J = 17.4, 3.1 Hz)	12.95 (s)	-	6.33 (s)	7.53 (m)		3.98 (s)		
49	5.74 (dd, J = 12.7, 3.1 Hz)	3.38 (dd, J = 17.2, 12.7 Hz)	3.07 (dd, J = 17.2, 3.1 Hz)	12.93 (s)	-	-	7.82 (s)	-	-		
50	5.67 (dd, J = 12.8, 3.0 Hz)	3.43 (dd, J = 17.2, 12.8 Hz)	2.95 (dd, J = 17.2, 3.0 Hz)	12.84 (s)	-	-	7.74 (s)	-	-		

3.1.3   Anti-dengue evaluation of halogenated flavonoids

The inhibition of infection DENV2 was conducted under the collaboration with Department of Microbiology, Faculty of Medicine, Chulalongkorn University. The percentage of inhibition, percentage of cell viability, EC_{50}, CC_{50} and TI value are presented in **Table 3.4**.

Table 3.4 Anti-dengue results of brominated and iodinated flavonoids.

Entry	Cpds	% Plaque inhibition (at 10 μM)	% Cell viability (at 10 μM)	EC_{50} (μM)	CC_{50} (μM)	TI
1	**34**	86.67 ± 6.67	79.87 ± 1.40	N.D	N.D	N.D
2	**19**	98.82 ± 0.42	89.08 ± 7.62	1.47[24]	44.28[24]	N.D
3	**18**	N.D	N.D	4.04 ± 1.37	>250	61.88
4	**42**	N.D	N.D	0.88 ± 0.14	256.43 ± 2.90	291.40
5	**7**	46.67 ± 9.43	97.84 ± 3.24	15.45[a]	>100[a]	6.47
6	**44**	99.93 ± 0.07	101.08 ± 4.91	2.06 ± 0.80	67.21 ± 0.80	32.56
7	**6**	95.11 ± 0.38	122.27 ± 3.12	10.76 ± 1.63	78.58 ± 3.29	7.30
8	**46**	99.90 ± 0.04	105.14 ± 1.17	5.83 ± 0.51	> 100	17.15
9	**47**	99.84 ± 0.10	129.51 ± 5.50	2.8 ± 1.55	N.A	N.A
10	**48**	97.11 ± 3.29	135.24 ± 1.36	4.1 ± 1.84	N.A	N.A
11	**12**	95.33 ± 0.67	108.02 ± 2.55	48.17*	311.3*	6.46
12	**49**	91.82 ± 2.73	101.98 ± 2.76	9.36*	39.74*	4.25
13	**50**	84.55 ± 1.57	102.07 ± 3.40	9.62*	35.77*	3.72
14	**51**	91.11 ± 3.58	111.40 ± 6.78	16.65*	92.66*	5.60

Tabl3 3.4 (Continue)

Entry	Cpds	% Plaque inhibition (at 10 μM)	% Cell viability (at 10 μM)	EC_{50} (μM)	CC_{50} (μM)	TI
15	**13**	N.D	N.D	8.34 ± 0.80	> 100	11.99
16	**43**	N.D	N.D	8.51 ± 0.43	> 100	11.75
17	**15**	N.D	N.D	>25	112.83 ± 7.59	4.51
18	**52**	N.D	N.D	6.84 ± 1.59	47.28 ± 1.58**	6.91

Data represent means ± standard error mean (SEM) of three independent experiment. The results were reported from three independent experiments. Compound **19** was used as positive control.

* The results were reported from one experiment.

** The results were reported from two independent experiments.

EC_{50} = Effective concentrations, the molar concentration of an agonist that produced 50% of the maximal possible effect of that agonist [40];

CC_{50}= Cytotoxic concentrations, the concentration of compound required for the reduction of 50% cell viability [41];

TI = Therapeutic Indices;   N.D = Not determine;   N.A = Not available.

[a] Srivarangkul, P. 2018.

The tabular results of biological indices of halogenated flavonoids pointed out some promising candidates for further studying on dengue inhibitors. The first preliminary screening of plaque inhibition at 10 μM displayed the efficiency of adding bromine atom into the flavonoid scaffold to the bioactivity. Most derivatives exhibited excellent inhibitory value of more than 90%, especially new compounds **44, 46** and **47** inhibited approximately more than 99% of virus infection (entries 6, 8 and 9). In comparison with the precursors, derivatization made a significant change for antiviral

activity of flavonoids, except the case of naringenin (**12**). Noticeably, 6,8-dibromopinocembrin (**44**) was doubled of inhibition comparing to its parent (46.67 % of pinocembrin (**7**) *versus* 99.93 % of **44**), which emphasized the contribution of bromine atom to the bioactivity (entries 5, 6). Naringenin (**12**) was previously reported as a promising inhibitor of dengue virus;[18] however, embedded bromine atom did not attain the enhancement of anti-dengue infection as expectation. The percentage of inhibition plaque of tetrabromo-, dibromo-, tetraiodo- derivatives **49-51** were 91.82, 84.55, 91.11, respectively, and lower than that of naringenin (**12**), though the value were still acceptable (entries 11-14). The difference between naringenin (**12**) and chrysin (**34**), baicalein (**18**), pinostrobin (**6**) and pinocembrin (**7**) is the appearance of substituent on phenyl ring B, which might affect the activity. Hesperetin (**15**) and quercetin (**13**) are also have alkoxy groups on phenyl ring B, therefore, they were not screened for inhibition at 10 μM. On the other hand, there is a negligible difference among brominated and iodinated derivatives in case of pinostrobin (**6**); however, the activity of 6,8-dibromopinostrobin (**46**) was slightly better than 6,8-diiodopinostrobin (**47**); 6,8-diiodopinostrobin (**47**) is more active than 6-iodopinostrobin (**48**) (entries 8-10).

To estimate the toxicity of tested compounds at 10 μM, the percentage of cell viability was accounted. The results showed that the series of halogenated flavonoids are relatively non-toxic with the cell as specific concentration. Most of the cases, more

than 90% of cells were viable, except for chrysin (**34**) and 6,8-dibromochrysin (**19**) (entries 1, 2).

Based on the preliminary screening results, to further evaluate the cytotoxicity and effective concentration of these synthesized products, appropriate experiments were conducted to determine EC_{50} and CC_{50} value. The illustration graph of the relationship was showed below (**Figure 3.5**).
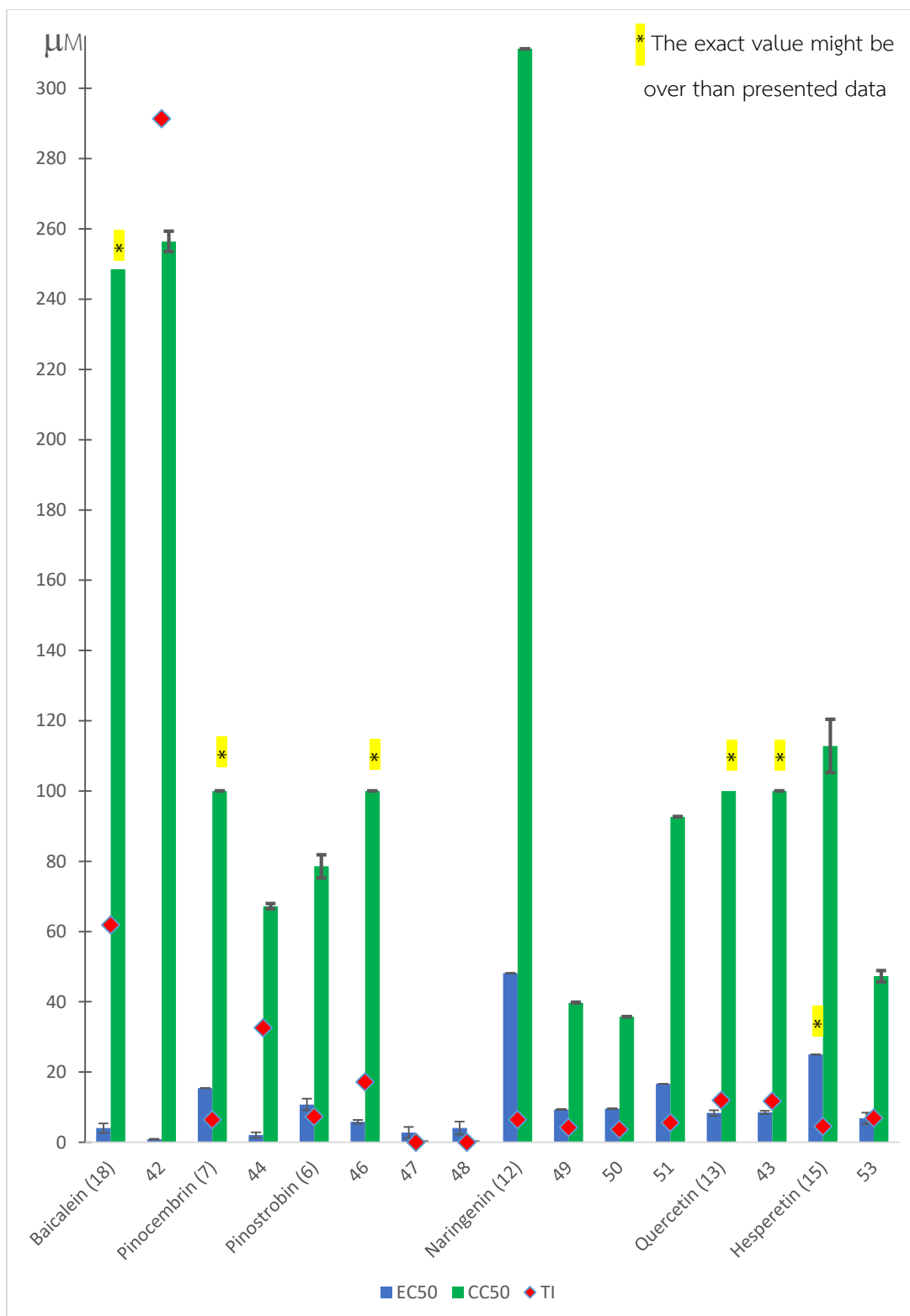
Figure 3.5 EC$_{50}$, CC$_{50}$ and TI value of flavonoids and their halogenated derivatives.

In accordance with the inhibitory results, most of the synthesized compounds displayed competitive EC_{50} and CC_{50} than the starting materials and could be considered as good anti-dengue agents. Among them, brominated baicalein (**42**) was the most effective compound with 0.88 µM of EC_{50} (entry 4). In addition, while screening the cytotoxicity concentration CC_{50}, the result also showed that **42** was less toxic to the cell with the highest CC_{50} of 256.43 µM in range, consequently TI was 291.40. Though baicalein (**18**) was investigated and proved to have remarkable antiviral effect on multiple stages of dengue virus infection before,[17] bromination made a tremendous enhancement of antiviral effect, along with the promising future as new dengue inhibitor in therapy. Furthermore, 6,8-dibromopinocembrin and pinostrobin (**44**) and (**46**) also possessed noticeably low EC_{50} 2.064 µM and 5.83 µM, respectively, which can be considered as potent anti-dengue agents. On the other hand, the appearance of substituents on the ring B of naringenin (**12**), quercetin (**13**) and hesperetin (**15**) might cause a drawback to the toxicity. The more substituents on ring B, the higher toxicity to the cell, as presented in CC_{50} of **49** and **50** at 39.74 and 35.77 µM, respectively, which made **50** is the most toxic compound to the cell (entries 12, 13). In addition, 6,8-dibromoquercetin (**43**) and 6,8-dibromohesperetin (**52**) though with acceptable CC_{50}, TI values are unattractive for further study. These suggested that the number of halogen atom need to be controlled and the ring B should be unsubstituted.

When comparing between brominated and iodinated products of pinostrobin (**6**), there is a small dissimilarity in effective concentration EC_{50} value. Generally, brominated derivatives exhibited better activity than iodinated ones;[24] however, among halogenated pinostrobins, 6,8-diiodopinostrobin (**47**) displayed the lowest EC_{50} of 2.8 μM, meanwhile the EC_{50} of 6,8-dibromopinostrobin (**44**) is 5.83 μM (entries 8, 9). Similarly, there is the modest distinction between tetrabromo- and tetraiodonaringenin, disclosing in the higher EC_{50} value of tetraiodonaringenin (**50**) 9.62 μM than tetrabromonaringenin (**49**) 9.36 μM (entries 12, 13). Nonetheless, the TI values showed that brominated derivative is more potent to be further investigated than iodinated one.

The graphic diagram also revealed the relationship between the EC_{50}, CC_{50} and TI as the important criteria for evaluating the bioactive potential of the compound. Some compounds such as naringenin (**12**), hesperetin (**15**) possessed high CC_{50} value, which represented for its low toxicity towards to the cells, however, since the EC_{50} index were relatively high, consequently leading to the low TI value, these compounds were not attractive to investigate deeply. In contrast, baicalein (**18**) and its derivative **42** exhibited either low EC_{50} or high CC_{50}, which resulted in extraordinary TI value, and will be considered to study intensely in the future. Besides, the graph could be used for comparison between unsubstituted phenyl ring B flavonoids with substituted ones, since there was a huge distance of baicalein (**18**), pinostrobin (**6**), pinocembrin (**7**) and

their derivatives **42**, **44**, **46**, **47** on the left side of the graph with naringenin (**12**), quercetin (**13**), hesperetin (**15**) and their derivatives **49**, **50**, **51**, **43**, **52** on the right side.

To discover the exact target and understand the important interactions of compound with protein in the cell, other computational calculation and bio-assays are necessary in the future.

3.2 Brominated O^7-ether chrysin as anti-dengue agents

3.2.1   Literature review of O^7-ether chrysin

Chrysin (**34**) is famous for its bioactivities such as anti-oxidant, anti-inflammatory, anti-cancer, neuroprotective, *etc.*[12] However, the applications of this compound in biological purposes are restricted because of low absorption and quick excretion in human body.[12] In order to overcome this limitation, a lot of structural modifications were applied like esterification, halogenation, etherification, *etc.*[24, 33, 36, 42] Since 1999, a research done by Shin and partners performed on eighteen chrysin derivatives and examined *in vivo* against the diabetes mellitus.[43] Among them, O^7-propylchrysin (**65**), O^7-butylchrysin (**38**), O^7-octylchrysin (**40**) (**Figure 3.6**) significantly dropped the glucose level in the blood as compared with that of the control, particularly O^7-butylchrysin (**38**) showed over 50% reduction in the blood glucose level at day-7.
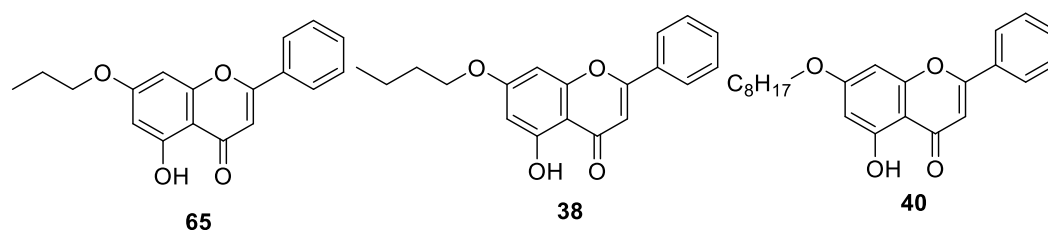
Figure 3.6 Structures of **65**, **38**, **40**.

In 2014, a study controlled by Cheng's group evaluated the relationship between chrysin (**34**), apigenin (**63**), luteolin (**64**), diosmetin (**66**) and their *O*-alkylated derivatives for the yeast α-glucosidase inhibitory activity.[44] The synthesized chrysins displayed a remarkable increasing of inhibition toward α-glucosidase enzyme comparing to their corresponding flavonoid, and as long as the alkyl were prolonged, up to six carbons of the chain, the IC_{50} value decreased significantly (**Figure 3.7**). The positive correlation between the length of side-chain and bioactivity showed the efficiency of structural modification on chrysin (**34**).
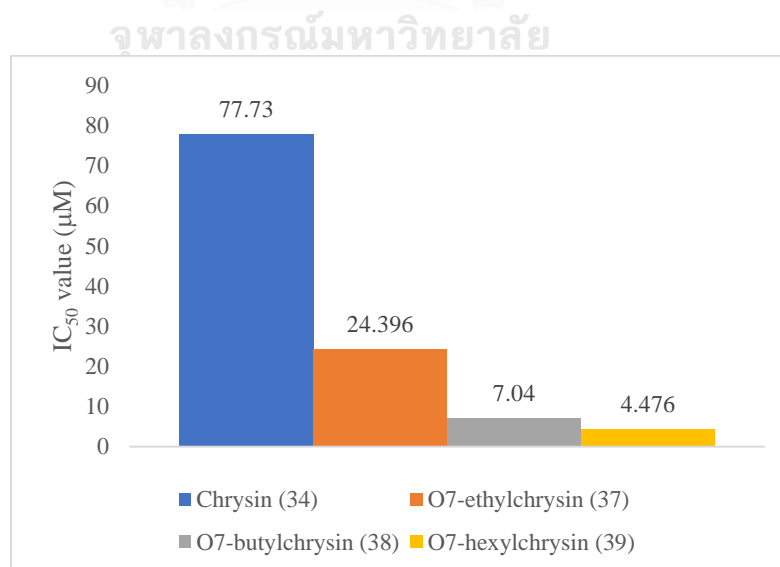
Figure 3.7 IC_{50} value of α-glucosidase inhibitory activity of chrysin and its derivatives.

A noticeable research in 2017 of Nile *et al.* investigated on some natural plant flavonoids, including chrysin (**34**), and their synthesized derivatives for anti-oxidant, anti-inflammatory and enzyme inhibition consolidated the prospect of O^7-chrysin derivatives.[45] The data obtained from this study implied that O^7-hexylchrysin (**39**) (**Figure 3.8**) might be used as anti-oxidant and anti-inflamatory candidate with low toxicity.
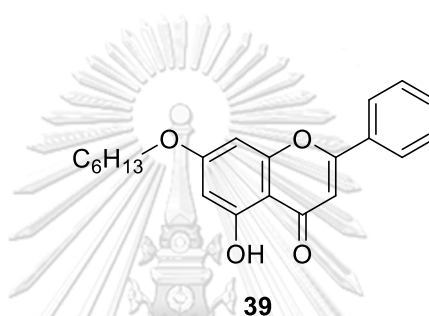
Figure 3.8 Structure of O^7-hexylchrysin (**39**).

Lastly, a study of Krongkan *et al.* on chrysin and nine synthesized derivatives with substituent modification on ring A was examined on five strains of pathogenic bacteria *P. acnes*, *S. aureus*, *S. sobrinus*, *S. typhi* and *S. mutans*.[28] Chrysin and its O^7-ether derivatives showed moderate inhibitory activity with all tested bacteria at 1 mM. Specifically, O^7-octylchrysin (**40**) exhibited very good effect against *S. sobrinus* (inhibition zone= 13.33 ± 0.47 mm) and good inhibition against *S. mutans* (inhibition zone= 11.33 ± 0.47 mm) and *S. aureus* (inhibition zone= 10.33 ± 0.47 mm); O^7- hexyl chrysin (**39**) displayed good value toward *S. sobrinus* (inhibition zone= 11.67 ± 0.47

mm) and *S. mutans* (inhibition zone= 13.00 ± 0.00 mm). This information suggested the promising bioactivity of O^7-ether chrysins for further investigation.

Based on the positive results of anti-dengue activity of 6,8-halogenated chrysin and literature reviews about biological potentials of O^7-ether chrysins, a series of brominated O^7-ether chrysins with a variety of certain alkyl chains length were generated in order to evaluate the relationship between structure and bioactivity.

3.2.2 Synthesis of brominated O^7-ether chrysins

Based on the success of using NaBr/H$_2$O$_2$/CH$_3$COOH for the synthesis of brominated flavonoids, at first, this condition was tested. There were two proposed pathways: bromination of chrysin (**34**) and then etherification at position 7 or reverse (**Figure 3.9**).
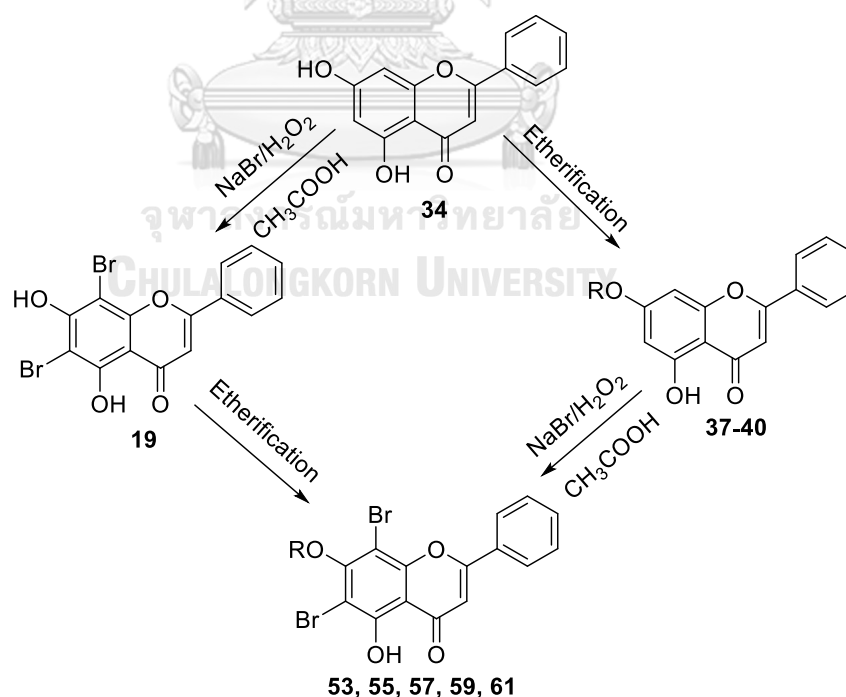
Figure 3.9 Two proposed synthetic pathways of dibromo O^7- ether chrysins **53**, **55**, **57**, **59**, **61**.

Opposed to expectation, none of the designed routes worked effectively. The first effort to synthesize the ether derivatives from **19** was failed because of the solubility of **19** in the solvent of etherification. Generally, etherification of flavonoids uses alkyl bromide and acetone as solvent with K_2CO_3 as base.[28] However, when applying these conditions, **19** did not dissolve well in acetone and after refluxing in 24 h, the starting material remained. To overcome this problem, the synthetic plan was reversed. Starting from chrysin (**34**), a series of desired ethers were synthesized first and then brominated by $NaBr/H_2O_2/CH_3COOH$. The selectivity toward hydroxyl group at position 7 instead of position 5 could be explained by the chelation of proton of 5-hydroxyl with the neighbor carbonyl group (**Figure 3.10**). This weak hydrogen bonding blocked the hydroxyl group, resulting in etherification occurred at 7-position only.
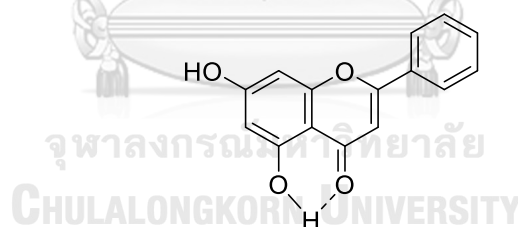
Hydrogen bonding of 5-hydroxyl group and C=O

Figure 3.10 Hydrogen bonding of hydroxyl group and adjacent carbonyl group.

This change expected to solve the obstacle since the condition operated smoothly with pinostrobin (**6**), which has the methoxy group at position 7. Once again, the procedure did not occur proficiently because of the solubility of O^7-ether chrysins (**37-40**) in used solvent. Acetic acid could not dissolve the ether chrysins, so after 24 h,

the reaction did not take place. In addition, the steric effect of the bulky side chains might obviate the attack of brominium ion Br^+ to positions 6 and 8. In summary, the application of bromination by $NaBr/H_2O_2/CH_3COOH$ was limited in the case of ether chrysin derivatives. Another approach to control the bromination of O^7-ether chrysins was to change the reagent to NBS, based on a study of Pan *et al.* in 2015.[33]

Synthetic process was followed previous method from a research of Pan and colleagues in 2015 with some modifications.[33] The series of O^7-ether chrysin were received from Kongkran *et al.* study.[28] The synthetic scheme including reaction conditions was summarized in **Figure 3.11**.

RO. ... NBS (2.0 equiv.), CH₂Cl₂ rt, 6 h ... Br

R= -C₂H₅: **37**
R= -C₄H₉: **38**
R= -C₆H₁₃: **39**
R= -C₈H₁₇: **40**
R= -C₁₂H₂₅: **41**

R= -C₂H₅: **53**
R= -C₄H₉: **55**
R= -C₆H₁₃: **57**
R= -C₈H₁₇: **59**
R= -C₁₂H₂₅: **61**
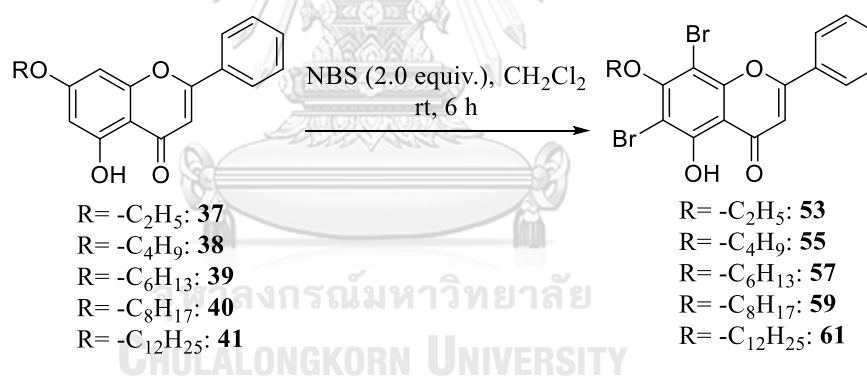
Figure 3.11 Synthetic scheme of dibromo O^7- ether chrysins **53**, **55**, **57**, **59**, **61**.

Briefly, in order to get dibromo substituted compounds as major component, the amount of NBS was increased up to 2.0 equiv. and the reaction mixture was checked every hour by TLC. Experiments using 1.0 equiv. of NBS were also carried out to obtain the 6-bromo derivatives for comparison in activity (**Figure 3.12**).
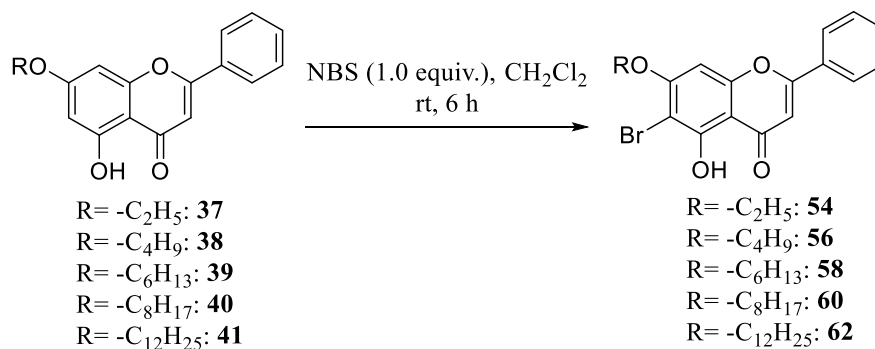
Figure 3.12 Synthetic scheme of monobromo O^7- ether chrysins **54**, **56**, **58**, **60**, **62**.

The regioselectivity of NBS to position 6 could be interpreted by the interaction of carbonyl group of NBS molecule with the hydroxyl at position 5 of ether derivatives, presenting in **Figure 3.13**.[39]
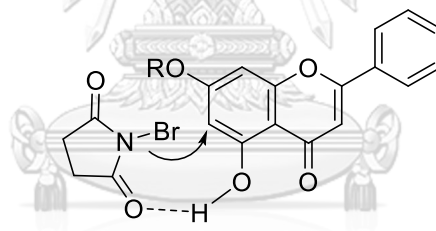
Figure 3.13 Regioselectivity of NBS to position 6 of O^7 - ether chrysins.

This chelation attracted two molecules close to each other in the rigid position, which supports for the specific selectivity. The experimental data is presented in **Table 3.5**.

Table 3.5 Bromination of O^7- ether chrysin series **53** - **62**.

Entry	R	Compound	NBS	% Yield
1		6,8-dibromo-O^7-ethylchrysin (**53**)	2.0 equiv.	86
2	C_2H_5	6-bromo-O^7-ethylchrysin (**54**)	1.0 equiv.	91
3		6,8-dibromo-O^7-butylchrysin (**55**)	2.0 equiv.	82
4	C_4H_9	6-bromo-O^7-butylchrysin (**56**)	1.0 equiv.	90
5		6,8-dibromo-O^7-hexylchrysin (**57**)	2.0 equiv.	87
6	C_6H_{13}	6-bromo-O^7-hexylchrysin (**58**)	1.0 equiv.	89
7		6,8-dibromo-O^7-octylchrysin (**59**)	2.0 equiv.	90
8	C_8H_{17}	6-bromo-O^7-octylchrysin (**60**)	1.0 equiv.	89
9		6,8-dibromo-O^7-dodecylchrysin (**61**)	2.0 equiv.	88
10	$C_{12}H_{25}$	6-bromo-O^7-dodecylchrysin (**62**)	1.0 equiv.	90

Similarly in the case of bromination of chrysin (**34**) to obtain **19**, the series of dibromo O^7-ether chrysins were confirmed by the missing of signals of two protons at positions 6 and 8 of phenyl ring A around δ_H 6.3 and 6.8 ppm, comparing to its precursors (Figures A30, A33, A36, *etc.*). On the other hand, the regioselectivity on position 6 was recognized by the missing of the proton signal at position 6, along with the correlation of proton at position 8 with carbon at position 9 on the 2D NMR spectra of monobromo derivatives **56** and **60** (**Figures 3.14**-**3.15**).
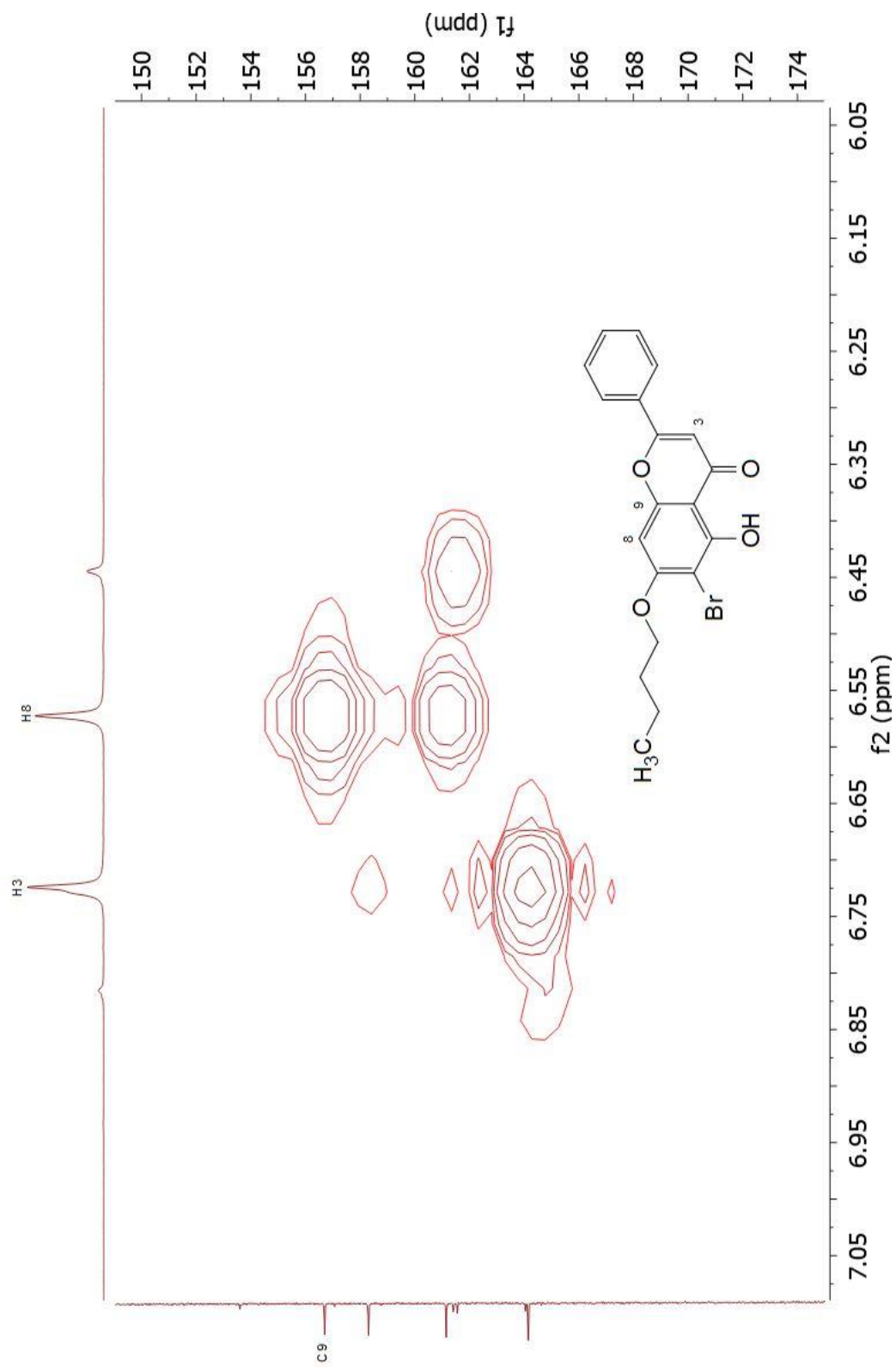
Figure 3.14 Correlation of proton 8 with carbon 9 on HMBC of **56** (Expansion)
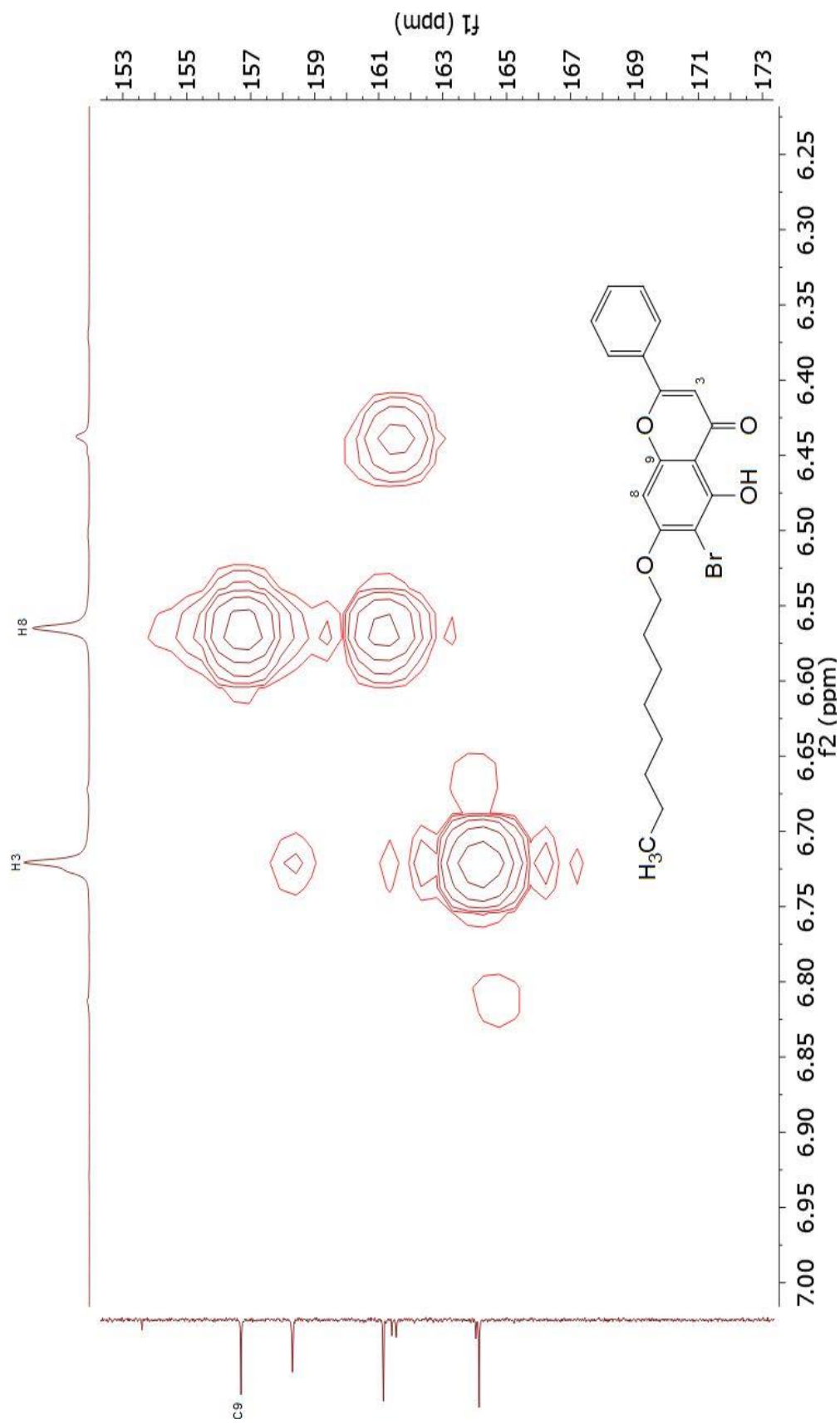
Figure 3.15 Correlation of proton 8 with carbon 9 on HMBC of **60** (Expansion).

In accordance with previous study,[33] the brominated O^7-ether chrysin was achieved in excellent yields (>82%). Moreover, the adjustment in ratio of NBS from 1.0 to 2.0 equiv. resulted in the excellent yield of dibromo derivatives indicating the efficiency of this modification. In fact, the bulky side chain at position 7 did not hinder positions 6- and 8- on the phenyl ring A, which implied that the bromination of O^7-ether flavonoids may not be affected by the steric effect. In addition, the existence of 6-bromo derivatives confirmed the crucial effect of hydroxyl group at 5-position on regioselectivity.[33, 39] Despite of the bulky alkyl side chains on position-7, the supporting of ethereal oxygen of fused heterocycle C and the chelated hydroxyl group activated the reactivity of position 6, as mentioned before.

The brominated ethers have not been synthesized before. The obtained compounds were elucidated by appropriate spectroscopic techniques. The ^{1}H NMR assignment and exact mass were displayed in **Table 3.6**.

Table 3.6 Tentative ^{1}H NMR spectral assignment and exact mass of compounds **53-62**.

Cpd	H-3	5-OH	H-6	H-8	H-2', 6'	H-3', 4', 5'	Alkyl chain	Exact mass Calcd.	Found
53	6.83 (s)	13.58 (s)	-	-	8.02 (dd, J = 8.0, 3.5 Hz)	7.58 (m)	4.24 (q, J = 7.0 Hz, 2H), 1.57 (t, J = 7.0 Hz, 3H)	460.90000	460.90024
54	6.73 (s)	13.46 (s)	-	6.56 (s)	7.89 (m)	7.41 (m)	4.22 (q, J = 7.0 Hz, 2H), 1.55 (t, J = 7.0 Hz, 3H)	389.98949	389.98970
55	6.82 (s)	13.57 (s)	-	-	8.01 (m)	7.58 (m)	4.15 (q, J = 6.5 Hz, 2H), 1.94 (m, 2H), 1.62 (m, 2H), 1.03 (t, J = 6.5 Hz, 3H)	488.93130	488.93206
56	6.69 (s)	13.46 (s)	-	6.54 (s)	7.88 (m)	7.54 (m)	4.12 (q, J = 6.4 Hz, 2H), 1.89 (m, 2H), 1.58 (m, 2H), 1.02 (t, J = 6.4 Hz, 3H)		
57	6.82 (s)	13.57 (s)	-	-	8.00 (d, J = 7.2 Hz)	7.58 (m)	4.14 (q, J = 6.6 Hz, 2H), 1.95 (m, 2H), 1.58 (s, 6H), 0.93 (t, J = 6.6 Hz, 3H)	516.96260	516.96247

Table 3.6 (Continue)

Cpd	H-3	5-OH	H-6	H-8	H-2', 6'	H-3', 4', 5'	Alkyl chain	Exact mass Calcd.	Exact mass Found
58	6.71 (s)	-	-	6.55 (s)	7.89 (m)	7.54 (m)	4.12 (q, J = 6.4 Hz, 2H), 1.91 (m, 2H), 1.53 (m, 2H), 1.38 (m, 4H), 0.93 (t, J = 6.4 Hz, 3H)		
59	6.82 (s)	13.53 (s)	-	-	8.00 (m)	7.56 (m)	4.12 (q, J = 6.6 Hz, 2H), 1.92 (m, 2H), 1.56 (m, 2H), 1.34 (m, 8H), 0.93 (t, J = 6.5, 3H)		
60	6.70 (s)	-	-	6.55 (s)	7.88 (d, J = 7.4)	7.54 (m)	4.11 (q, J = 6.4 Hz, 2H), 1.90 (m, 2H), 1.54 (m, 2H), 1.35 (m, 8H), 0.89 (t, J = 6.4, 3H)		
61	6.82 (s)	13.58 (s)	-	-	8.02 (m)	7.58 (m)	4.14 (q, J = 6.6 Hz, 2H), 1.95 (m, 2H), 1.27 (s, 18H), 0.88 (t, J = 6.5 Hz, 3H)	601.0564	601.0573
62	6.73 (s)	13.48 (s)	-	6.57 (s)	7.89 (m)	7.55 (m)	4.13 (q, J = 6.0 Hz, 2H), 1.89 (m, 2H), 1.27 (s, 18H), 0.88 (t, J = 6.0 Hz, 3H)	501.16405	501.16529

3.2.3 Anti-dengue activity of brominated O^7-ether chrysins

The percentage of plaque inhibition, percentage of cell viability of brominated O^7-ether chrysins are shown in **Table 3.7**.

Table 3.7 % plaque inhibition and % cell viability of brominated O^7- ether chrysins series.

Entry	Compounds	R	% plaque inhibition (at 10 μM)	% cell viability (at 10 μM)
1	Chrysin (**34**)		86.67 ± 6.67	79.87 ± 1.40
2	6,8-dibromochrysin (**19**)	H	98.82 ± 0.42	89.08 ± 7.62
3	6,8-dibromo-O^7-ethylchrysin (**53**)	C_2H_5	79.09 ± 6.86	109.98 ± 3.86
4	6-bromo-O^7-ethylchrysin (**54**)	C_2H_5	74.45 ± 8.18	104.66 ± 2.49
5	6,8-dibromo-O^7-butylchrysin (**55**)	C_4H_9	87.27 ± 6.30	112.27 ± 3.70
6	6-bromo-O^7-butylchrysin (**56**)	C_4H_9	In.	In.
7	6,8-dibromo-O^7-hexylchrysin (**57**)	C_6H_{13}	90.00 ± 3.15	98.67 ± 2.18
8	6-bromo-O^7-hexylchrysin (**58**)	C_6H_{13}	In.	In.
9	6,8-dibromo-O^7-octylchrysin (**59**)	C_8H_{17}	In.	In.
10	6-bromo-O^7-octylchrysin (**60**)	C_8H_{17}	97.11 ± 0.77	93.83 ± 1.24
11	6,8-dibromo-O^7-dodecylchrysin (**61**)	$C_{12}H_{25}$	84.55 ± 1.57	116.74 ± 8.86
12	6-bromo-O^7-dodecylchrysin (**62**)	$C_{12}H_{25}$	In.	In.

In.: Insoluble.

Data represent means ± standard error mean (SEM) of three independent experiment.

The results were reported from three independent experiments.

The preliminary screening at 10 μM displayed unpredictable structure-activity relationship of the brominated ether chrysins. Results showed that increasing the length of alkyl chain did not increase the percent viral inhibition as previously hypothesized by docking.[46] To be specific, 6,8-dibromochrysin (**19**) (98.82% of inhibition) was the most efficient dengue inhibitor among dibromo compounds. Among the dibromo ether chrysins, there was an ambiguous trend when increasing the number of carbon in the alkyl chains. From ethyl to hexyl, the percentage of plaque inhibition went up gradually from 79.09% of **53** to 90.00% of **57**. However, since the missing value of **60** made the trend was interrupted, the optimized number of carbons of side chain stimulating the activity could not be given out clearly. The difference of percentage inhibition between **57** and **61** was not remarkable, 90.00% of **57** *versus* 84.55% of **61**, but the decreasing showed that the longer side chain, the worse activity is. Literature review also showed the suitable side chain to obtain the best activity was normally hexyl.[28, 44, 45] On the other hand, the inhibition pattern was inconclusive in monobromo compounds. Although most of the dibromo derivatives were able to be dissolved in DMSO or DMSO with 1% acetone – solvents used for the bioactive test – more than half of monobromo ether chrysins were insoluble. The monobromo long alkyl side chains were hardly tested therefore results could not be obtained obviously. However, 6-bromo-O^7-octylchrysin (**60**) possessed 97.11% of inhibition could be considered as the best monobromo ether derivative. Moreover, in comparison with other derivatives, **60** was the most active compound in the series, with negligible

distinction in percentage plaque inhibition with **19**, 97.11% and 98.82% respectively. It was speculated that the monobromo series benefited from increasing alkyl chain more than the dibromo series. The bulky nature of dibromo series did not allow addition of another bulky group adjacent to them; whereas the monobromo still allow a room for the bulky hydrocarbon and still fit all additional structures into the binding pocket. However, it was obvious that the side chain C_2H_5 contributed to the lowest inhibition efficiency in both mono and dibromo compounds suggesting steric hindrance (or incompetency) towards the binding pocket.

Cell viability assay was used to confirm that all soluble compounds were relatively non-cytotoxic at the tested concentration (10 μM). Despite of the moderate inhibition at examined concentration comparing to **19**, all the synthesized compounds were less toxic with cells than chrysin (**34**) and 6,8-dibromochrysin (**19**). Noticeably, there was a consistence between the inhibitory activity and the toxicity of the series: the more active compound, the more toxic to the cells it is. **60** was observed as the best inhibitor with 97.11% inhibition, but it also had the lowest percentage cell viability among derivatives with 93.83%. To combine the inhibitory efficiency and the toxicity for evaluation, the best candidates for further study were 6,8-dibromo-O^7-butylchrysin (**55**) and 6,8-dibromo-O^7-hexylchrysin (**57**).

On the other hand, the results also implied the drawback of this modification, as the solubility of the derivatives were significantly decreased. The etherification could not enhance the antiviral activity as assumption.

Chapter 4

Conclusions

Derived from corresponding flavonoids, twenty-two halogenated derivatives were synthesized and elucidated their structures by appropriate spectroscopic techniques. Among them, eighteen compounds were obtained for the first time. These substrates were characterized by using ^{1}H, ^{13}C and HR-ESI-MS, and the summary of their structures is displayed below.
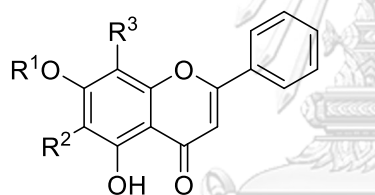
(**53**) $R^1 = C_2H_5$, $R^2 = R^3 =$ Br
(**54**) $R^1 = C_2H_5$, $R^2 =$ Br, $R^3 =$ H
(**55**) $R^1 = C_4H_9$, $R^2 = R^3 =$ Br
(**56**) $R^1 = C_4H_9$, $R^2 =$ Br, $R^3 =$ H
(**57**) $R^1 = C_6H_{13}$, $R^2 = R^3 =$ Br
(**58**) $R^1 = C_6H_{13}$, $R^2 =$ Br, $R^3 =$ H
(**59**) $R^1 = C_8H_{17}$, $R^2 = R^3 =$ Br
(**60**) $R^1 = C_8H_{17}$, $R^2 =$ Br, $R^3 =$ H
(**61**) $R^1 = C_{12}H_{25}$, $R^2 = R^3 =$ Br
(**62**) $R^1 = C_{12}H_{25}$, $R^2 =$ Br, $R^3 =$ H
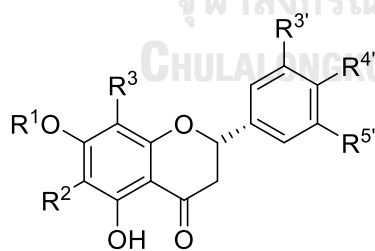
(**44**) $R^1 = R^3 = R^{4'} = R^{5'} =$ H, $R^2 = R^3 =$ Br
(**45**) $R^1 = R^3 = R^{3'} = R^{4'} = R^{5'} =$ H, $R^2 =$ I
(**46**) $R^1 = CH_3$, $R^{3'} = R^{4'} = R^{5'} =$ H, $R^2 = R^3 =$ Br
(**47**) $R^1 = CH_3$, $R^{3'} = R^{4'} = R^{5'} =$ H, $R^2 = R^3 =$ I
(**48**) $R^1 = CH_3$, $R^3 = R^{3'} = R^{4'} = R^{5'} =$ H, $R^2 =$ I
(**49**) $R^1 =$ H, $R^{4'} =$ OH, $R^2 = R^3 = R^{3'} = R^{5'} =$ Br
(**50**) $R^1 =$ H, $R^{4'} =$ OH, $R^2 = R^3 = R^{3'} = R^{5'} =$ I

To evaluate the potential of halogenated flavonoids as new anti-dengue agents, selected derivatives were examined the inhibitory activity against dengue virus serotype 2 DENV-2 infection. The preliminary screening at 10 μM demonstrated the

efficiency of halogen atom contributing to the bioactivity. New compounds **44**, **46**, **47** displayed competitive % inhibition plaque comparing with the precursors. All the tested compounds also relatively low toxic with cells at 10 µM. When inspecting the EC_{50} and CC_{50}, 8-bromobaicalein (**42**) was the most active compounds in the series, along with brominated and iodinated pinostrobin and pinocembrin (**44**), (**46**), (**47**) as the promising anti-dengue agents. These halogenated derivatives can be further studied for the interaction mechanism with virus in the future.

Suggestion for future work

The possible development relating to this study is the conjugation of potent compounds with soluble components such as esters or amines in order to enhance the bioavailability as well as bioactivity of these derivatives. Further study about the mechanism of interaction between the substrates and virus should be considered. On the other hand, the new brominated and iodinated flavonoids in this research are possibly investigated other biological activities such as anticancer or anti-inflammatory.

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APPENDIX
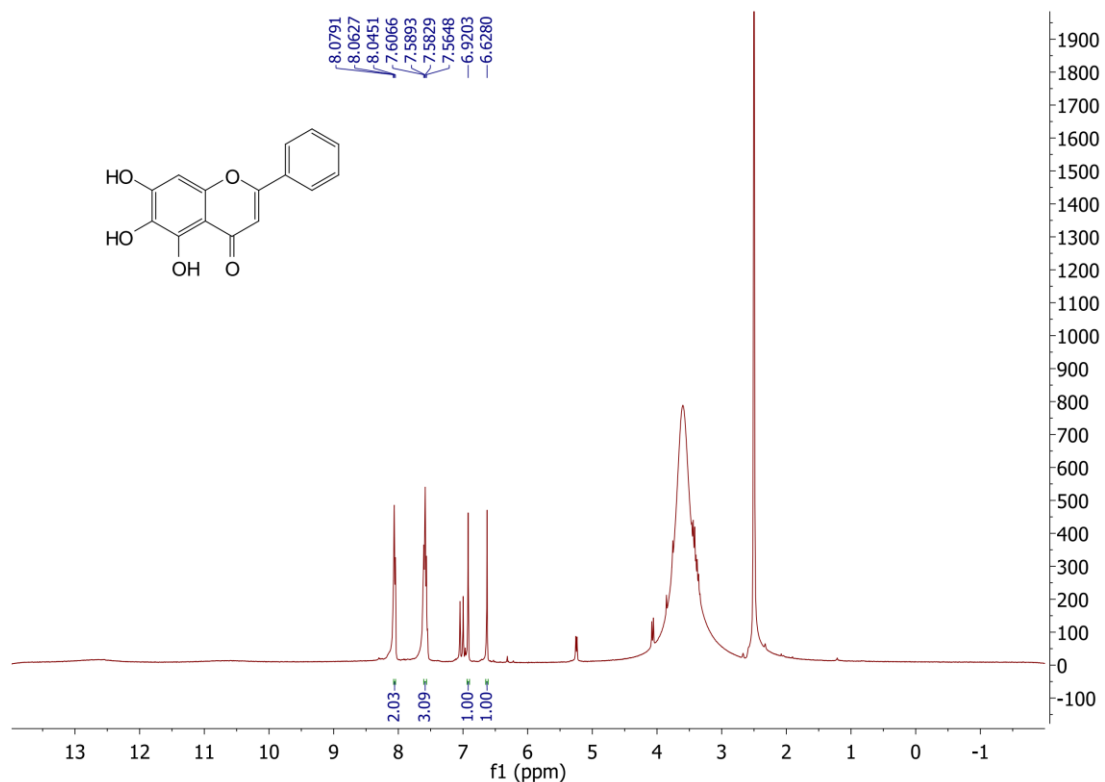
Figure A1 The ^{1}H NMR spectrum (DMSO-d$_6$, 400 MHz) of 18.
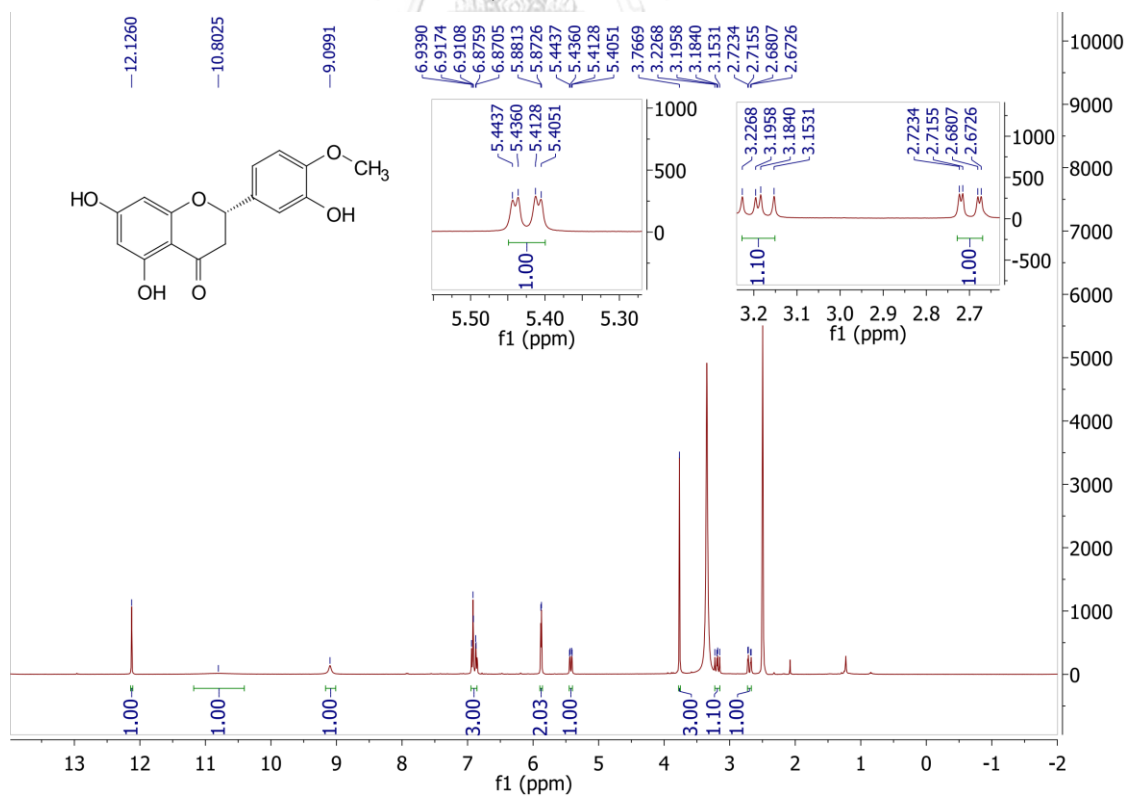
Figure A2 The ^{1}H NMR spectrum (DMSO-d$_6$, 400 MHz) of 15.
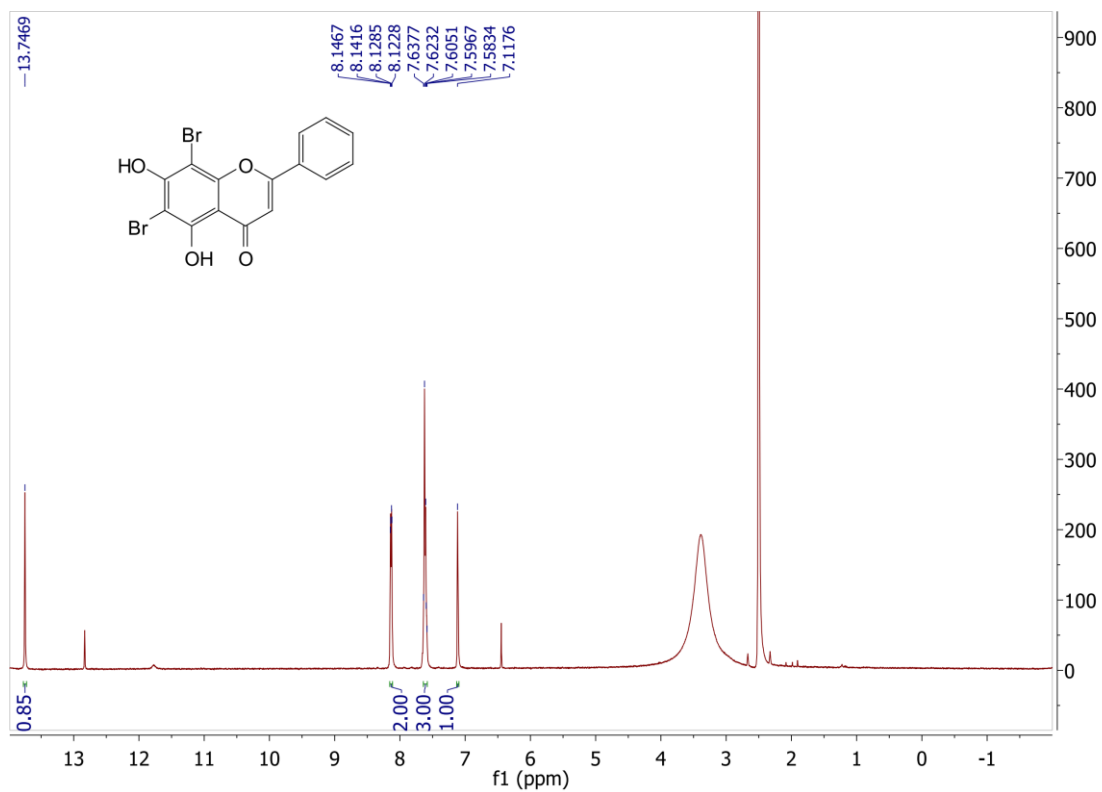
Figure A3 The ^{1}H NMR spectrum (DMSO-d$_6$, 400 MHz) of **19**.
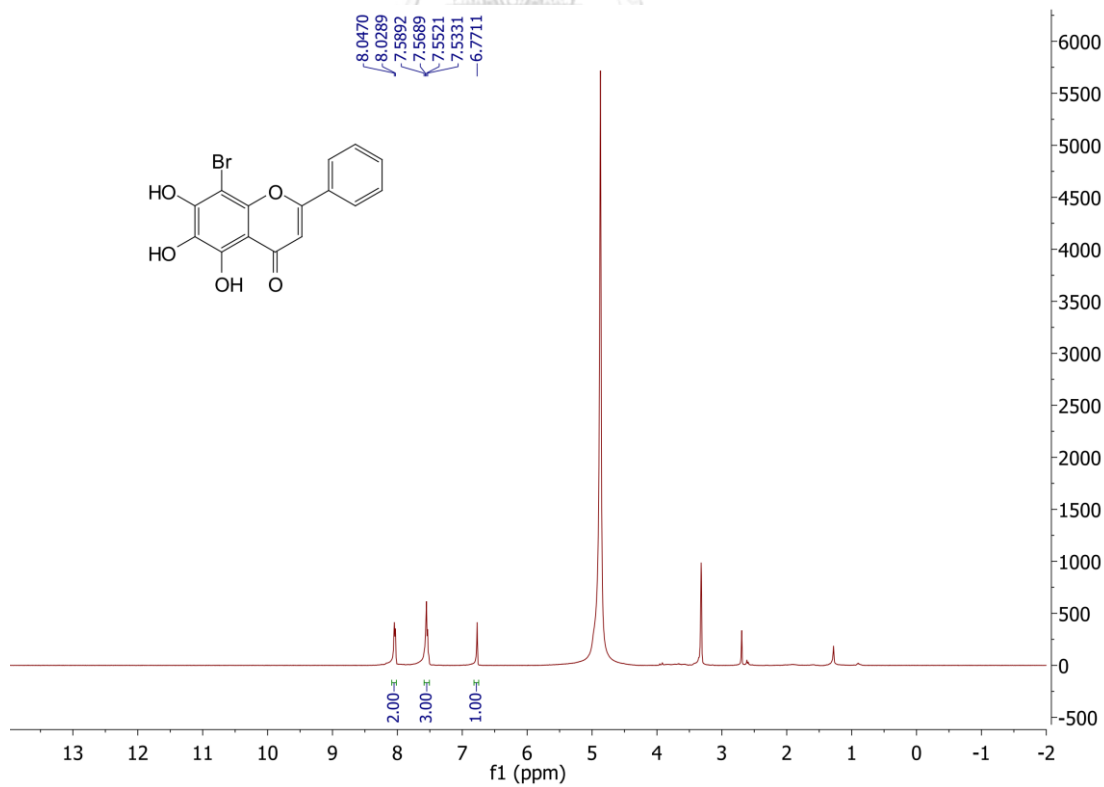
Figure A4 The ^{1}H NMR spectrum (MeOD, 400 MHz) of **42**.
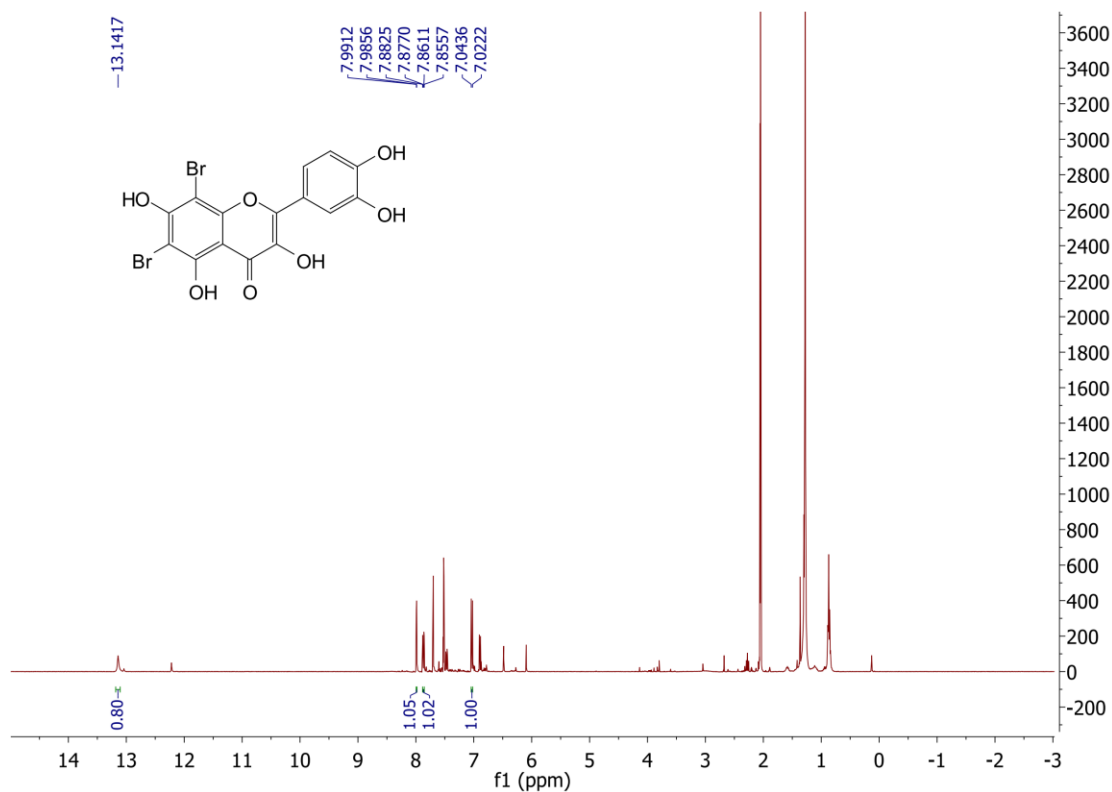
Figure A5 The ^{1}H NMR spectrum(Acetone-d$_6$) of 43.

Mass Spectrum List Report

Analysis Info

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Comment		

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Operator   CU.
Instrument / Ser#   micrOTOF-Q II 10335

Acquisition Parameter

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Scan End	1500 m/z	Set Collision Cell RF	250.0 Vpp	Set Divert Valve	Waste

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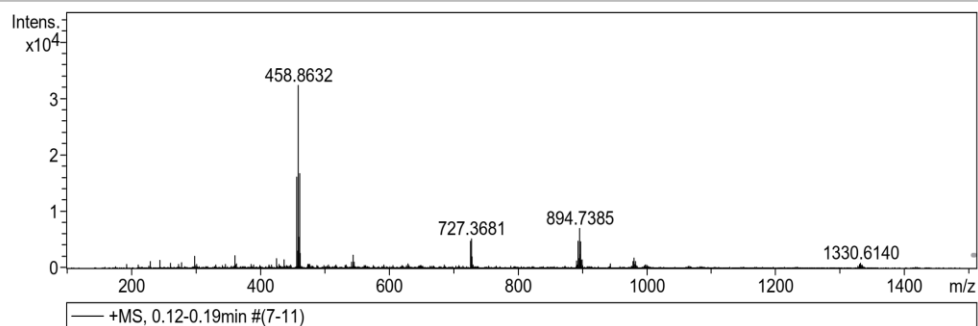
Figure A6 The HR-ESI-MS of 44.

Figure A7 The ^{1}H NMR spectrum (Acetone-d$_6$, 400 MHz) of **44**.
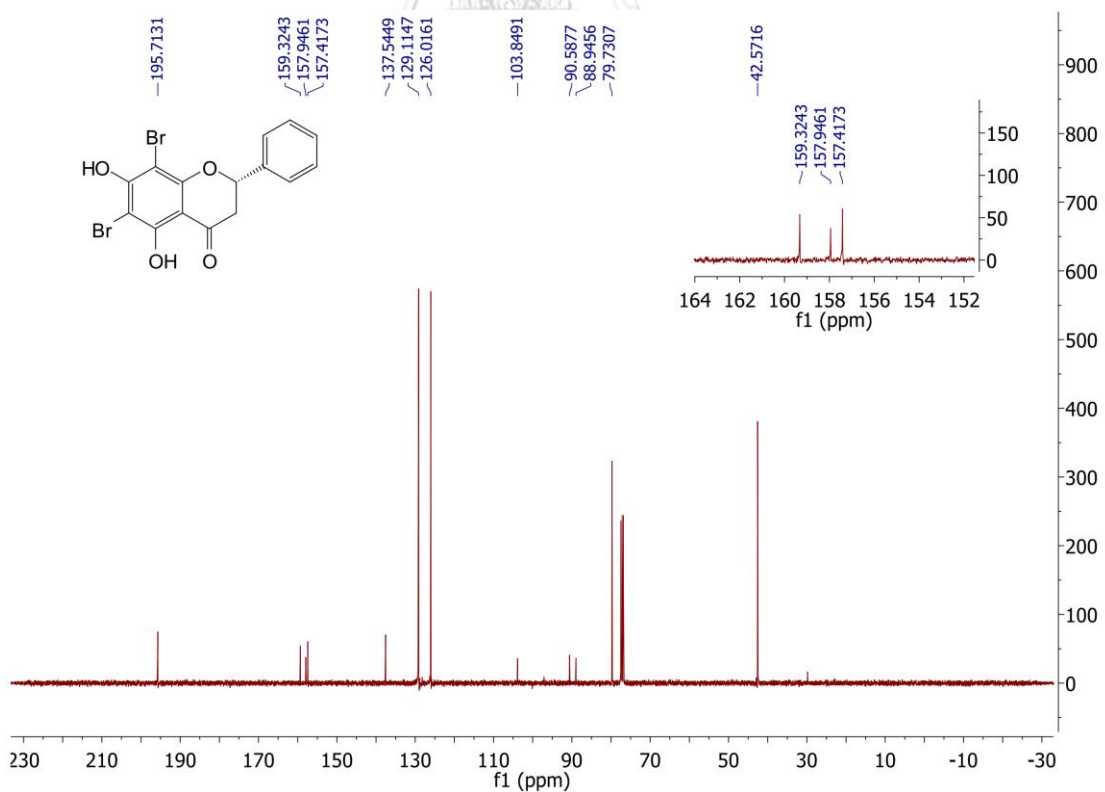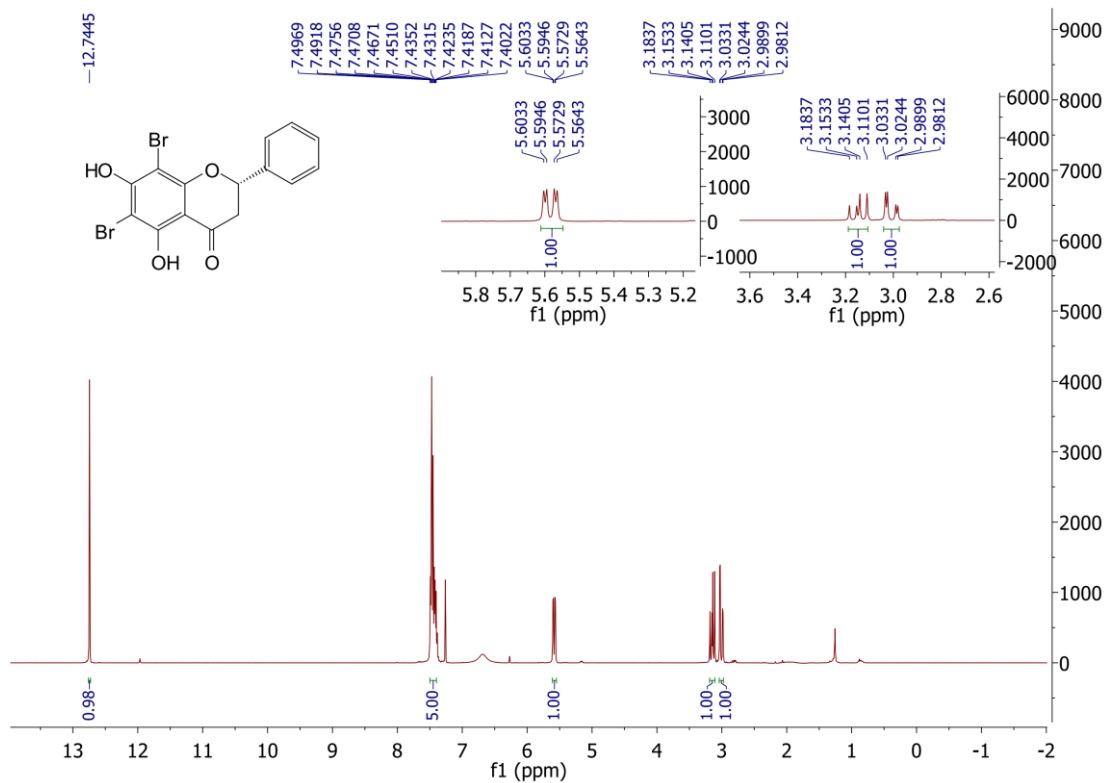
Figure A8 The ^{13}C NMR spectrum (Acetone-d$_6$, 100 MHz) of **44**.
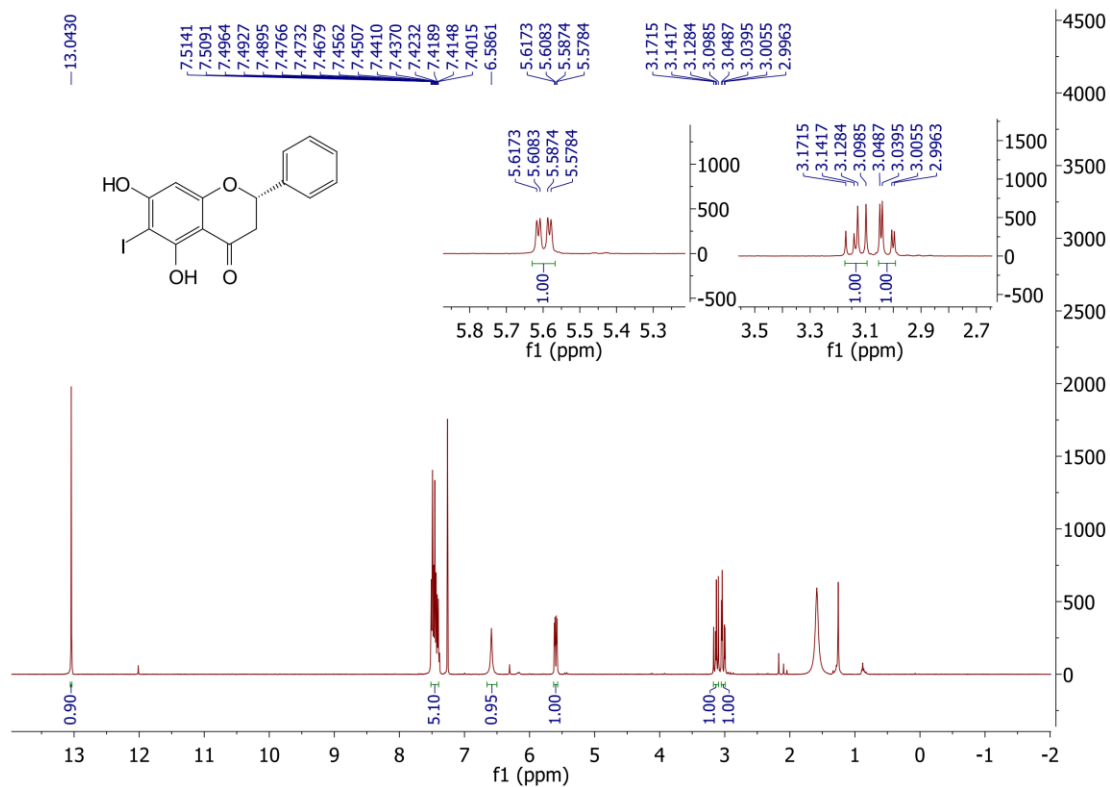
Figure A9 The ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of **45**.
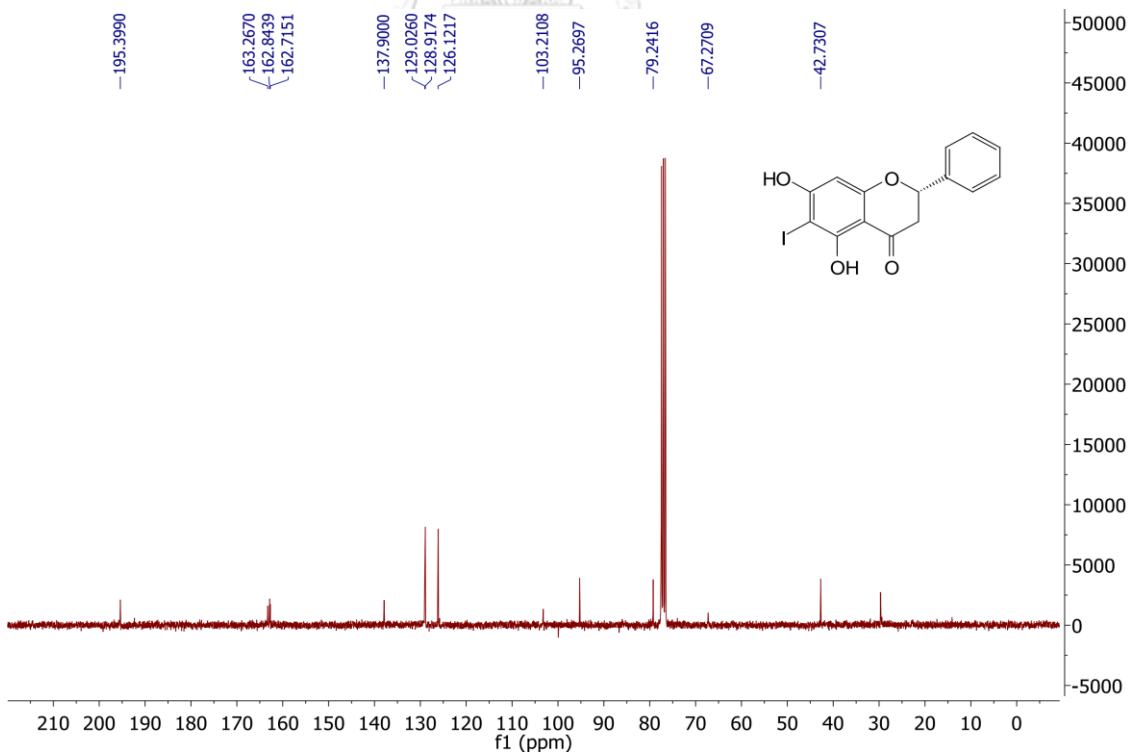
Figure A10 The ^{13}C NMR spectrum (CDCl$_3$, 100 MHz) of **45**.

Mass Spectrum List Report

Analysis Info

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Comment

Acquisition Date 9/24/2018 1:20:00 PM

Operator CU.
Instrument / Ser# micrOTOF-Q II 10335

Acquisition Parameter

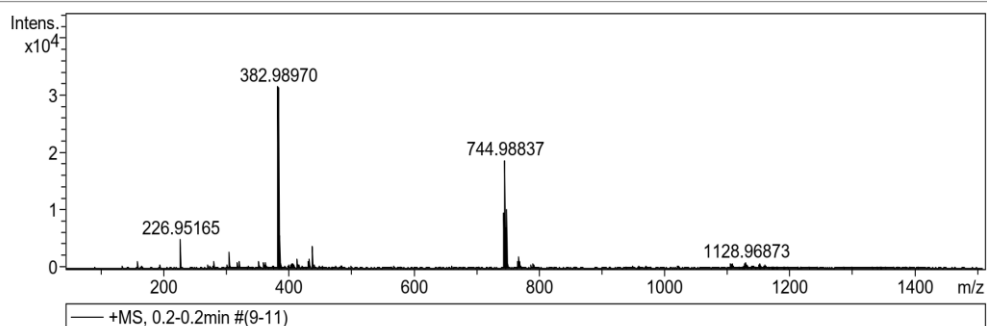|					
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Scan End	1500 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste

Figure A11 The HR-ESI-MS of 46.
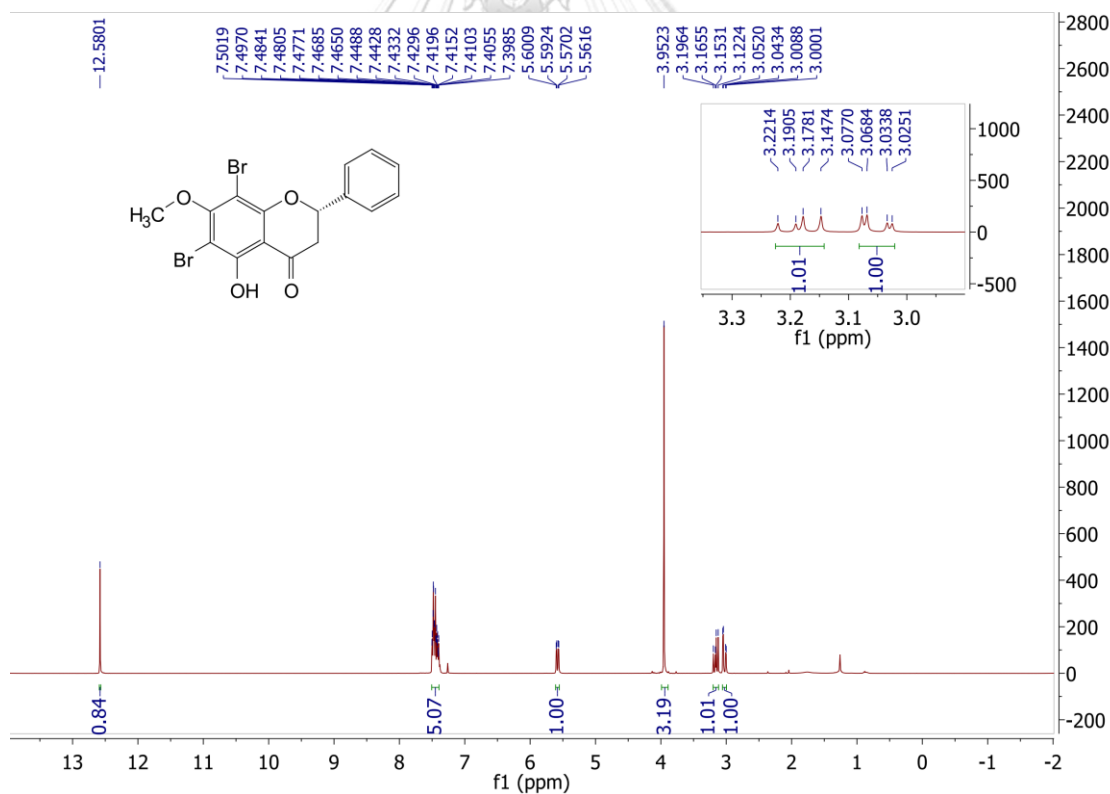
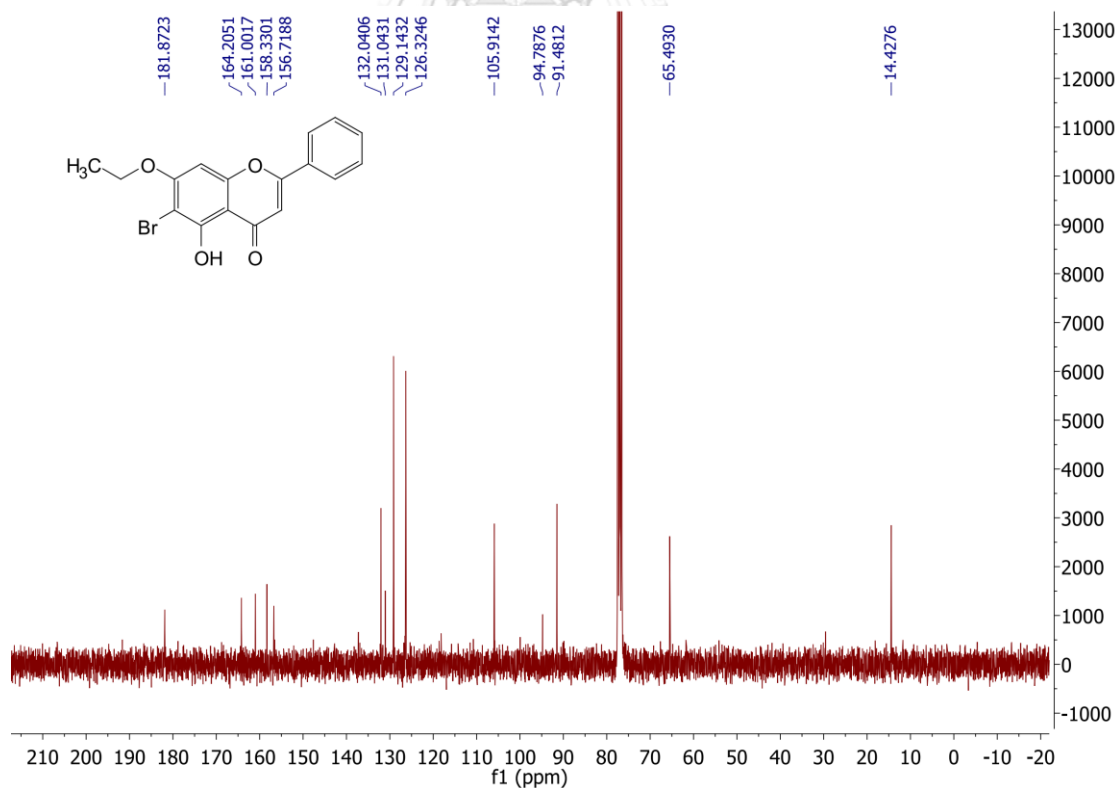
Figure A12 The ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of 46.
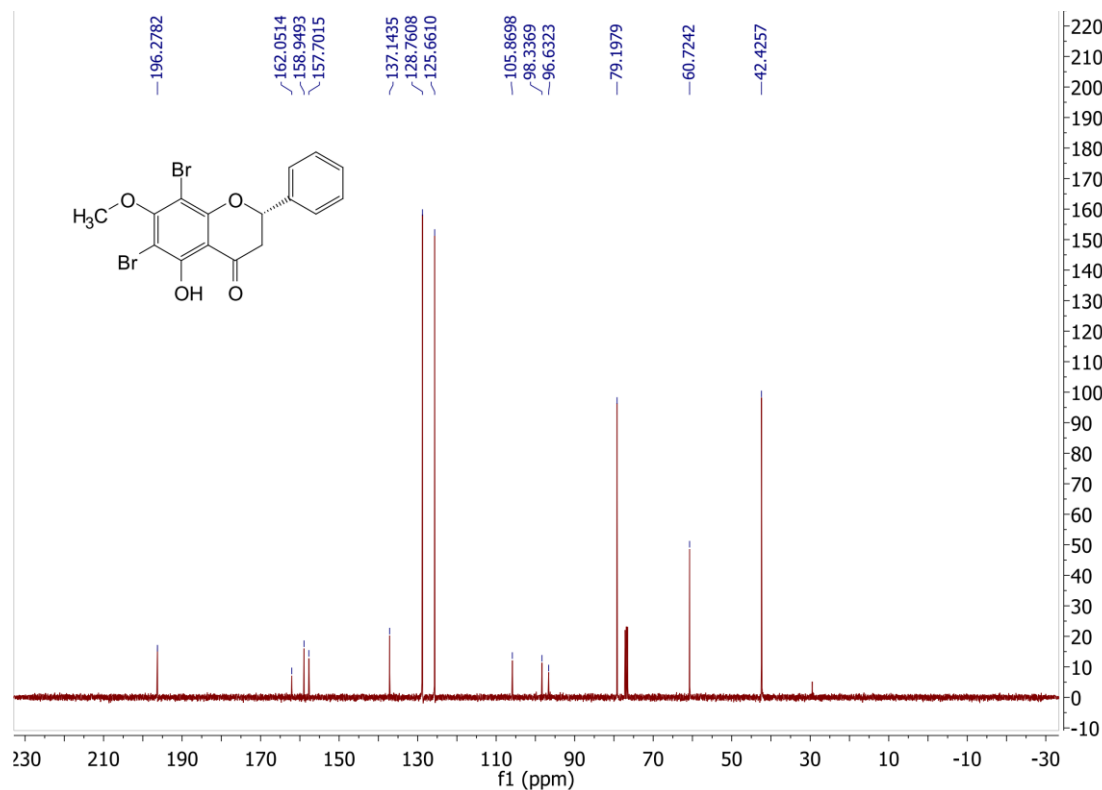
Figure A13 The ^{13}C NMR spectrum (CDCl$_3$, 100 MHz) of **46**.

Mass Spectrum List Report

Analysis Info

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Comment

Operator CU.
Instrument / Ser# micrOTOF-Q II 10335

Acquisition Parameter

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Figure A14 The HR-ESI-MS of **47**.
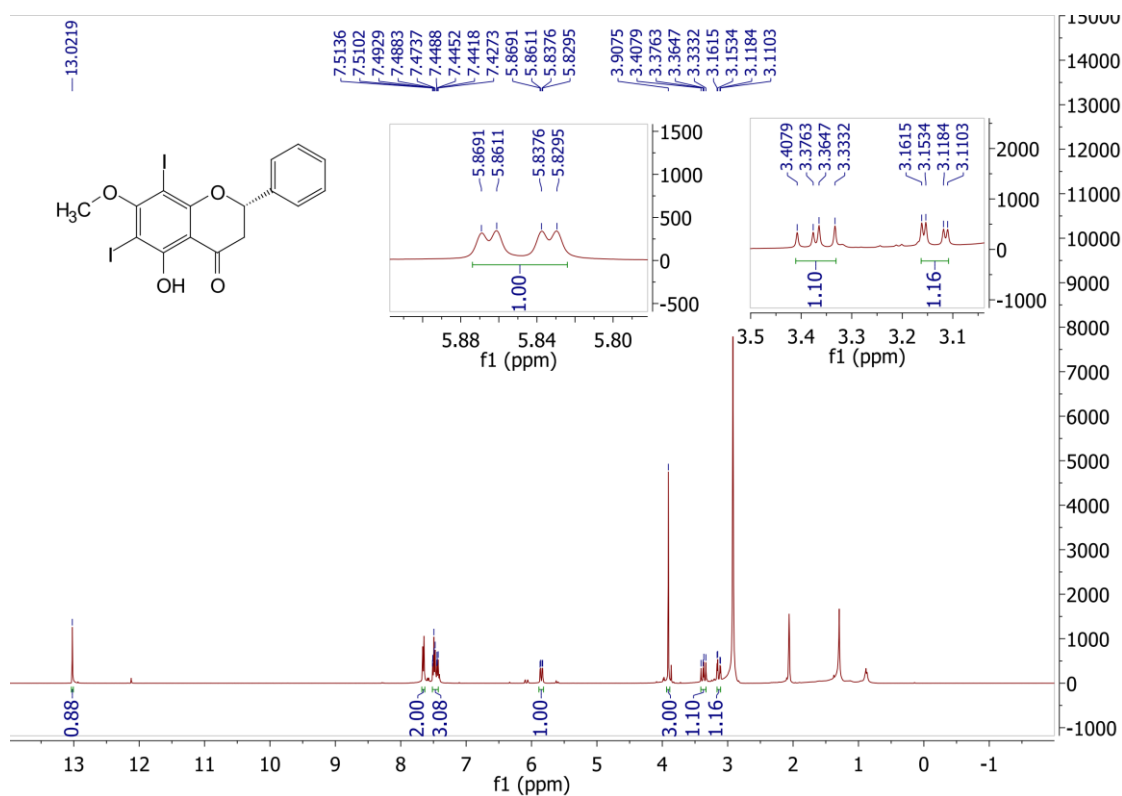
Figure A15 The ^{1}H NMR spectrum (Acetone-d$_6$, 400 MHz) of **47**.
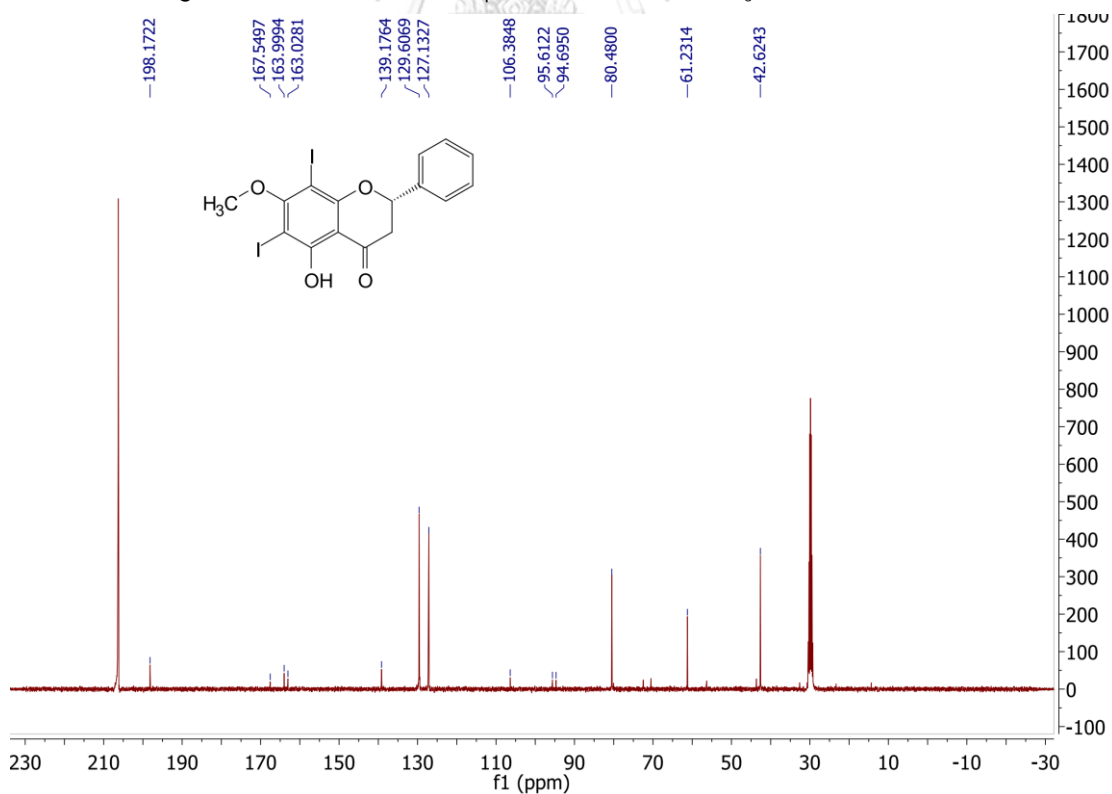
Figure A16 The ^{13}C NMR spectrum (Acetone-d$_6$, 100 MHz) of **47**.
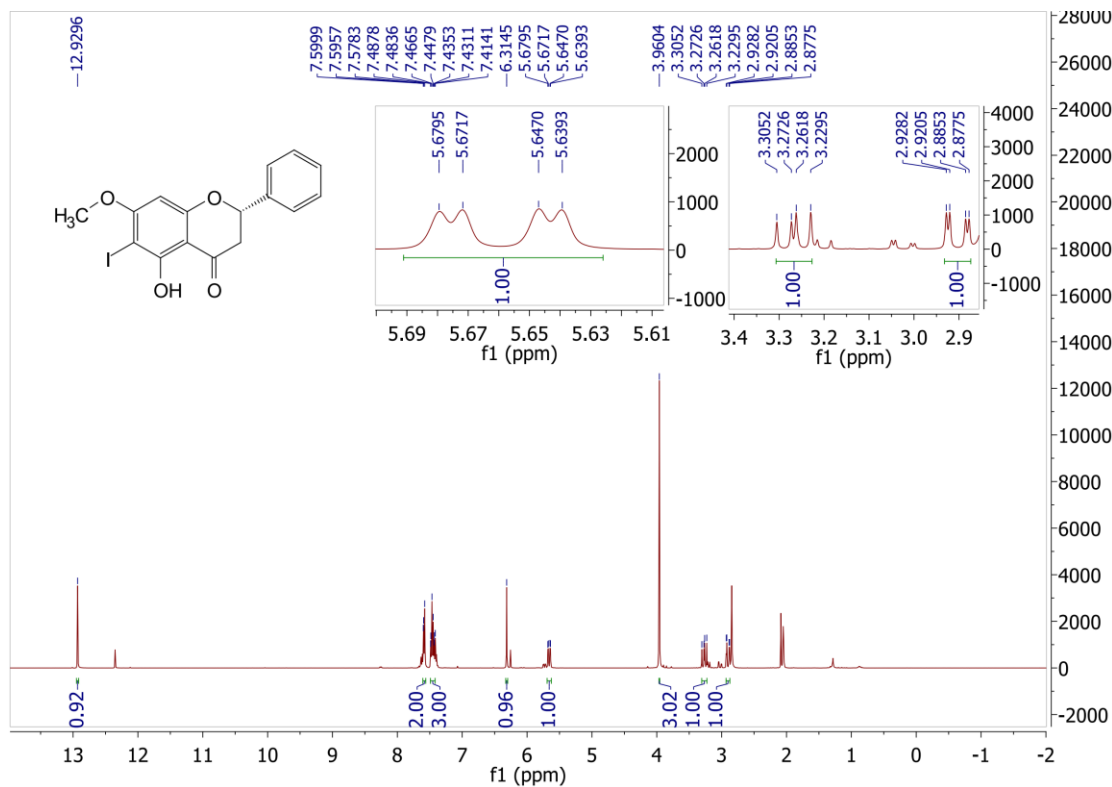
Figure A17 The ^{1}H NMR (Acetone-d$_6$, 400 MHz) of **48**.
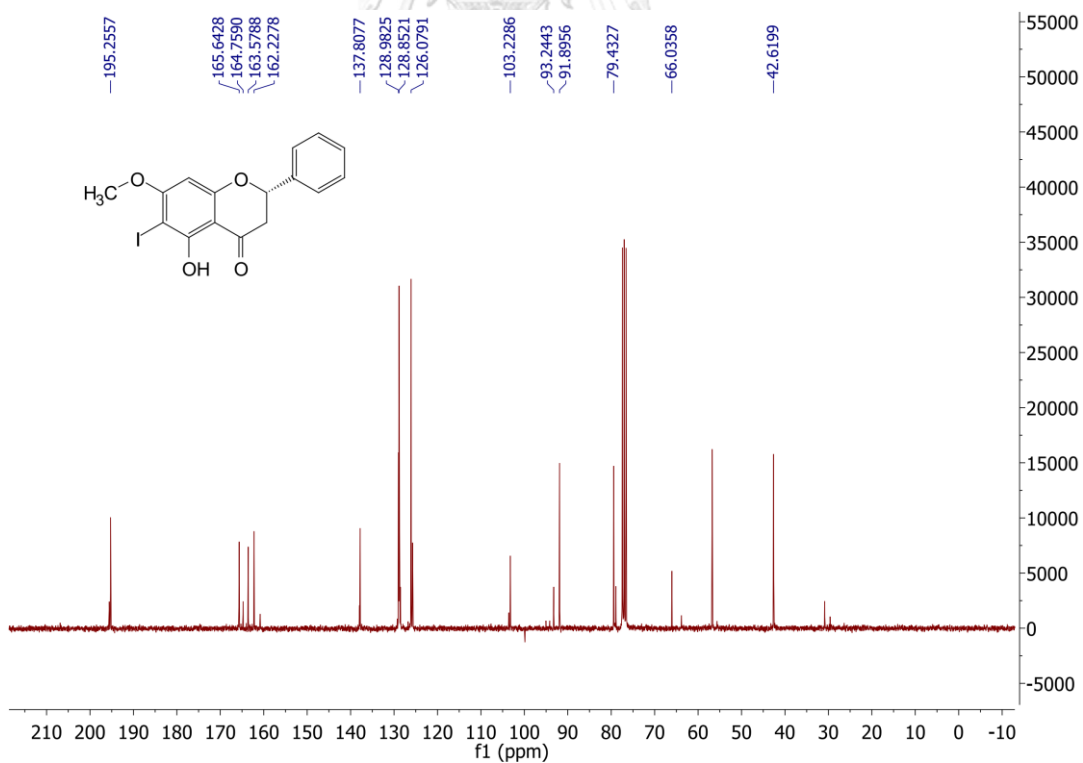
Figure A18 The ^{13}C NMR (Acetone-d$_6$, 100 MHz) of **48**.
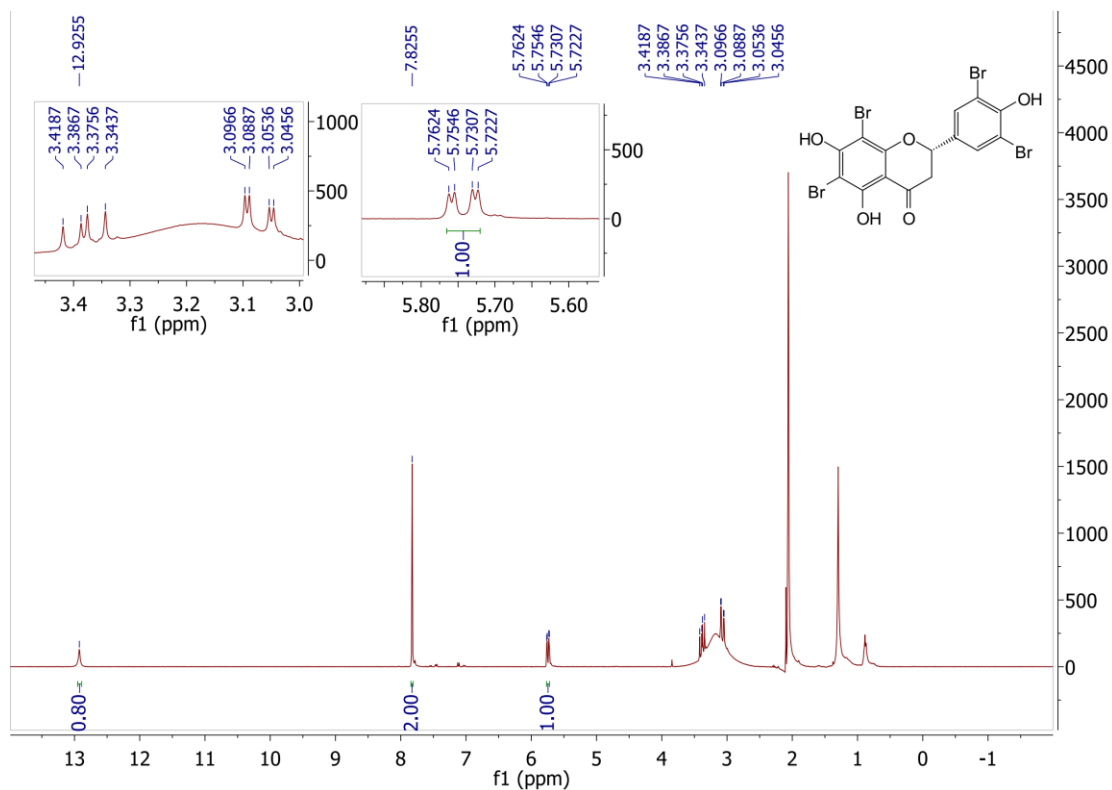
Figure A19 The ^{1}H NMR spectrum (Acetone-d$_6$, 400 MHz) of **49**.
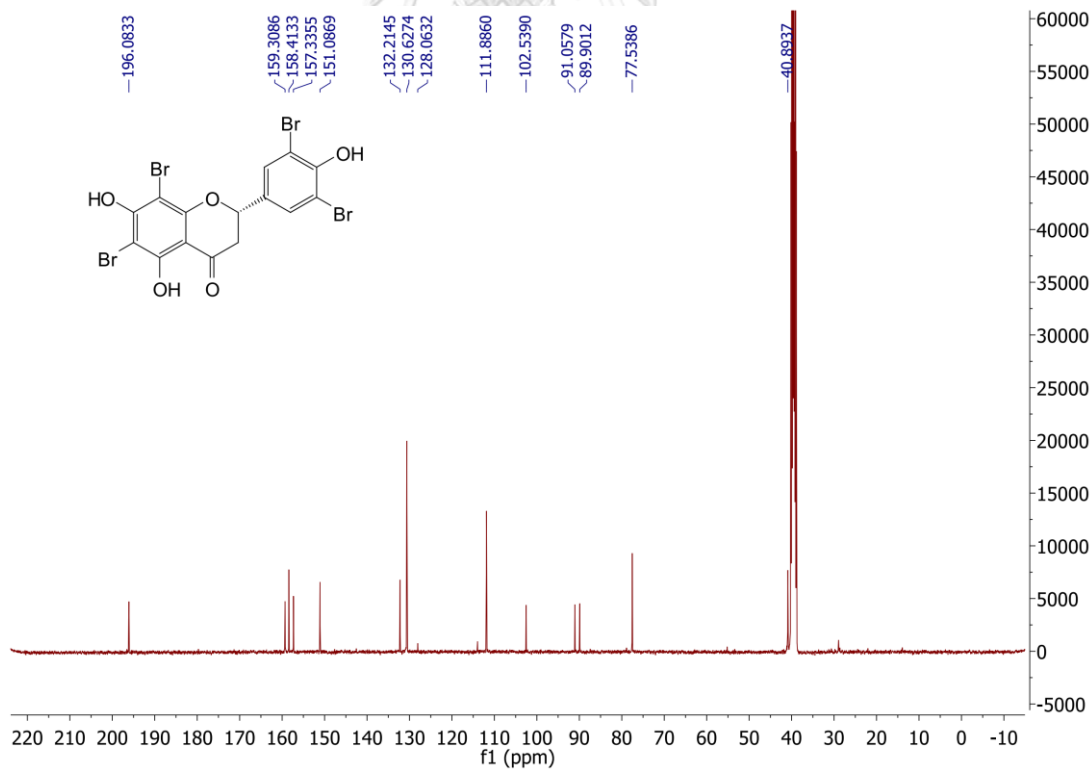
Figure A20 The ^{13}C NMR spectrum (Acetone-d$_6$, 100 MHz) of **49**.
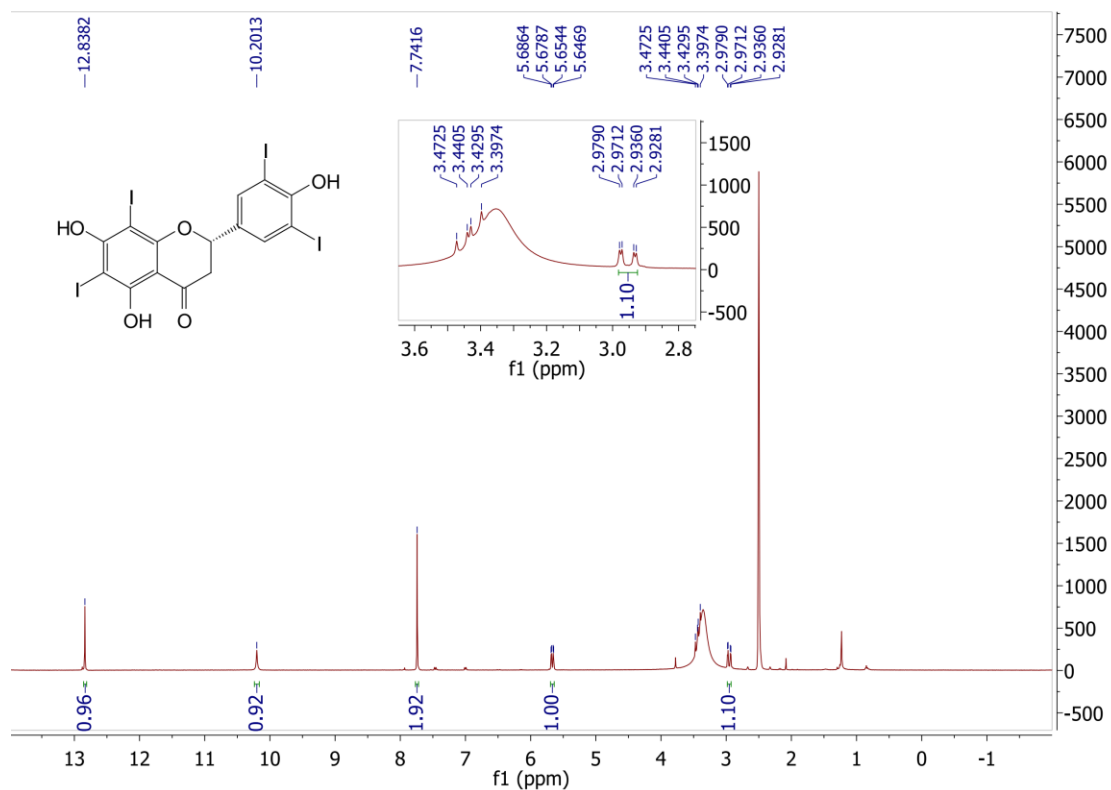
Figure A21 The ^{1}H NMR spectrum (DMSO-d$_6$, 400 MHz) of **50**.
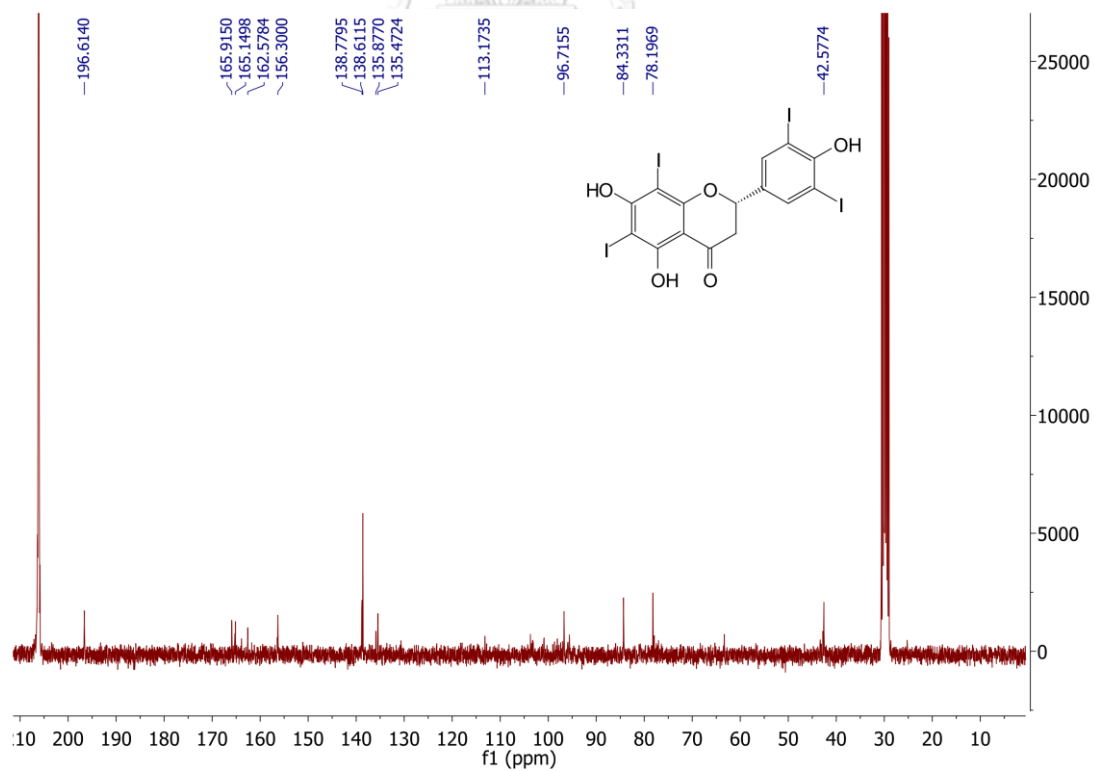
Figure A22 The ^{13}C NMR spectrum (DMSO-d$_6$, 100 MHz) of **50**.
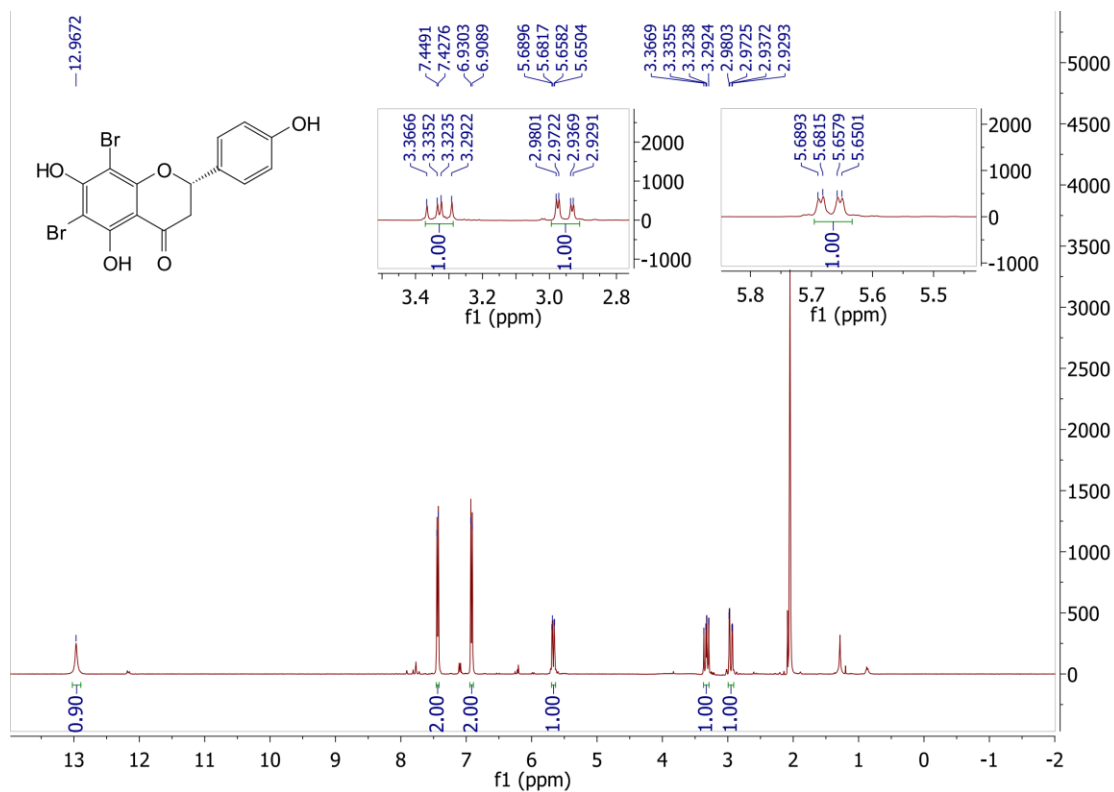
Figure A23 The ^{1}H NMR spectrum (Acetone-d$_6$, 400 MHz) of **51**.
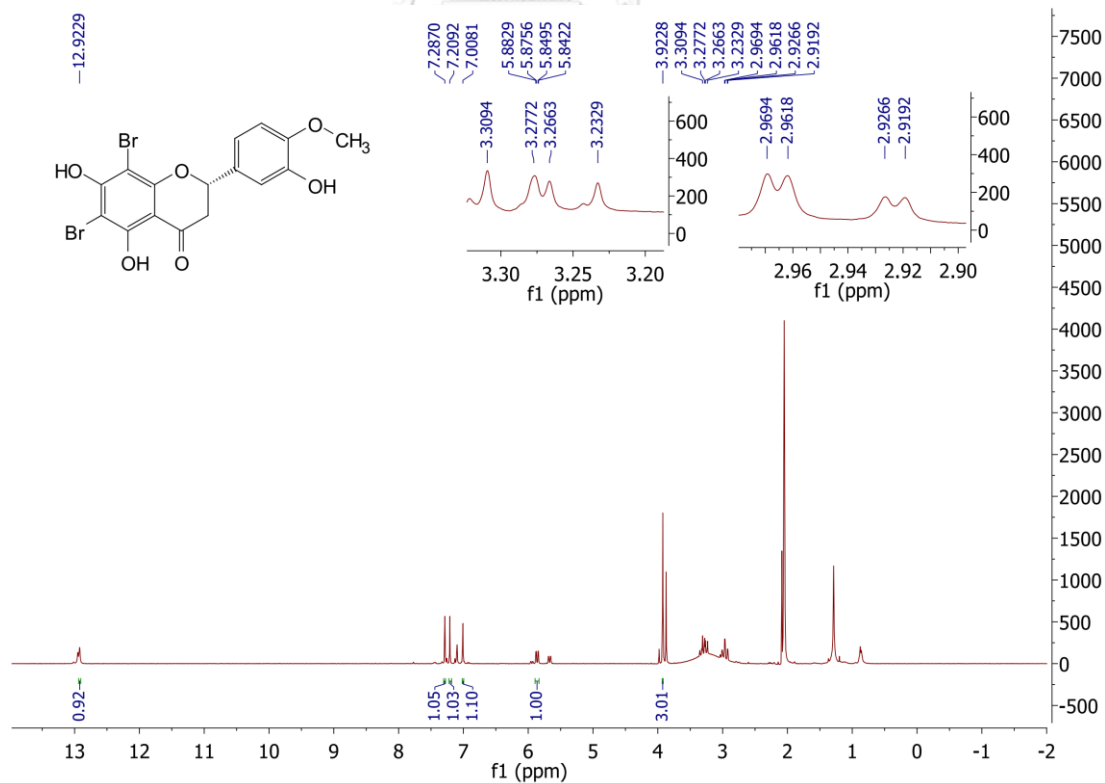
Figure A24 The ^{1}H NMR spectrum (Acetone-d$_6$, 400 MHz) of **52**.

Mass Spectrum List Report

Figure A25 The HR-ESI-MS of **53**.
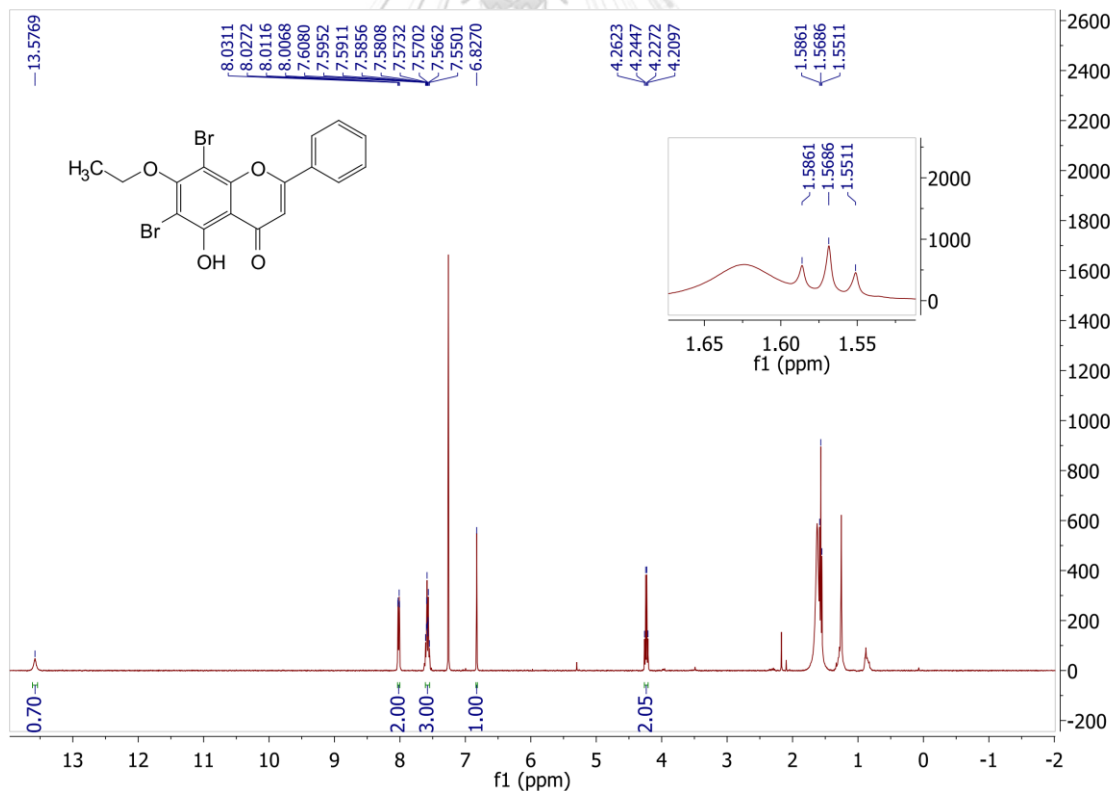
Figure A26 The ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of **53**.
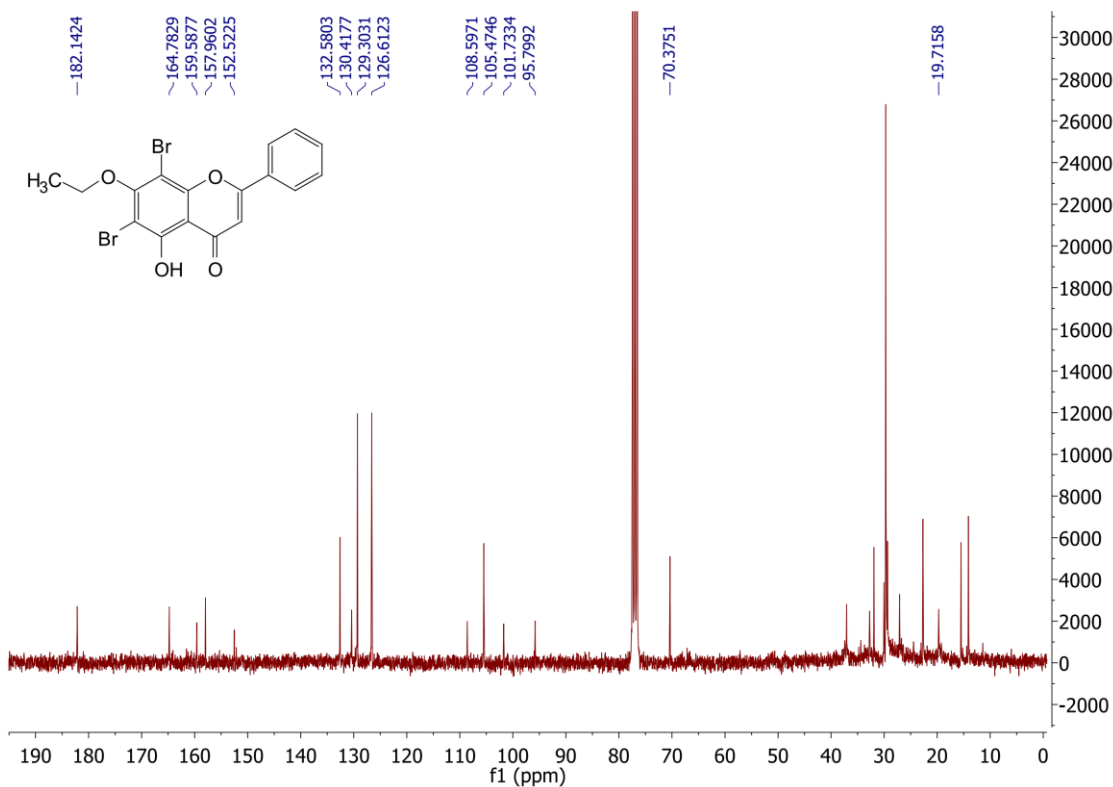
Figure A27 The ^{13}C NMR spectrum (CDCl$_3$, 100 MHz) of **53**.

Mass Spectrum List Report

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Comment			

Acquisition Parameter

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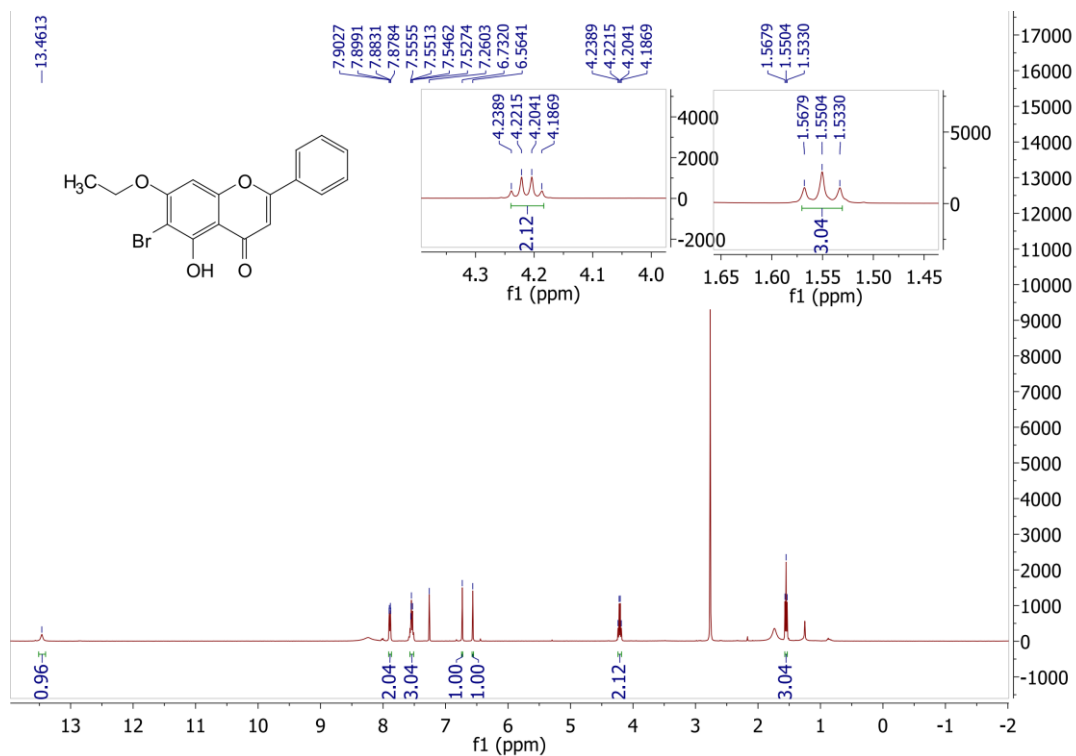
Figure A29 The ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of **54**.

Figure A30 The ^{13}C NMR spectrum (CDCl$_3$, 100 MHz) of **54**.

Mass Spectrum List Report

Analysis Info

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Comment			

Acquisition Parameter

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Figure A31 The HR-ESI-MS of **55**.
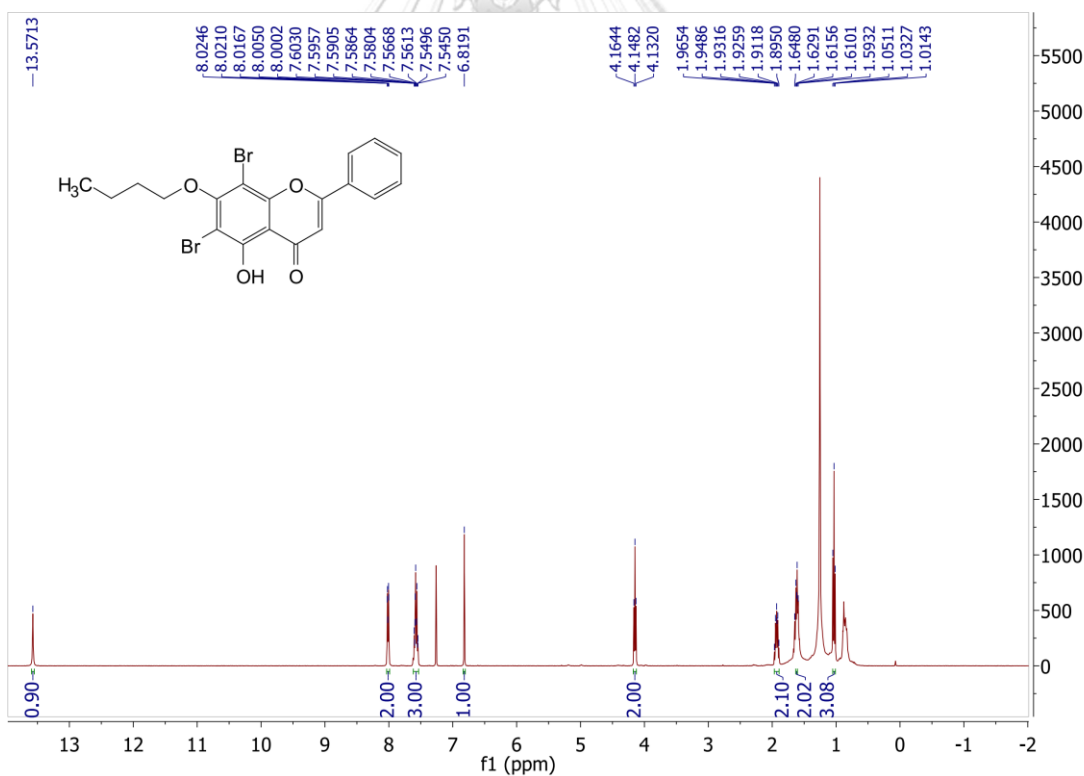
Figure A32 The ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of **55**.
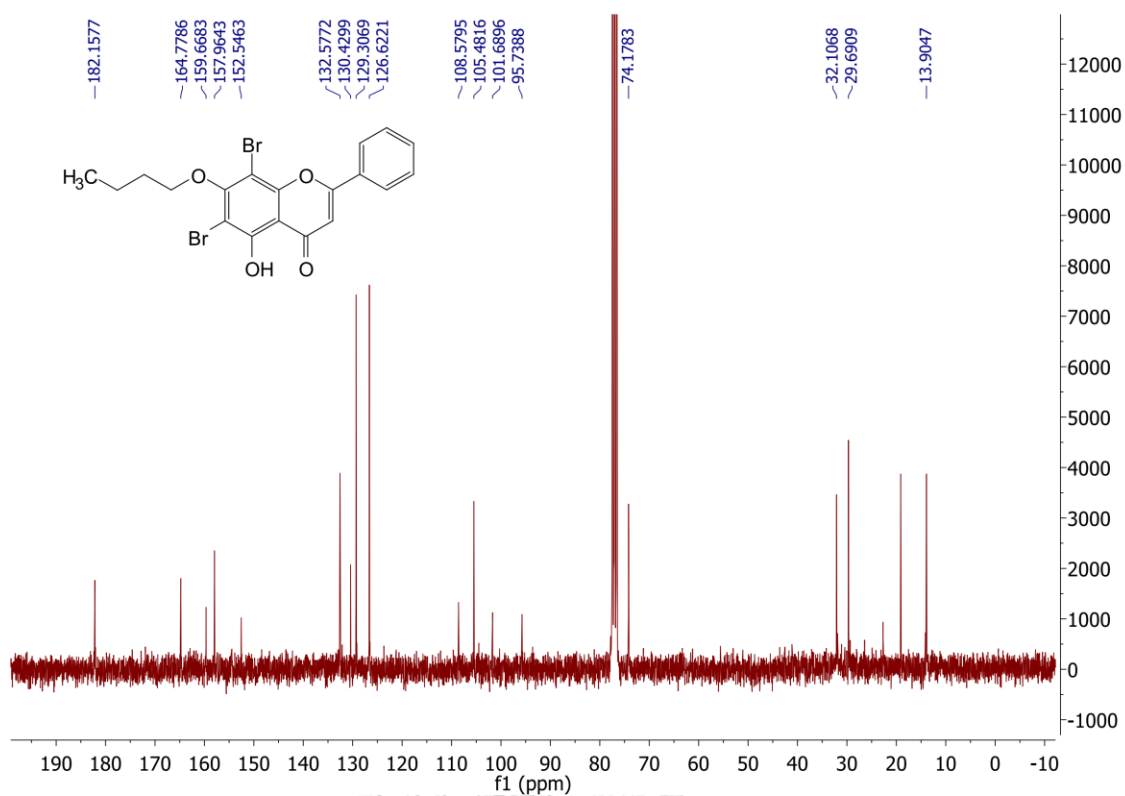
Figure A33 The ^{13}C NMR spectrum (CDCl$_3$, 100 MHz) of **55**.
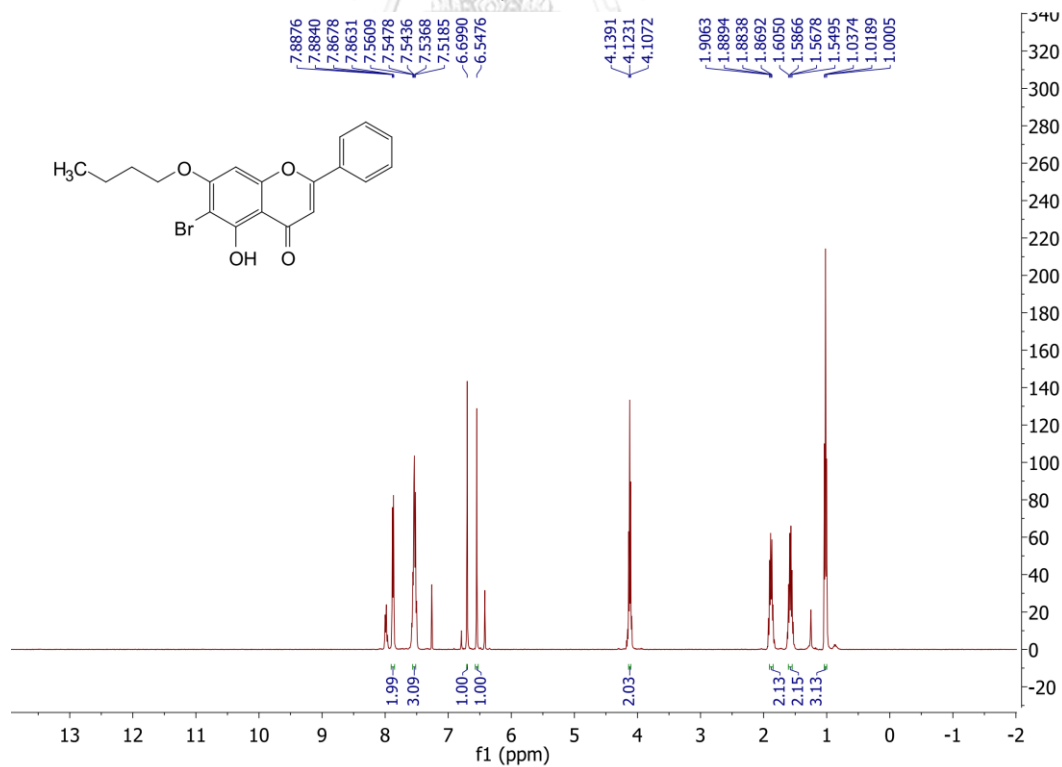
Figure A34 The ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of **56**.
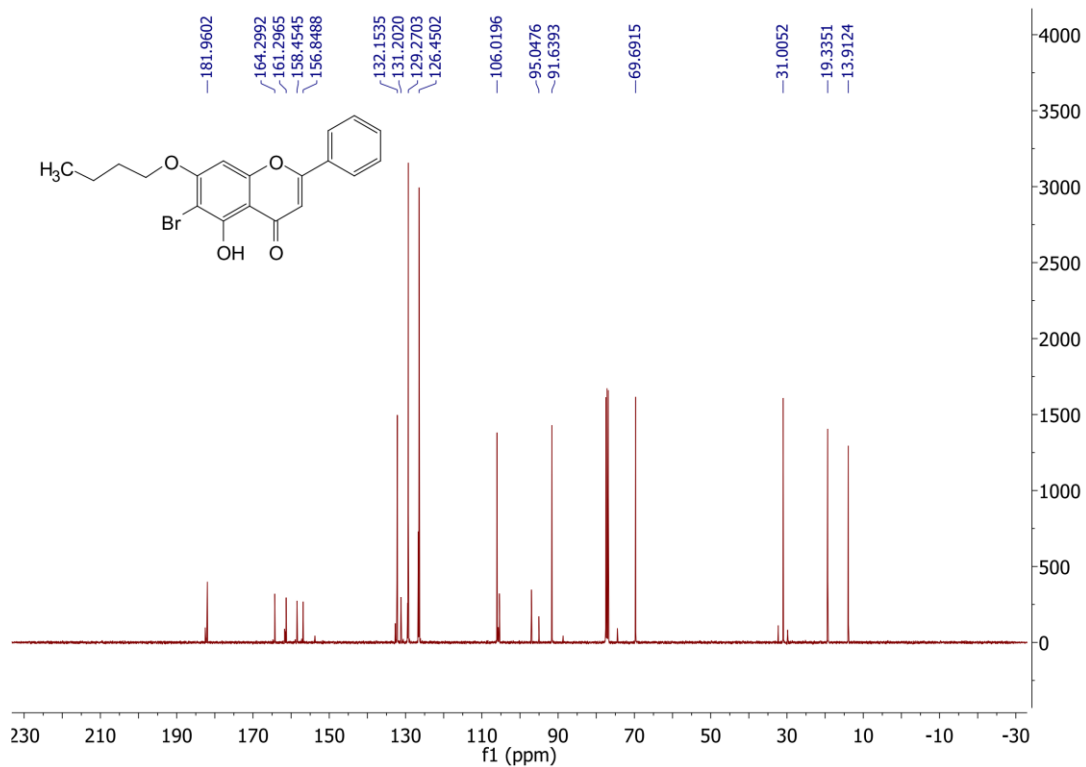
Figure A35 The ^{13}C NMR spectrum (CDCl$_3$, 100 MHz) of **56**.
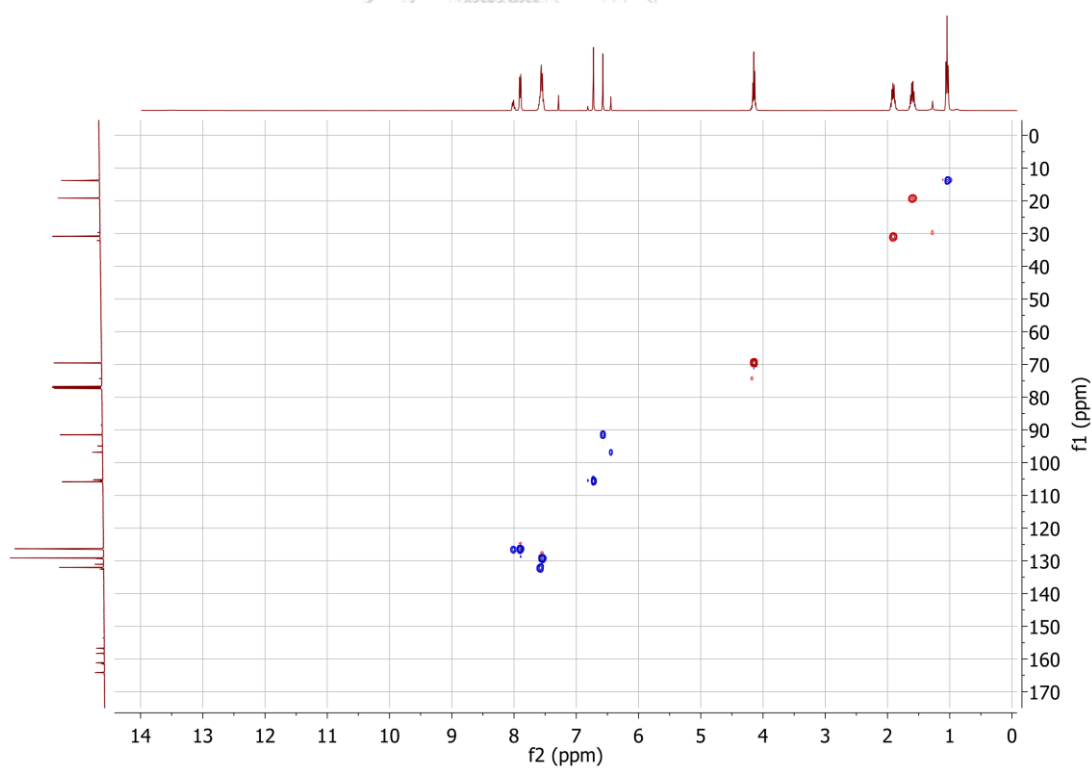
Figure A36 The HSQC correlation (CDCl$_3$) of **56**.
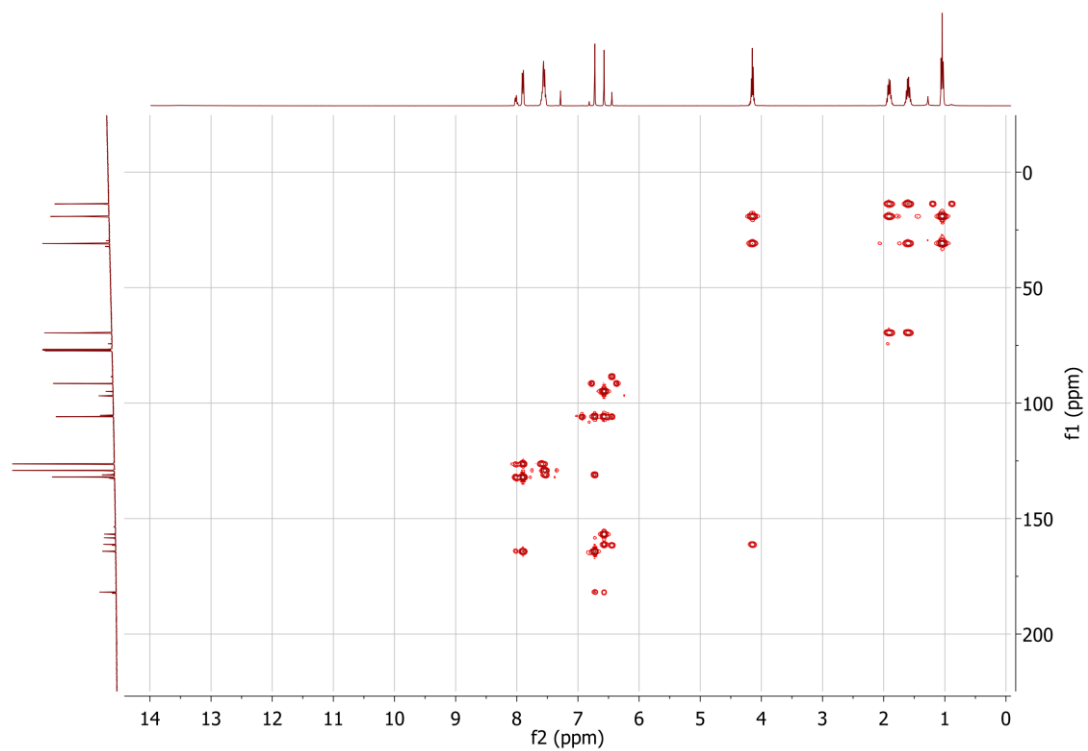
Figure A37 The HMBC correlation (CDCl$_3$) of **56**.
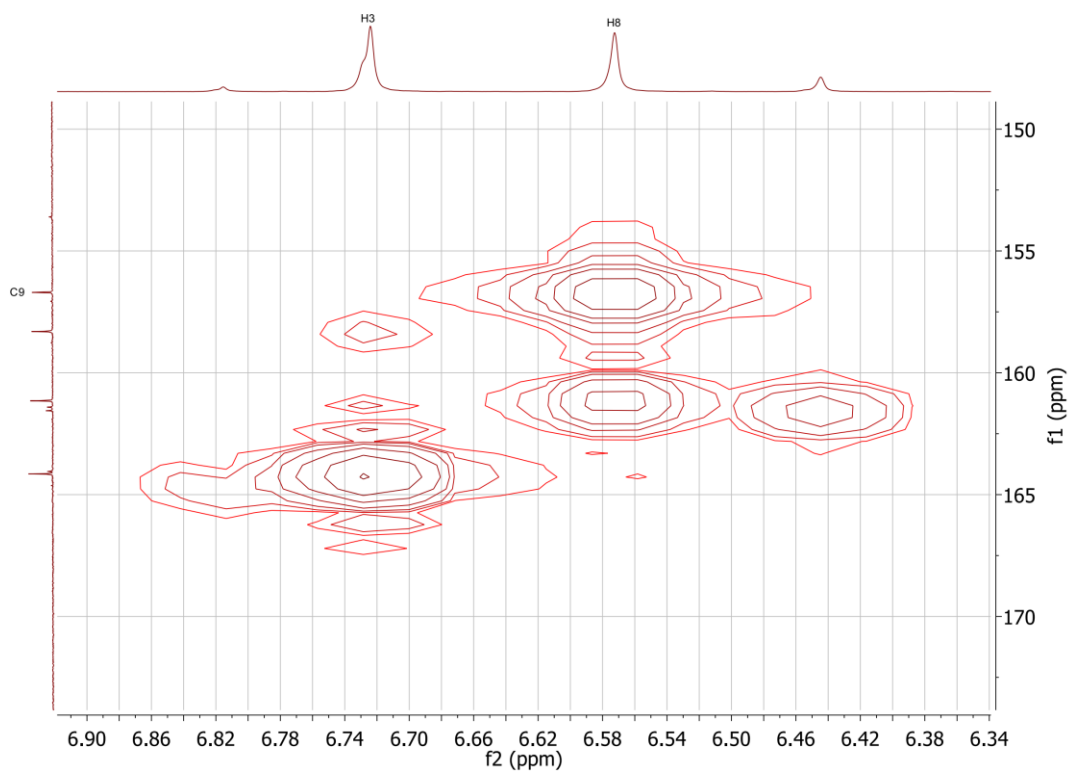
Figure A38 The HMBC (expansion) correlation (CDCl$_3$) of **56**.

Mass Spectrum List Report

Analysis Info

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Acquisition Date   9/24/2018 3:24:22 PM

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Instrument / Ser#   micrOTOF-Q II 10335

Acquisition Parameter

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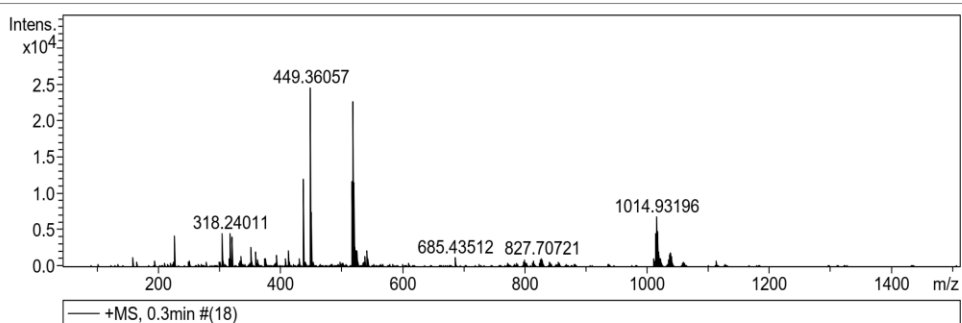
Figure A39 The HR-ESI-MS of 57.
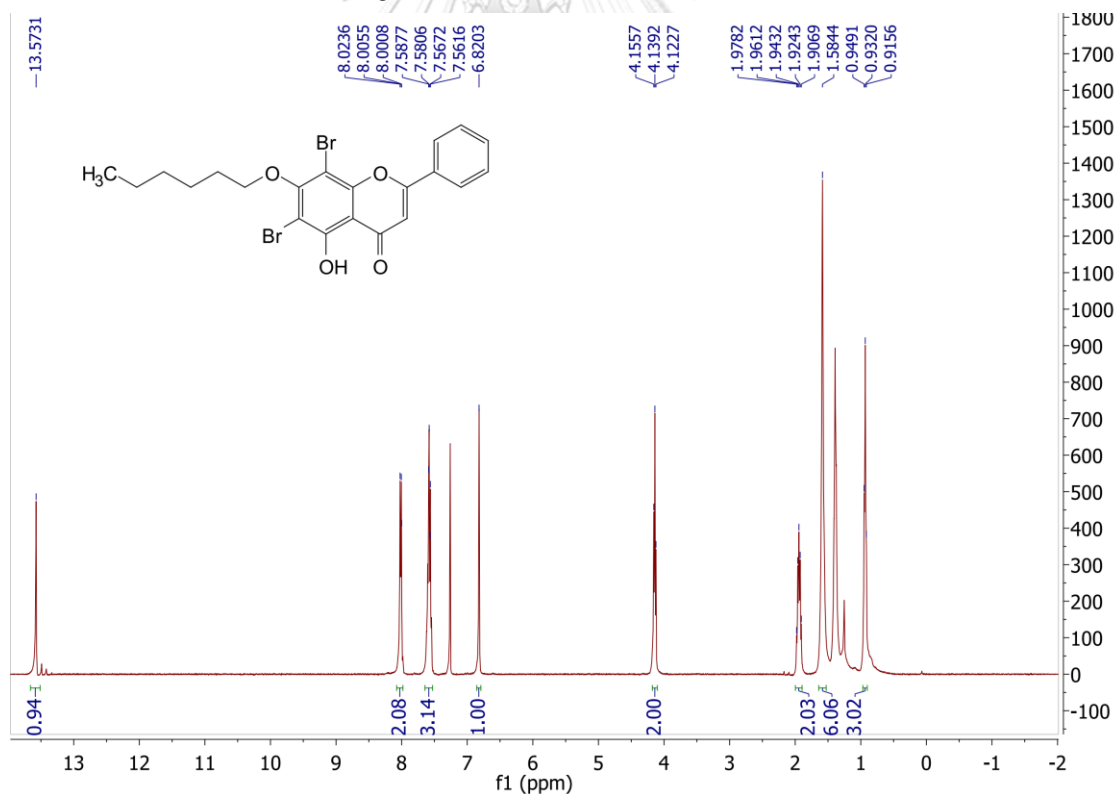
Figure A40 The ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of 57.
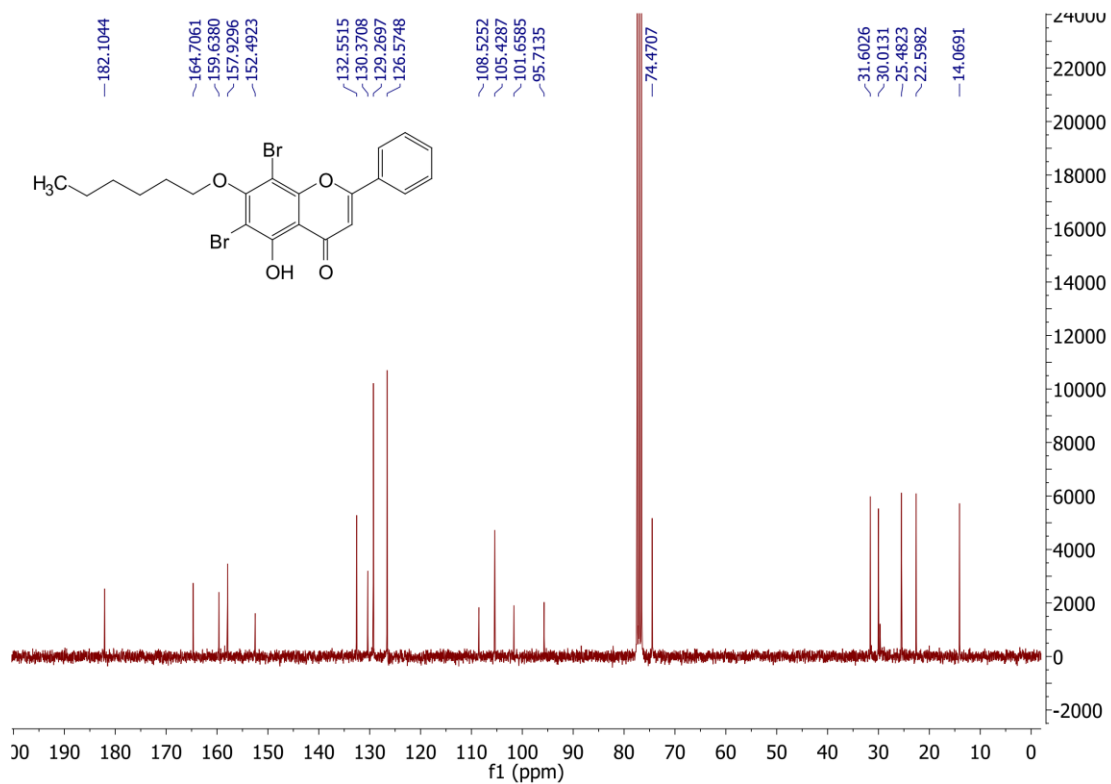
Figure A41 The ^{13}C NMR spectrum (CDCl$_3$, 100 MHz) of **57**.
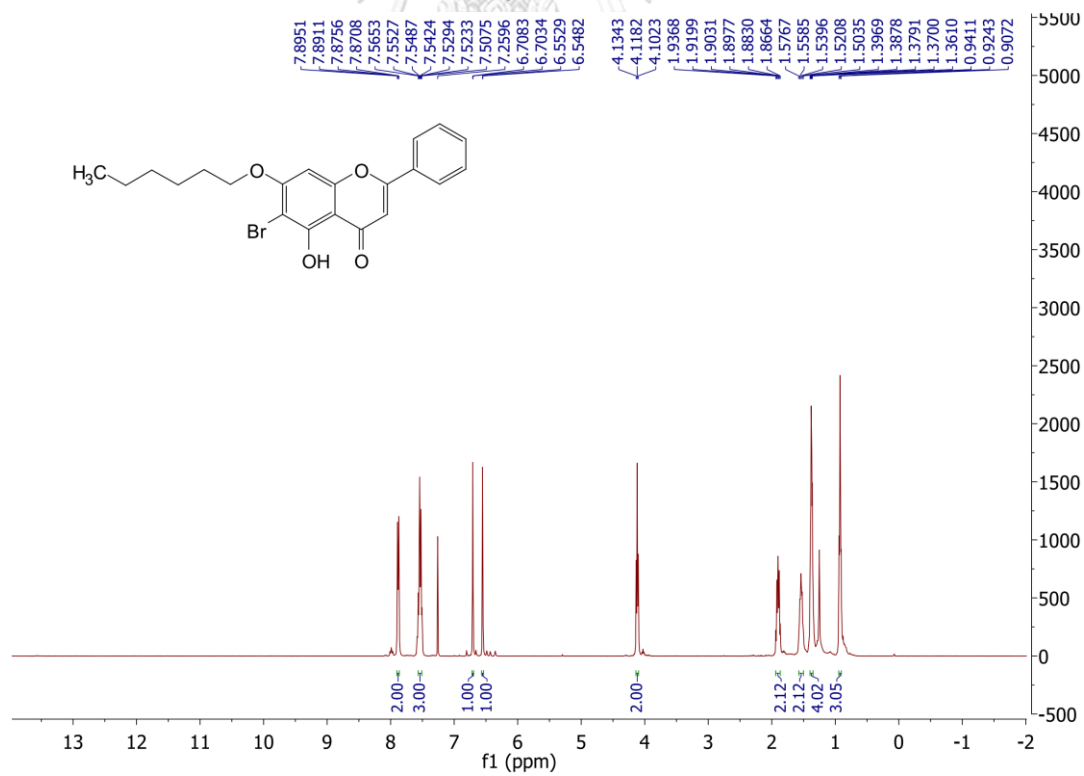
Figure A42 The ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of **58**.

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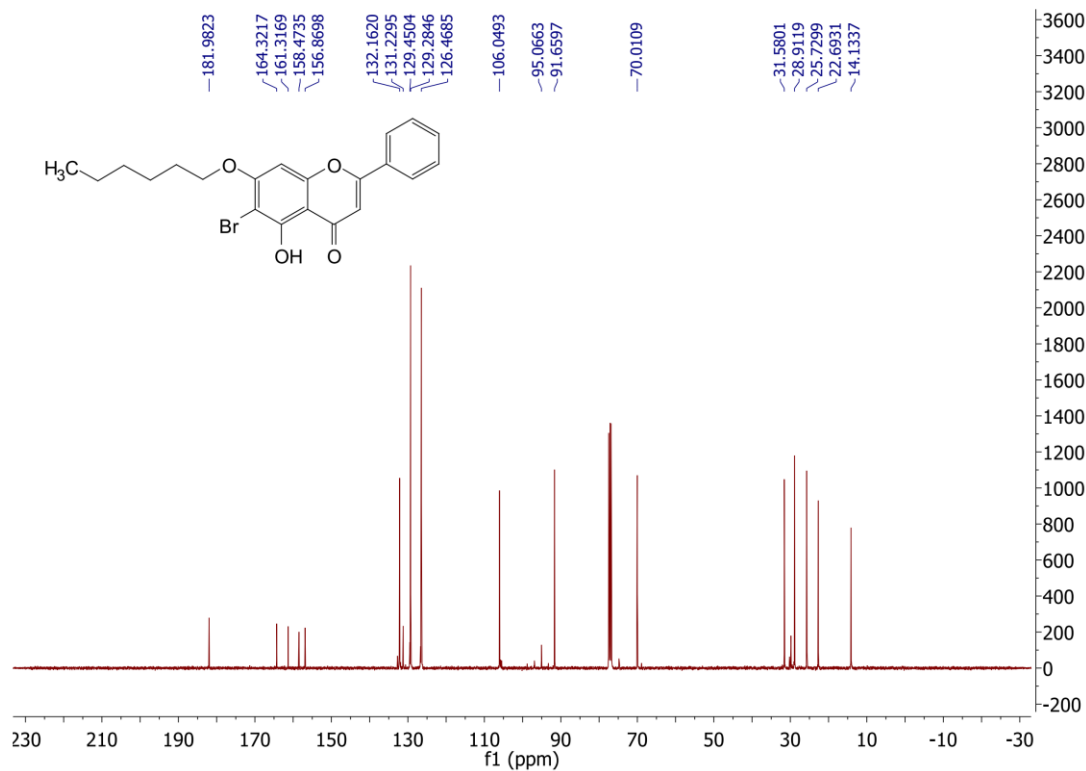
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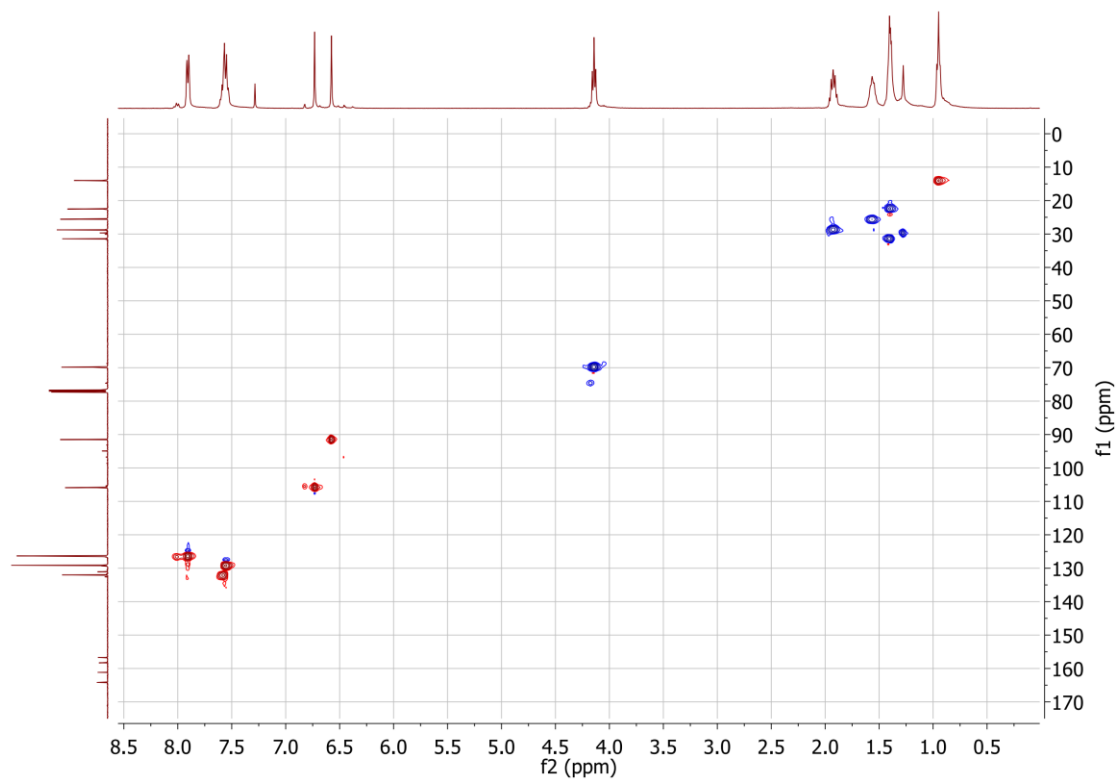
Figure A44 The HSQC correlation (CDCl$_3$) of **58**.
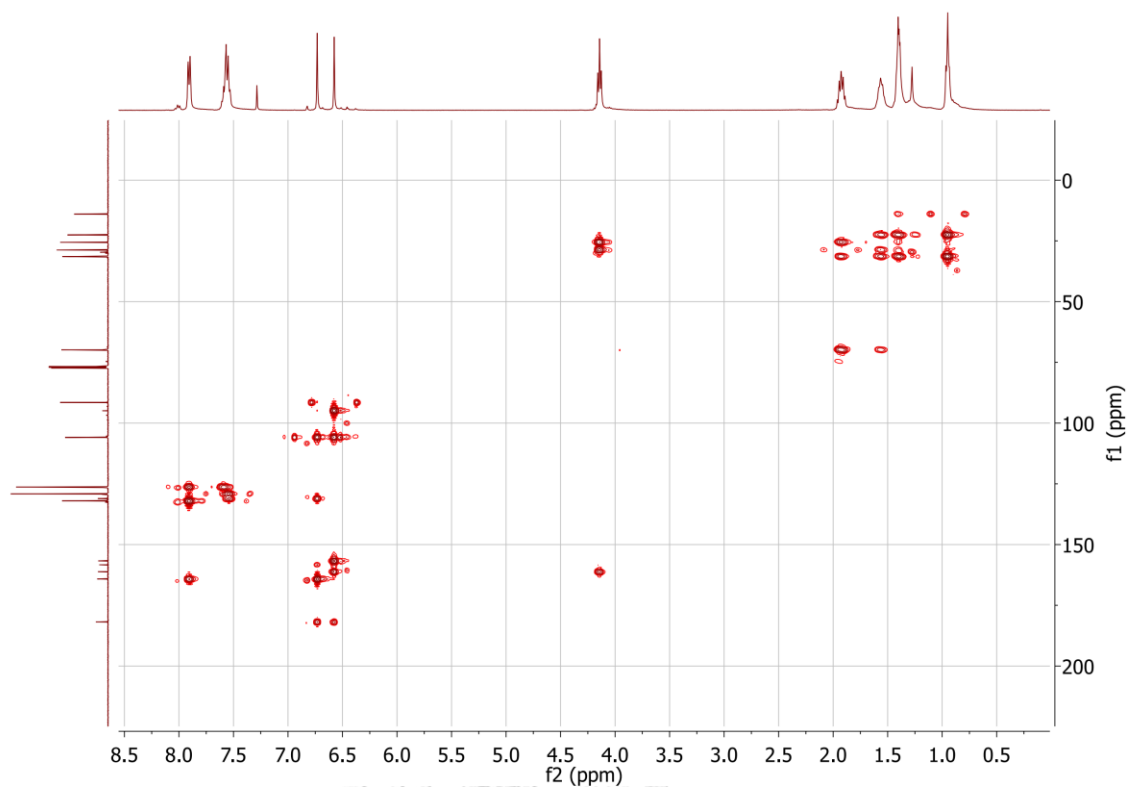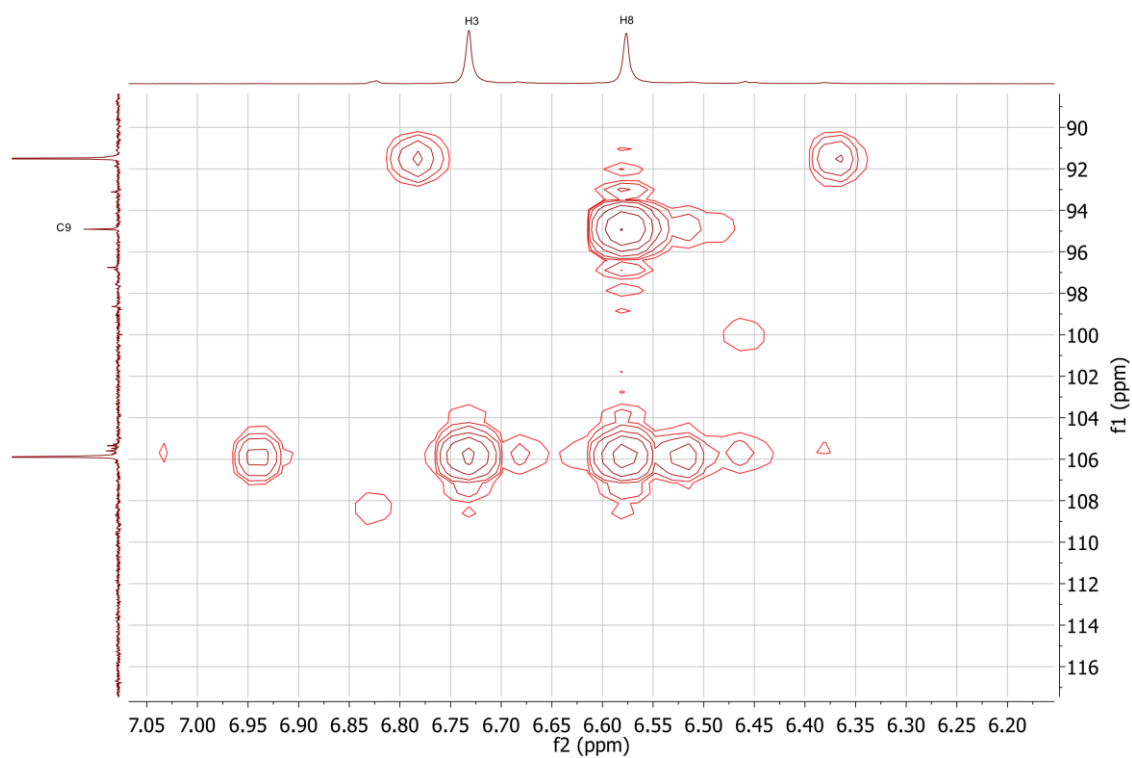
Figure A45 The HMBC correlation (CDCl$_3$) of **58**.

Figure A46 The HMBC (expansion) correlation (CDCl$_3$) of **58**.
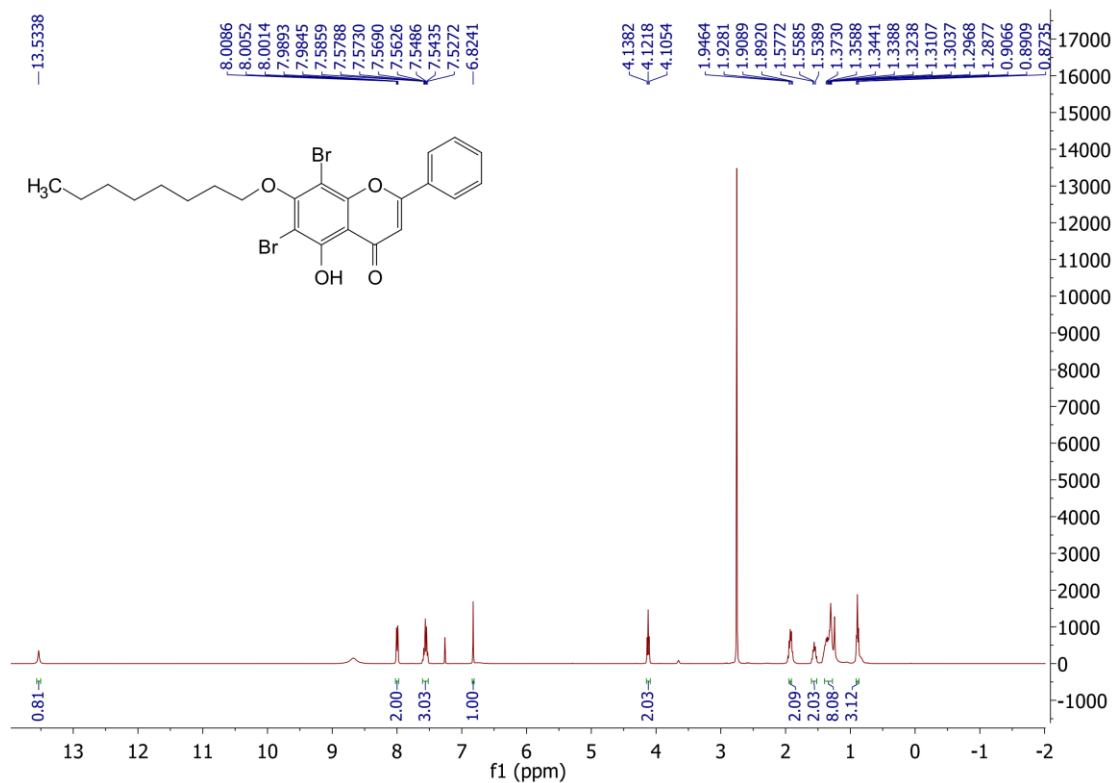
Figure A47 The ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of **59**.
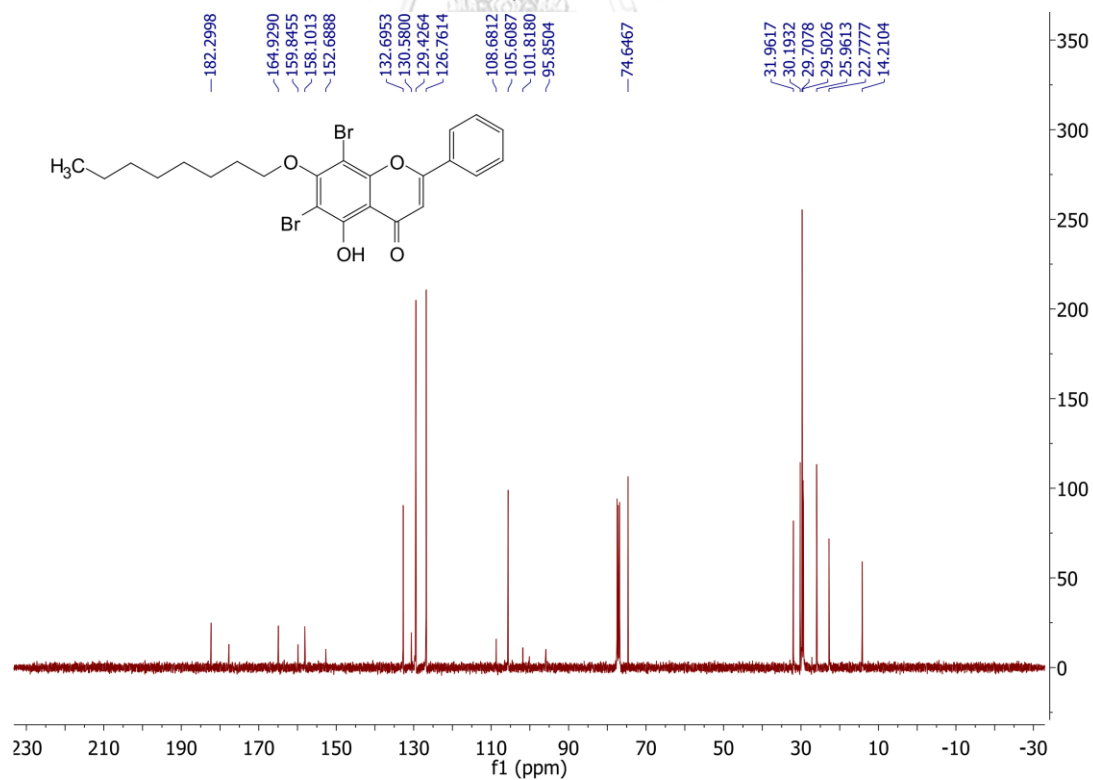
Figure A48 The ^{13}C NMR spectrum (CDCl$_3$, 100 MHz) of **59**.
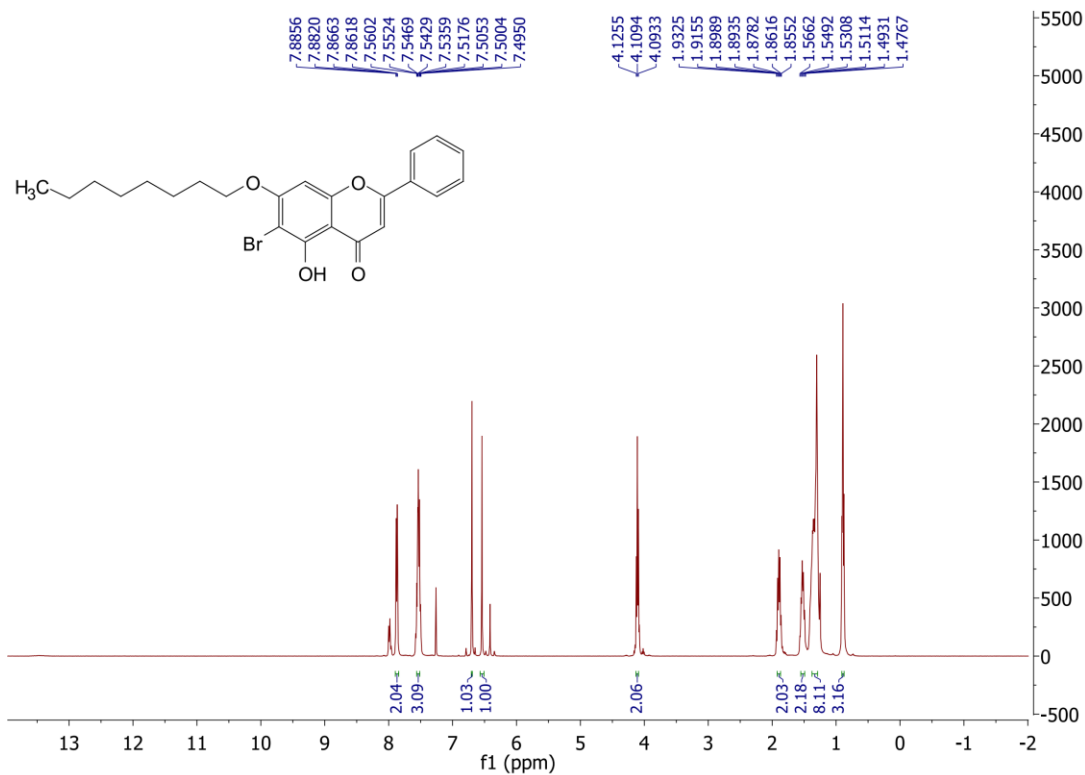
Figure A49 The ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of **60**.
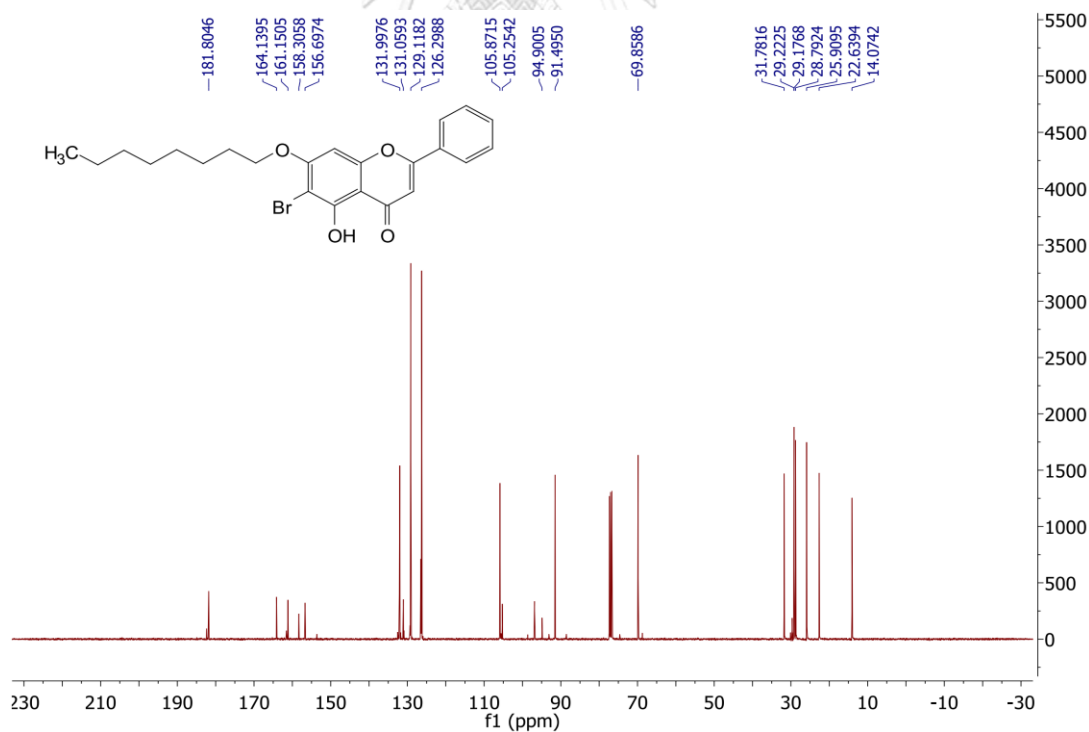
Figure A50 The ^{13}C NMR spectrum (CDCl$_3$, 100 MHz) of **60**.

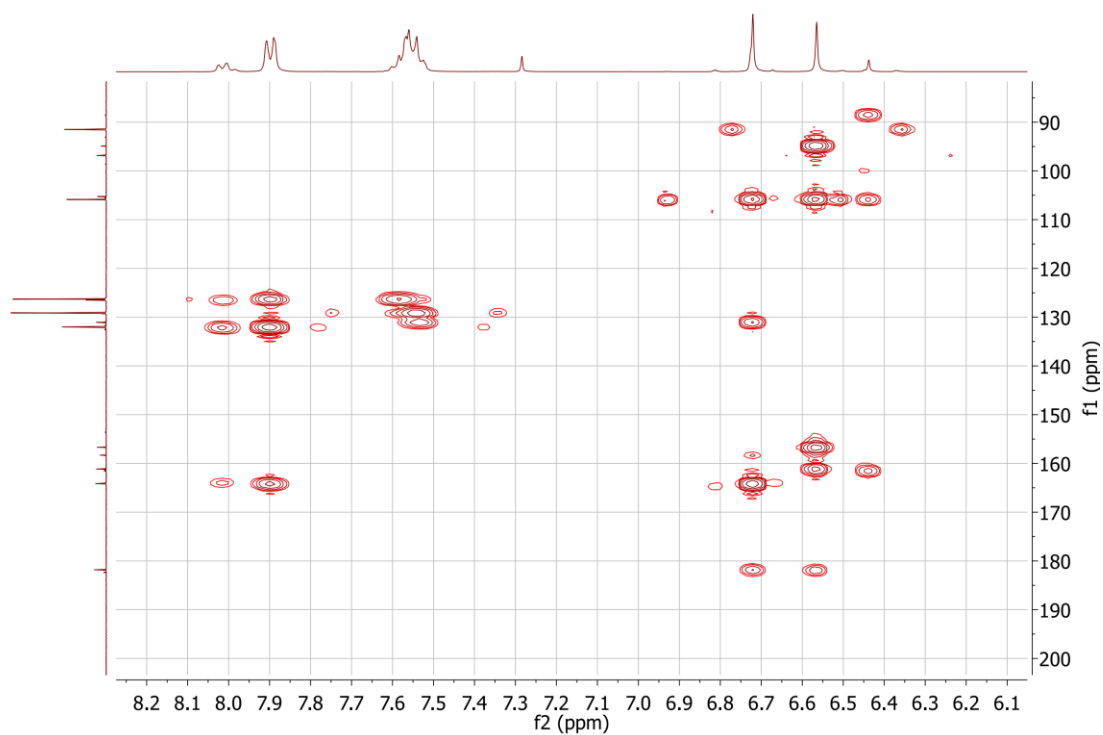**Figure A51** The HSQC correlation (CDCl$_3$) of **60**.

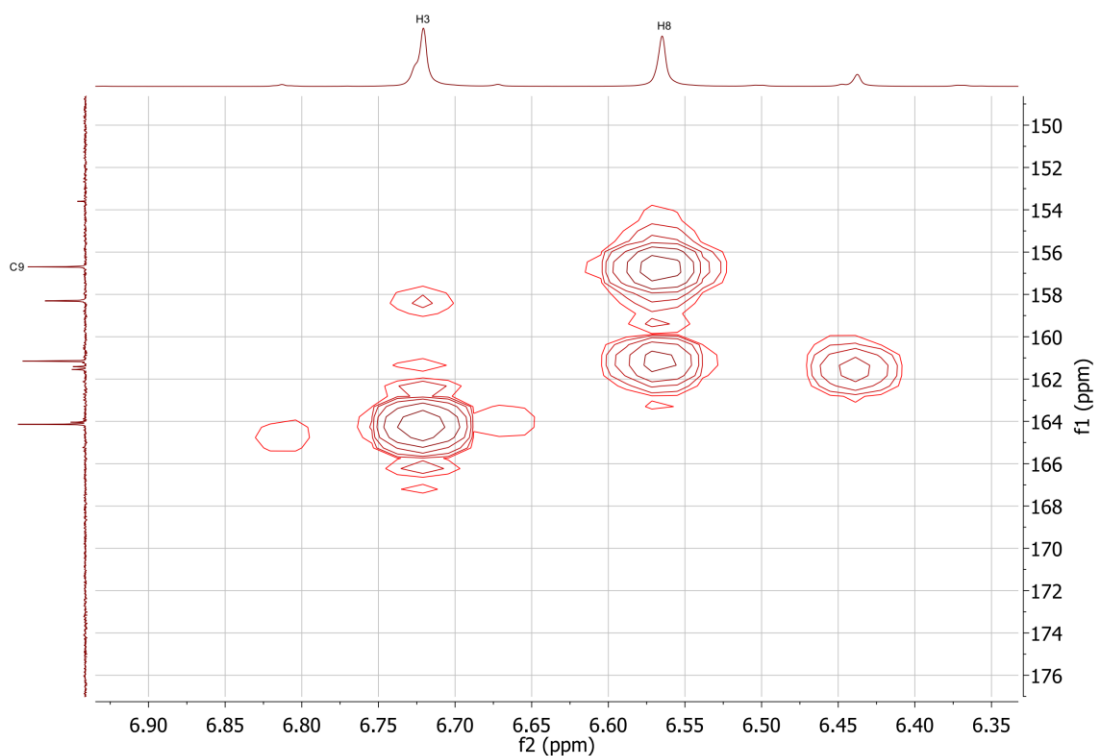**Figure A52** The HMBC correlation (CDCl$_3$) of **60**.

Figure A53 The HMBC (expansion) correlation (CDCl$_3$) of **60**.

Mass Spectrum List Report

Analysis Info   Acquisition Date   9/17/2018 3:12:45 PM

Analysis Name   D:\Data\Data Service\180917_pos_T46..d
Method   NV_pos_0.3min_profile_1segment_lowNubulizerDrygas.m   Operator   CU.
Sample Name   180917_pos_T46.   Instrument / Ser#   micrOTOF-Q II 10335
Comment

Acquisition Parameter

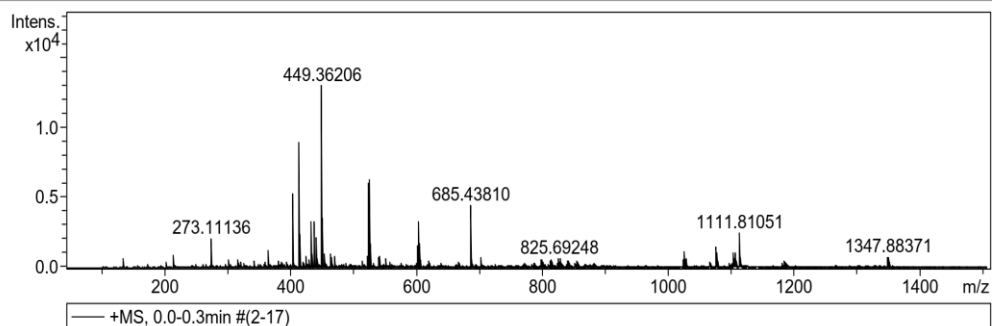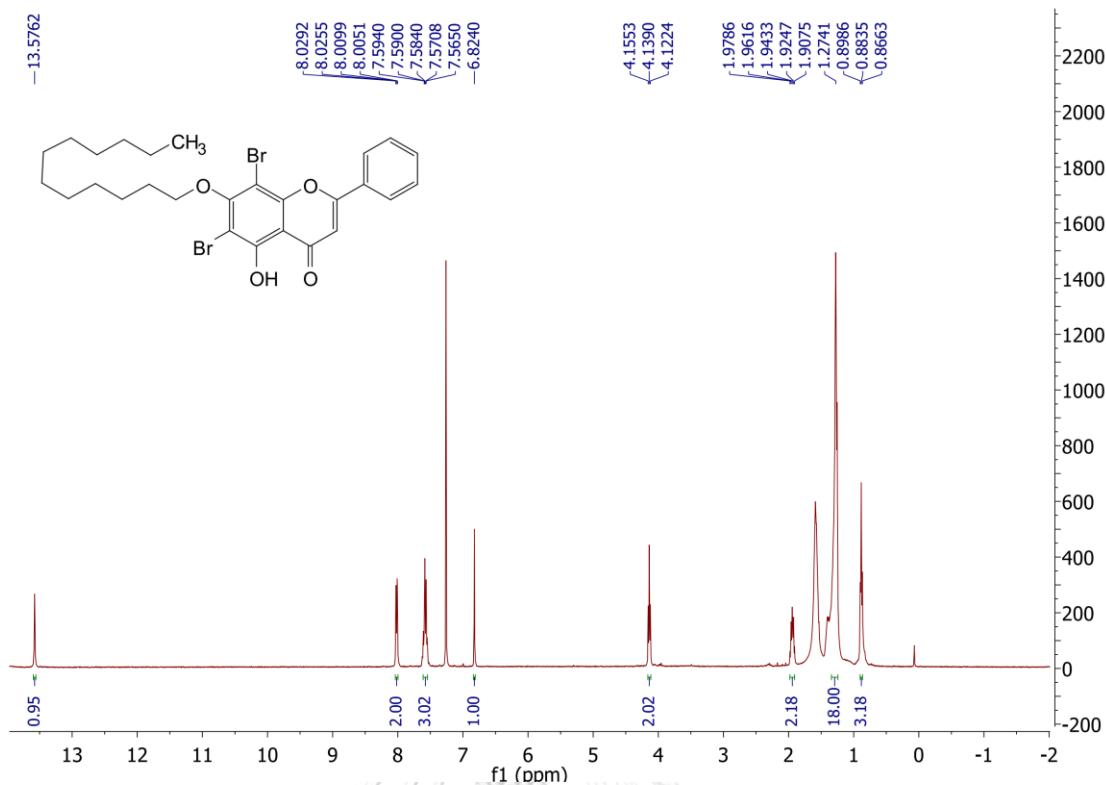| Source Type | ESI | Ion Polarity | Positive | Set Nebulizer | 0.4 Bar |
Focus	Not active	Set Capillary	4000 V	Set Dry Heater	200 ℃
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste

Figure A54 The HR-ESI-MS of **61**.

Figure A55 The ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of **61**.

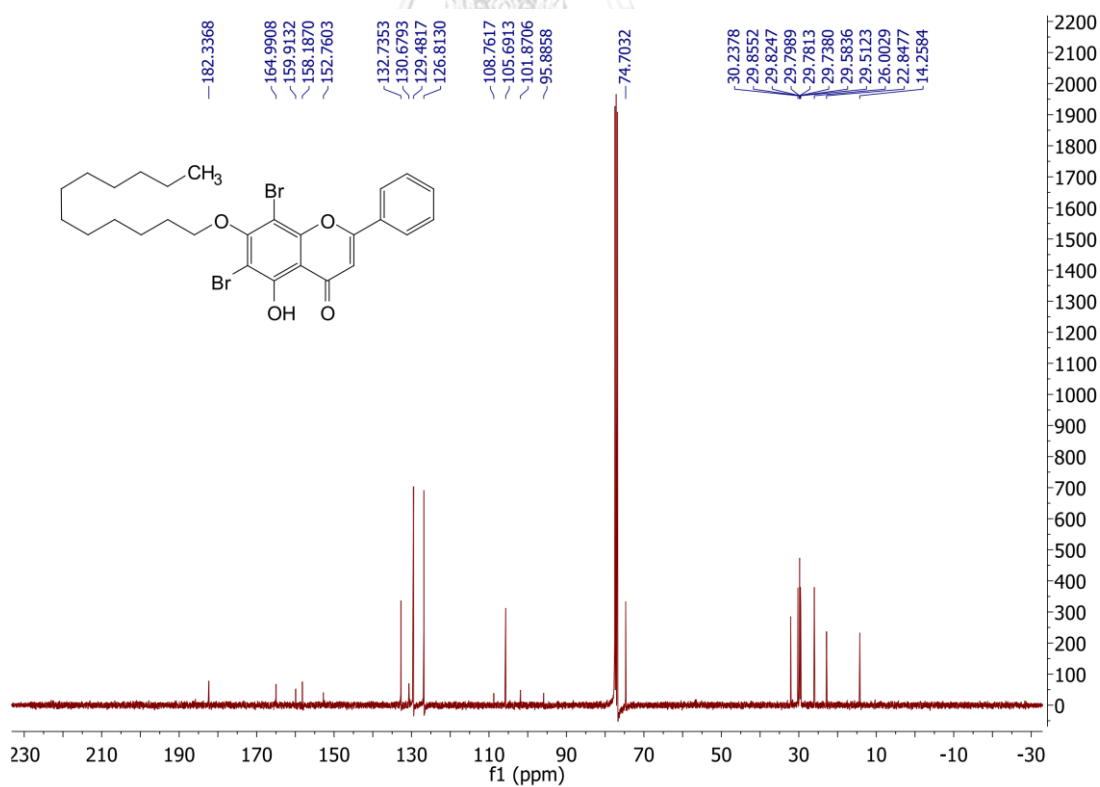**Figure A56** The ^{13}C NMR spectrum (CDCl$_3$) of **61**.

Mass Spectrum List Report

Analysis Info

Analysis Name D:\Data\Data Service\180924_pos_T47.d
Method NV_pos_0.3min_profile_1segment_lowNubulizerDrygas.m
Sample Name 180924_pos_T47
Comment

Acquisition Date 9/24/2018 3:01:16 PM

Operator CU.
Instrument / Ser# micrOTOF-Q II 10335

Acquisition Parameter

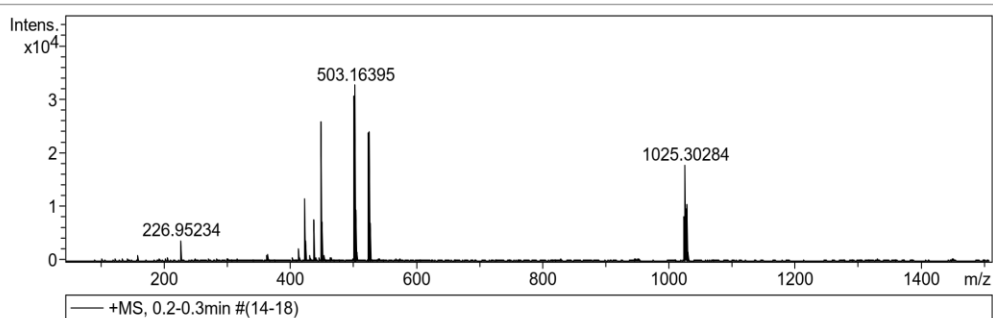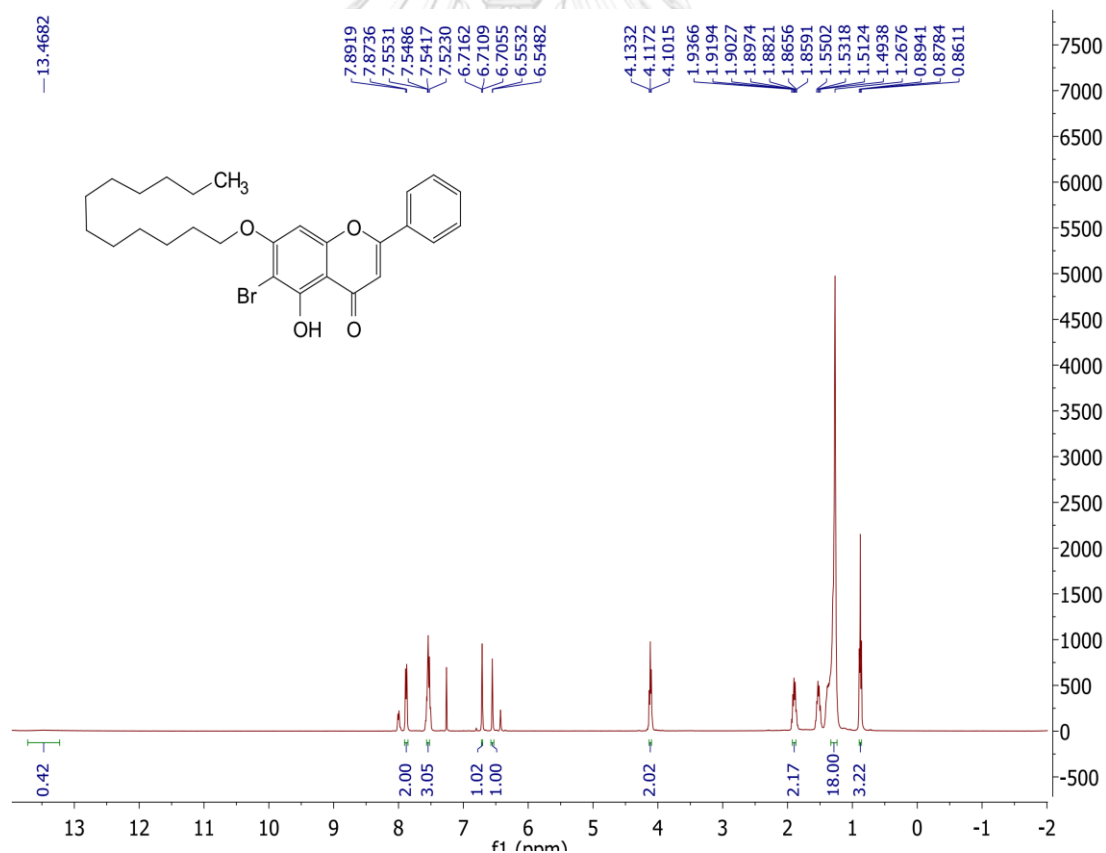| Source Type | ESI | Ion Polarity | Positive | Set Nebulizer | 0.4 Bar |
Focus	Not active	Set Capillary	4000 V	Set Dry Heater	200 ℃
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste

Figure A57 The HR-ESI-MS of **62**.

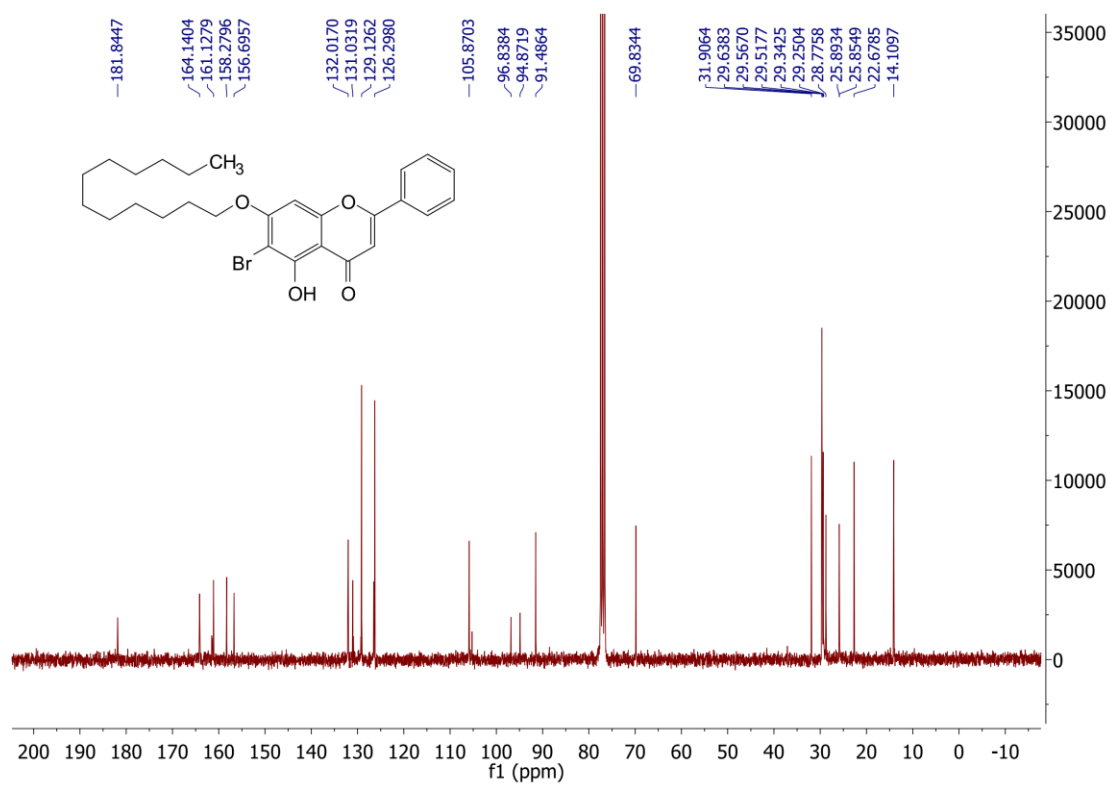**Figure A58** The ^{1}H NMR spectrum (CDCl$_3$) of **62**.

Figure A59 The ^{13}C NMR spectrum (CDCl$_3$) of **62**.

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