การสังเคราะห์อนุพันธ์โรทีนอยด์ที่มีฤทธิ์ยับยั้งเซลล์มะเร็งและโทโพไอโซเมอเรส II

นางสาวสุปราณี แสงทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

SYNTHESIS OF ROTENOID DERIVATIVES WITH CANCER AND TOPOISOMERASE II INHIBITORY ACTIVITIES

Miss Supranee Sangthong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic year 2008

Thesis Title	SYNTHESIS OF ROTENOID DERIVATIVES WITH CANCER AND TOPOISOMERASE II INHIBITORY ACTIVITIES
By	Miss Supranee Sangthong
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Nongnuj Muangsin, Ph.D.
Thesis Co-Advisor	Associate Professor Nattaya Ngamrojanavanich, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

S. Hannongbua ..... Dean the Faculty of the Science

(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

Warinthorn Chavasiri ..... Chairman

(Assistant Professor Warinthorn Chavasiri, Ph.D.)

N. Muangsin ..... Thesis Advisor

(Associate Professor Nongnuj Muangsin, Ph.D.)

N. Ngamrojanavanich ..... Thesis Co-Advisor

(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

Polkit Sangvanich ..... Examiner

(Associate Professor Polkit Sangvanich, Ph.D.)

..... External Examiner

(Nalena Praphairaksit, D.V.M., Ph.D.)

สุปราณี แสงทอง : การสังเคราะห์อนุพันธ์โรทีนอยด์ที่มีฤทธิ์ยับยั้งเซลล์มะเร็งและโทโพไอโซเมอเรส II (SYNTHESIS OF ROTENOID DERIVATIVES WITH CANCER AND TOPOISOMERASE II INHIBITORY ACTIVITIES) อ.ที่ปรึกษาวิทยานิพันธ์หลัก: รศ. ดร. นงนุช เหมืองสิน, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. นาตยา งามโรจนวณิชย์, 126 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อหาความสัมพันธ์ระหว่างโครงสร้างและการออกฤทธิ์ ของสารประกอบโรทีนอยด์ต่อการออกฤทธิ์ทางชีวภาพและการยับยั้งโทโปไอโซเมอร์เรส II ได้มีรายงานว่า 6-ดีออกซีไคลโทไรอะซิทาล ซึ่งเป็นสารประกอบโรทีนอยด์ที่สกัดจากรากแห้งของ *Stemona collinse* Craib. หรือหนอนตายอยาก มีฤทธิ์ยับยั้งเซลล์มะเร็งของมนุษย์หลายชนิด และมีฤทธิ์ยับยั้งที่ดีกว่าดอกซอรูบิซินซึ่งเป็นยาต้านมะเร็งที่จำหน่ายเชิงการค้า ในงานวิจัยนี้ สารประกอบ 6-ดีออกซีไคลโทไรอะซิทาลถูกเปลี่ยนเป็นสารอนุพันธ์อิพอกไซด์โดยการทำปฏิกิริยากับอีพิคลอโรไฮดริน สารอนุพันธ์อิพอกไซด์ถูกเปิดวงด้วยนิวคลีโอไฟล์, สารประกอบประเภทมอร์โฟลิน และเบนซิลลามีน นำอนุพันธ์ทุกตัวที่ได้มาทดสอบการออกฤทธิ์ต่อเซลล์ KB, MCF-7 และ NCI-H187 นอกจากนี้ได้ทดสอบการยับยั้งเอนไซม์โทโปไอโซเมอร์เรส II โดยมีดอกซอรูบิซินและอีโทโปไซด์เป็นสารอ้างอิง การทดลองนี้พบว่าสารอนุพันธ์อิพอกไซด์ (2)ให้การออกฤทธิ์ที่ดีต่อเซลล์ KB, MCF-7 และ NCI-H187 โดยมีค่า IC_{50} 2.87, 7.33 และ 3.21μg/ml ตามลำดับ สารประกอบของ 6-ดีออกซีไคลโทไรอะซิทาลที่มีไดเอทาโนลามีน (**10**) ไม่มีฤทธิ์ต่อเซลล์ไลน์ทั้งสามชนิด สาร **8** และ **9** ออกฤทธิ์ที่ดีต่อเซลล์ NCI-H187 โดยมีค่า IC_{50} 2.93 และ 4.70 μg/mlตามลำดับ เพื่อเพิ่มการออกฤทธิ์ทางชีวภาพของสารที่ **8**, จึงทำให้สาร **8** โดยทำให้อยู่ในรูปของเกลือด้วยการเติมกรดไฮโดรคลอริกเข้มข้น สารอนุพันธ์มอร์โฟลิน 6-ดีออกซีไคลโทไรอะซิทาลที่อยู่ในรูปเกลือไฮโดรคลอไรด์ให้การออกฤทธิ์ที่ดีต่อเซลล์ KB,และ NCI-H187 โดยมีค่า IC_{50} 0.68, และ 0.63 μg/ml ตามลำดับ ซึ่งให้การออกฤทธิ์ที่ดีกว่าอนุพันธ์สารอนุพันธ์มอร์โฟลิน (IC_{50} 13.25, และ 3.12 μg/ml) ตามลำดับ นอกจากนี้ยังให้การออกฤทธิ์ที่ดีต่อเซลล์ KB ซึ่งดีกว่าดอกซอรูบิซิน (IC_{50} 2.01 μg/ml) จากผลการทดสอบการออกฤทธิ์ยับยั้งโทโปไอโซเมอร์เรส II พบว่า อนุพันธ์เกือบทุกตัวของ 6-ดีออกซีไคลโทไรอะซิทาล สามารถยับยั้งเอนไซม์โทโปไอโซเมอร์เรส II ได้มากกว่า 50% ยกเว้นอนุพันธ์ตัวที่ **5** และ **9** และสารอนุพันธ์มอร์โฟลิน 6-ดีออกซีไคลโทไรอะซิทาล (**8**) สามารถยับยั้งเอนไซม์โทโปไอโซเมอร์เรส II ได้ถึง 93.5%

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....สุปราณี แสงทอง.....

ปีการศึกษา ..... 2551..... ลายมือชื่ออาจารย์ที่ปรึกษา.....นงนุช เหมืองสิน.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....นาตยา งามโรจนวณิชย์.....

5072524823 : MAJOR BIOTECHNOLOGY

KEYWORDS: ANTICANCER AGENTS/ ROTENOID / TOPOISOMERASE II / STRUCTURE-ACTIVITY RELATIONSHIP

SUPRANEE SANGTHONG: SYNTHESIS OF ROTENOID DERIVATIVES WITH CANCER AND TOPOISOMERASE II INHIBITORY ACTIVITIES. THESIS PRINCIPAL ADVISOR: ASSOC. PROF. NONGNUJ MUANGSIN, Ph. D, THESIS COADVISOR: ASSOC. PROF. NATTAYA NGAMROJANAVANICH, Ph. D., 126 pp.

This thesis aims to investigate the structure-activity relationship of rotenoid compounds on cytotoxicity and topoisomerase II inhibition. It has been reported that 6-deoxyclitoriacetal, a rotenoid substance extracted from the dried root of *Stemona collinse* Craib., showed strong cytotoxic activity against various human cell lines and better than that of doxorubicin as a commercial anticancer drug. In this work, 6-deoxyclitoriacetal was converted to epoxide derivative by reacting with epichlorohydrin. The epoxide derivative was ring-opened with nucleophiles, morpholine and benzylamine compounds. All derivatives were tested for their cytotoxicity against KB, MCF-7, and NCI-H187 cell lines. In addition, the topoisomerase II inhibitory assay were evaluated using doxorubicin and etoposide as references. It has been found that the epoxide derivetivesv (**2**) showed strong cytotoxicity against all three cell lines with IC_{50} of 2.87, 7.33, and 3.21 μg/ml for KB, MCF-7, and NCI-H187, respectively. Diethanolamine containing 6-deoxyclitoriacetal (**10**) was inactive for all three cell lines. The morpholine derivatives of **8** and **9** showed strong activity against NCI-H187 with IC_{50} of 2.93 and 4.70 μg/ml, respectively. In order to enhance bioavailability of the synthesized compounds, an acid addition salt of **8** was prepared by adding strong hydrochloric acid. The salt of morpholine containing 6-deoxyclitoriacetal hydrocholide showed stronger cytotoxicity against KB and NCI-H187 with IC_{50} of 0.68 and 0.63 IC_{50} μg/ml, respectively, than that of its free base (IC_{50}=13.28 and 3.12 μg/ml, respectively. Its salt also has a stronger cytotoxity against KB than that of doxorubicin (IC_{50}=2.01 μg/ml). From results of Topoisomerase II assay revealed that most of 6-deoxyclitoriacetal inhibited Topoisomerase II with %inhibition rate over 50%, except for **5** and **9** cannot be complete inhibited the activity of Topoisomerase II activity. The morpholine derivative **8** at 100 μg exerted the most potent Topoisomerase II enzyme with 93.5%inhibition.

Field of study:.....Biotechnology... Student's signature:.....Supranee Sangthong.....

Academic year:.....2008..... Advisor's signature.....N. Muangsin.....

Co-advisor's signature:.....N. Ngamrojanavanich.....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis advisor Associate Professor Dr. Nongnuj Muangsin for her kindness, providing valuable advice, guidance, suggestions and assistance throughout the course of this research. I am also grateful to thesis examiners: Associate Professor Dr. Nattaya Ngamrojnavanich, Associate Professor Dr. Polkit Sangvanich for their valuable comments and suggestions.

Moreover, I would like to thank the Research Centre for Bioorganic Chemistry (RCBC); Department of Chemistry, Chulalongkorn University for the use of facilities, equipment, glassware, and chemicals; the Halal Science Center, Chulalongkorn University for initiating a generous support of the gel electrophoresis equipment.

Finally, I am very appreciative of my family and my good friends whose names are not mentioned here for their love, assistance and encouragement and social support throughout my study period.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS AND SYMBOLS

δ	chemical shift
μL	microliter
μmol	micromole
aq	aqueous
c	concentration
C	cytosine
calcd	calculated
$CDCl_3$	deuterated chloroform
d	doublet
D_2O	deuterium oxide
DCM	dichloromethane
DMAP	4-dimethylaminopyridine
DMF	N,N′-dimethylformamide
DMSO-d_6	deuterated dimethylsulfoxide
DNA	deoxyribonucleic acid
g	gram
G	guanine
h	hour
J	coupling constant
M	multiplet
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
MeOH	methanol
mg	milligram
MHz	megahertz
min	minute
mL	milliliter
mM	millimolar
mmol	millimole
mp.	melting point
MS	mass spectrometry
NMR	nuclear magnetic resonance

°C	celcius degress
ppm	part per million
s	singlet
t	triplet
TLC	thin layer chromatography
T_m	melting temperature
Ts	*p*-toluenesulfonyl (=tosyl)
UV	ultraviolet

CHAPTER I

INTRODUCTION

1. Introduction

Rotenoid [Fig. 1.1a], a natural product extracted from medicinal plants in tropical countries, has been used by native fisherman as a poisons to stupefy fish, e.g. the use of *Lonchocarpus* species by South American Indians [1]. Even though the constituents were unknown, ground root preparations from another rotenoid plant, *Derris elliptica* were used as garden insecticides in the East Indies [2], and are still commercially available today as "Derris Powder". Its status as a botanical insecticide and its high potency for control of some pests which have become resistant to some pyrethroids has led to continued interest [3, 4].

One of the interesting activities of rotenoid compounds is anticancer activity. For example, 6-deoxyclitoriacetal from the roots of *Clitoria macrophylla* showed strong cytotoxic activity against culture P388 lymphocytic leukemia cell [5, 6]. Rotenoids from *Amorpha fruticosa* are found to be inhibitors of human cancer cell line [7-9]. In Thailand, the rotenoid compound, 6-deoxyclitoriacetal [Fig. 1.1b], was isolated from the dried roots of *Stemona collinsae* Craib [5-6]. It has been known to have cytotoxic activity against various types of human carcinoma such as breast carcinoma (BT474), lung carcinoma (CHAGO), hepato carcinoma (HEP-G2) gastric carcinoma (KATO3), and colon carcinoma (SW620) at IC_{50} 0.2, 0.9, 0.1, 0.3, and 0.1 μg/ml, respectively [10].

Topoisomerase II (Top II) is an enzyme which regulated the supercoiling of DNA and plays important roles in replication, translation, recombination, and segregation. This enzyme is the primary target of variety of many anticancer drugs such as epipodophyllotoxins, antrapyrozles, acridines, ellipticines, and anthracyclines. The anthracycline drugs such as doxorubicin (Fig. 1.1c) and daunomycin (Fig. 1.1e) are widely used anticancer agents. The major mechanisms of the anthracyclin antibiotics are noncovalent DNA interaction, formation of covalent DNA adults, free radical effects, and topoisomerase II inhibition. In cancer cells, the levels of Top II are associated with higher with rates of replication of tumor cells. These drugs interfere

the normal functioning of the enzyme by trapping with Top II-DNA complex which is then stabilized as Top II-DNA-drug complex. They cause accumulation of Top II cleavage complex by inhibition DNA relegation. However, the time of drugs in this complex is very important for their efficacy as anticancer agents. Thus, the compounds that irreversibly trap the Top II-DNA complex are likely to have the greatest cytotoxic potency and perhaps more selective for cancer cells [11]. In addition, natural compounds such as Psorospermin (Fig. 1.1d) isolated from the roots and stem bark of the African plant *Psorospermum febrifugum*, has been shown to intercalate into the DNA helix and to covalently modify guanine at the N-7 position in the major groove through an epoxide-mediated electrophilic attack [12, 13].

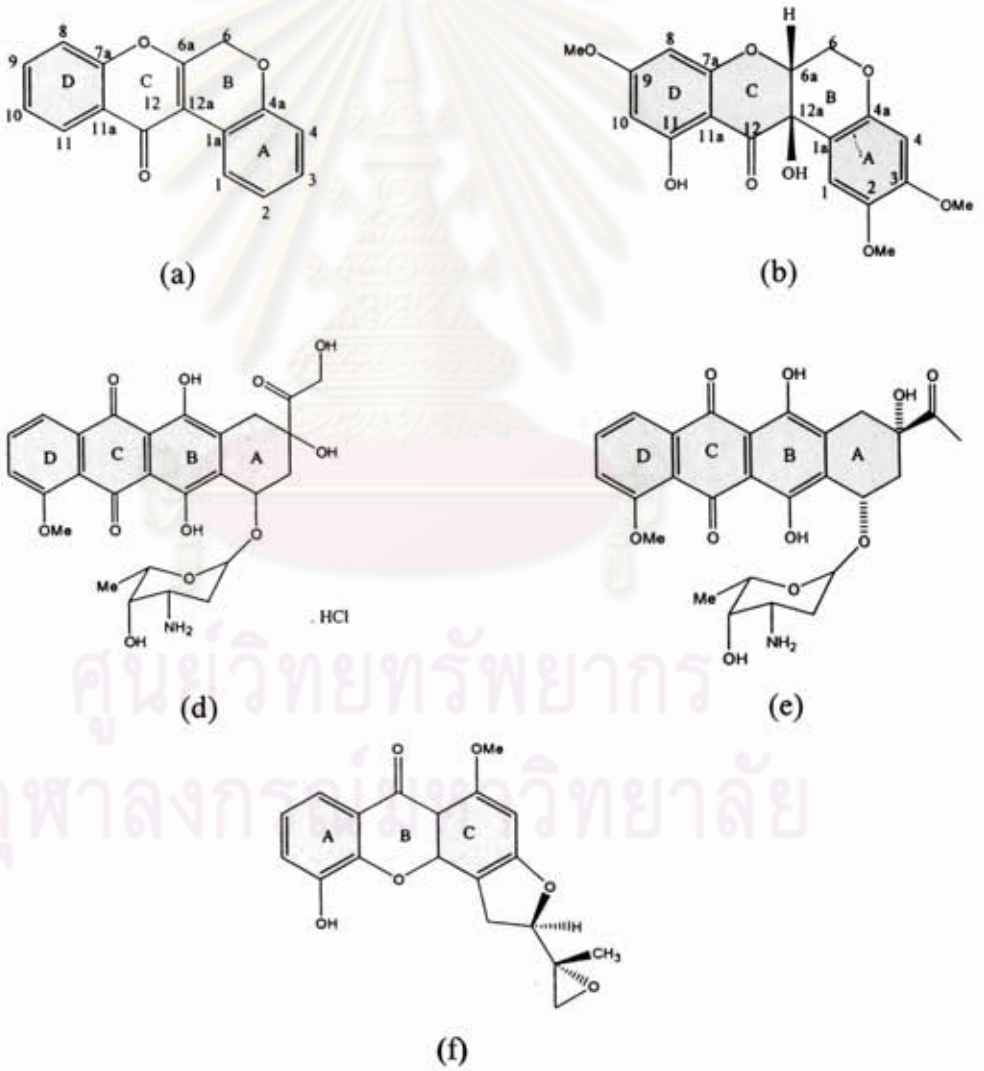


Fig. 1.1 Chemical structures of (a) rotenoid structure, (b) 6-deoxyclitoriacetal (c) doxorubicin HCl, (d) daunomycin and (e) psorospermin

In this research, we hope to define the cytotoxic and topoisomerase II inhibitory activities of the rotenoids by synthesizing and evaluating a systematic set of analogues which have specific modifications based on the rotenoid core structure [Fig 1.1 b]. We therefore wised to investigate the role of hydroxyl group at C11-OH on cytotoxicity by preparing a set of hydroxyl substituted core structure of 6-deoxyclitoriacetal based on the hypothesis that hydroxyl group at C-11 position in the D-ring may play an important role in binding with DNA and hence deform the DNA double strand, subsequently inhibits the proliferation of cells.

In this research, we aim to modify the structure of 6-deoxyclitoriacetal by using epoxidation reaction with epichlorohydrin, and then ring-opened with nucleophiles, morpholine and benzylamine compounds. The cytotoxic and topoisomerase II inhibitory activities were evaluated for all compounds using doxorubicin and etoposide as references. Furthermore, the most potent topoisomerase II inhibitor obtained from the rotenoid derivatives will subject to increase water solubility in order to enhance bioavailability and able to apply in pharmaceutical field. This can be performed by converting the free base to as acid addition salt.

2. Objectives of this research

To synthesis 6-deoxyclitoriacetal and its derivative

To evaluate the cytotoxic activity of 6-deoxyclitoriacetal and its derivatives.

To enhance the water solubility of the synthesized compound by converting to be an acid addition salt.

To investigate the potency of 6-deoxyclitoriacetal derivatives on inhibiting of topoisomerase II.

CHAPTER II

BACKGROUND AND LITERATURE REVIEWS

2. Background and literature reviews

2.1 Cancer

Cancer is a disease caused by unregulated proliferation of cells (Fig. 2.1). According to World Health Organization (WHO), it is causing 7 million deaths every year or 12.5% of deaths worldwide. In addition, it is the second major cause of death following cardiovascular deseases [15]. The total number of cases of cancer is predicted to increase in between 2000 and 2020 by 73% in the developing countries and by 27% in the developed countries [12]. In Thailand, the rate of people dying from cancer is still increasing every year and it is the first leading cause of death [14].

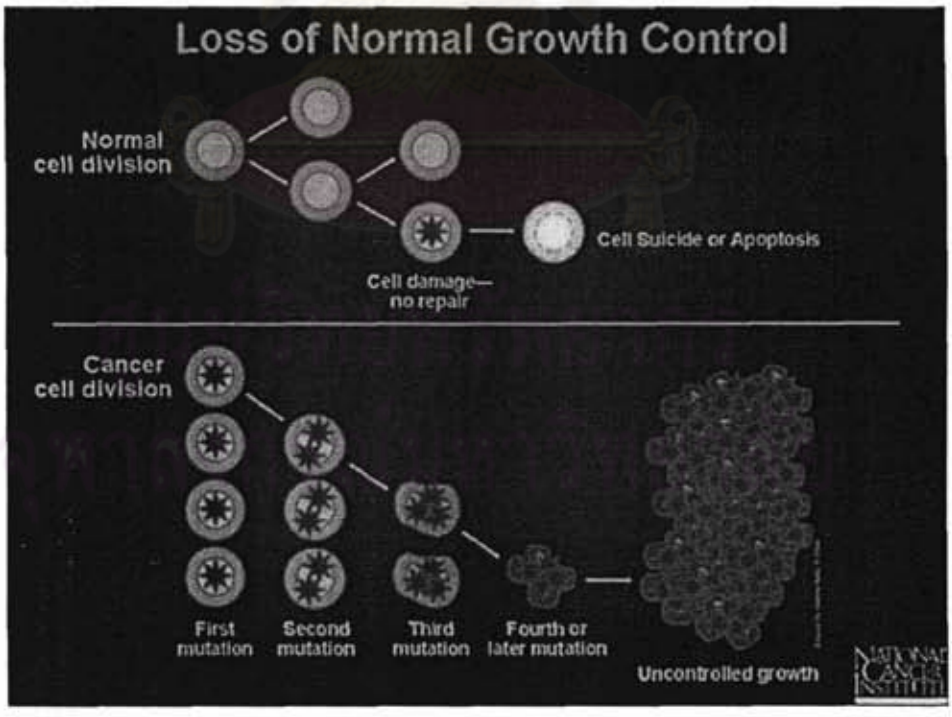


Fig 2.1 Cell division of normal and cancer cell

2.2 Natural product

Natural products play a major role in anticancer drug discovery as a unique source of original structure which can provide models in the future drug design. In addition, it has long been an important source of medicinal agents and have been used in many countries such as China, India and Thailand. Many anticancer drugs have been isolated or derived from natural sources [15]. The well-known compound is vinca alkaloid (vinblastin and vincristine) drug which is isolated from the Madagascar periwinkle, *Catharanthus roseus* (Linn.) [16]. Some anticancer drugs, such as etoposide and tenopotide are semi-synthesis derivatives of epipodophyllotoxin [17].

Flavonoids are natural products which have very broad bioactivities such as antibacterial, antimalarial and antifungal activities. Some of flavonoid compounds have been used as supplement medicines or vitamins for a long time. However, many flavonoid compounds have been reported to have antitumor activities and can inhibit DNA topoisomerase enzyme, for example, quercetin (Fig 2.2a) and rutin (Fig 2.2b).

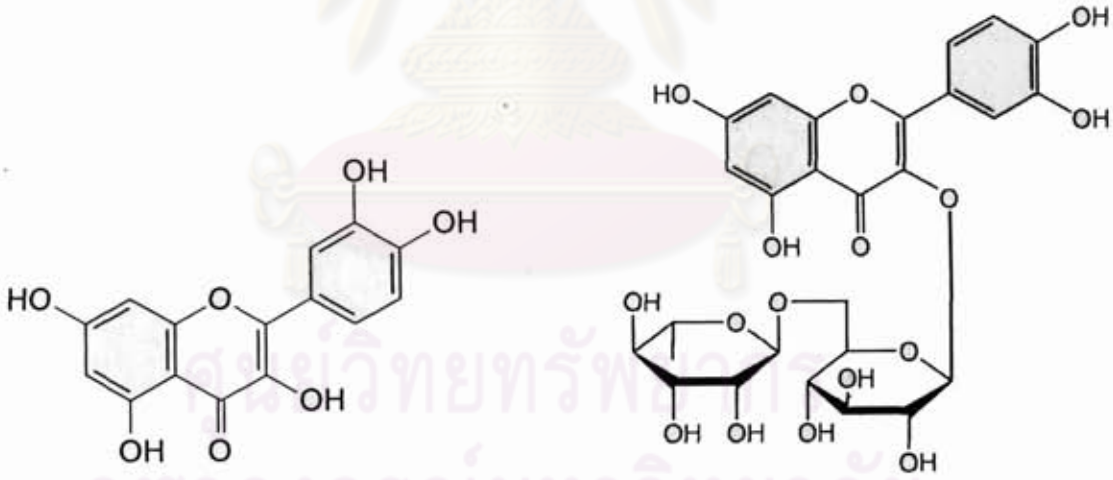


Fig 2.2 The structures of flavonoid compounds (a) Quercetin and (b) Rutin

Coumarins is also natural compound which is found in a variety of plant source. Coumarin has important effects in plant biochemistry and physiology as anti-inflamatory, antioxidant, antiallergic, antiviral, and anticarcinogenic activities [18]. This compound shows topoisomerases II inhibition [19].

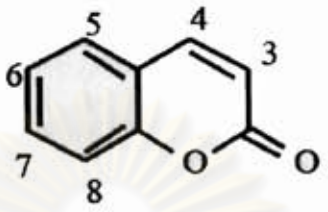


Fig 2.3 Structure of coumarin

As can be seen from the many successful discoveries of commercial anticancer drugs from natural products, this thesis focus on explorting new approaches for natural product discovery based on rotinoid compound.

2.3 Stemonaceae

Stemonacese is classified into three genera including *Stemona, Croomia,* and *Stichoneuron*. *Stemona* is the largest genus which has about 25 species. *Stemona* species have long been used in China, Japan, and Thailand for various medicinal and biological properties. Especially extracts from the fleshy tuberous roots are still used to treat respiratory disorders. In addition, *S. collinsar* Craib, the extract showed very high insect toxicity. In Thailand, it has been used as a traditional Thai medicine with a wide range of applications such as liver cancer, skin infections, and anti-parasitic agent [20-23].

2.4 Rotenoid compound

Rotenoid compounds are a class of plant secondary metabolites that derived from isoflavones. These compounds have long been used as insecticides and fish poison in South America and East Africa [20].

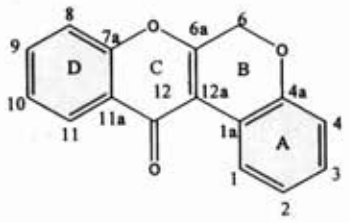


Fig 2.4 General structure of rotenoid

6-Doxyclitoriacetal [Fig 1.1b] is a rotenoid compound from the roots of *Clitoria macrophylla* [4]. It has strong cytotoxic activity against culture P388 lymphocytic leukemia cell [6].

In Thailand, 6-deoxyclitoriacetal was isolated from the dried roots of *Stemona collinsae* Craib. It has been known to have cytotoxic activity against various types of human carcinoma such as breast carcinoma (BT474), lung carcinoma (CHAGO), hepatocarcinoma (HEP-G2) gastric carcinoma (KATO3), and colon carcinoma (SW620) at IC_{50} 0.2, 0.9, 0.1, 0.3, and 0.1 μg/ml, respectively [27].

2.5 Topoisomerase

Topisomerases are nuclear enzymes that catalyze changes in the topological state of DNA by breaking and rejoining of DNA stands. Theses enzymes have important roles in DNA metabolism such as replication, recombination, transcription, and chromosome condensation. In human, DNA topoisomerase are classified into two types; type I and type II. All physiological functions of DNA depend on its tertiary configuration. DNA is a double helix and the supercoiled DNA which require relaxation before replication and translation. A variety of antitumor agents currently used in chemotheraphy are known to inhibit DNA toposiomerase.

DNA topoisomerase II is the target of various anti-tumor agents such as doxorubicin, amsacrine, and etoposide which stabilize the 'cleavable complex' between the enzyme and DNA. For example, psorospermin (Fig. 2.5) is a natural product isolated from the roots and stem bark of the African plant *Psorospermum febrifugum*. It can intercalate into the DNA helix and covalently bond to guanine at the N-7 position in the major groove through an epoxide-mediated electrophilic attack (12, 13).

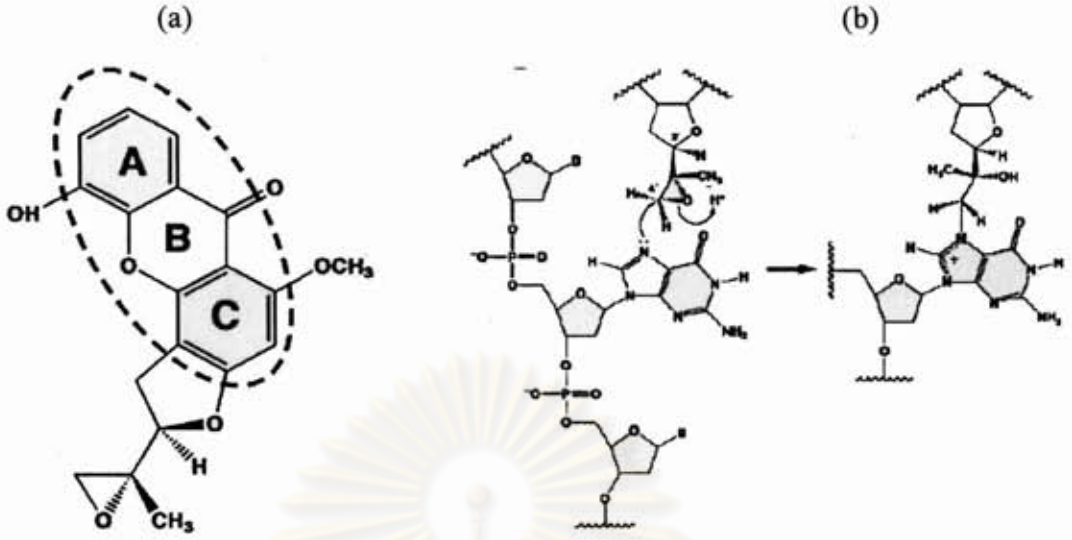


Fig 2.5 (a) Structures of psorospermin (b) Summary of the proposed mechanism of covalent modification of DNA by psorospermin to form the psorospermin (N7 guanine)-DNA adduct. The drug molecule intercalates the DNA molecule to position the reactive epoxide into the major groove to perform site-directed electrophilic addition on N7 of guanine.

2.6 Epoxide

The basic structure of an epoxide contains an oxygen atom attached to two adjacent carbon atom of a hydrocarbon [Fig. 2.6]. It is particularly useful in many industries. The example of the bioactivity substances bearing the epoxide are as follows;

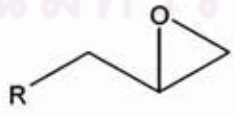
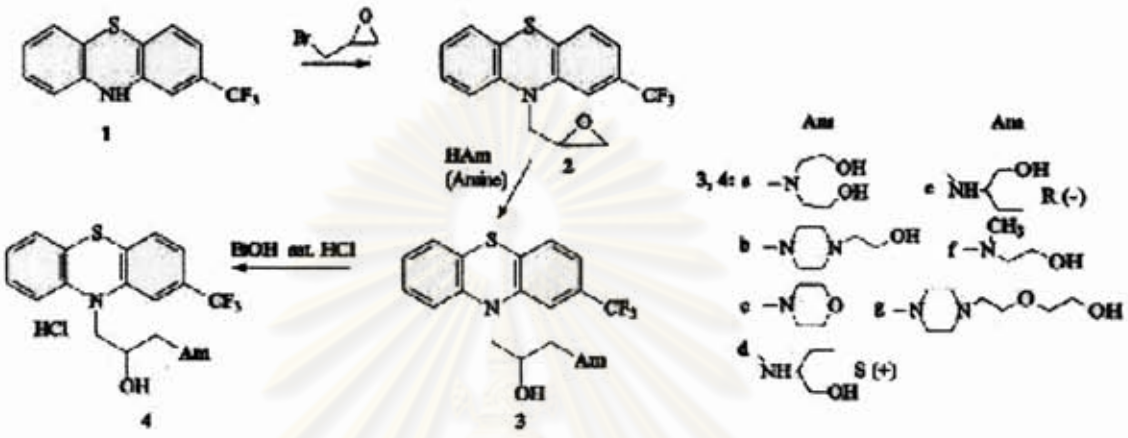


Fig 2.6 Structure of epoxide

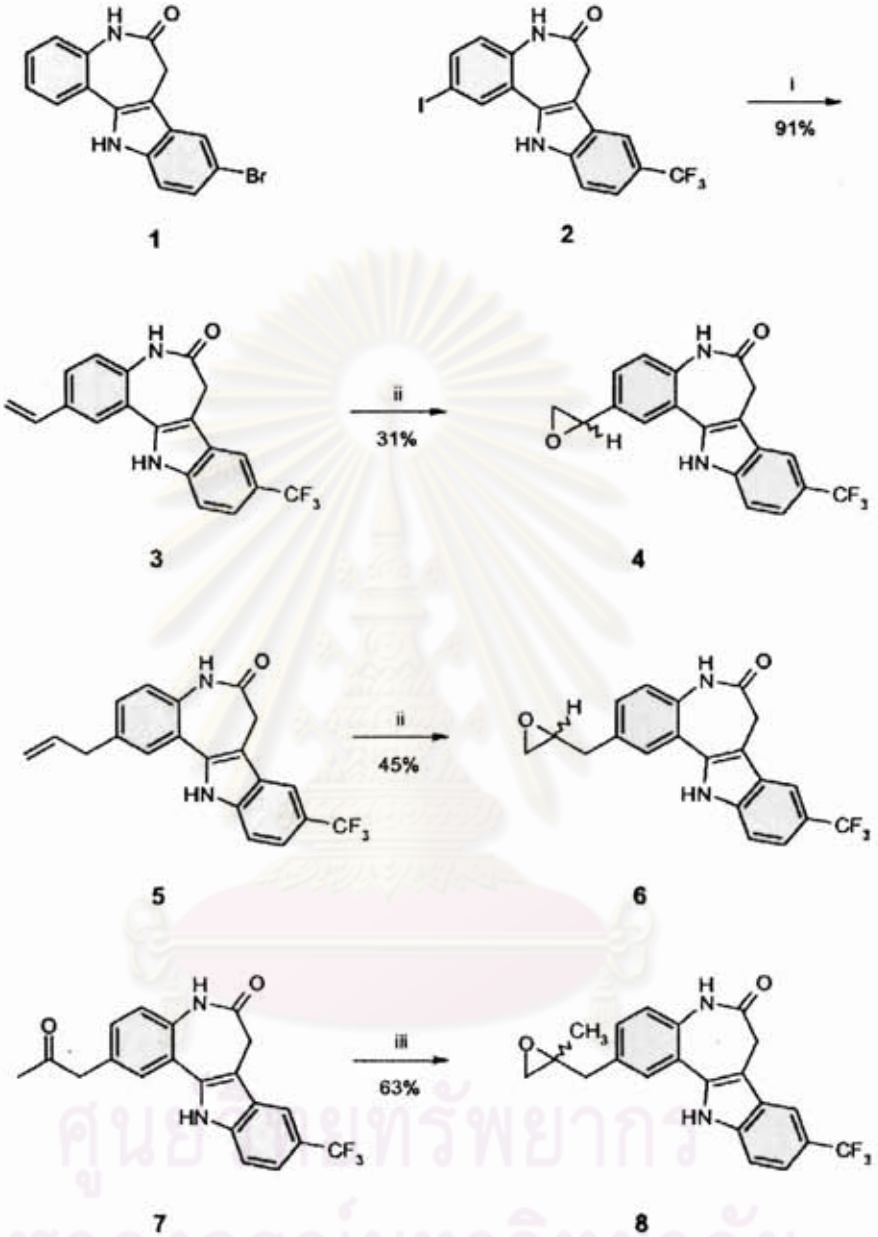
In 2003, Gasiorowski *et al.* [30] prepared the modification of fluphenazine, **1** (Scheme 2.1) in order to decrease its psychotropic effects. The derivatives were

synthesized based on the *in silico* study using hydroxyethylamine, hydroxyethylpiperazine or morpholine as new candidates for chemopreventive drugs as shown in scheme 2.1. They suggested that the more hydrophilic nature of the analogues will effect on a lower penetration through the blood-brain barrier.



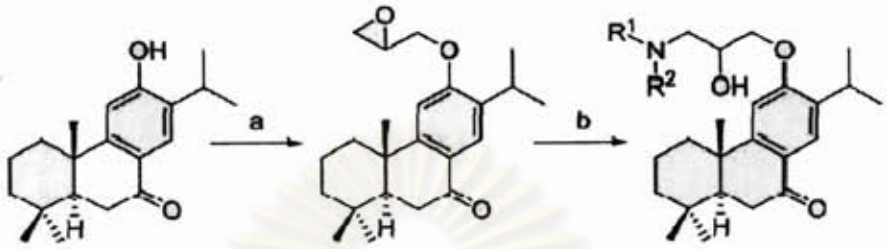
Scheme 2.1 The synthesis route of the preparation of the fluphenazine analogues.

In 2005, Xie *et al.* [31] synthesized the structure-based design of paullone (**1**) derivatives with side chains bearing epoxide groups in order to increase the anticancer activity. Compounds with epoxide groups were recently reported to exhibit antitumor activity in nude mice bearing protrate tumor xenografts. The reactions of paullone derivatives are shown in scheme 2.2.



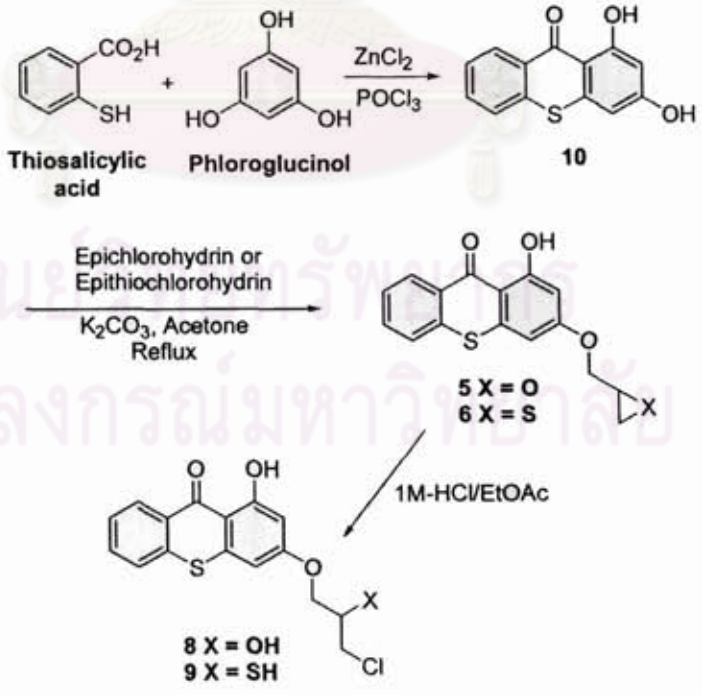
Scheme 2.2 (i) Tributylvinyltin, 10 mol% $PdCl_2(PPh_3)_2$, DMF, 60°C,N_2; (ii) H_2O_2 (35%),MeCN, K_2CO_3,MeOH, rt. (iii) $((CH3)_{3S}O)$+ I–, NaH, DMSO, N_2, 50°C.

In 2006, *Cordava et al.* [32] reported that a series of β-amino alcohol analogues of sugiol were synthesized. The reaction scheme of sugiol derivatives are shown in Fig. 2.3. They suggested that the β-amino alcohol fragments play significant role in the antiproliferative activity of sugiol.



Scheme 2.3 Reagents and conditions: (a) i. NaH, DMF, 0°C; ii. (±)-epibromohydrin, 55°C, 20 h, 93%; (b) R_1R_2NH, MeOH, 70 °C, 20 h.

In 2007, Woo *et al.* [40] reported that some epoxypropoxy xanthones were synthesized. There has been found that the epoxide ring is an important part for the cytotoxicity. The reaction scheme of xanthone derivatives are shown in Fig. 2.4.



Scheme 2.4. Synthetic method for xanthone derivatives.

In addition, there have been reported that the compounds having functional groups such as morpholine, aminomorpholine do not only enhance anticancer activity, but also decrease a side effect of parent compound. For example, gefitinib **A** [Fig. 2.7] is a drug that is used to treat several types of cancer [33]. Doxorubicin morpholine **B** [Fig. 2.8] is also used for caner treatment, also shows good cytotoxicity than doxorubicin [34].

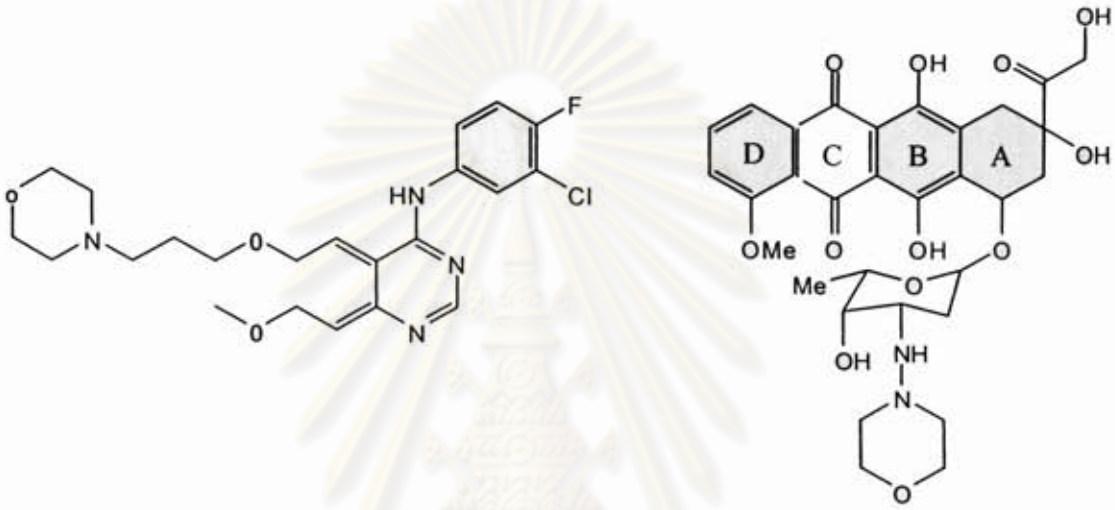
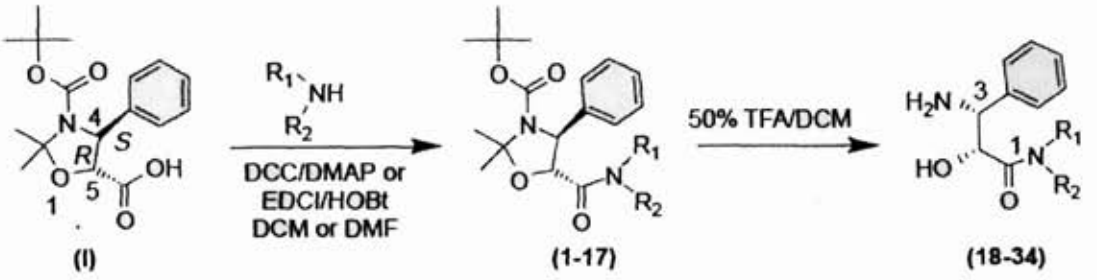


Fig 2.7 Structure of gefitinib **A** and morpholine antracycline **B**

In 2008, Kumar *et al.* [35]. reported that oxazolinding-5-carboxamide derivatives were synthesized and evaluated for their anticancer activities. The results showed that molecules which can be used as a new anticancer pharmacophore should be the molecule having *N*-aryl or *N*-benzyl. The reaction scheme is shown in Scheme 2.5.



Scheme 2.5 Synthesis of tested compounds

Therefore, in this work, we aim to synthesize novel 6-deoxyclitoacetal derivatives containing an epoxy group, a morpholine group or a benzyl group to enhance cytotoxic activity.

CHAPTER III

EXPERIMENTAL

3. Experimental

3.1 General

3.1.1 Materials

All organic solvents were reagent grade. All of the chemical reagents were purchased from Aldrich Chem. Co. Deionized double distilled water and analytical grade reagents were used throughout. All other reagents were obtained form Sigma. Etoposide (positive control drug for topoisomerase II study) and plasmid PBR 322 DNA were purchased from Sigma. Topoisomerase II was purchased from Amersham (UK). *Escherchai coli* strains HB101 from Botany faculty of science, Chulalongkorn University. Nutrient agar (NA) medium was 0.5% peptone, 0.5% NaCl, 0.3% beef extract, 2% agar (pH 7± 0.2), Ampicilin trihydrate (TP drug).

3.1.2 Analytical instruments

3.1.2.1 NMR studies

For the characterization of 6-deoxyclitoriacetal derivatives, 5 mg of compounds were prepared by dissolved in deuterated chloroform ($CDCl_3$). The solution was added to the NMR tube. ^1H and ^{13}C NMR spectra were recorded using Varian Mercury NMR spectrometer openated at 400 MHz for ^1H and 100 MHz for ^{13}C nuclei (Varian Company, CA, USA). The chemical shifts were referenced to the signal of sodium 2,2,-dimethyl-2-silapentan-5-sulfonate (DSS) with resonance at δ_H 0.00 and reported in parts per million (ppm) relative to the residual $CHCl_3$ peak (7.26 ppm for ^1H-NMR and 77.0 ppm for ^{13}C-NMR. The coupling constant (*J*) are reported

in Hertz (Hz) and then the ^{1}H and ^{13}C data were processed with the MESTREC software.

3.1.2.2 Mass spectrometry

Mass spectra were recorded on Mass spectrometer; Waters Micromass Quattamicro API ESCi (Water, MA, USA.). [Samples were dissolved in ethyl acetate and direct injected into Mass Spectrometer in 50 μL (**1-14**), and mass spectra of the 6-deoxyclitoriacetal derivatives were recorded by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS).

3.2 Extraction of 6-deoxyclitoriacetal from the dried roots of *Stemona collinsae* Craib.

3.2.1 The dried root of *Stemona collinsae* Craib (12 kg) was extracted from CH_2Cl_2.

3.2.2 The combined CH_2Cl_2 extract were concentrated to dryness under reduced pressure to obtain a brown crude extract.

3.2.3 The crude extract was isolated on silica gel column using CH_2Cl_2: hexane: EtOAc = 2:2:1 (v/v) as an eluent. Each fraction was analyzed by TLC visualize using UV light.

3.2.4 The fraction showing similar spots were obtained from the combined and then concentrated to dryness. 6-deoxyclitoriracetal was obtained from the elution of CH_2Cl_2: hexane: EtOAc = 2:2:1 (v/v)

3.2.5 Re-crystallization 6-deoxyclitoriracetal with CH_2Cl_2: MeOH = 1:2 (v/v) to obtain colorless needles (3.2 g).

3.2.6 The structure of 6-deoxyclitoriracetal was characterized by NMR spectroscopic techniques [10].

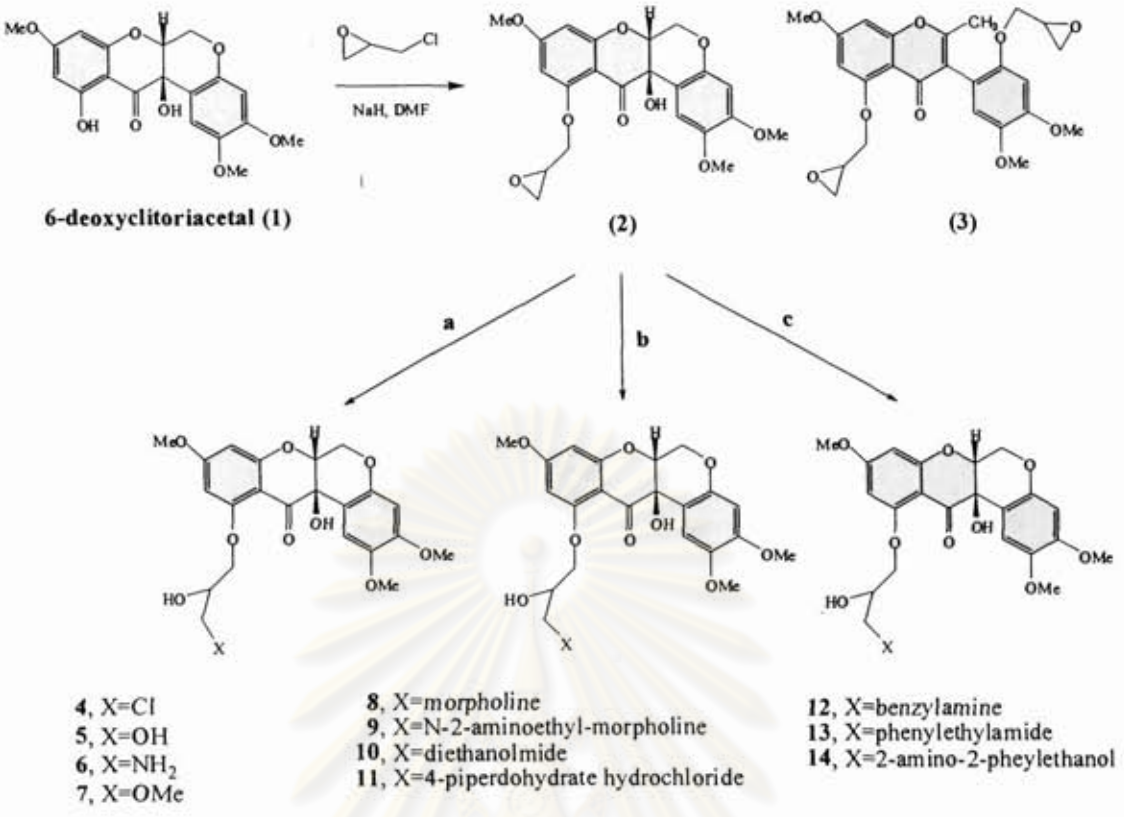
3.3 Synthesis of 6-deoxyclitoriacetal derivatives

6-Deoxyclitoriacetal has been reported that showed good anticancer activity. In 2007, Chimsook *et al* [36] reported that the modifications of 6-deoxyclitoriacetal

with amino acid, aromatic carboxylic acid and pyrimidine bases at C11-OH positions can enhance the anticancer activity by improving the binding ability or stabilizing intercalation with DNA, and the 6-deoxyclitoriacetal derivatives also showed the cytotoxicity better than parent compound [26]. Therefore, in this research, the 6-deoxyclitoriacetal is a model anticancer substance being used to study. 6-Deoxyclitoriacetal derivatives were synthesized to enhance cytotoxic activity and to study the effect of functional groups on cytotoxicity by preparing the epoxide derivatives and ring opening the epoxide using nucleophiles, morpholine and benzylamine compounds. After that, the compound of the best cytotoxicity was synthesized as acid addition salts in order to increase solubility and bioavariability.

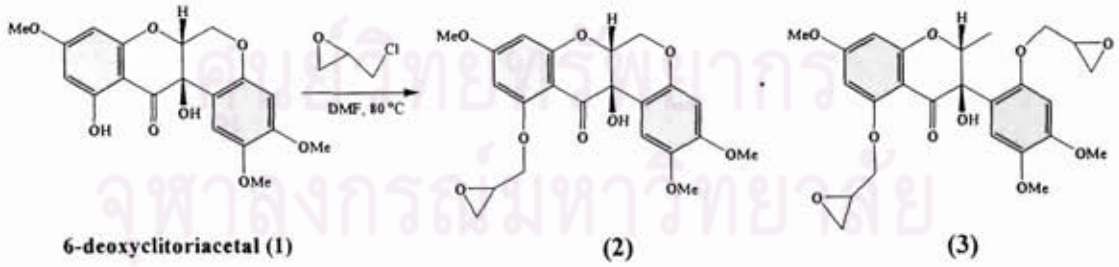
3.3.1 Epoxidation of 6-deoxyclitoriacetal

In order to study the effect of functional group of epoxide ring on cytotoxicity, the 6-deoxyclitoriacetal (**1**) was modified with epoxide group in present of dimethylformamide (DMF) as a solvent and anhydrous sodium hydride as a strong base to deprotonate at C11-OH positions. Each compound was refered to **2** to **3**. The reaction scheme of the epoxidation 6-deoxyclitoriacetal is show in scheme **3.1**.



Scheme 3.1 Synthesis of 6-deoxyclitoriacetal derivatives

Synthesis of 12a-Hydroxy-2,3,9-trimethoxy-11-oxiranylmethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one (**1**) and 3-(4,5-Dimethoxy-2-oxiranylmethoxy-phenyl)-3-hydroxy-7-methoxy-2-methyl-5-oxiranylmethoxy-chroman-4-one (**2**)



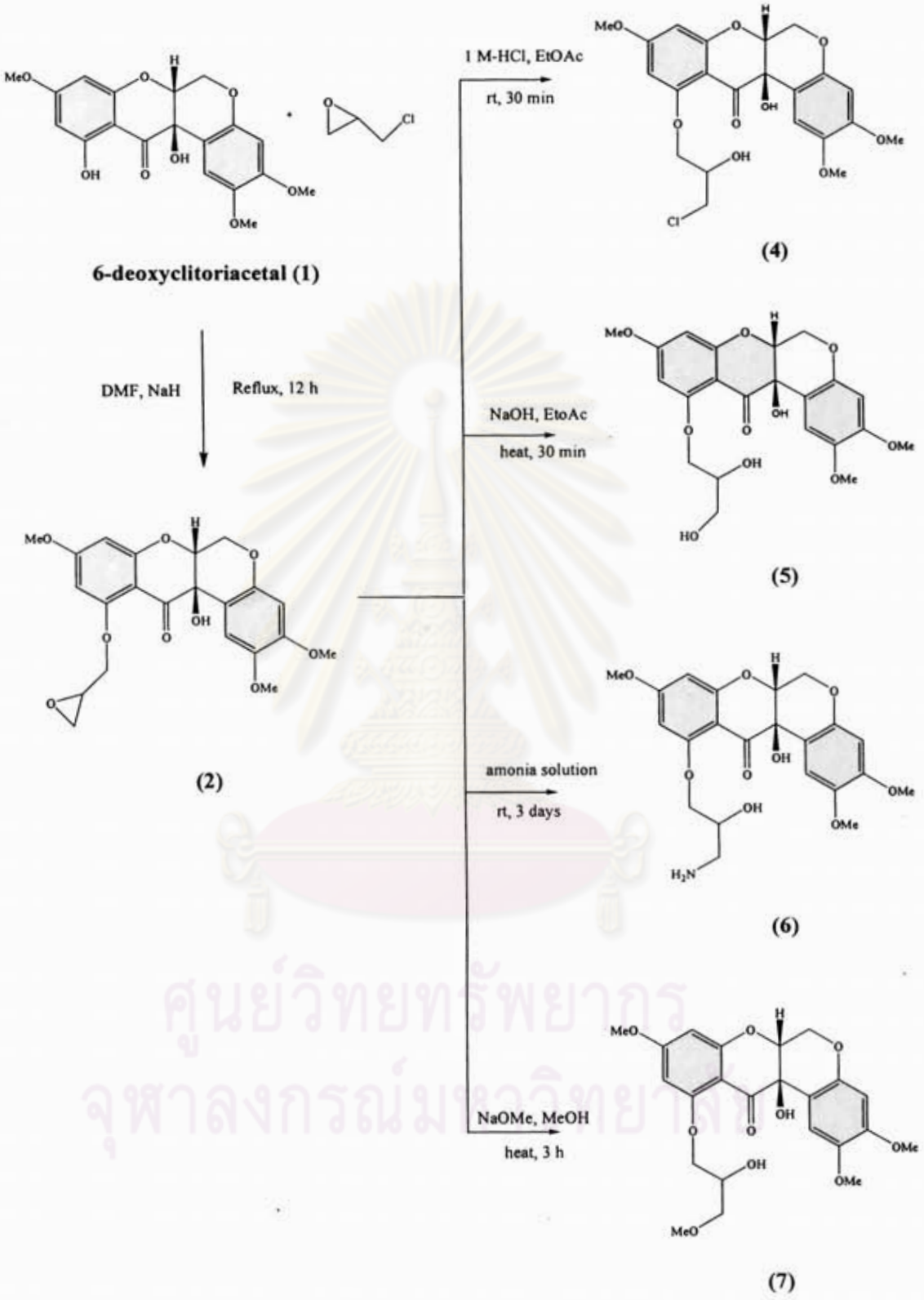
6-Deoxyclitoriracetal, **1** (0.05 g, 0.13 mmol) was dissolved in aqueous DMF (6 ml) under nitrogen condition and added NaH (0.03 g, 1.25 mmol). The reaction mixture was refluxed, added epichlorohydrin (10 µl, 0.13 mmol) and stired for 6 hours. After cooling down to room temperature, the excess of epichlorohydrin was

successfully washed with water, brine and extracted with dichloromethane. The combined organic layers were dried over anhydrous sodium sulfate, after that solvent was evaporated to dryness. The resulting crude product was dissolved in dichloromethane and purified by silica gel column chromatography using CH_2Cl_2: Hexane: EtOAc (2:1:2) as eluent to give **2** and **3** as a yellow solid compound (40% yield and 10% yield) [33].

3.3.2 The nucleophilic ring opening of epoxide derivatives

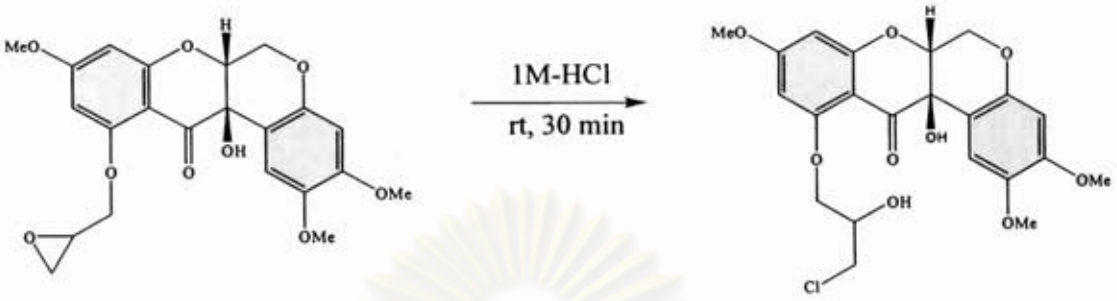
In order to study the effect of functional group of nucleophilic group and the length of side chain of necleophilic group on cytotoxicity, the 6-deoxyclitoriacetal (**1**) was modified with epoxide groups (OH, Cl, NH_2, and OMe). The 6-deoxyclitoriacetal was added with epoxide ring (**2**) before opening it with each nucleophiclic groups. Each compound was refered to **4** to **7**

The reaction scheme of nucleophilic substituented analogues is shown in scheme **3.2**.



Scheme 3.2 Synthesis of 6-deoxyclitoriacetal-neclitophilic analogues

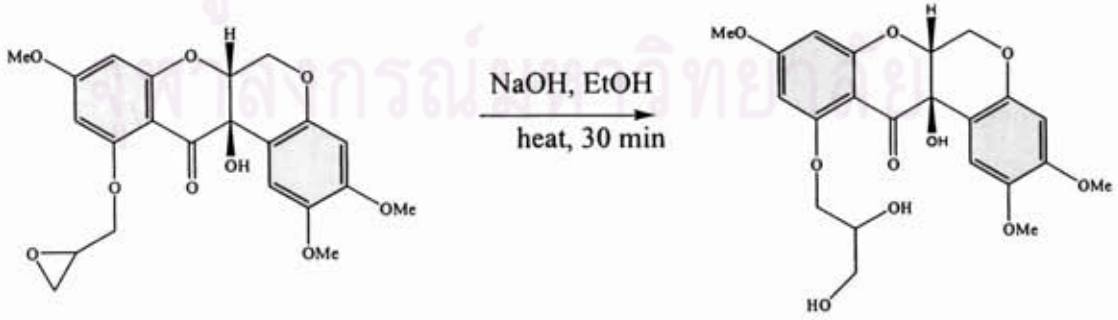
3.3.2.1 Synthesis of 11-(3-Chloro-2-hydroxy-propoxy)-12a-hydroxy-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one (**4**)



(2) **(4)**

To the reaction mixture of **2** (20 mg, 0.046 mmol) in 3 ml of aqueous ethyl acetate was added 1M-HCl (3 ml) and this solution was stirred for 30 min at room temperature. The solvent was removed under reduced pressure and the residue was washed with water, organic layer was extracted with dichloromethane and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography with dichloromethane: ethyl acetate: dichloromethane (2:1:2, v/v) as elutent to give **4** as yellow solid compound (70% yield).

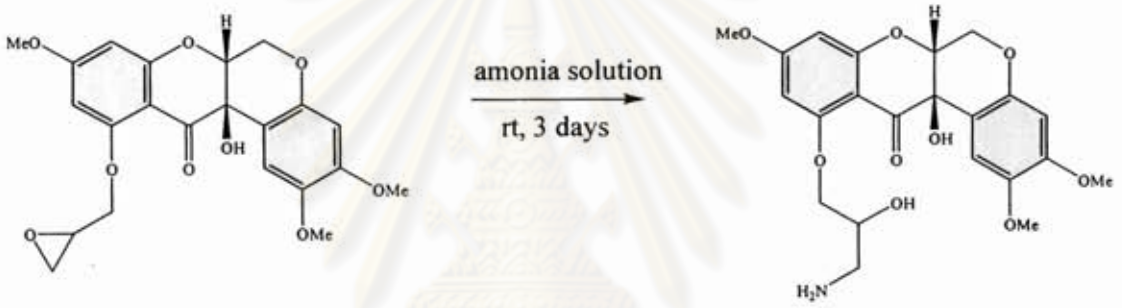
3.3.2.2 Synthesis of 11-(2,3-Dihydroxy-propoxy)-12a-hydroxy-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one (**5**)



(2) **(5)**

To the reaction mixture of **2** (20 mg, 0.045 mmol) in 3 ml of ethanol was added 1M NaOH. The mixture was refluxed at 80 °C and stirred for 30 min. The solvent was removed under reduced pressure and the residue was washed with water and organic layer was extracted with dichloromethane and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography with dichloromethane: ethyl acetate: dichloromethane (2:1:2, v/v) as eluent to give **5** as yellow solid compound (63% yield).

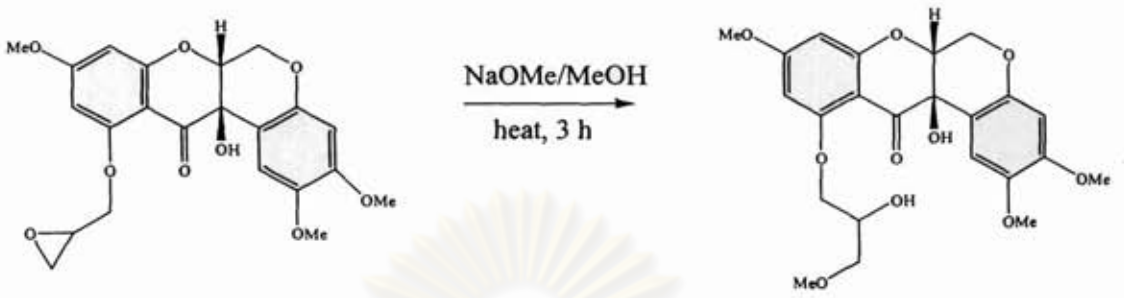
3.3.2.3 Synthesis of 11-(3-Amino-2-hydroxy-propoxy)-12a-hydroxy-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one (**6**)



(2) **(6)**

The reaction mixture of **2** (20 mg, 0.045 mmol) in 3 ml of ammonia solution was stirred at room temperate for 3 days. The solvent was removed under reduced pressure and the residue was washed with water, organic layer was extracted with dichloromethane and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography with dichloromethane: ethyl acetate: dichloromethane (2:1:2, v/v) as elutent to give **6** as yellow solid compound (45% yield).

3.3.2.4 Synthesis of 12a-Hydroxy-11-(2-hydroxy-3-methoxy-propoxy)-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one (7)



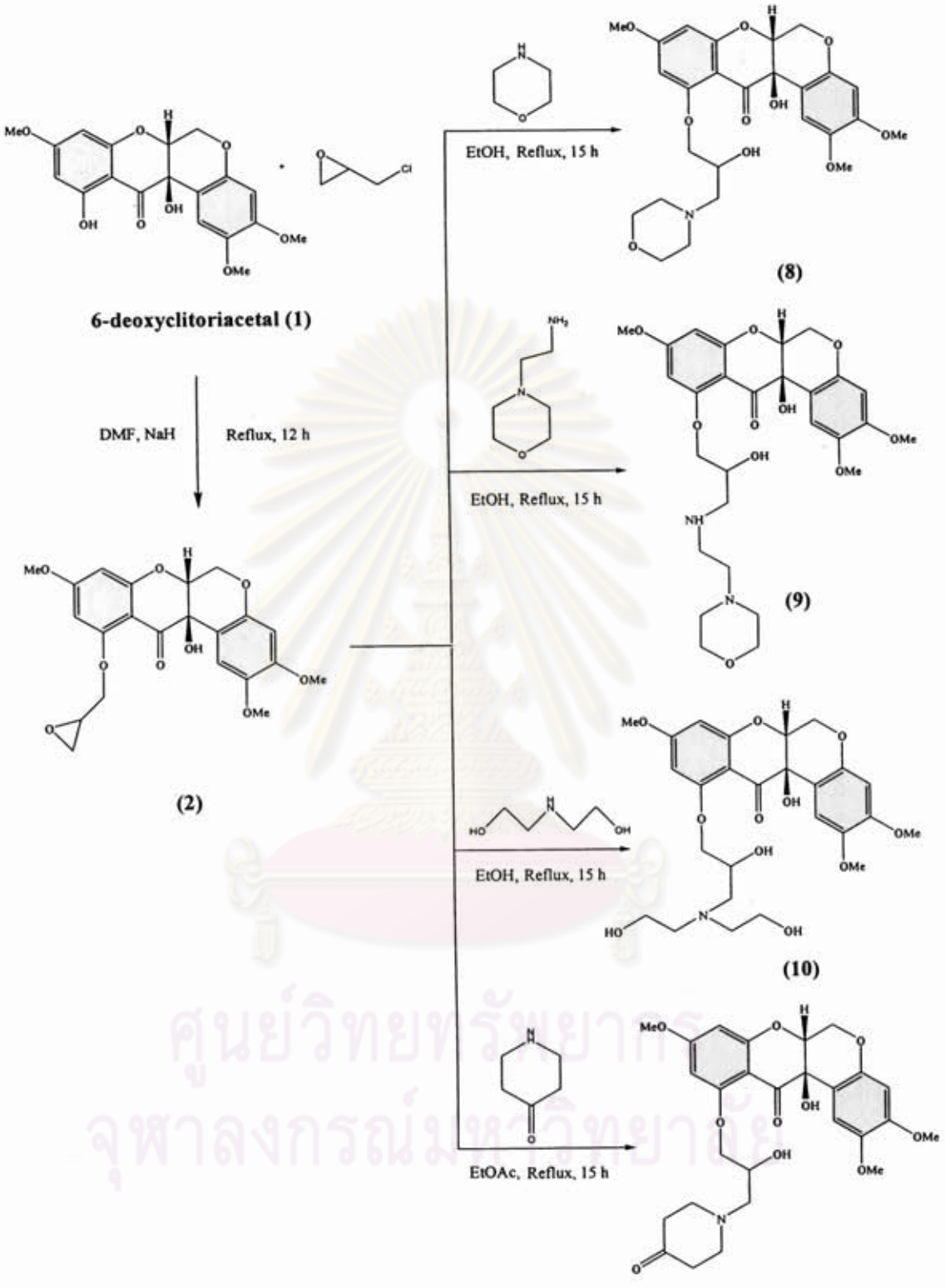
(2) (7)

To the reaction mixture of **2** (20 mg, 0.045 mmol) in 3 ml of NaOMe/MeOH was refluxed and stirred for 3 h at 80 °C. The solvent was removed under reduced pressure and the residue was washed with water and organic layer was extracted with dichloromethane and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography with dichloromethane: ethyl acetate: dichloromethane (2:1:2, v/v) as elutent to give **7** as yellow solid compound (78% yield).

3.3.3 Preparation of 6-deoxyclitoriacetal-morpholine analogues

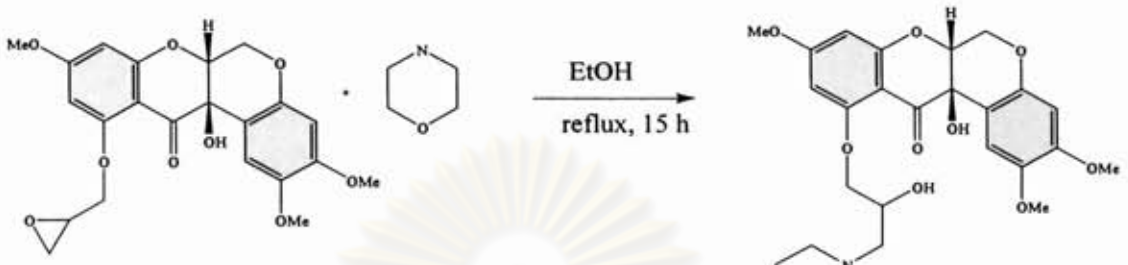
In order to study the effect of functional group of morpholine compound and the length of side chain of morpholine group on cytotoxicity, four morpholine analogues were chosen to synthesize with 6-doxyclitoriacetal namely morpholine, N-2-aminoethyl-morpholine, diethanolmide, and piperidine. The 6-deoxyclitoriacetal was added with epoxide ring (**2**) before opening it with each morpholine analogues. Each compound was refered to **8** to **11**

The reaction scheme of 6-deoxyclitoriacetal-morpholine analogues is show in scheme **3** [30].



Scheme 3.3 Synthesis of 6-deoxyclitoriacetal-morpholine analogues

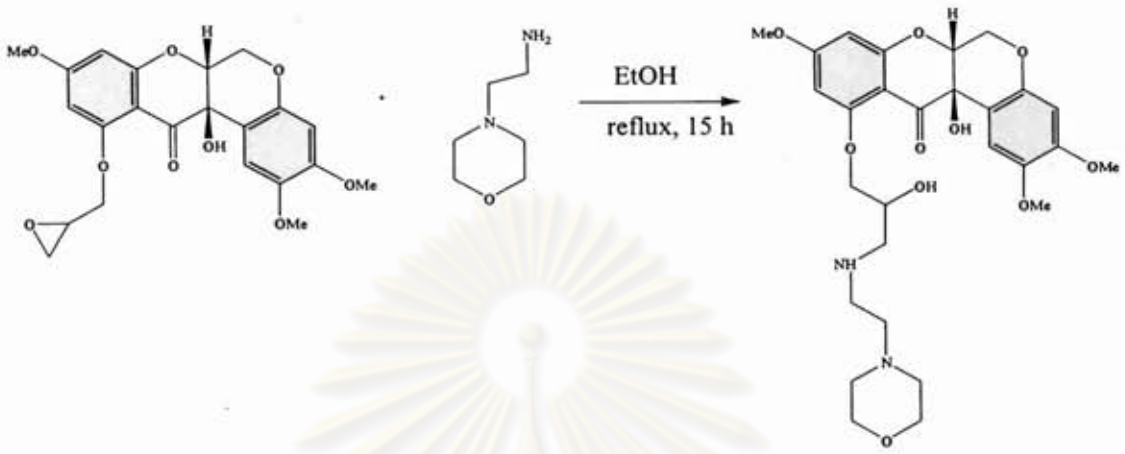
3.3.3.1 Synthesis of 12a-Hydroxy-11-(2-hydroxy-3-morpholin-4-yl-propoxy)-2, 3, 9- trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one (**8**)



(2) **Morpholine** **(8)**

To a solution of epoxide **2** (20 mg, 0.045 mmol) in 5 ml of Ethanol was added the morpholine (0.2 ml, 0.225 mmol). The reaction mixture was refluxed for 15 hours and then allowed to cool down to room temperature. The solvent was removed under pressure and the residue was washed with water and extracted organic layer with dichloromethane and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography with dichloromethane: ethyl acetate: dichloromethane (2:1:2, v/v) as elutent to give **8** as yellow solid compound (86% yield).

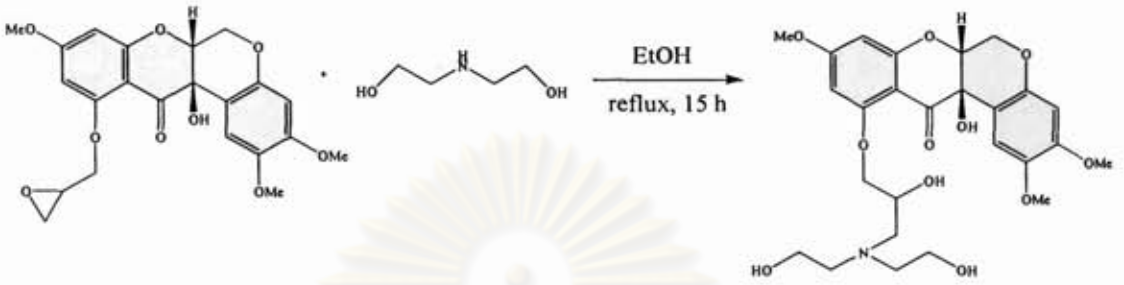
3.3.3.2 Synthesis of 12a-Hydroxy-11-[2-hydroxy-3-(2-morpholin-4-yl-ethylamino)-propoxy]-2, 3, 9-trimethoxy-6a, 12a-dihydro-6H-chromeno [3, 4-b]chromen-12-one (**9**)



(2) **N-2-aminoethyl-morpholine** **(9)**

To a solution of epoxide **2** (20 mg, 0.045mmol) in 5 ml of Ethanol was added the N-2-aminoethyl-morpholine (0.01 ml, 0.09 mmol). The reaction mixture was refluxed for 15 hour and then allowed to cool down to room temperature The reaction mixture was refluxed for 15 hours and then allowed to cool down to room temperature. The solvent was removed under pressure and the residue was washed with water and extracted organic layer with dichloromethane and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography with dichloromethane: ethyl acetate: dichloromethane (2:1:2, v/v) as elutent to give **9** as yellow solid compound (94% yield).

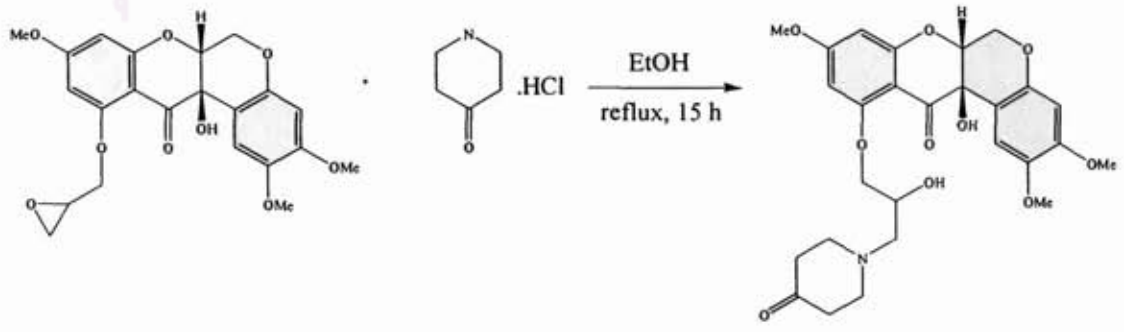
3.3.3.3 Synthesis of 12a-Hydroxy-11-[2-hydroxy-3-(2-hydroxy-1-hydroxymethyl-ethylamino)-propoxy]-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno [3,4-b]chromen-12-one (**10**)



(**2**) **Diethanolmide** (**10**)

To a solution of epoxide **2** (20 mg, 0.045 mmol) in 5 Ethanol was added the diethanolmide (0.2 ml, 10.4 mmol). The reaction mixture was refluxed for 15 hour and then allowed to cool down to room temperature. The reaction mixture was refluxed for 15 hours and then allowed to cool down to room temperature. The solvent was removed under pressure and the residue was washed with water and extracted organic layer with dichloromethane and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography with dichloromethane: ethyl acetate: dichloromethane (2:1:2, v/v) as elutent to give **10** as yellow solid compound (91% yield).

3.3.3.4 Synthesis of 1-[2-Hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a, 12,12a-tetrahydro-chromeno[3,4-b]chromen-11-yloxy)-propyl]-piperidin-4-one (**11**)



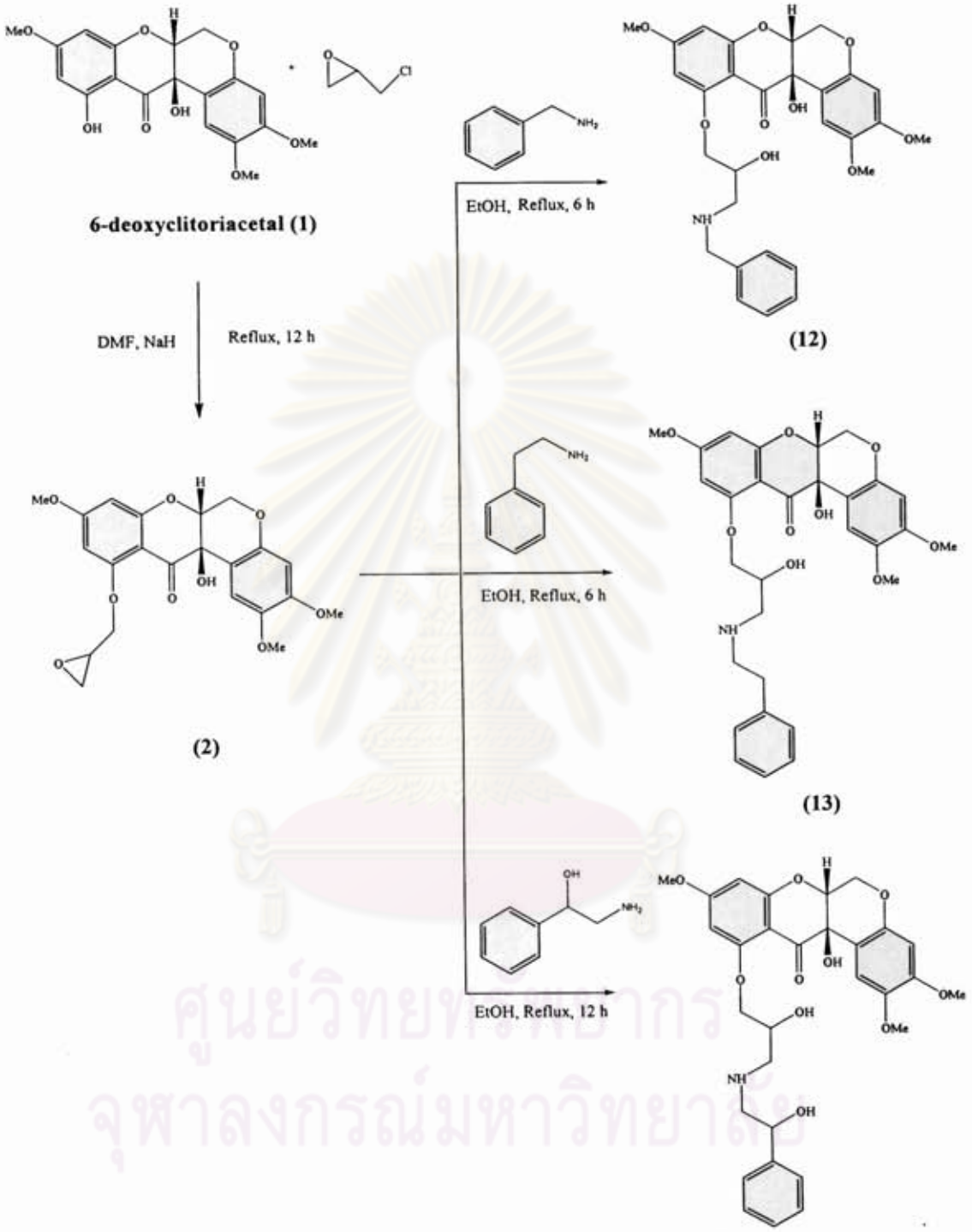
(**2**) **4-Piperidon hydrate hydrochlouide** (**11**)

To a solution of epoxide **2** (20 mg, 0.045 mmol) in 5 ml of ethyl acetate was added the 4-piperidon hydrate hydrochloride (0.015 ml, 0.09 mmol). The reaction mixture was refluxed for 15 hours and then allowed to cool down to room temperature. The reaction mixture was refluxed for 15 hour and then allowed to cool down to room temperature. The solvent was removed under pressure and the residue was washed with water and extracted organic layer with dichloromethane and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography with dichloromethane: ethyl acetate: dichloromethane (2:1:2, v/v) as elutent to give **11** as yellow solid compound (68% yield).

3.3.4 Preparation of 6-deoxyclitoriacetal-aromatic ring analogues

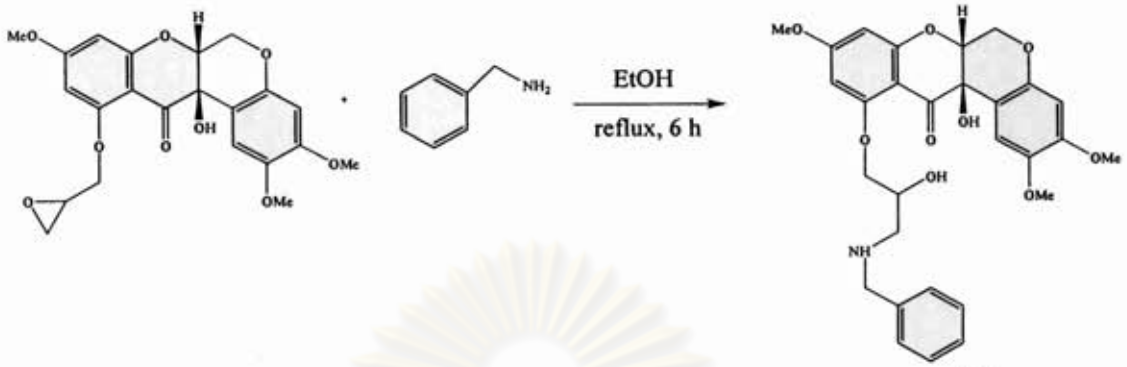
In order to study the effect of functional group of aromatic ring and the length of side chain of aromatic ring on cytotoxicity, three aromatic ring analogues were chosen to synthesize with 6-doxyclitoriacetal namely benzylamind, 2-phenylethylamine, and (*R*)-(-)-2-amino-2-phenylethanol. The 6-deoxyclitoriacetal was added with epoxide ring (**2**) before opening it with each aromatic ring analogues. Each compound was refered to **12** to **14**.

The reaction scheme of 6-deoxyclitoriacetal-aromatic analogues is shown in scheme **3.4** [35].



Scheme 3.4 Synthesis of 6-deoxyclitoriacetal-aromatic analogues

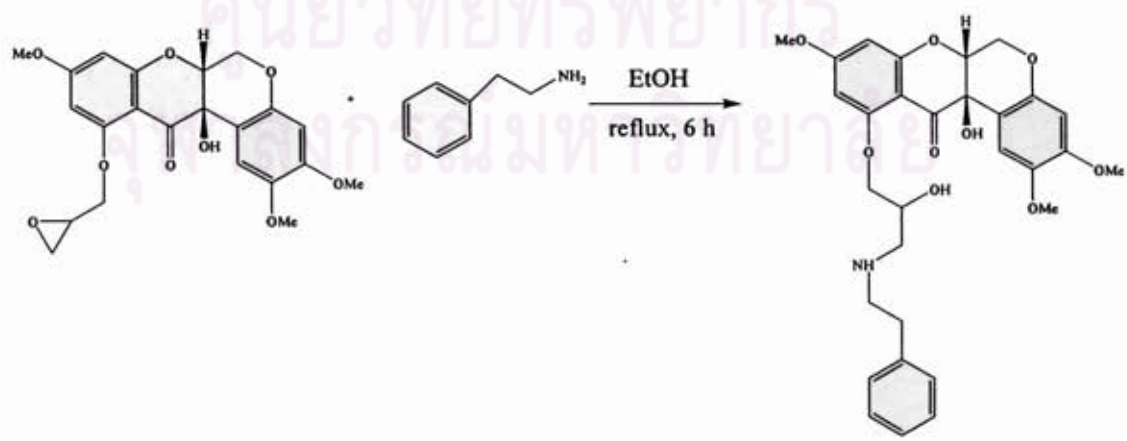
3.3.4.1 Synthesis of 11-(3-Benzylamino-2-hydroxy-propoxy)-12a-hydroxy-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one (**12**)



(2) **Benzylamind** **(12)**

To a solution of epoxide **2** (20 mg, 0.045 mmol) in 5 ml of Ethanol was added benzylamind 0.03 g, 0.140 mmol). The reaction mixture was refruxed for 6 hours and then allowed to cool down to room temperature. The solvent was removed under pressure and the residue was washed with water and extracted organic layer with dichloromethane and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column with dichloromethane: ethyl acetate: dichloromethane (2:1:2, v/v) as elutent to give **12** as yellow solid compound (83% yield).

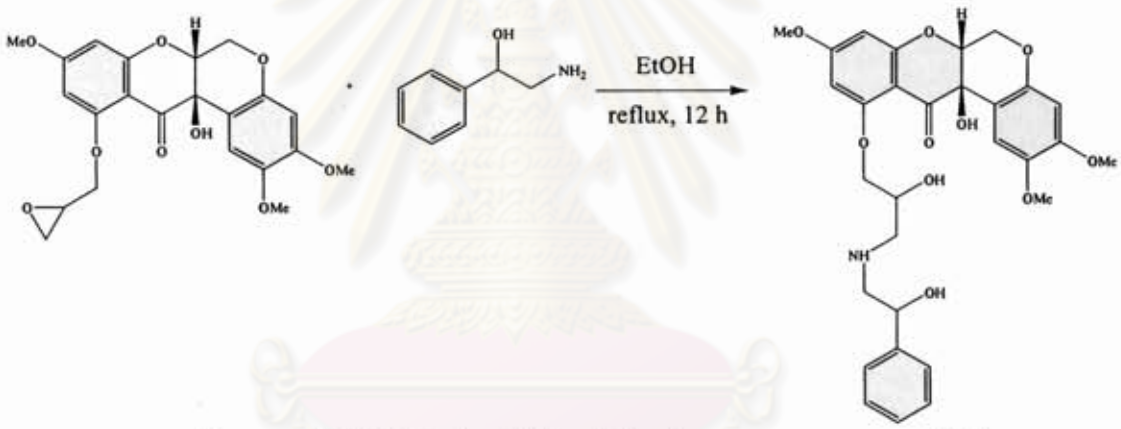
3.3.4.2 Synthesis of 12a-Hydroxy-11-(2-hydroxy-3-phenethylamino-propoxy)-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one (**13**)



(2) **2-Phenylethylamine** **(13)**

To a solution of epoxide **2** 20 mg, 0.045 mmol) in 5 ml of MeOH was added the 2-phenylethylamine (0.01 ml, 0.09 mmol). The reaction mixture was refluxed for 6 hour and then allowed to cool down to room temperature. The solvent was removed under pressure and the residue was washed with water and extracted organic layer with dichloromethane and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column with dichloromethane: ethyl acetate: dichloromethane (2:1:2, v/v) as elutent to give **13** as yellow solid compound (69% yield).

3.3.4.3 Synthesis of 12a-Hydroxy-11-[2-hydroxy-3-(2-hydroxy-2-phenyl-ethylamino)-propoxy]-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one (**14**)



(2) **(*R*)-(-)-2-Amino-2-phenylethanol** **(14)**

To a solution of epoxide **2** (20 mg, 0.045 mmol) in 5 ml of ethyl acetate was added the (*R*)-(-)-2-amino-2-phenylethanol (0.03 g, 0.140 mmol). The reaction mixture was refluxed for 12 hours and then allowed to cool down to room temperature. The solvent was removed under pressure and the residue was washed with water and extracted organic layer with dichloromethane and dried over $MgSO_4$. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography with dichloromethane: ethyl acetate: dichloromethane (2:1:2, v/v) as eluent to give **14** as yellow solid compound (78% yield).

dichloromethane (2:1:2, v/v) as eluent to give **14** as yellow solid compound (78% yield).

3.4 Acid addition salt

(8) **(15)**

0.5 g of **8** (0.97 mmol) was added with conc.HCl (1 ml), after that the solvent was epvaporated to dryness. The crude was subjected to test solubility, 0.1g of **15** (solid substance must be pulverized) in a glass-stopped 10 ml graduated cylinder, increasing volumes of distilled water at room temperature are added according to the step shown in the Table 3.1. After each addition of the indicated amount of water, the mixture is strirr vigorously for 30 minutes and is visually checked for dissolved completely of the sample [36]. The product was tested cytotoxicity and topoisomerase II assay.

3.5 Topoisomerase II inhibition

3.5.1 Preparation of pBR322 plasmid DNA

Strain of *E. coli* HB101 was routinely grown at 37 °C for 18 h in LB medium containing 100 μg/ml of ampiciline for isolation of single colony of *E. coli*. plsmid DNA isolation from bacteria cells. Cell from overnight growth in fresh LB medium at 37°C to exponential phase (A_{600} = 0.4-0.6) was taken in an Eppendorf kit and centrifuged for 2 min. Cell lysis was achieved by adding 400 μl of lysis buffer.

The suspension was kept on ice for 5 min. The lysate was centrifuged at 13,000 rpm for 2 min to pellet the cell DNA and other bacterial debris. Plasmid DNA was washed with 400 µl of wash buffer and centrifuged at 13,000 rpm for 2 min , the pellet was dried briefly by centrifuging for 2 min in an Eppendorf centrifuge and finally dissolved in 10 µl of dH_2O. Plasmid DNA was visualized in 1% agarose gel electrophoresis [25].

3.5.2 Topoisomerase II assay

Topoisomerase II is an important nuclear enzyme controlling DNA topology through catalysis of a breakage of double-stranded DNA, allowing for the passage of double-stranded DNA followed by a resealing of the DNA. Relaxation of DNA supercoils by topoisomerase II is considered crucial to its role in DNA replication and in transcription. To further elucidate the mechanism of action of 6-deoxyclitoriacetal, stemonal and 6-deoxyclitoriacetal analogues for cytotoxicity, the relaxation of supercoiled plasmid pBR322 DNA was evaluated. Topoisomerase II relaxation assay was conducted using human topoisomerase II (Amersham). Etoposide are selective topoisomerase II inhibitors was used as positive control.

Rotenoid compounds were screened for the topoisomerase II inhibition function. The activity of the compounds on the relaxation of DNA topoisomerase IIα was determined by measuring the conversion of supercoiled PBR 322 plasmid DNA to its relaxed form. Topoisomerase II relaxation assay was conducted by using human topoisomerase II with etoposide as a positive control.

Rotenoid compounds and etoposide were prepared as stock solutions (10, 25 mM, respectively) in DMSO and stored at -20 °C. The reaction mixture contained 10 mM Tris-HCl (pH 7.9), 175 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2.5 % glycerol, 1 mM ATP, 0.5 mM dithiothreitol, 30 µg/mL bovine serum albumin, 0.2 µg pBR322 plasmid DNA, 0.3 U DNA topoisomerase IIα, and test compounds in a final volume of 50 µL. The reactions were incubated for 30 min at 37 °C and terminated by the addition of 3 µL of solution containing 0.77 % sodium dodecyl sulfate, and 77 mM EDTA. Samples were mixed with 2 µL of solution containing 30 % sucrose, 0.5 % bromophenol blue and 0.5 % xylene cyanol, and subjectded to electrophoresis on a 1 % agarose gel at 1.5 V/cm for 10 h with a running buffer of Tris-borate-EDTA.

Gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 μg/mL). DNA bands were visualized by transillumination with UV light and quantities by an image analyzer and Syngene software [40].

CHAPTER IV

RESULTS AND DISCUSSION

4. Results and discussion

4.1 Extraction of 6-deoxyclitoriacetal from the dried roots of *Stemona collinsae* Craib.

6-deoxyclitoriacetal (1) was obtained as colorless needles. Its melting point is 131–132 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.57 (s, 3H), 3.70 (s, 3H) 3.74 (s, 3H), 4.33 (dd, J_1 = 12.3, J_2 = 1.6 Hz, 1H), 4.47 (dd, J_1 = 12.3 Hz, J_2 = 2.3 Hz, 1H), 4.67 (d, J = 2.3 Hz, 1H), 6.01 (d, J = 2.3 Hz, 1H), 6.06 (d, J= 2.3 Hz, 1H), 6.68 (s, 1H), 6.71 (s, 1H), 11.95 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.8, 55.9, 56.3, 63.6, 66.9, 75.5, 94.5, 95.6, 100.1, 101.1, 108.2, 109.2, 143.9, 148.3, 151.3, 161.6, 164.3, 169.0, 195.0 and MS (m/z) calcd for $C_{19}H_{19}O_8$ ($M+H^+$) 375.12. The data are in agreement with those reported in the literatures [8-10]. The structure composed of four fused ring A, B, C and D, which is a characteristic of rotenoid. The molecule has a bent-shaped conformation at C6a to C12a [Fig. 4.1].

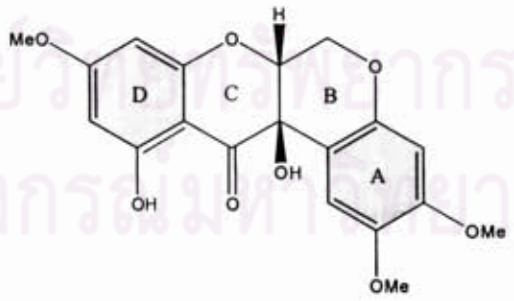


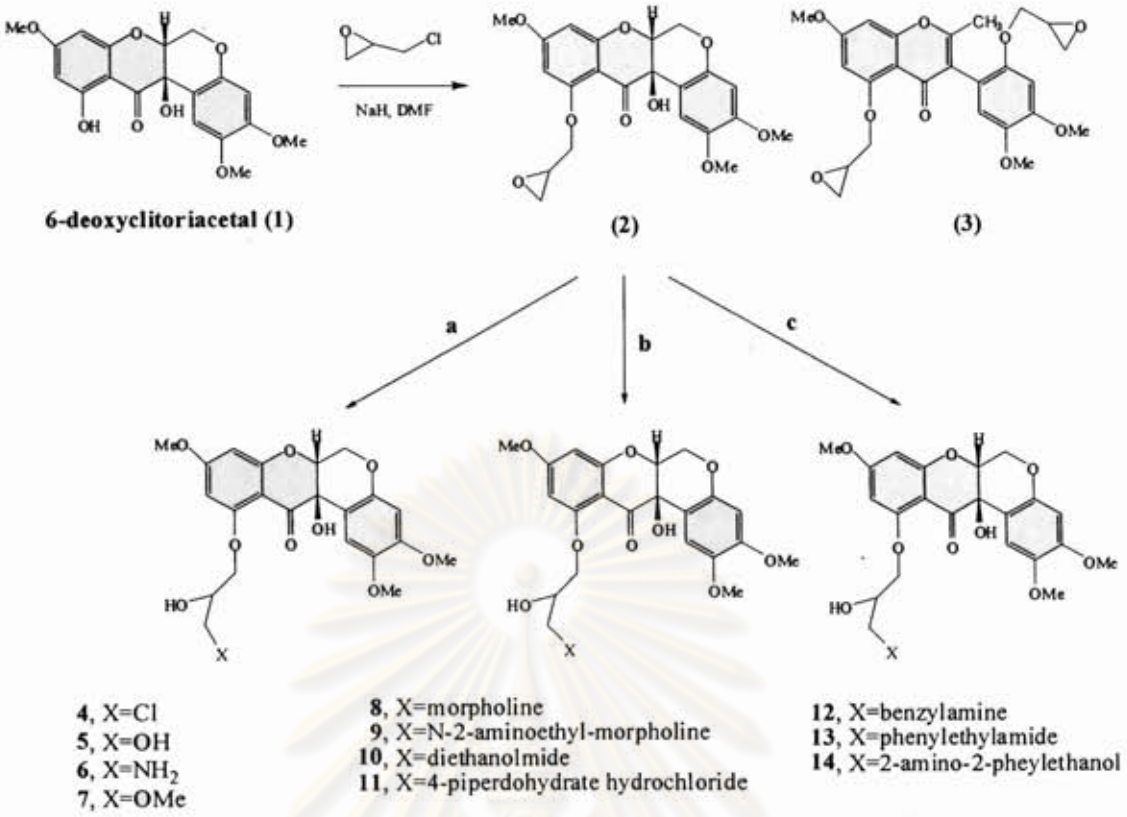
Fig 4.1 Chemical structure of 6-deoxyclitoriacetal

4.2 Synthesis of 6-deoxyclitoriacetal analogues

6-Deoxyclitoriacetal is a model anticancer substance being used for this reseach, the results of cytotoxicity against three cancer cell lines, KB (Human mouth carcinoma), MCF7 (Breast cancer) and NCL-H187 (Human small lung cancer) cell lines. It was found that 6-deoxyclitoriacetal showed the IC_{50} value of 0.08, 0.26 and 0.04 μg/ml when compared with the IC_{50} value of positive control, doxorubicin, with 0.33, 0.82 and 0.04 μg/ml in KB (Human mouth carcinoma), MCF7 (Breast cancer) and NCL-H187 (Human small lung cancer) cell lines, respectively [9].

As a knowledge of the intercalation and topoisomerase II inhibition mechanism of doxorubicin to inhibit the cytotoxic activity, this compound is composed of a planar aromatic moiety and a six-member daunosamine sugar moiety, forming a bent-shape molecule. The planar aromatic moiety can intercalate between two base pairs of DNA, while daunosamine sugar moiety can increase binding activity with base pairs of DNA at the intercalating sit by hydrogen bonding. Intercalation is also essential for ability of doxorubicin to trap topoisomerase II intermediates. We propose that not only the planar aromatic molecule is the important factor in the intercalation process, but also the functional group (sugar ring) can help to stabilize the topo II-DNA-drug complex via the intermolecular interaction such as hydrogen bonding, π-π interaction. Therefore, the derivatives of 6-deoxyclitoriacetal were synthesis to study the abovementioned factors, and then study the structure-relationship by testing for their cytotoxicities with various cell lines, furthermore, study the ability to inhibit the Topoisomerase II. The preferred functional groups are the flexible functional groups with strongly hydrogen bonding to the base pairs of DNA and help stabilization of the substance-DNA intercalation

The chemical structure of 6-deoxyclitoriacetal was modified at C11-OH position to increase flexibility and then enhance its anticancer activity. The reaction is shown in Scheme 4.1. Firstly, 6-deoxyclitoriacetal (**1**) is converted to an epoxide derivative by in reacting with epichlorohydrin (**2**), then the epoxide derivative was ring opened by nucleophiles, morpholine and benzylamine compounds. The method use of epichlorohydrin as starting material was investigated.



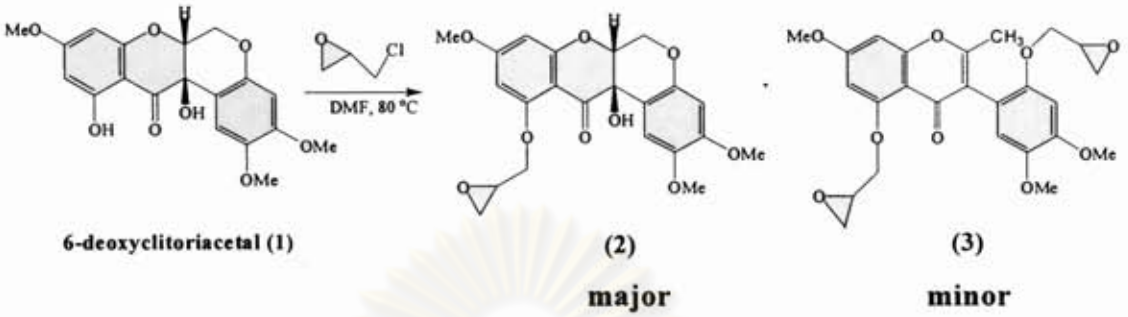
Scheme 4.1 Synthesis of 6-deoxyclitoriacetal derivatives

The 6-deoxyclitoriacetal derivatives were screened for their *in vitro* cytotoxicity on tumor cell lines using doxorubicin HCl as positive control. The IC_{50} values were determined in micro molar (μM) concentrations. The human tumor cell lines used in the screening were human breast (MCF-7), oral (KB), and small cell lung (NCI-H187) cancer cell lines. The cytotoxic activity data is summarized in Table 4.1. The compounds having IC_{50} < 10, 10-20 and > 20 μM have been designated as high, moderate and low cytotoxic derivatives, respectively.

4.2.1 6-Deoxyclitoriacetal-epoxidation

Epoxide ring plays an important role in organic synthesis. Many anticancer substances compose of an epoxide ring as a part of molecules such as psorospermin and mitomycin [12, 13]. Therefore, in this work the structure of 6-deoxyclitoriacetal (**1**) was modified by epoxidation with epichlorohydrin. Compound **2** is a major product of the epoxidation reaction. The reaction scheme is shown in Scheme 4.2. The

details of the spectroscopy characterization including ^{1}H NMR, ^{13}C NMR and LC-ESI mass spectroscopy for all compounds were given in Table 4.1



6-deoxyclitoriacetal (1) **(2)** **major** **(3)** **minor**

Scheme 4.2 Synthesis pathway for **2** and **3**

Table 4.1 Characterization of (**2**)

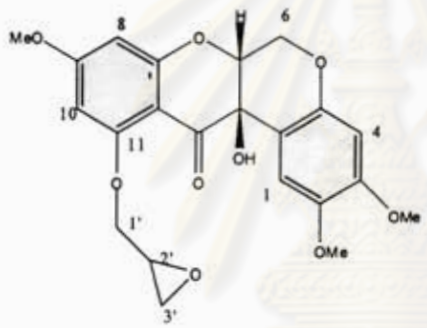
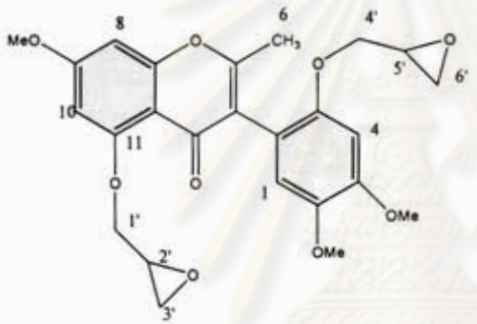
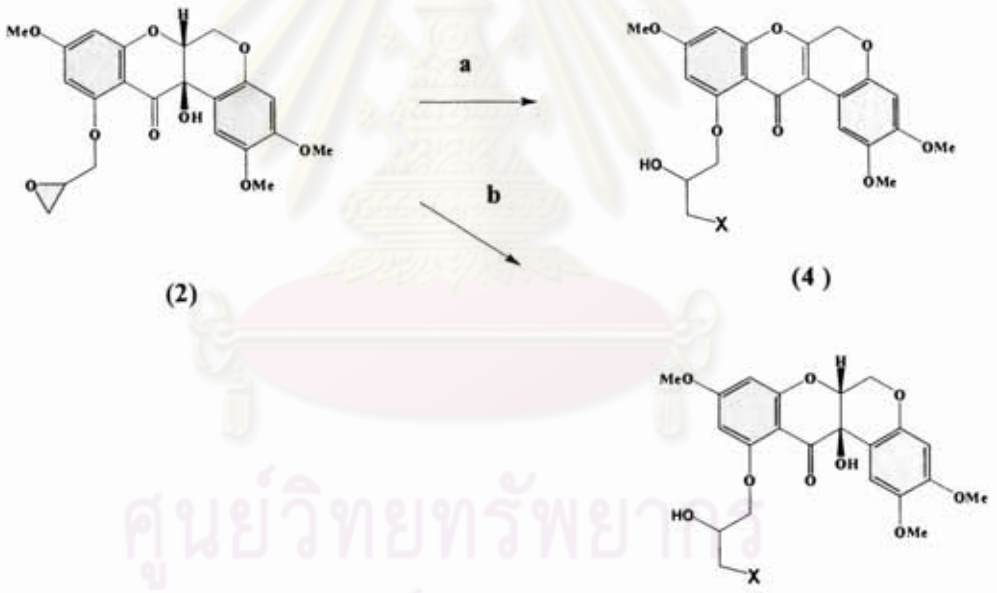
Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
2	12a-Hydroxy-2,3,9-trimethyl-11-oxiranylmethoxy-6a,12a-dihydro-6*H*-chromeno[3,4-*b*]chromen-12-one		$C_{22}H_{22}O_9$= 429.0 $[M+Na]^+$= 452.6	3.67 (s, 3H, OMe-2), 3.68 (s, 3H, OMe-9), 3.73 (s, 3H, OMe-3), 4.38 (dd, *J* = 12.0, 12.0 Hz, 1H, H-6), 4.46 (d, *J* = 2.3 Hz, 1H, H-6a), 4.51 (dd, *J* = 12.0, 12.0 Hz, 1H, H-6), 5.96 (d, *J* = 1.8 Hz, 1H, H-8), 6.40 (d, *J* = 1.7 Hz, 1H, H-10), 6.44 (s, 1H, H-4), 6.46 (s, 1H, H-1), 2.96 (dd, *J* = 2.5, 5.0 Hz, 1H, C3′-Ha), 3.02 (dd, *J* = 5.0, 5.0 Hz, 1H, C3′-Hb), 3.32-3.35 (m, 1H, C-2′H), 3.95 (dd, *J* = 11.3 11.3, 11.3 Hz, 1H, C1′-Ha), 4.04 (dd, *J* = 4.7, 11.3 Hz, 1H, C1′-Hb)	44.6 (C3′), 49.9 (C-2′), 55.6 (OMe-9), 55.8 (OMe-3), 56.4 (OMe-2), 63.8 (C-6), 67.1 (C-12a), 75.7 (C-6a), 75.7 (C-1′) 94.0 (C-8), 94.5 (C-10), 101.0 (C-4), 102.2 (C-1a), 109.4 (C-1), 142.1 (C-2), 148.2 (C-4a), 151.0 (C-3), 161.4 (C-7a), 164.2 (C-11), 168.8 (C-9), 195.0 (C-12)

Table 4.1 (continue) Characterization of (**3**)

Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
3	(2R,3R)-3-(4,5-dimethoxy-2-(oxiran-2-ylmethoxy)phenyl)-3-hydroxy-7-methoxy-2-methyl-5-(oxiran-2-ylmethoxy)chroman-4-one		$C_{25}H_{28}O_{10}$= 469.15 $[M+Na]^+$ = 492.15	2.65 (s, 2H, H-6'), 2.67 (s, 2H, H-3'), 2.87 (s, 2H, H-2'), 2.95 (s, 2H, H-5'), 3.68 (s, 3H, OMe-3), 4.69 (s, 3H, OMe-9), 3.75 (s, 3H, OMe-2), 3.80-4.00 (m, 3H, H-4'), 4.10-4.20 (m, 2H, H-1'), 4.51 (d, *J*=12 Hz, 3H, H-6), 4.58 (dd , *J*= 8.4, 8.4 Hz, H-6a), 6.17 (s, 1H, H-8), 6.27 (s, 1H, H-10), 6.37 (s, 1H, H-4), 6.46 (s, 1H, H-1)	14.5 (C-6) 44.6 (C-3'), 44.8 (C-6'), 49.9 (C-2'), 50.0 (C-5') 55.6 (OMe-9), 55.8 (OMe-3), 56.4 (OMe-2), 67.1 (C-12a), 75.7 (C-6a), 75.7 (C-1'), 76.0 (C-4), 94.0 (C-8), 94.5 (C-10), 101.0 (C-4), 102.2 (C-1a), 109.4 (C-1), 142.1 (C-2), 148.2 (C-4a), 151.0 (C-3), 161.4 (C-7a), 164.2 (C-11), 168.8 (C-9), 195.0 (C-12)

4.2.2 6-Doxyclitoriacetal- nucleophilic derivatives

In general, the target for several anticancer agents is deoxyribonucleic acid (DNA), especially involving in inhibit replication and transcription. It composed of nucleotide base as a part of molecule including pyrimidine and purine base. Therefore, in this research to study propability of good leving group by in stead of unstable epoxide group on cytotoxicity activities [33] the structure (**1**) was modified with certain nucleophilic analogues to study the effect of functional groups (Cl, OH, NH_2, and OMe) on the cytotoxic activities. The epoxide derivative (**2**) was ring opened by nucleophiles; namely, HCl, NaOH, NH_3 and $NaOCH_3$. The reaction scheme is shown in Scheme 4.3. The details of the spectroscopy characterization including ^1H NMR, ^{13}C NMR and LC-ESI mass spectroscopy for all compounds were given in Table 4.1



4, X=Cl; 5,X=OH; 6, X=NH_2; 7, X=OMe

Scheme 4.3 Synthesis pathway for **4** to **7**; (a) 1m-HCl, EtOAc, 30 min, (**4**), NaOH, EtOH, heat, 30 min (**5**), (b); ammonia solution, 3 day (**6**), NaOMe/MeOH, heat, 3 h (**7**)

Table 4.1 (continue) Characterization of (**4**)

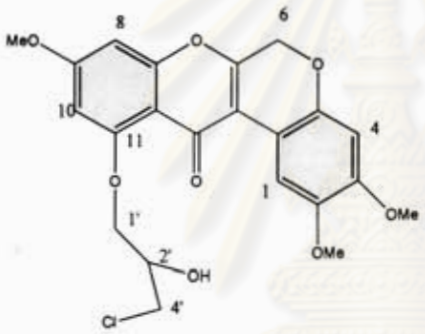
Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
4	11-(3-Chloro-2-hydroxy-propoxy)-12a-hydroxy-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one		$C_{22}H_{23}ClO_9$= 448.61	6.42 (s, 1H, H-1), 6.46 (s, 1H, H-4), 6.02 (2, 2H, H-10, 8), 4.68 (s, 1H, H-3'), 4.56 (dd, J= 12.0, 12.0 Hz, 2H, H-6), 4.45 (d, J= 12 Hz, 1H, H-6a), 4.08-4.30 (dd, J= 14.4, 14.4 Hz, 1H, H-2'), 3.80 (s, 3H, OMe-2), 3.76 (s, 3H, OMe-9), 3.71 (s, 3H, OMe-3)	44.6 (C3'), 49.9 (C-2'), 55.6 (OMe-9), 55.8 (OMe-3), 56.4 (OMe-2), 63.8 (C-6), 67.1 (C-12a), 75.7 (C-6a), 94.0 (C-8), 94.5 (C-10), 101.0 (C-4), 102.2 (C-1a), 109.4 (C-1), 142.1 (C-2), 148.2 (C-4a), 151.0 (C-3), 161.4 (C-7a), 164.2 (C-11), 168.8 (C-9), 75.7 (C-1'), 195.0 (C-12)

Table 4.1 (continue) Characterization of **(5)**

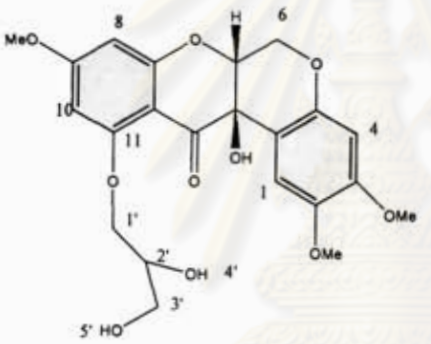
Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
5	11-(2,3-Dihydroxy-propoxy)-12a-hydroxy-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one		$C_{22}H_{24}O_{10}$= 448.17 $[M+Na]^+$= 488.56	3.71 (s, 3H, OMe-3), 3.76 (s, 3H, OMe-9), 3.80 (s, 3H, OMe-2), 3.81-3.85 (dd, *J*= 6.0, 6.0 Hz, 1H, H-4'), 4.12-4.18 (m, 1H, H-2'), 4.21-4.24 (m, 2H, H-1'), 4.45 (d, *J*= 12 Hz, 1H, H-6a), 4.56 (dd, *J*= 12.0, 12.0 Hz, 2H, H-6), 6.05 (s, 1H, H-8), 4.74 (s, 1H, H-5'), 6.06 (s, 2H, H-10), 6.47 (s, 1H, H-4), 6.48 (s, 1H, H-1)	44.6 (C3'), 49.9 (C-2'), 55.6 (OMe-9), 55.8 (OMe-3), 56.4 (OMe-2), 63.8 (C-6), 67.1 (C-12a), 75.7 (C-6a), 75.7 (C-1'), 94.0 (C-8), 94.5 (C-10), 101.0 (C-4), 102.2 (C-1a), 109.4 (C-1), 142.1 (C-2), 148.2 (C-4a), 151.0 (C-3), 161.4 (C-7a), 164.2 (C-11), 168.8 (C-9), 195.0 (C-12)

Table 4.1 (continue) Characterization of (**6**)

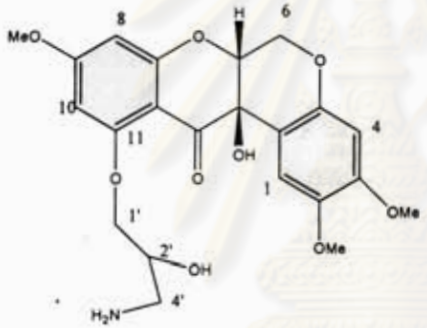
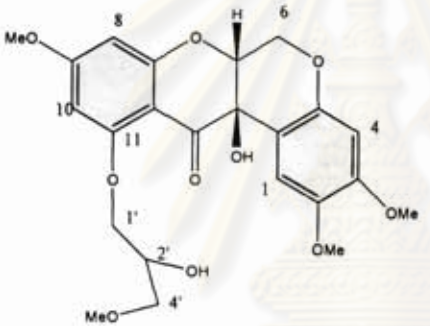
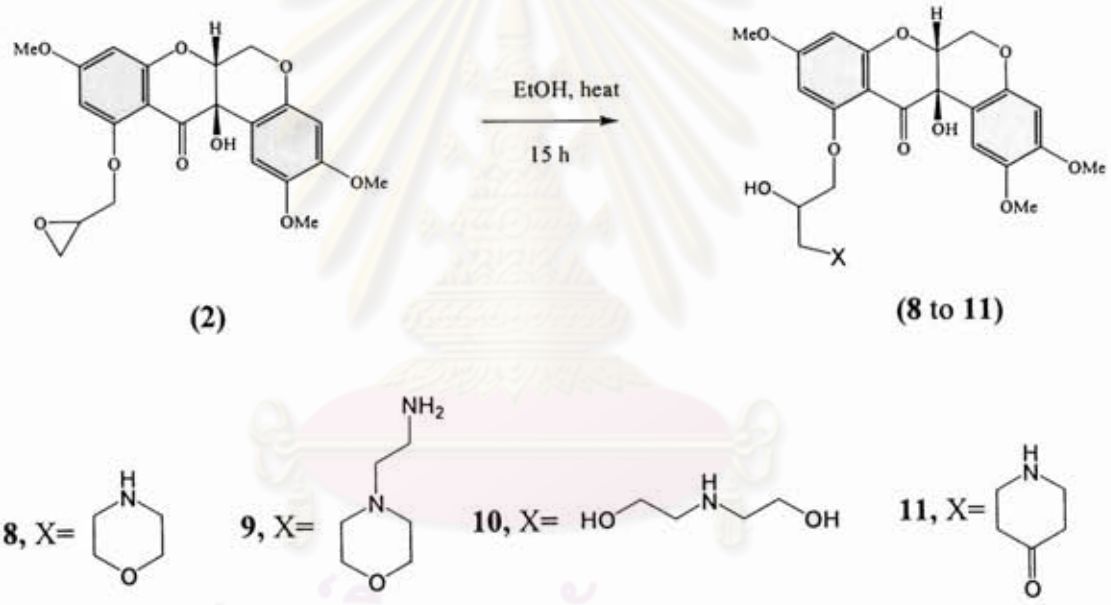
Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
6	11-(3-Amino-2-hydroxy-propoxy)-12a-hydroxy-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one		$C_{22}H_{25}NO_9$= 447.1 $[M+Na]^+$= 489.1	2.85-3.01 (m, 2H, H-5'), 3.62 (s, 3H, OMe-3), 3.68 (s, 3H, OMe-9), 3.71 (OMe-2), 4.35-4.25 (m, 1H, H-2'), 4.34-4.39 (m, 2H, H-1'), 4.46 (d, *J*= 12 Hz, 1H, H-6a), 4.51 (dd, *J*= 12.0, 7.2 Hz, 2H, H-6), 5.59 (s, 2H, H-10, 8), 6.39 (s, 1H, H-4), 6.40 (s, 1H, H-1)	44.6 (C3'), 49.9 (C-2'), 55.6 (OMe-9), 55.8 (OMe-3), 56.4 (OMe-2), 63.8 (C-6), 67.1 (C-12a), 75.7 (C-1'), 75.7 (C-6a), 94.0 (C-8), 94.5 (C-10), 101.0 (C-4), 102.2 (C-1a), 109.4 (C-1), 142.1 (C-2), 148.2 (C-4a), 151.0 (C-3), 161.4 (C-7a), 164.2 (C-11), 168.8 (C-9), 195.0 (C-12)

Table 4.1 (continue) Characterization of (7)

Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
7	12a-Hydroxy-11-(2-hydroxy-3-methoxy-propoxy)-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one		$C_{23}H_{26}O_{10}$= 462.1 $[M+H]^+$= 460.3	3.41 (s, 3H, OMe-5'), 3.61 (dd, *J*= 3.6, 3.6 Hz, 2H, H-4'), 3.71 (s, 3H, OMe-3), 3.75 (s, 3H, OMe-9), 3.39-4.02 (m, 2H, H-1'), 3.79 (OMe-2), 4.08-4.17 (m, 1H, H-2'), 5.43 (d, *J*= 11.6 Hz, 1H, H-6a), 5.43 (d, *J*= 11.6 Hz, 1H, H-6a), 5.56 (dd, *J*= 10.0, 10.0 Hz, 2H, H-6), 6.04 (s, 2H, H-10, 8), 6.47 (s, 1H, H-4), 6.50 (s, 1H, H-1)	44.6 (C3'), 49.9 (C-2'), 55.6 (OMe-9), 55.8 (OMe-3), 56.4 (OMe-2), 59.6 (C-5'), 63.8 (C-6), 67.1 (C-12a), 75.7 (C-6a), 75.7 (C-1') 94.0 (C-8), 94.5 (C-10), 101.0 (C-4), 102.2 (C-1a), 109.4 (C-1), 142.1 (C-2), 148.2 (C-4a), 151.0 (C-3), 161.4 (C-7a), 164.2 (C-11), 168.8 (C-9), 195.0 (C-12)

4.2.3 6-Doxyclitoriacetla-morpholine derivatives

Morpholine groups play an important role in many industries. In particular, the use of morpholine group in pharmaceutical fields which can increase antimalarials, antipasmodies and anticancer activities. Therefore, in this work the structure of 6-deoxyclitoriacetal (**1**) was modified with certain morpholine groups in order to enhance anticancer activity. Four morpholine analogues were synthesized, namely morpholine, N-2-aminoethyl-morpholine, diethanolmine, and piperidine. The reaction scheme is shown in Scheme 4.3. The details of the spectroscopy characterization including ^{1}H NMR, ^{13}C NMR and LC-ESI mass spectroscopy for all compounds were given in Table 4.1



Scheme 4.4 Synthesis pathway for **8** to **11**, the condition ; EtOH, heat, 15 h ; morpholine (**8**), N-2-aminoethyl-morpholine (**9**), diethanolmine (**10**), and piperidine (**11**)

Table 4.1 (continue) Characterization of (**8**)

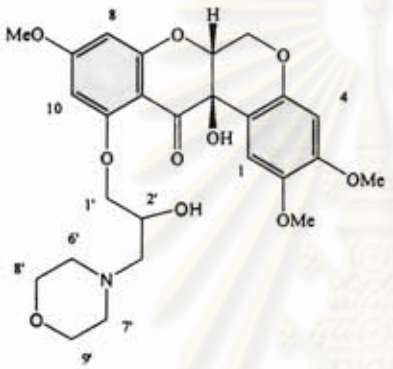
Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
8	12a-Hydroxy-11-(2-hydroxy-3-morpholin-4-yl-propoxy)-2, 3, 9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one		$C_{26}H_{31}NO_{10}$= 517.26 $[M+Na]^+$= 540.3	2.42-2.75 (m, 4H, H-5',6'), 3.85-3.40 (m, 2H, H-4a',4b'), 3.61 (s, 3H, OMe-2), 3.65 (dd, J = 7.2,7.2 Hz, 2H, H-8a',8b'), 3.69 (d, J =7.6 Hz, 1H, H-7a), 3.70 (s, 3H, OMe-9), 3.71 (d, J = 7.6 Hz, 1H, H7-b), 3.74 (s, 3H, OMe-3), 3.82 (m, 2H, H-4'), 4.02-4.18 (m, 2H, H-1a',1b'), 4.38 (dd, J = 12.0, 12.0 Hz, 1H, H-6), 4.46 (d, J = 2.3 Hz, 1H, H-6a), 4.68 (m, 1H, H-2'), 4.51 (dd, J = 12.0, 12.3 Hz, 1H, H-6),5.97 (s, 1H,H-8), 5.99 (s, 1H, H-10), 6.41 (s, 1H, H-4), 6.44 (s, 1H, H-1)	54.10 (C-6'), 54.15 (C-5'), 56.20 (C-3'), 56.45 (OMe-3), 56.5 (OMe-9), 57.0 (OMe-2), 60.2 (C-6), 64.0 (C-3'), 66.5 (C-8'), 66.8 (C-7'), 67.0 (C-12a), 72.0 (C-1'), 74.0 (C-6a), 94.0 (C-8), 94.5 (C-10), 101.5 (C-4), 103.5 (C-4a), 108.0 (C-1a), 108.5 (C-1), 144.0 (C-2), 148.0 (C-3a), 150.5 (C-3), 162.0 (C-7a), 164.0 (C-11), 166.5 (C-9), 190.5 (C-12)

Table 4.1 (continue) Characterization of (**9**)

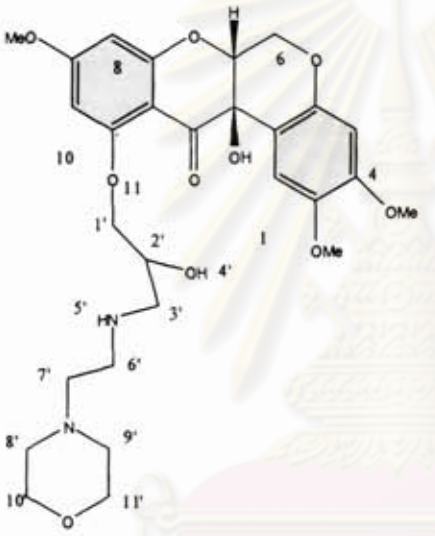
Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
9	12a-Hydroxy-11-[2-hydroxy-3-(2-morpholin-4-yl-ethylamino)-propoxy]-2, 3, 9-trimethoxy-6a, 12a-dihydro-6H-chromeno [3, 4-b]chromen-12-one		$C_{28}H_{36}N_2O_{10}$= 560.24 $[M+H]^+$= 560.74	2.46 (s,2H, H-8a',8b'), 2.48 (s, 2H, H-9a', 9b') 2.51 (dd, *J*= 6.4, 6.0 Hz, 2H, H-7a'-7b'), 2.73-2.80 (m ,2H, H-6a', 6b'), 2.82-2.92 (m, 2H, H-4a',4b'), 3.71 (s, 3H, OMe-2), 3.76 (s, 3H, OMe-9), 3.80 (s, 3H, OMe-3), 3.90-4.0 (q, 1H, H-2a',2b'), 4.09-4.17 (m, 2H, H-1a',1b'), 4.51 (d, *J*=12 Hz, 2H, H-6), 4.56 (dd, *J*=12.0, 12.0 Hz, 1H, H-6a), 6.01 (s, 1H,H-8), 6.03 (s, 1H, H-10), 6.47 (s, 1H, H-4), 6.50 (s, 1H, H-1)	46.0 (C-6'), 50.2 (C-3'), 52.5 (C-9'), 56.5 (C-8'), 54.0 (OMe-9), 55.0 (OMe-3), 55.5 (OMe-2), 64.0 (C-6), 66.5 (C-10'), 67.5 (C-11'), 72.5 (C-1'), 72.0 (C-2'), 74.5 (C-6a), 94.0 (C-8), 94.5 (C-10), 101.5 (C-4), 103.5 (C-4a), 108.0 (C-1a), 108.5 (C-1), 144.0 (C-2), 148.0 (C-3a), 150.5 (C-3), 162.0 (C-7a), 164.0 (C-11), 166.5 (C-9), 190.5 (C-12)

Table 4.1 (continue) Characterization of (**10**)

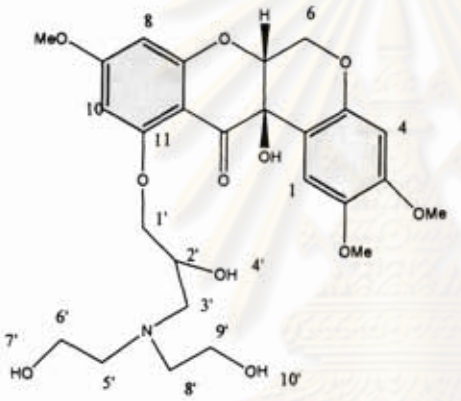
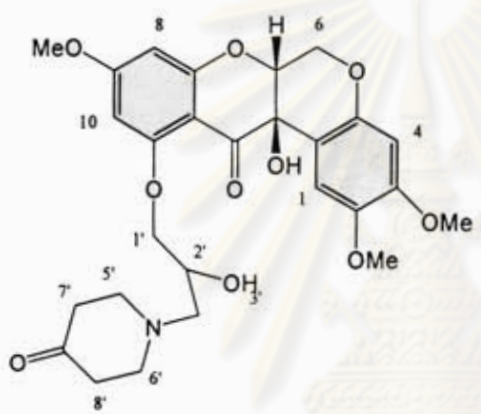
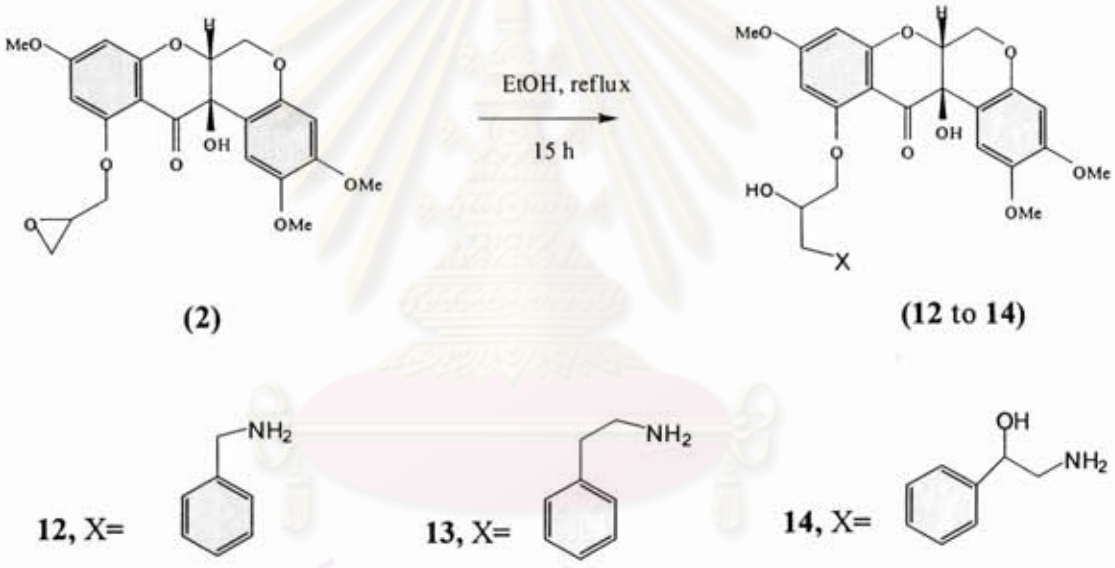
Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
10	12a-Hydroxy-11-[2-hydroxy-3-(2-hydroxy-1-hydroxymethyl-ethylamino)-propoxy]-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno [3,4-b]chromen-12-one		$C_{25}H_{31}NO_{11}$= 535.21 $[M+H]^+$= 535.71	2.66 (dd, *J*= 13.6, 13.6 Hz, 2H, H-8a', 8b'), 2.82 (dd, *J*= 9.2, 9.2 Hz, 2H, H-5a', 5b'), 2.95 (d, *J*= 8.8 Hz, 1H, H-4b'), 2.98 (d, J= 8.8 Hz, 1H, 4a'), 3.61 (s, 2H, H-6a',6b'), 3.64 (s, 2H, H-9a', 9b'), 3.71 (s, 3H, OMe-2), 3.74 (s, 3H, OMe-9), 3.78 (s, 3H, OMe-2), 3.88-4.80 (m, 1H, H-2'), 4.10-4.25 (m, 2H, H-1a',1b'), 5.99 (d, J= 3.2 Hz, 1H, H-8), 6.04 (dd, *J*= 7.2, 7.2 Hz, H1, H-4), 6.51 (s, 1H, H-1)	54.0 (C-8'), 54.5 (C-5'), 56.5 (OMe-9), 56.7 (OMe-3), 58.0 (OMe-2), 64.0 (C-9'), 64.5 (C-6'), 65.5 (C-2'), 72.0 (C-1'), 75.0 (C-6a), 94.0 (C-8), 94.5 (C-10), 101.5 (C-4), 103.5 (C-4a), 108.0 (C-1a), 108.5 (C-1), 144.0 (C-2), 148.0 (C-3a), 150.5 (C-3), 162.0 (C-7a), 164.0 (C-11), 166.5 (C-9), 190.5 (C-12)

Table 4.1 (continue) Characterization of (**11**)

Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
11	1-[2-Hydroxy-3-(12a-hydroxy-2,3,9-trimethoxy-12-oxo-6,6a,12,12a-tetrahydro-chromeno[3,4-b]chromen-11-yloxy)-propyl]-piperidin-4-one		$C_{27}H_{31}NO_{10}$= 529.28 $[M+Na]^+$= 552.3	1.14-1.22 (m, 2H, H-9'), 1.80 (s, 1H, H-4'), 2.46-2.60 (m 2H, H-8'), 2.87-2.92 (m, 2H, H-6'), 4.42 (d, *J*=12 Hz, H-1'), 3.68 (s, 3H, OMe-2), 3.74 (s, 3H, OMe-9), 3.71 (s, 3H, OMe-3), 4.09-4.24 (m, 1H, H-2'), 4.43 (d, *J*=12 Hz, 2H, H-6), 4.54 (dd, *J*= 12.0, 12.0 Hz, 1H, H-6a), 6.00 (s,1H, H-8), 6.02 (s, 1H, H-10), 6.45 (s, 1H, H-4), 6.48 (d, *J*= 7.6 Hz, 1H, H-1),	54.10 (C-6'), 54.15 (C-5'), 56.20 (C-3'), 56.45 (OMe-3), 56.5 (OMe-9), 57.0 (OMe-2), 60.2 (C-6), 64.0 (C-3'), 66.5 (C-8'), 66.8 (C-7'), 67.0 (C-12a), 72.0 (C-1'), 74.0 (C-6a), 94.0 (C-8), 94.5 (C-10), 101.5 (C-4), 103.5 (C-4a), 108.0 (C-1a), 108.5 (C-1), 144.0 (C-2), 148.0 (C-3a), 150.5 (C-3), 162.0 (C-7a), 164.0 (C-11), 166.5 (C-9), 190.5 (C-12) 210.0 (C-9')

4.2.4 6-Deoxyclitoriaceal benzylamine derivatives

In terms of structure-relationship, the insertion of functional groups into the molecule such as N-aryl or N-benzyl can enhance cytotoxic activities against ovary and oral cancers [32]. As there are will introduce into structure as well as increase size of structure of the analogues. Therefore, in this work the structure of 6-deoxyclitoriacetal (**1**) was modified with certain aromatic ring in order to enhance anticancer activity. The reaction scheme is shown in Scheme 4.5. The details of the spectroscopy characterization including ^{1}H NMR, ^{13}C NMR and LC-ESI mass spectroscopy for all compounds were given in Table 4.1



Scheme 4.5 Synthesis pathway for **12** to **14**; benzylamine, EtOH, reflux, 15 h (**12**), 2-phenylethylamine, reflux, 15 h (**13**), 2-amino-phenylethanol, reflux, 15 h (**14**)

Table 4.1 (continue) Characterization of (**12**)

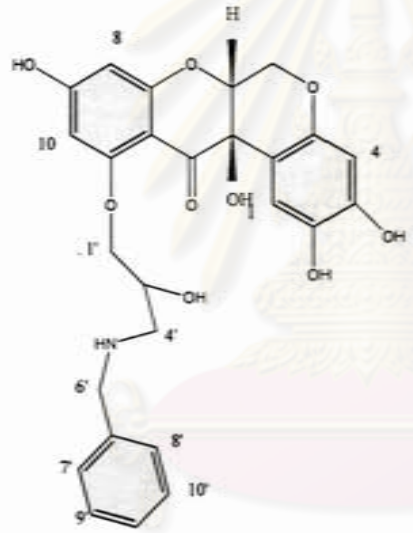
Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
12	11-(3-Benzylamino-2-hydroxy-propoxy)-12a-hydroxy-2,3,9-trimethoxy-6a-methyl-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one		$C_{28}H_{29}NO_9$= 525.5, $[M+Na]^+$= 538.15	2.74 (dd, *J*= 6.8, 6.8 Hz, 2H, H-6'), 2.93 (s, 2H, H-4'), 2.95 (d, *J*=6.4, 2H, H-1') , 3.69 (s, 3H, OMe-2), 3.74 (s, 3H, OMe-9) , 3.78 (s, 3H, OMe-3), 4.55 (dd, *J*=8.4, 8.4, 2H, H-6), 4.46 (d, *J*=12 Hz, 1H, H-6a), 4.05-4.25 (m, 1H, H-2'), 6.10 (s, 1H, H-8), 6.01 (s, 1H, H-10), 6.46 (s, 1H, H-4), 6.50 (s, 1H, H-1), 7.17 (s, 1H, H-11'), 7.19 (s, 1H, H-8'), 7.21 (s, 1H, H-7), 7.27 (s, 1H, H-10'), 7.29 (s, 1H, H-9')	44.6 (C3'), 49.9 (C-2'), 55.6 (OMe-9), 55.8 (OMe-3), 56.4 (OMe-2), 63.8 (C-6), 67.1 (C-12a), 75.7 (C-1'), 75.7 (C-6a), 94.0 (C-8), 94.5 (C-10), 101.0 (C-4), 102.2 (C-1a), 109.4 (C-1), 142.1 (C-2), 148.2 (C-4a), 151.0 (C-3), 161.4 (C-7a), 164.2 (C-11), 168.8 (C-9), 195.0 (C-12)

Table 4.1 (continue) Characterization of (**13**)

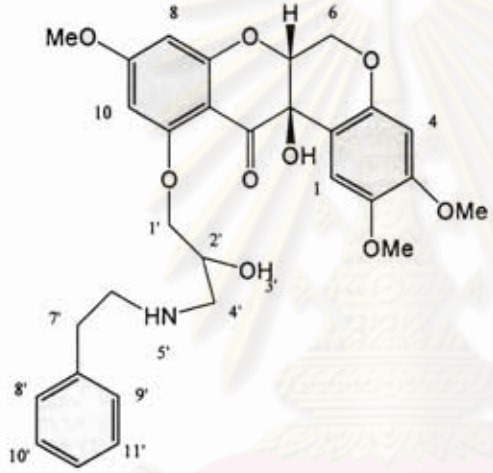
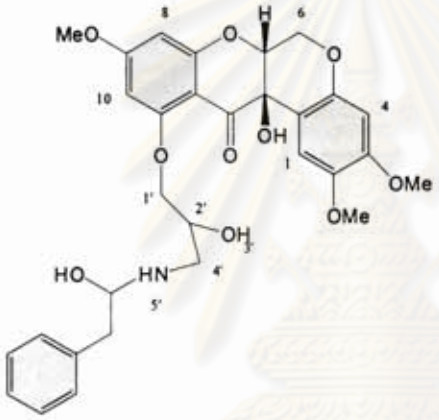
Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
13	12a-Hydroxy-11-(2-hydroxy-3-phenethylamino-propoxy)-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one		$C_{30}H_{33}NO_9$= 551.28, $[M+H]^+$= 552.29	2.74 (dd, *J*=6.8, 6.8 Hz, 2H, H-4'), 2.95 (dd, *J*= 6.4, 6.8 Hz, 2H, H-6'), 3.69 (s, 3H, OMe-2), 3.74 (s, 3H, OMe-9), 3.78 (s, 3H, OMe-3), 3.90-3.98 (m, 1H, H-2'), 4.04-4.20 (m, 2H, H-1), 4.45 (d, *J*= 12, 2H, H-6), 4.54 (dd, *J*= 7.2, 7.2 Hz, 1H, H-6a), 6.01 (s, 1H, H-8), 6.04 (s ,1H, H-10), 6.46 (s, 1H, H-4), 6.52 (dd, *J*= 8.0, 8.0 Hz, 1H, H-1), 7.17 (s, 1H, H-9'), 7.21 (s, 1H, H-8'), 7.72 (s, 1H, 12'), 7.28 (s,1H, H11'), 7.30 (s, 1H, H-10')	30.6 (C-7'), 40,0 (C-6'), 42.5 (C-4'), 56.0 (OMe-9), 56.2 (OMe-3), 56.5 (OMe-2), 64.0 (C-6), 68.2 (C-12a), 70.0 (C-2'), 72.5 (C-1'), 76.0 (C-6a), 94.0 (C-8), 94.5 (C-10), 100.5 (C-4), 108.4 (C-1a), 108.6 (C-1), 126.0 (C-12'), 128.0 (C-8'), 128.4 (C-9'), 128.5 (C-11'), 128.6 (C-10'), 140.0 (C-2), 144.0 (C4a), 148.0 (C-3), 150.0 (C-13), 160.5 (C-7a), 162.0 (C-11), 166.5 (C-9), 190.0 (C-12)

Table 4.1 (continue) Characterization of (**14**)

Compound	Name	Structure	MW.	^{1}H NMR	^{13}C NMR
14	12a-Hydroxy-11-[2-hydroxy-3-(2-hydroxy-2-phenyl-ethylamino)-propoxy]-2,3,9-trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one		$C_{30}H_{33}NO_{10}$= 567.2 $[M+Na]^+$= 604.4	3.54 (dd, *J*= 7.2, 7.2 Hz, 2H, H-4'), 3.59-3.66 (m, 2H, H-6'), 3.69 (s, 3H, OMe-2), 3.74 (s, 3H, OMe-9), 3.78 (s,3H, OMe-3), 4.00-4.01 (m, 1H, H-2'), 4.20 (dd, *J*= 5.6, 6.0 Hz, 2H, H-1'), 4.42 (d, *J*= 12 Hz, H-6), 4.55 (d, *J*= 12 Hz, 1H, H-6a), 4.81-4.9 (m ,1H, H-7'), 6.02 (s,1H, H-8),6.03 (s, 1H, H-10), 6.46 (s, 1H, H-4), 6.50 (s, 1H, H-1), 7.51 (s, 2H, H-9', 10'), 7.69 (s, 2H, H-11',12')	42.5 (C-4'), 44.5 (C-6'), 56.0 (OMe-9), 56.2 (OMe-3), 56.5 (OMe-2), 64.0 (C-6), 68.2 (C-12a), 70.0 (C-2'), 70.5 (c-7'), 72.5 (C-1'), 76.0 (C-6a), 94.0 (C-8), 94.5 (C-10), 100.5 (C-4), 108.4 (C-1a), 108.6 (C-1), 126.0 (C-12'), 128.0 (C-8'), 128.4 (C-9'), 128.5 (C-11'), 128.6 (C-10'), 140.0 (C-2), 144.0 (C4a), 148.0 (C-3), 150.0 (C-13), 160.5 (C-7a), 162.0 (C-11), 166.5 (C-9), 190.0 (C-12)

4.3 Solubility test

Water solubility is an important factor in the bioavailability of pharmaceutical products. The hydrochloride salt of **15** is high water solubility at room temperature. 4 mg of 15 was completely dissolved in 40 μL of water (water solubility = 1000 g/L).

4.4 X-ray Structure Determination and Refinement for 11-(3-Chloro-2-hydroxy-propoxy)-2,3,9-trimethoxy-6H-chromeno[3,4-b]chromen-12-one (4)

Suitable single crystals of **4** were crystallized from methanol by slow evaporation in refrigerator. X-ray analysis including data collection, cell refinement and data reduction was performed on a Bruker SMART APEX2 area detector (Scientific and Technology Research Equipment Centre, Chulalongkorn University) covering the full sphere of 0.96Å resolution. A total of 14,308 reflections were integrated by image processing with 99.0% completeness, and were merged into 4,517 reflections for the triclinic system with a R_{int} of 0.0269. The crystal and structure-refinement data are summarized in Table 4.2. The full crystal and structure-refinement data are given in Appendix A. The structure was solved by direct methods and refined anisotropically by full-matrix least-squares with non-hydrogen atoms using SHELXS-97 [5]. Hydrogen atom was found from difference Fourier maps and refined isotropically. CCDC-727200 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via (please use the link below) by e−mailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK, 716905. Fax: +44(0)1223−336033.

Result and Discussion

An ORTEP view of **4** (*50% probability* thermal ellipsoids) is shown in Fig 4.2. The molecule consists of 6-deoxyclitoriacetal moiety. The molecular structure confirms that the epoxide is ring-opened by hydrochloric acid to give the title compound. The C7-C15 distance of 1.347(4) Å is consistent with C=C double bond and hence the molecular structure is planar. The bond distances and angles were

given in appendixA The bond distances and angles are in the normal ranges expected for compounds of this type. The hydroxyl group form hydrogen bonds with the quinone oxygen atom [O8A...O3(-x-1, -y+2, -z), 2.757Å]. The geometry for hydrogen bonding is given in Table 4.2. The molecular packing diagram of **4** is shown in Fig. 3, the hydrogen bonded dimer is formed between the hydroxyl group and the quinone oxygen atom.

Table 4.2 The crystal and structure-refinement data for **4**

Crystal data

$C_{22}H_{20}ClO_8$	$V = 1010.30\ (9)$ Å^3
$M_r = 447.83$	$Z = 2$
Triclinic, $P\bar{1}$	$F_{000} = 466$
Hall symbol: -P 1	$D_x = 1.472$ Mg m^{-3}
$a = 7.1534\ (4)$ Å	Mo $K\alpha$ radiation $\lambda = 0.71073$ Å
$b = 11.7904\ (6)$ Å	$\theta = 1.6–27.4°$
$c = 12.7661\ (7)$ Å	$\mu = 0.24$ mm^{-1}
$\alpha = 76.901\ (3)°$	$T = 293$ K
$\beta = 86.991\ (3)°$	Prism, colourless
$\gamma = 74.455\ (3)°$	0.4 × 0.25 × 0.25 mm

Data collection

Bruker SMART APEX2 diffractometer	2879 reflections with $I > 2\sigma(I)$
Radiation source: Mo	$R_{int} = 0.027$
Monochromator: graphite	$\theta_{max} = 27.4°$
$T = 293$ K	$\theta_{min} = 1.6°$
Absorption correction: none	$h = -8 \rightarrow 9$
14308 measured reflections	$k = -15 \rightarrow 14$
4517 independent reflections	$l = -16 \rightarrow 16$

Refinement

Refinement on F^2	Secondary atom site location: difference Fourier map

Least-squares matrix: full	Hydrogen site location: inferred from neighbouring sites
$R[F^2 > 2\sigma(F^2)] = 0.073$	Contr
$wR(F^2) = 0.244$	$w = 1/[\sigma^2(F_o^2) + (0.1253P)^2 + 0.9163P]$ where $P = (F_o^2 + 2F_c^2)/3$
$S = 1.03$	$(\Delta/\sigma)_{max} < 0.001$
4517 reflections	$\Delta\rho_{max} = 0.78$ e Å^{-3}
280 parameters	$\Delta\rho_{min} = -0.54$ e Å^{-3}
Primary atom site location: structure-invariant direct methods	Extinction correction: none

Table 4.3 The hydrogen bonding geometry for **4**.

D-H	d(D-H)	d(H..A)	<DHA	d(D..A)	A
O8A-H8OA	0.850	1.922	167.46	2.757	O3 [-x-1, -y+2, -z]

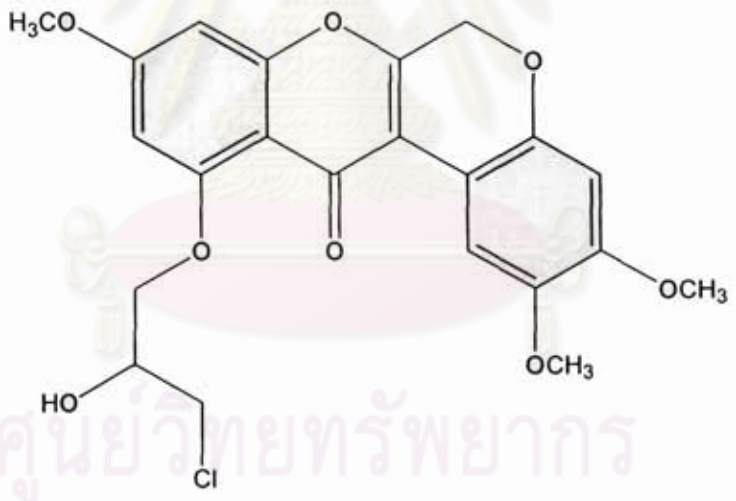


Fig 4.2 Chemical structure of 11-(3-Chloro-2-hydroxy-propoxy)-2,3,9-trimethoxy-6H-chromeno[3,4-b]chromen-12-one (**4**)

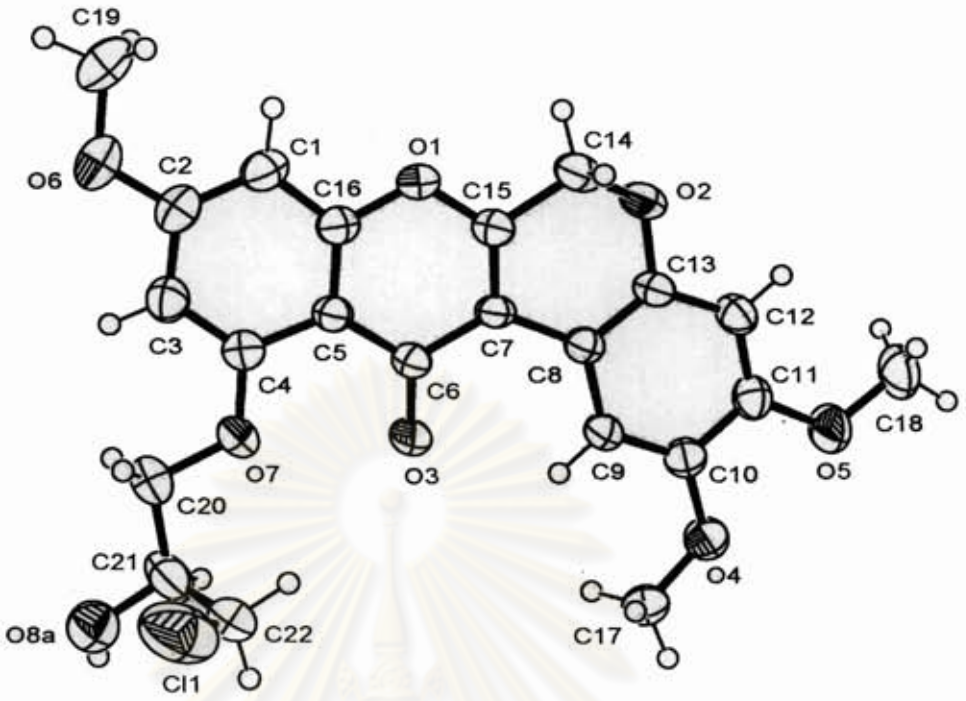


Fig 4.3 *ORTEP view* of crystal structure of **4**. Thermal ellipsoids are drawn at the *50%* probability level.

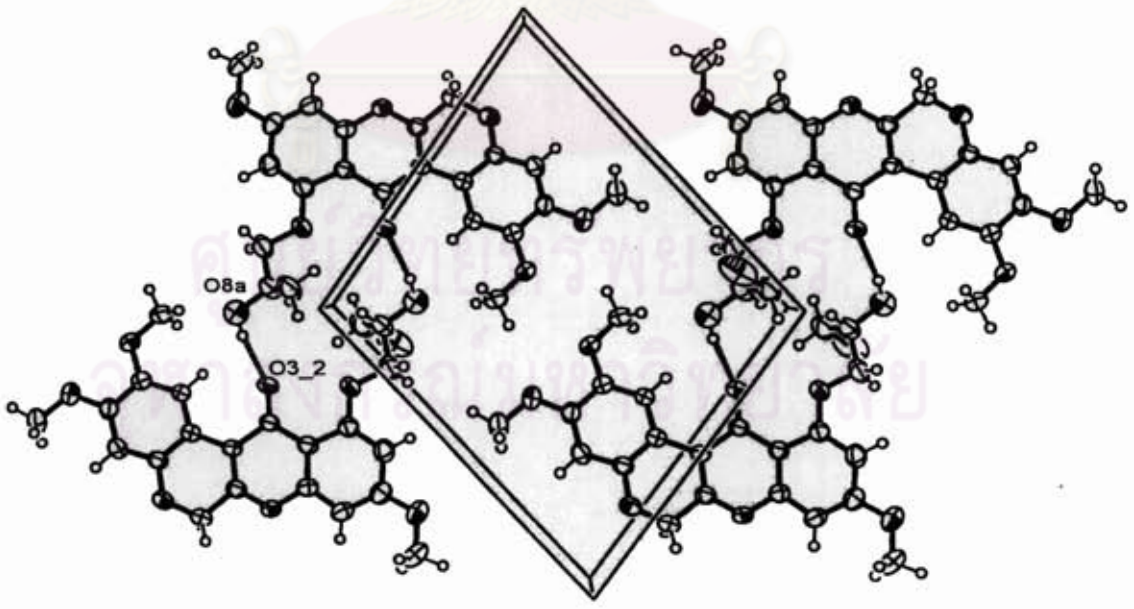


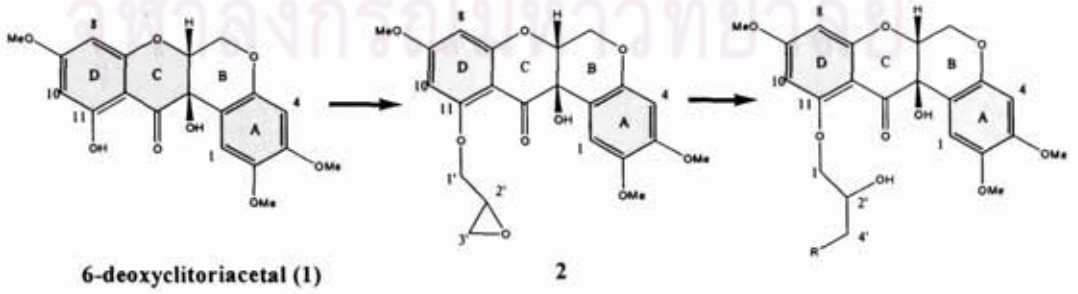
Fig 4.4 Packing diagram of **4** viewed along *a*-axis, dashed lines indicated hydrogen bonds.

4.5 Cytotoxic activities of 6-deoxyclitoriacetal and its analogues

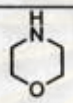
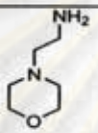
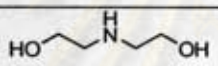
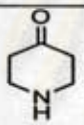
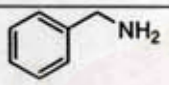
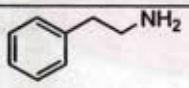
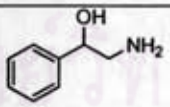
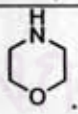
In this research, the cytotoxic activities of 6-deoxyclitoriacetal and its analogues were evaluated to investigate the structure-activity relationship. As mentioned in the previous section, we propose that important factors for 6-deoxyclitoriacetal to have good cytotoxicity are (i) at lease one part of the molecule should be planar (to intercalate part with DNA), (ii) the molecule adopts a bent shape or V shape (to stabilize the intercalation by locking a part of molecule in the minor or major groove of a DNA double strand) and the last factor is (iii) the molecule should have functional groups that can trap topoisomerase II-DNA complex (to enhance interfere with the normal functioning of the enzyme, leading to inhibit DNA relaxation). Therefore, the derivatives of 6-deoxyclitoriacetal were synthesized to study the above mentioned factors, and then tested for their cytotoxicities.

All synthesized compounds (**2 to 15**) were tested for cytotoxicity against KB (Human oral carcinoma), MCF7 (Breast cancer) and NCL-H187 (Human small lung cancer) cell lines using doxorubicin and ellipticine as the positive control. The cytotoxic activities of 6-deoxyclitoriacetal (**1**), and their 6-deoxyclitoriacetal analogues were reported in IC_{50} value. The IC_{50} is the half maximal (50%) inhibitory concentration (IC) of a substance. In general, higher IC_{50} means lower cytotoxic activity. The cytotoxic activity results of all compounds were tabulated in Table 4.4.

Table 4.4 Cytotoxic activities of 6-deoxyclitoriacetal (**1**), and 6-deoxyclitoriacetal analogues (**2** to **15**) against KB (Human oral carcinoma), MCF7 (Breast cancer) and NCL-H187 (Human small lung cancer) cell lines.



6-deoxyclitoriacetal (1) **2**

Compounds	R	IC_{50} (μg/ml)		
		Anti-KB	Anti-MCF7	Anti-NCI-H187
Doxorubicin	-	2.01	42.52	0.03
1	-	13.28	23.65	3.12
2	-	2.87	7.33	3.21
3	-	32.87	5.63	27.82
4	Cl	14.77	24.91	6.61
5	OH	13.36	Inactive	13.94
6	NH_2	43.24	Inactive	10.96
7	OMe	46.92	Inactive	11.69
8		26.51	31.71	2.93
9		26.31	36.82	4.70
10		Inactive	Inactive	Inactive
11		Inactive	48.63	Inactive
12		32.29	34.63	35.53
13		23.53	3.05	13.62
14		46.44	Inactive	22.08
15	 .HCl	0.68	Inactive	0.63

Doxorubicin (Adriamycin) is a commercial drug, generally used in cancer chemotherapy (Fig. 4.5). It is an anthracycline antibiotic. It can intercalate into base pairs of DNA. This manner is similar in anthracyclines. It is commonly used to treat some leukemia as well as cancers of the breast, lung, stomach, bladder, ovaries and others [38, 39]. Doxorubicin is known to interact with DNA by intercalation and then

inhibit the progression of the topoisomerase II, enzyme that unwinds DNA for transcription. The planar aromatic chromophore moiety intercalates between two base pairs of the DNA, while the six-member daunosamine sugar sits in the minor groove and interacts with base pairs adjacent to the intercalation site [39].

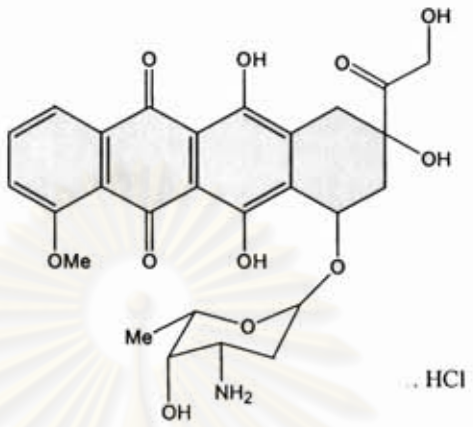
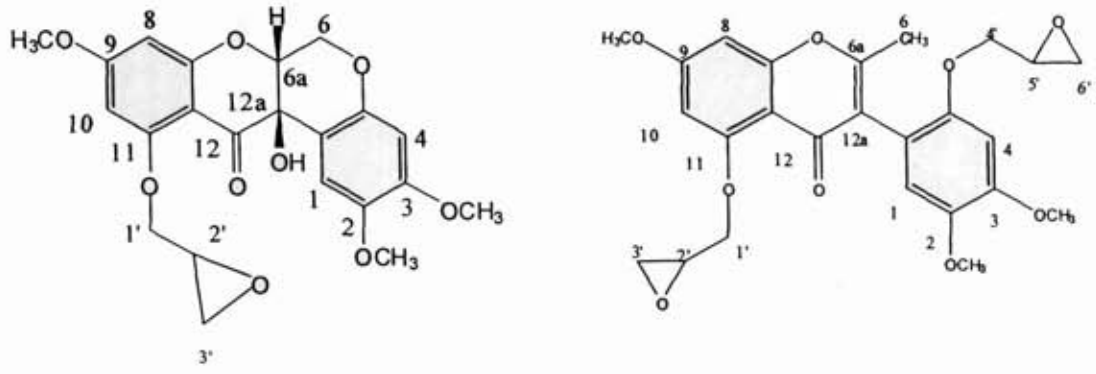


Fig 4.5 Chemical structures of doxorubicin HCl

Doxorubicin HCl is a positive control drug that has cytotoxic activities against three cancer cell lines, i.e. MCF7 (Breast cancer), KB (Human mouth carcinoma), and NCL-H187 (Human small lung cancer). The results indicate significant cytotoxic activities with the IC_{50} value of 42.52, 2.01 and 0.03 μg/ml against MCF-7, KB, and NCI-H187, respectively.

4.5.1 6-deoxyclitoriacetal-epoxidation derivatives

In order to study effect of functional group on cytotoxicities, three groups of different substituents of 6-deoxyclitoriacetal analogues have been synthesized. Firstly, 6-deoxyclitoriacetal (**1**) was treated with epichlorohydrin in the presence of sodium hydride at 80°C in dimethyformamide (DMF) to give the epoxide derivative of **2** and **3** (48% and 10% yield, respectively). The result of cytotoxicity is indicated in Table 4.4.



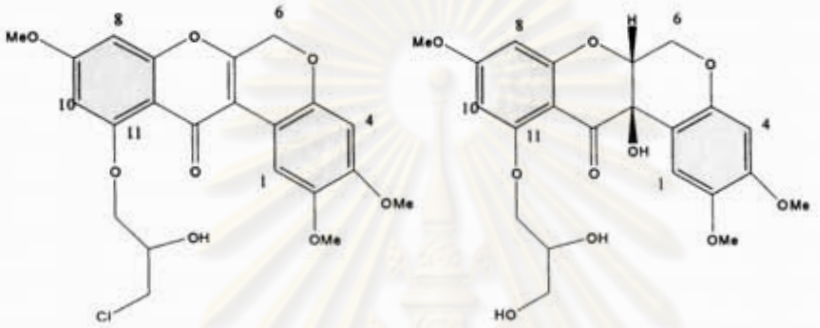
2 3

Fig 4.6 The chemical structures of (a) **2**, (b) **3**

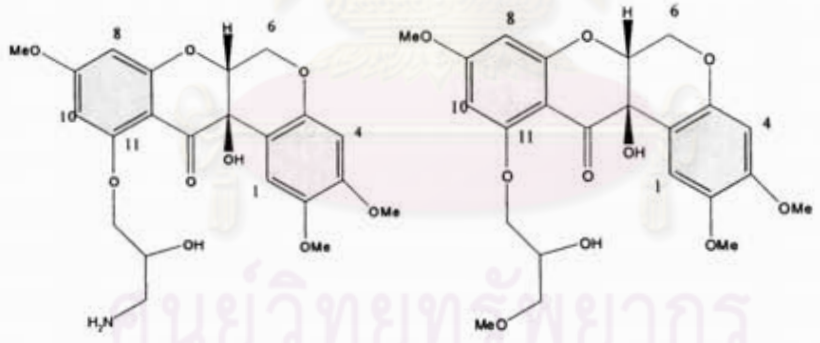
The epoxide intermediate of **2** and **3** was used to study the effect of the number of epoxide groups and the effect of its molecular planarity on cytotoxicity. It was shown that **2** (IC_{50} = 2.87, 7.33 and 3.21 μg/ml for KB, NCI-H187 and MCF7, respectively) has higher cytotoxic activities against all three cell lines than that of the parent compound (IC_{50} = 13.28, 23.65 and 3.12 μg/ml) and **3** (IC_{50} = 32.87, 5.63 and 27.83 μg/ml). Compound 3 has highest cytotoxicity against MCF-7 cell line (IC_{50} = 5.63 μg/ml) when compare to doxorubicin (IC_{50} = 42.52 μg/ml) and its parent compound (IC_{50} = 23.65 μg/ml), while it was less potent inhibitor than **2** and the parent compound against KB and NCl-H187 cell lines, respectively. We assumed that the epoxide group can increase its cytotoxicity by undergoing chemical reaction between the epoxide group of the tested compounds and the amine group of DNA, leading to the deformation of DNA structure and hence preventing the DNA replication process. Moreover, the molecular planarity of **2** can help the intercalation of the tested compound with the DNA increasing the deformation of DNA structure. In contrast to **3**, the compound bearing two epoxide groups which should be more potent inhibitor than **2**, however, the cytotoxic activity of **3** against the KB and NCl-H187 cell lines is significantly lower than **2,** because the molecule is lack of planarity according to the tetrahydropyran ring was opened by the epoxidation reaction. The results suggested that the molecular planarity is an important factor for inhibit the proliferation of KB and NCl-H187 cell lines, but less effect on against the MCF7 cell line.

4.5.2 6-Deoxyclitoriacetal – hucleophiles analogues

To investigate the cytotoxicity of the ring opened epoxide derivative, four nucleophiles (-Cl, -OH, -NH_2, and -OMe) groups containing compounds were synthesized, namely compounds **4** to **7,** respectively, (Fig. 4.7). All compounds were evaluated for their cytotoxicitis against MCF7 (breast cancer), KB (human mouth carcinoma), and NCL-H187 (human small lung cancer) cell lines. The cytotoxic activities results of each compound shown in Table 4.4



4 **5**



6 **7**

Fig 4.7 The chemical structures of **4**, **5**, **6** and **7**

Attempts to open the epoxide ring with nucleophiles for enhancing its cytotoxic activity, the results of cytotoxic activities for **4** to **7** showed that all ring opened epoxide derivatives have less cytotoxicity against all three cell lines than that of doxorubicin and their parent compound. Compounds **5**, **6** and **7** are inactive for MCF7 cell lines.

Among the nucleophile ring opened epoxide derivatives, the chloride substituent containing compound (**4**) with the IC_{50} of 14.77, 24.91 and 6.62 μg/ml for KB, MCF7 and NCl-H187 cell lines, respectively, is a more potent inhibitor than that of the rest of the tested compounds. For the KB cell lines, the $-NH_2$ (IC_{50} = 43.24 μg/ml) and -OMe (IC_{50} = 46.92 μg/ml) derivatives exhibited less cytotoxic activity than that of the -Cl (IC_{50} = 14.77 μg/ml) and the -OH (IC_{50} = 13.36 μg/ml) containing derivatives. The ability to inhibit the NCl-H187 cell line for these four ring opened epoxide derivatives are similar levels of cytotoxic activity (IC_{50} = 6.62, 13.94, 10.96 and 11.69 μg/ml for **4**, **5**, **6** and **7**, respectively). The -Cl and -OH groups are favorite to be a hydrogen bonded acceptor whereas the $-NH_2$ and -OMe groups are more preferably to be a hydrogen bonded donor. The $-NH_2$ group in DNA which is a hydrogen bonded donor prefer to form a intermolecular hydrogen bonded complex with a tested compound having hydrogen bonded acceptor, therefore, The compounds containing -Cl (**4**) and -OH (**5**) group showed higher activity than the compounds having $-NH_2$ (**6**) and -OMe group (**7**). This is may imply that the hydrogen bonded acceptor group is one of factor for improving cytotoxic activtiy against the KB cell line, but this factor dose not influence the inhibition of the cytotoxic activity against the NCl-H187 cell line.

4.5.3 6-Deoxyclitoriacetal – morpholine analogues

As mentioned hereinbefore, many morpholine containing compounds exhibit good cytotoxic acitivity. In this study, four morpholine groups were synthesized with 6-deoxyclitoriacetal, namely morpholine, N-2-aminoethyl-morpholine, diethanolmide, and 4-piperidon hydrate hydrochloride.the morpholine derivative in order to study the steric effect of side chain on cytotoxic activity. The modifications of 6-deoxyclitoriacetal derivative-morpholine analogues were prepared from the epoxide derivative. Compounds **8**, **9**, **10** and **11** (Fig. 4.8) were isolated in good yield with 86%, 94%, 91% and 68%yield, respectively. The new derivatives of **8** to **11** were assayed for cytotoxic activities against KB, MCF-7 and NCI-H187 cell lines. The results of cytotoxic activity of **8** to **11** were shown in Table 4.4

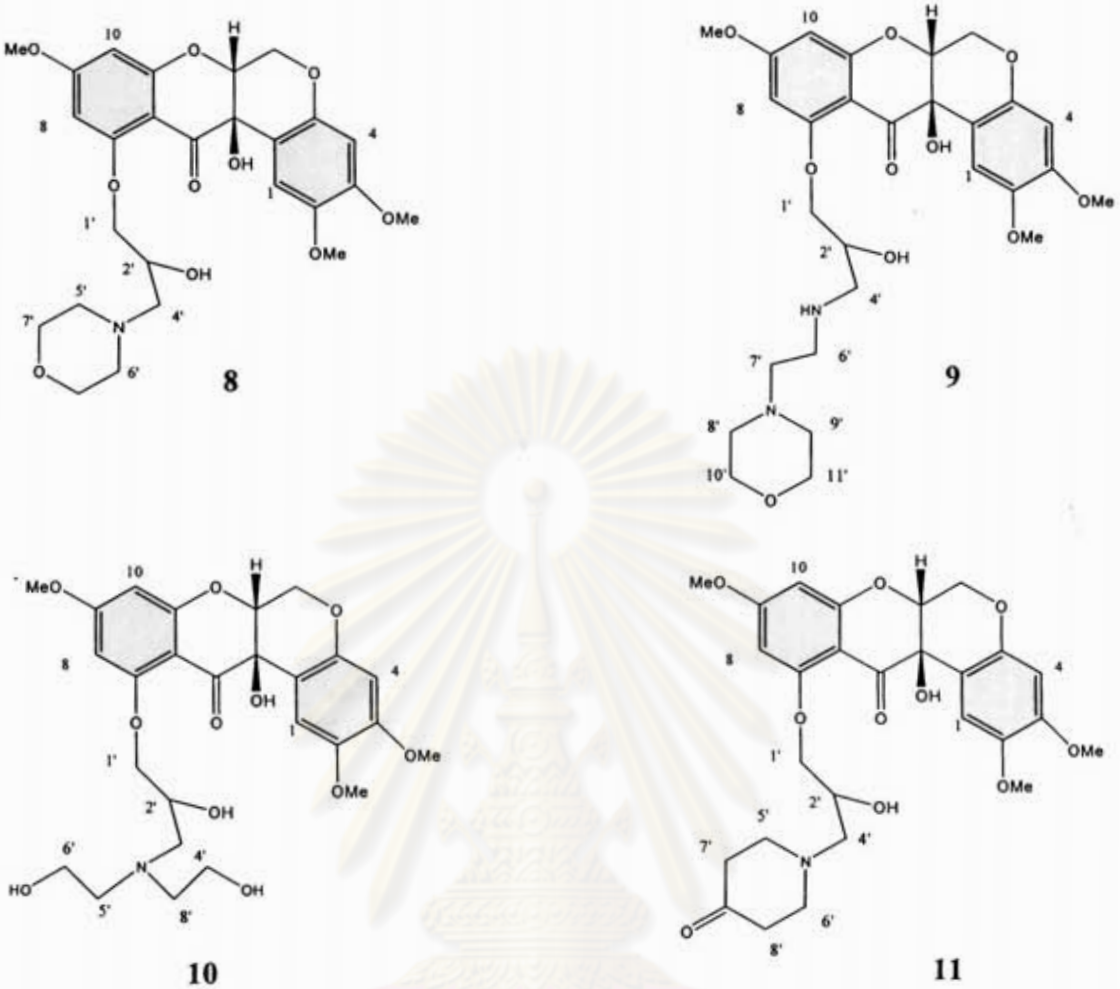


Fig 4.8 The chemical structures of **8**, **9**, **10**, and **11**

In the series of morpholine derivatives, the cytotoxic activities of **8** to **11** against the KB, MCF7 and NCI-H187 cell lines are not better than that of doxorubicin and their parent compound. Compounds **8** and **9** have comparative cytotoxic activities against all three KB, MCF7 and NCI-H187 cell lines with the IC_{50} of 26.51, 31.71 and 2.93 μg/ml for **8** and IC_{50} of 26.31, 36.82 and 4.70 μg/ml for **9**. The compound containing diethanolamine group (**10**) is inactive against all three cell lines. The results indicate that the compound containing morpholine group does noto improve the cytotoxicity. Moreover, the molecule having a bulky and flexible side chain like diethanolamine is inactive. This would seem that the steric effect of a side chain is one of factors for inhibiting the cytotoxic activity.

4.5.4 6-Deoxyclitoriacetal – aromatic ring analogues

In order to study the effect functional group of aromatic ring and the length of side chain on cytotoxic activity of 6-deoxyclitoriacetal derivatives, three aromatic ring were selected to be synthesized with 6-deoxyclitoriacetal, namely benzylamine, 2-phenylethylamine, and (R)-(-)-2-amino-2-phenylethanol. The modifications of aromatic ring containing 6-deoxyclitoriacetal derivatives using the epoxide derivative as starting material were prepared. Compounds **12**, **13** and **14** (Fig. 4.9) isolated from column chromatrography gave 83%, 69% and 78%yield, respectively. The new derivatives of **12** to **14** were tested cytotoxic activity against MCF7 (Breast cancer), KB (Human mouth carcinoma), and NCL-H187 (Human small lung cancer). The result of cytotoxicity is indicated in Table 4.4

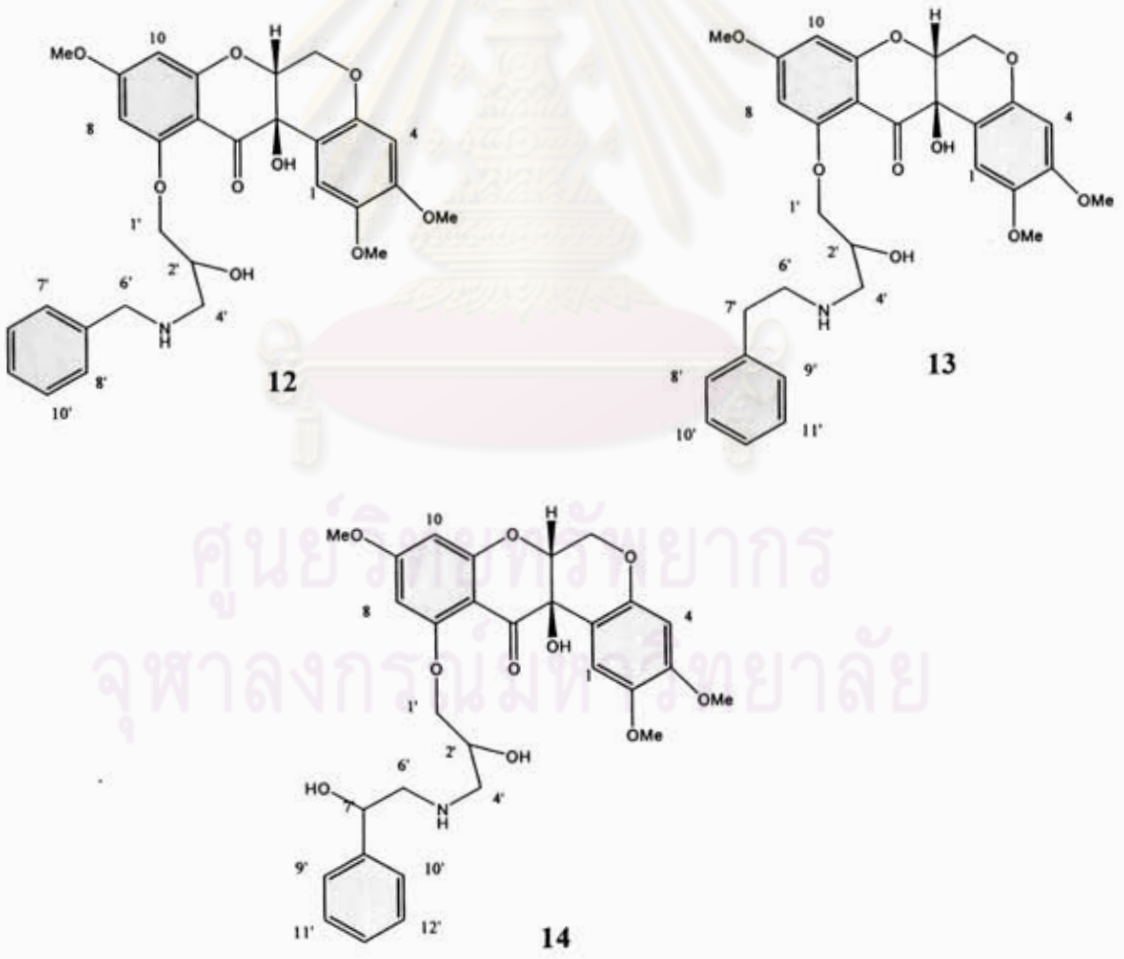


Fig 4.9 The chemical structures of **12**, **13**, and **14**

The benzylamine derivatives of 6-deoxyclitoriacetal showed less cytotoxic activity against all KB, MCF-7 and NCI-H187 cell lines than that of doxorubicin and the parent compounds, except **13** (IC_{50} = 3.05 μg/ml) has the highest cytotoxic activity against MCF-7. It is interesting that the phenylethylamine containing molecule (**13**) with IC_{50} of 23.53, 3.05 and 13.62 μg/ml for KB, MCF-7 and NCI-H187, respectively, showed higher cytotoxicity than that of the benzylamine containing compound (**12**) (IC_{50} = 32.29, 34.63 and 35.53 μg/ml), even though the side chain of **13** is longer than that of **12**.

Furthermore, the overall structure of **14** is similar to that of **13**, except the side chain of **14** bearing the hydroxyl group which is expected to increase the cytotoxic activity because having one more a hydrogen bonded acceptor in the molecule, however, the IC_{50} values of **14** against the KB, MCF-7 and NCI-H187 (46.44, inactive, 22.08 μg/ml, respectively) is even more higher than that of **13** (23.53, 3.05, 13.62 μg/ml, respectively).

For all derivatives of 6-deoxyclitoriacetal, the most potent inhibitor against KB cell line is the molecule containing epoxide group (**2**) (IC_{50} = 2.87μg/ml), while that of MCF7 and NCI-H-H187 are **13** (IC_{50} = 3.05 μg/ml) and **8** (IC_{50} = 2.93 μg/ml), respectively. It is possible that the mechanisms in these three human cell lines are different.

As a result, it can be pointed out that there is a divergence of the cytotoxicity for the MCF-7 and NCI-H187 cell lines. As can be seen from the IC_{50} values are not correlated with the functional groups, the length of side chain and the hydrogen bonded group.

However, the hydrogen bonded acceptor is one of factors for increasing the cytotoxic activity against the KB cell line. The less steric effect of the side chain is on the molecule, the higher cytotoxic activity against the KB cell line.

The molecule bearing the functional group that is able to undergo chemical bonding with the DNA; like an epoxide group, will promote the formation of a compound-DNA complex, and then the DNA is deformed. The proliferation of some cancer cells may be controlled.

4.5.5 Salt of 12a-Hydroxy-11-(2-hydroxy-3-morpholin-4-yl-propoxy)-2, 3, 9- trimethoxy-6a,12a-dihydro-6H-chromeno[3,4-b]chromen-12-one hydrochloride (15)

The above mentioned results show that the most potent inhibitor is the epoxide derivative (**2**), because it can undergo chemical reaction with DNA and then deform the DNA strains. In addition, we can assume that the derivatives consisted of functional groups that can participate non-covalent interactions with DNA have lower cytotoxic activity against human cell lines than the epoxide derivative. Therefore, we attempt to increase the cytotoxic activity of the obtained derivatives along with their water solubility. This can be performed based on the knowledge that its water solubility form has higher bioavailability than that of its free base form. Therefore, the free base form of **8** was converted to be an acid addition salt (**15**) by adding conc. HCl. It was found that the acid addition salt form (**15**) has higher water solubility (1000g/L), whereas its free form is water insoluble. Moreover, the salt form also has stronger cytotoxicity against KB and NCI-H187 cell lines (IC_{50} = 0.68μg/ml and 0.63 μg/ml, respectively) than that of its free base form (**8**) (IC_{50} = 26.51 μg/ml and 2.93 μg/ml, respectively).

Interestingly, several compounds were inactive against MCF7 (breast cancer cell line). MCF7 cell line is the estrogen receptor-positive cell line. It is used to a prominent model system for the study of breast cancer. Despite the fact that many tumors initially respond to chemotherapy, breast cancer cells can subsequently survive and gain resistance to the treatment. Tamoxifen (Fig. 4.10) is the anticancer drug used to treat the breast cancer [41]. Therefore, several compounds were inactive against MCF7 probably because this cell line was easy to resistant with many compounds. Moreover, the competitive compounds to be a breast anticancer should have a pharmacophore similar to estrogen receptor. Therefore, it can be noticed that our tested compounds has different structure to Tamoxifen, which is an anticancer drug which specific with the breast cancer, resulting in inactive activity for MCF7 cell lines.

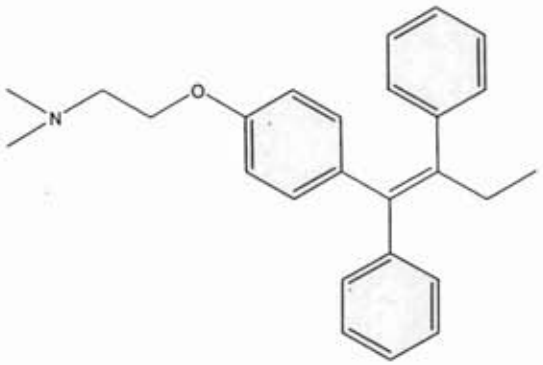


Fig 4.10 The chemical structure of Tamoxifen

4.6 Preparation of pBR322 plasmid DNA

pBR322 Plasmid DNA was prepared from gram-negative bacteria (*E. coli* HB101). FastPlasmid® mini was used for precipitation and separation of plasmid. The *HindIII*-digested λ DNA (commercially obtained) was used as a marker (100 bp-1 kb). The resulted products were analyzed by 1% agarose gel electrophoresis. The obtained plasmid DNA composed of both nicked DNA form and supercoiled pBR322 form [Fig. 4.11]. Plasmid sizes were calculated using computer software (*Syngene*-Gene Genius, Bio Imaging Systems, *Syngene*). The mobility of supercoiled form corresponds to the marker with 4.3 kb.

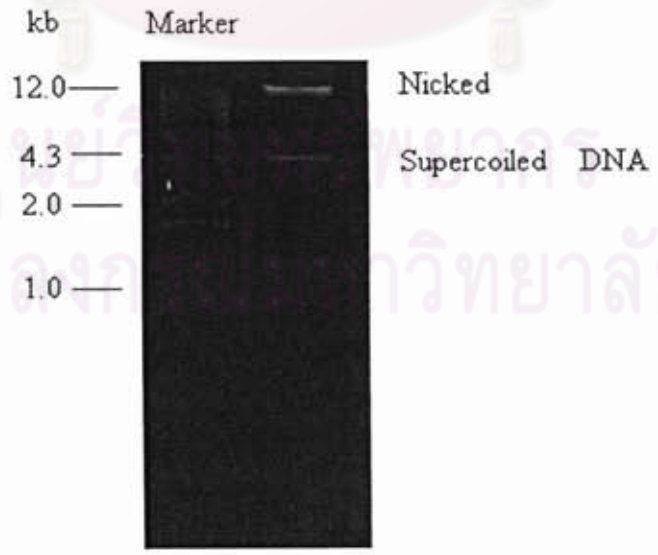


Fig 4.11 Representative of agarose gel profile of pBR322 plasmid DNA band

4.7 Topoisomerase II assay

4.7.1 Topoisomerase II inhibition by 6-deoxyclitoriacetal and its derivatives (**1** to **14**)

Topoisomerase II is nuclear enzyme that catalyzes changes in the topological state of DNA by breaking and rejoining of double stand DNA. This enzyme has important roles in DNA metabolism such as replication, recombination, transcription, and chromosome condensation. As the structure of DNA is a double helix and is in a supercoiled DNA form. The supercoild DNA must be relaxed by Topoisomerase II enzyme before processing the transcription and translation. To investigate the cytotoxic mechanism of 6-deoxyclitoriacetal and its derivatives (**1** to **14**), the effect of the synthesized compounds for inhibiting the relaxation of Topisomerase II were evaluated. The data were analyzed and %inhibition was calculated using Syngene software [33]. The results of topoisomerase II inhibiton of tested compounds and etoposide are shown in Fig 4.12, respectively.

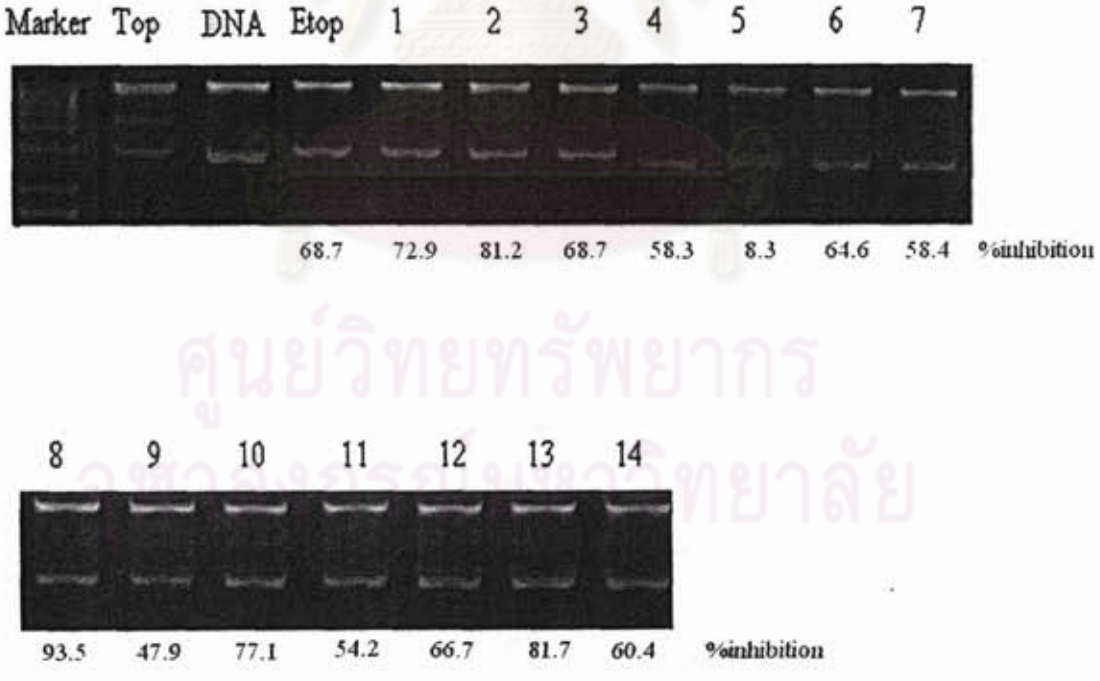


Fig 4.12 Inhibitory effects of tested compounds on human DNA topoisomerse II, supercoiled pBR322 plasmid DNA was mixed with topoisomerase II and 100 μM compounds dissolved in DMSO (land 4 to 19). Lane 2; TopoII + plasmid DNA, Lane

3; DNA only, Lane 4; TopII+ plasmid DNA + test compounds and Lane 5 to 19; TopII+ plasmid DNA + test compounds

To further investigate the mechanism of action of 6-deoxyclitoriacetal dervivatives (**2** to **14**) regarding to cytotoxicity, the relaxation of supercoiled pBR322 plasmid DNA by topoisomerase II were evaluated using etoposide, a commercial topoisomerase II inhibitor, as a positive control (Fig 4.12). The topoisomerase II assay was performed with the tested compound concentration of 100 μM and then monitored by 1% agarose gel electrophoresis. In Fig 4.11, the first lane is the *HindIII*-digested λ DNA marker, lane 2 is DNA alone, lane 3 is DNA and Top II, and lane 4 is DNA + Top II + etoposide as a reference. The results show that etoposide inhibits 68.7% of Top II reaction which is close to that of 6-Deoxyclitoriacetal **(1)** (72.9% inhibition)

Among the epoxidation derivatives (**2** and **3**), compound **2** and **3** show the inhibition rate of topoisomerase II with 81.2% and 68.7%, respectively. The topoisomerase II inhibition rates are correlated with the results of cytotoxic activities against KB, MCF-7 and NCI-H187 cell lines. Compound **2** is much higher than that of 6-deoxyclitoriacetal and **3** (see, Table 4.4).

Series of the nucleophile ring-opened epoxide derivatives (**4** to **7**) reveal similar inhibition rates of Topoisomerase II, which are in the range of about 58.3% to 64.6%inhibition, except for the hydroxyl derivative (**5**) gives only 8.3% inhibition. This indicates that **5** is inactive to topoisomerase II inhibition but exhibits good cytotoxic activity against the KB and NCF-H187 cell lines. This is also an evidence of a non-correlation between the topoisomerase II activity and cytotoxic acitivity against some human cell lines.

Series of morpholine derivatives (**8** to **11**) reveal that the morpholine derivative **8** is the most potent inhibitor of topoisomerase II activity with 93.5%inhibition. It is interesting that the diethanolamine containing compound (**10**), which is inactive for all three cell lines, but it can strong inhibit the topoisomerase II reaction with 77.1%inhibition,

The topoisomerase II inhibition of benzylamine series is in the range of 60.4% to 81.7%inhibition. Therefore, the inhibition rate of topoisomerase II reaction induced by benzylamine derivatives not correlated to the cytotoxic activity.

Compound **13** showed the strongest inhibition on topoisomerase II with 81.7%inhibition and it is the most potent inhibitor against MCF-7 (IC_{50} = 3.05μg/ml).

As results of cytotoxicity and Topoisomerase II inhibition, compounds **2**, **8**, and **13** are not only exhibited good cytotoxicity, but also shown good topoisomerase II inhibition. In particular, **2** might show cytotoxicity via topoisomerase II inhibition. These results confirm that topoisomerase II inhibition might help to increase cytotoxic activity. However, the ability of 6-deoxyclitoriacetal derivatives to inhibit topoisomerase II is thought to be important for the biological properties. Therefore, 6-deoxyclitoriacetal derivatives-induced topoisomerase II inhibition may originate not only from topoisomerase II targeting but also from other mechanisms.

4.7.2 Topoisomerase II inhibition by water solubility compound (**15**)

The effect of compound **8** (free base form) and **15** (salt form) on topoisomerase II inhibition (Fig 4.13, lanes 4 to 7) were investigated. The effect of concentrations of compounds **8** and **15** at 100 μM and 20 μM were evaluated. The results show that the salt form (54.3%inhibition) can inhibit the topoisomerase II reaction better than that of its free base (66.7%inhibition) at the same concentration (100μM) (Fig. 4.13, lanes 4 and 6, respectively). At lowering concentration (20μM) of compounds **8** and **15,** both compounds showed lower % inhibition (17.1% and 14.3% inhibition for **8** and **15**, respectively) than that of their higher concentration (67.7% and 54.3% inhibition for **8** and **15**, respectively (Fig 4.13, lane 5 and 7).

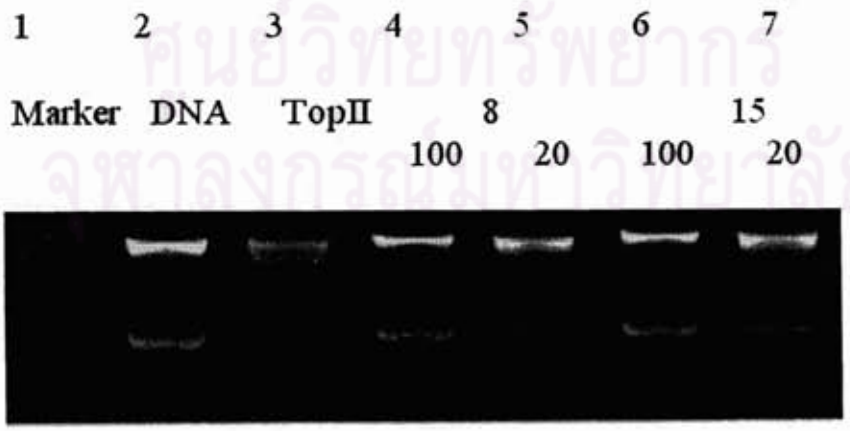
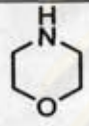
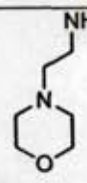
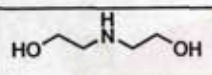
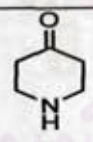
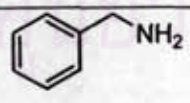
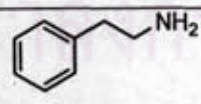
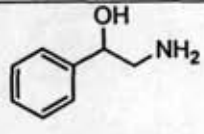
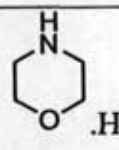


Fig 4.13 Inhibitory effects of 6-deoxyclitoriacetal derivatives (**8** and **15**) on human DNA topoisomerse II, supercoiled plasmid DNA was mixed with topoisomerase II compounds. Lane 1; Marker, Lane 2; DNA only, Lane 3; TopoII + plasmid DNA, Lane 4 and 5; TopII+ plasmid DNA + compound **8** at 100 and 20 μM, Lane 6 and 7; ; TopII+ plasmid DNA + compound **15** at 100 and 20 μM.

As a result, it can be assumed that the activity of topoisomerase II inhibition induced by tested compounds depending on their concentration. In addition, comparison of cytotoxicity between both compounds was indicated that the salt form (**15**) has stronger cytotoxicity than that of its free base (**8**), as well as its topoisomerase II inhibition. The salt form exhibited high cytotoxicity against NCI-H187 and KB cell lines with IC_{50} values of 0.63 and 0.68 μg/ml, respectively, whereas **8** shown only high cytotoxicity against NCI-H187 cell line with IC_{50} value of 2.93 μg/ml. From the results, although **15** shown good cytotoxicity, it show less topoisomerse II inhibition than **8**. Its mechanism might show other.

Table 4.5 The summary of the characteristics, cytotoxic activities and % inhibition topoisomerase II of all compounds

Compounds	R	% topII Inhibition	IC_{50} (µg/ml)		
			Anti-KB	Anti-MCF7	Anti-NCI-H187
Doxorubicin	-	-	2.01	42.52	0.03
Etoposide	-	68.7	-	-	-
1	-	72.9	13.28	23.65	3.12
2	-	81.2	2.87	7.33	3.21
3	-	68.7	32.87	5.63	27.82
4	Cl	58.3	14.77	24.91	6.61
5	OH	8.3	13.36	Inactive	13.94
6	NH_2	64.6	43.24	Inactive	10.96
7	OMe	58.4	46.92	Inactive	11.69
8		93.5	26.51	31.71	2.93
9		47.9	26.31	36.82	4.7
10		77.1	Inactive	Inactive	Inactive
11		54.2	Inactive	48.63	Inactive
12		66.7	32.29	34.63	35.53
13		81.7	23.53	3.05	13.62
14		60.4	46.44	Inactive	22.08
15	 .HCl	54.3	0.68	Inactive	0.63

CHAPTER V

CONCLUSION

Conclusion

6-Deoxycliltoriacetal (**1**) is a rotenoid compound extracted from the dried root of *Stemona collinse* Craib. 6-deoxyclitoriacetal has good cytotoxicity against various types of human carcinoma. Therefore, in order to enhance its cytotoxic activity and seeking for new Topoisomerase II inhibitor, the derivatives of 6-deoxycliltoriacetal was synthesized by converting 6-deoxyclitoriacetal to epoxide derivative by reacting with epichlorohydrin. The epoxide derivative was ring-opened with nucleophiles, morpholine and benzylamine compounds. All compounds were tested cytotoxicity against human breast (MCF-7), oral (KB), and small cell lung (NCI-H187) cancer cell lines.

Among the 6-deoxyclitoriacetal derivatives (**2** to **3**), **2** exhibited strong cytotoxicity against all cell lines including KB, NCI-H187 and MCF7 with the IC_{50} value of 2.87, 3.21, and 7.33 μg/ml, respectively, whereas **3** exhibited high cytotoxicity against only MCF-7 cell line with the IC_{50} value of 5.63 μg/ml. However, both compounds also strongly inhibited the topoisomerase II activity with 81.2% and 68.7%inhibition, respectively. This is probably due to the epoxide group reacated with an amine group in DNA and then form a stable complex, and deform the DNA double strands.

Among the 6-deoxyclitoriaceta-nucleophilic derivatives (**4** to **7**), the compound **4** showed the best cytotoxicity against NCI-H187 cell line with the IC_{50} value of 6.61 μg/ml whereas other compounds elicited moderate cytotoixcity. The results suggest that the present of a high electronegativity atom of chloride atom reduces steric effects and enhanecs the stable binding compound-DNA complex.

Among the 6-deoxyclitoriaceta-morpholine derivatives (**8** to **11**), compounds containing morpholine (**8**) and amino-morpholine (**9**) exhibited strong cytotoxicity and selective against NCI-H187 cell line with IC_{50} value of 2.93 and 4.70 μg/ml, respectively. In addition, both compounds are also good topoisomerase II inhibitors

with 93.5% and 47.9%inhibition, respectively. Especially, **8** is the most potent topoisomerase II inhibitor. However, diethanolamine containing 6-deoxyclitoriacetal (**10**) was inactive for all three cell lines. It might be owing to the steric effect.

Among the 6-deoxyclitoriacetal-aromatic derivatives (**12** to **14**), **13** exhibited a high cytotoxicity against MCF-7 cell line with the IC_{50} value of 3.05 μg/ml and had good topoisomerase II inhibitory activity with 81.7%inhibition. In contrast, **12** showed lower cytotoxic activities for all cell lines and less inhibit topoisomerase II activtiy than that of **13**. The results suggest that the aliphatic chain of benzylamine increase with decreasing of cytotoxic and Topoisomerase II activity.

In order to enhance bioavailability of the synthesized compounds, an acid addition salt of **8** was prepared by adding strong hydrochloric acid. The salt of morpholine containing 6-deoxyclitoriacetal hydrocholide showed stronger cytotoxicity against KB and NCI-H187 with IC_{50} of 0.68 and 0.63 μg/ml, respectively, than that of its free base with the IC_{50} of 26.51 and 2.93 μg/ml, respectively. Its salt also has a stronger cytotoxity against KB than 2.01 IC_{50} μg/ml of doxorubicin. In addition, it also inhibits topoisomerase II with 54.3% and 14.3%inhibition at 100 and 20 μM, respectively, than that of **8** with 67.7% and 17.1% inhibition.

From results of Topoisomerase II assay revealed that most of 6-deoxyclitoriacetal inhibited Topoisomerase II with %inhibition over 50%, except for **5** and **9** cannot be complete inhibited the activity of Topoisomerase II enzyme. The morpholine derivative **8** at 100 μM exerted the most potent Topoisomerase II enzyme with 93.5% inhibition.

REFERENCES

[1]. Chun, K.H.; and Kosmeder, S. H. Effects of Deguelin on the Phosphatidylinositol 3-Kinase/Akt Pathway and Apoptosis in Premalignant Human Bronchial Epithelial Cells. J. Natl. Cancer Inst 95 (2003): 291-302

[2]. Ching, S.C.; Lai, H.M.; Leung, P. T.; and Young, K. Dielectric Microspheres as Optical Cavities. Proceedings of 3rd Asia-Pacific Physics Conference 1 (1986): 80-81.

[3]. Kapoor, L.D. Handbook of Ayurvedic Medicinal Plants. CRC Pres Boca Raton, 1990.

[4]. Subcharoen, P. Handbook of anticancer: Thai Traditional medicine: new concept for treated cancer. Thai Traditional Medicine Institute: Ministry of Public Healt, 1998.

[5]. Lin, L. J.; Ruangsusngsi, N.; Cordell, G. A.; Shieh, H. L.; and Pezzuto, J. 6-deoxyclitoriacetal from clitoria-macrophylla. Phytochemistry 31 (1992): 4329-4331.

[6]. Fang, N.; and Casida, J.E. Anticancer action of cube insecticide: correlation for rotenoid constituents between inhibition of NADH: ubiquinone oxidoreductase and induced orinithine decarboxylase activities. Proc. Natl. Acad. Sci 95 (1998): 3380-3384.

[7]. Silva, B. P.; Bernardo, R.R.; and Perente, J.P. Clitoriacetal 11-O-β-D glucopyranoside from *Clitoria fairchildiana*. Phytochemistry 47 (1998): 121-124.

[8]. Lin, L.; Wang H. K.; Chang, J. J.; McPhail, A. T.; McPhail, D. R.; Terada, H.; Konoshima, T.; Kokumai, M.; Kozuka, M.; Estes, J. R.; and Lee, K. H. Antitumor agents, 138 rotenoids and isoflavones as cytotoxic constituents from *Amorpha fruticosa*. J. Nat. Prod 56 (1993): 690-698.

[9]. Konoshima, T.; Terada, H.; Kokumai, M.; Kozuka, M.; Tokuda, H.; Estes, J.R.; Li, L.P.; Wang, H. K.; and Lee, K.H. Studies on inhibitors of skin tumor promotion, XII. Rotenoids from *Amorpha fruticosa*. J. Nat. Prod 56 (1993): 843-848.

[10]. Roengsumran, S.; Khorphueng, P.; Chaichit, N.; Muangsin, N.; and Petsom, A. Crystal structure of 6-deoxyclitoriacetal, $C_{19}H_{18}O_8$. Z. Kristallogr. NCS 28 (2003): 105-106.

[11]. Vladu, B.; Woynarowski, J. M.; Manikumar, G.; Wani, M. C.; Wall, M. E.; Von-Hoff, D. d.; and Wadkins, R. M. 7- and 10-Substituted camptothecins; dependence of topoisomerase I-DNA cleavable complex formation and stability on the 7- and 10-substituents. Mol. Pharmacol 57 (2000): 243-251.

[12]. Masthers, C.D.; Boschi-Pinto, C.; Lopez, A. D.; and Murray, C. J. L. Cancer incidence, mortality and surivival by site for 14 regions of the world. World Health Organization, 2001.

[13]. Kwok, Y.; Hurley, L.H. Topoisomerase II site-directed alkylation of DNA by Psorospermin and its effect on toposiomerase II-mediated DNA cleavage. J.Biol.Chem. 273 (1998): 3020-3026.

[14]. National Statisical Office. Key Statistics of Thailand 2003. Ministry of Information and Communication Technology, 2003

[15]. Parkin, D.M. Global cancer statistics in the year 2000. Lancet Oncol 2 (2001): 533-543.

[16]. Hartwell, J. L. Plants Used Against Cancer, Quarterman, Lawrence, Massachusetts (1982): 89-93.

[17]. Cragg, G. M.; Boyd, M. R.; Cardellina, J. H. Newman, D. J.; Snader, K. M.; and Mccloud, T. G. Ethnobotany and the Search for New Drugs in Ciba Foudation Symposium. Wiey & Sons, Chichester, U. K185 (1994): 178-196

[18]. Kostava, I. Synthetic and Natural Coumarins as Cytotoxic Agents. Curr. Med. Chem-Anti Cancer Agents 5 (2005): 29-46.

[19]. Bergerat, A.; Gadelle, D.; and Forterre, P. Purification of a DNA topoisomerase II from the hyperthermophilic archaeon Sulfolobus shibatae: a thermostable enzyme with both bacterial and eucaryal features. J. Biol. Chem 269 (1994): 27663–27669.

[20]. Crombie, L. Reviw article number 135 biosynthesis in the rotenoid group of natural products: applications of isotope methodology. Phytochemistry 6 (1998): 476-507.

[21]. Pacher, C.; Seger, D.; Engelmeier, S.; Vajrodaya, O.; and Hofer, H. Antifungal stilbenoids from Stemona collinsae. Journal of Natural Products. 65 (2002): 820–827.

[22]. Brem, C.; Seger, T.; Pacher, O.; Hofer, S.; Vajrodaya and Greger, H. Feeding deterrence and contact toxicity of *Stemona* alkaloids—a source of potent natural insecticides. Journal of Agricultural and Food Chemistry 50 (2002): 83–88.

[23]. Kaltenegger, E.; Brem, B.; Mereiter, K.; Kalchhauser, H.; Kählig, H.; Hofer, O.; Vajrodaya, S.; and Greger, H. Insecticidal pyrido[1,2-a]azepine alkaloids and related derivatives from *Stemona* species. Phytochemistry 63 (2003): 803–816.

[24]. Mosquera, R.; and Orijales, A. Synthesis of two new diosmetin ethers. Indian. J. Chemistry 34B (1995): 1083-1084.

[25]. Saha, B.; Saha, D.; Niyogi, S.; and Bal, M. A New Method of Plasmid DNA Preparation by Sucrose-Mediated Detergent Lysis from Escherichia coli (Gram-Negative) and Staphylococcus aureus (Gram-Positive). Analytical BioChem 176 (1989): 344-349.

[26]. Fang, N.; and Casida, J. W. Anticancer action of cube insecticide: Correlation for rotenoid constituents between inhibition of NADH: ubiquinone oxidoreductase and induced ornithine decarboxylase activities. Proc. Natl. Acad. Sci 95 (1998): 80-84.

[27]. Ito, C.; Itoigawa, M.; Kajima, N.; Tan, H.T.-W.; Takayasu, J.; Tokuda, H.; Nishino, H.; and Furukawa, H. Cancer chemopreventive activity of rotenoids fromDerris trifoliata. PlantaMedica 70 (2004): 585–588.

[28]. Suffness, M.; and Pezzuto, J. M. Assays related to cancer drug discovery. Method in Plant Biochemistry, Assays for Bioactivity, Acadamic Press 6 (1991): 71-133.

[29]. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assay. J. Jmmuno. Met 65 (1983): 55-63.

[30]. Gasiorawski, K.; Malinka, W. Swiatek, P.; and Jaszczyszyn, A. Antimutagenic activity of new analogues of fluphenazine. Cellular & Molecular Biology Leters 8 (2003): 927-942.

[31]. Xie, X.; Lemcke, T.; Gussio, R.; Zaharevitz, W.D.; Leost, M.; Meijer, L.; and Kunick, C. Epoxide-containing side chains enhance antiproliferative activity of paullones 40 (2005): 655-661.

[32]. Cordova, I.; Leon, L.; Leon, F.; Andres L.; and Luis, J. Synthesis and antiproriferative activity of novel sugiol β-amino alcohol analogues. J. Med. Chem 41 (2006): 1327-1332.

[33]. Suenaqa, M.; Yamaquchi, A.; Soda, H.; Orihara, K.; Sakaki, Y.; Umehara, M.; Terashi, K.; Kawamata, N.; Oka, M.; Kohno, S.; and Tei, C. Antiproliferative effects of gefitinib are associated with suppression of E2F-1 expression and telomerase activity.Pub. Med 26 (2006): 3387-91.

[34]. Wasowska, M.; Oszczapowicz, I.; Wietrzyk, J.; Opolski, A.; Madej, J.; Dzimira, S.; and Oszczapowicz, J. Influence of the structure of new anthracycline antibiotics on their biological properties. Anticancer Res 25 (2005): 2043–2048.

[35]. Kumar, V.; Mudgal, M.; Rani, N.; Jha, A.; Jaggi, M.; Singh, A.; Sanna, V.; Singh, P.; Sharma, P.; Irchhaiya, R.; and Curman, A. Synthesis of functionalized amino acid derivatives as new pharmacophores for designing anticancer agents. J. Enzyme inhibition and Medicinal Chemistry (2008): 1-8.

[36]. Hodgson, D. M.; Gibbs, A. R.; and Lee, G. P. On the mechanism of lithium amide-induced rearrangements of 4-substituted cyclopentene oxides to cyclopentenols. Tetrahedron 52 (1996): 14861-14384.

[37]. Hanson, R. M. The synthetic methodology of nonracemic glycidol and related 2,3-epoxy alcohols. Chem. Rev 91 (1991): 437-475.

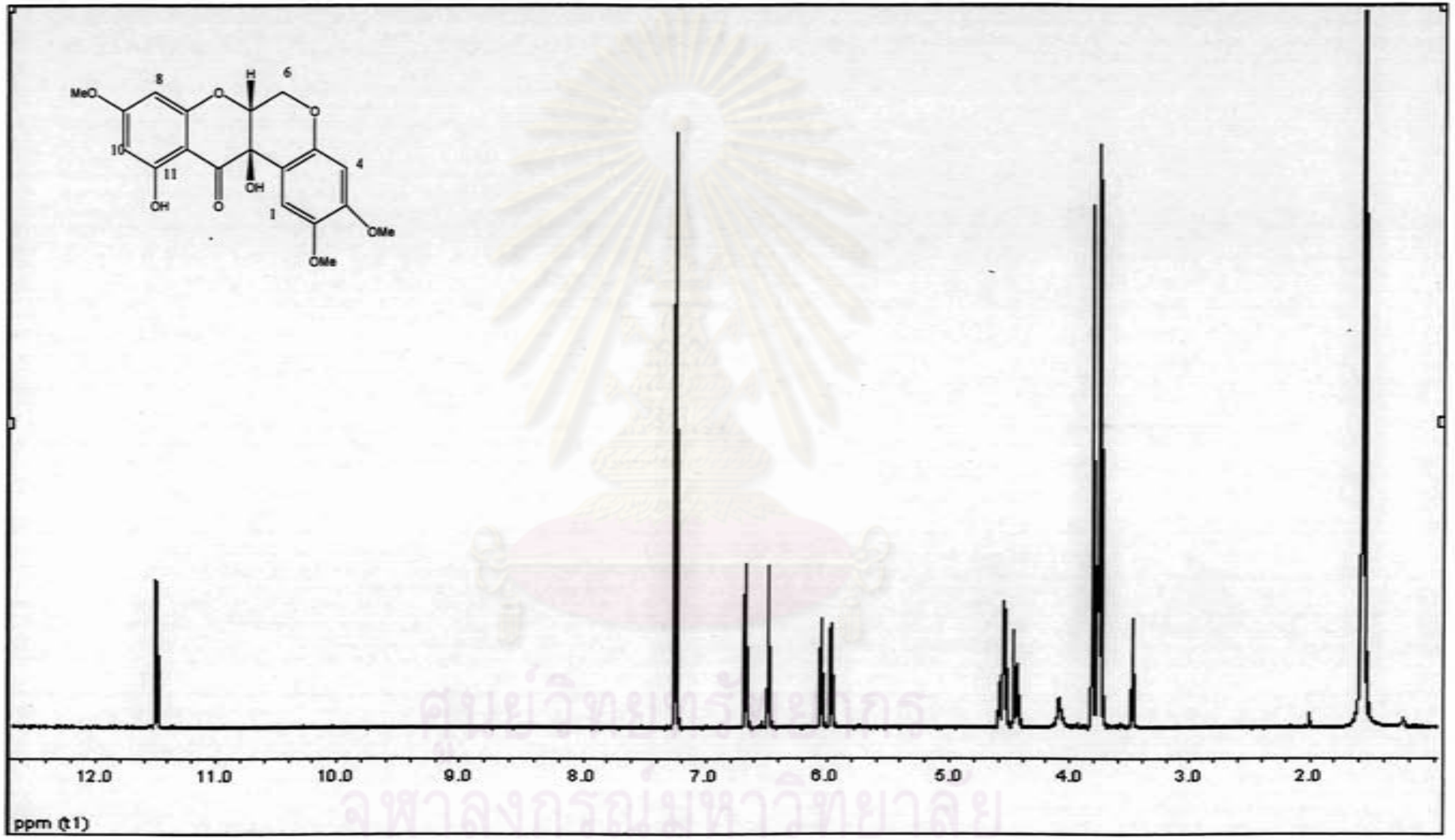
[38]. Temperini, C.; Messori, L.; Orioli, P.; Bugno, C. D.; Animati, F.; and Ughetto, G. Thecrytal sructure of the complex between a disacchairde anthracycline and the DNA hexamer d(CGATCG) reveals two different binding sites involving two DNA duplexes. Nucleic Acids Res 31 (2003): 1464-1469.

[39]. Frederick, C. A.; Williams, L. D; Ughetto, G.; Van der Marel, G. A.; van Boom, J. H.; Rich, A.; and Wang Andrew, H. J. Sturture comparison of anticancer drug-DNA complexed: adriamyckin and daunomycin. Biochemistry 29 (1990): 2538-2549.

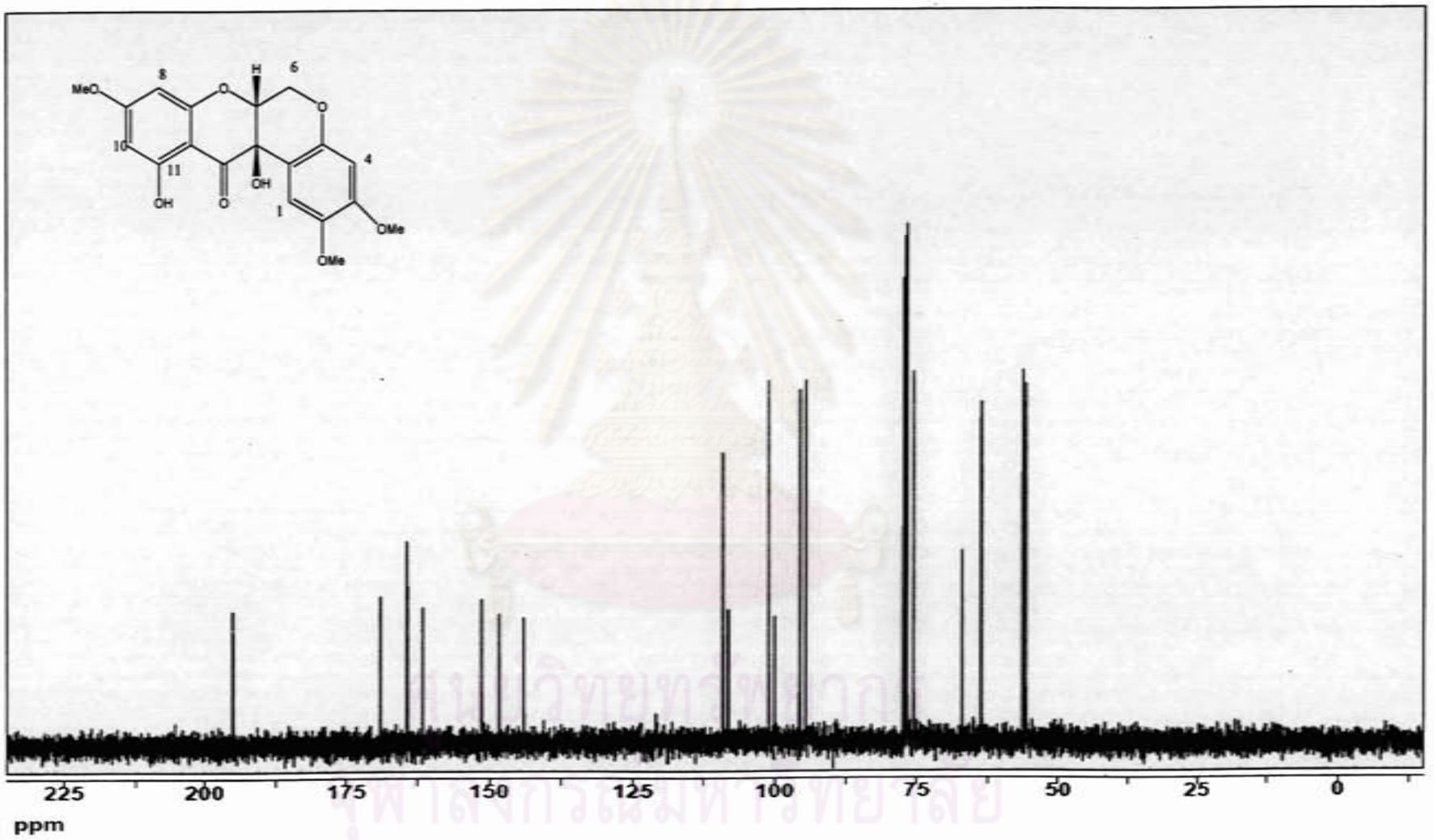
[40]. Woo, S.; Jung, J.; Lee, C.; Kwon, Y.; Na, Y. Synthesis, Cytotoxicity and Topoisomerase II Inhibition Study of New Thioxanthone Analogues. Bioorg. Med. Chem. Lett 17 (2007): 471-474.

[41]. Jordan, V.C. Tamoxifen (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. Br. J. Pharmacol 147 (2006): 269-276.

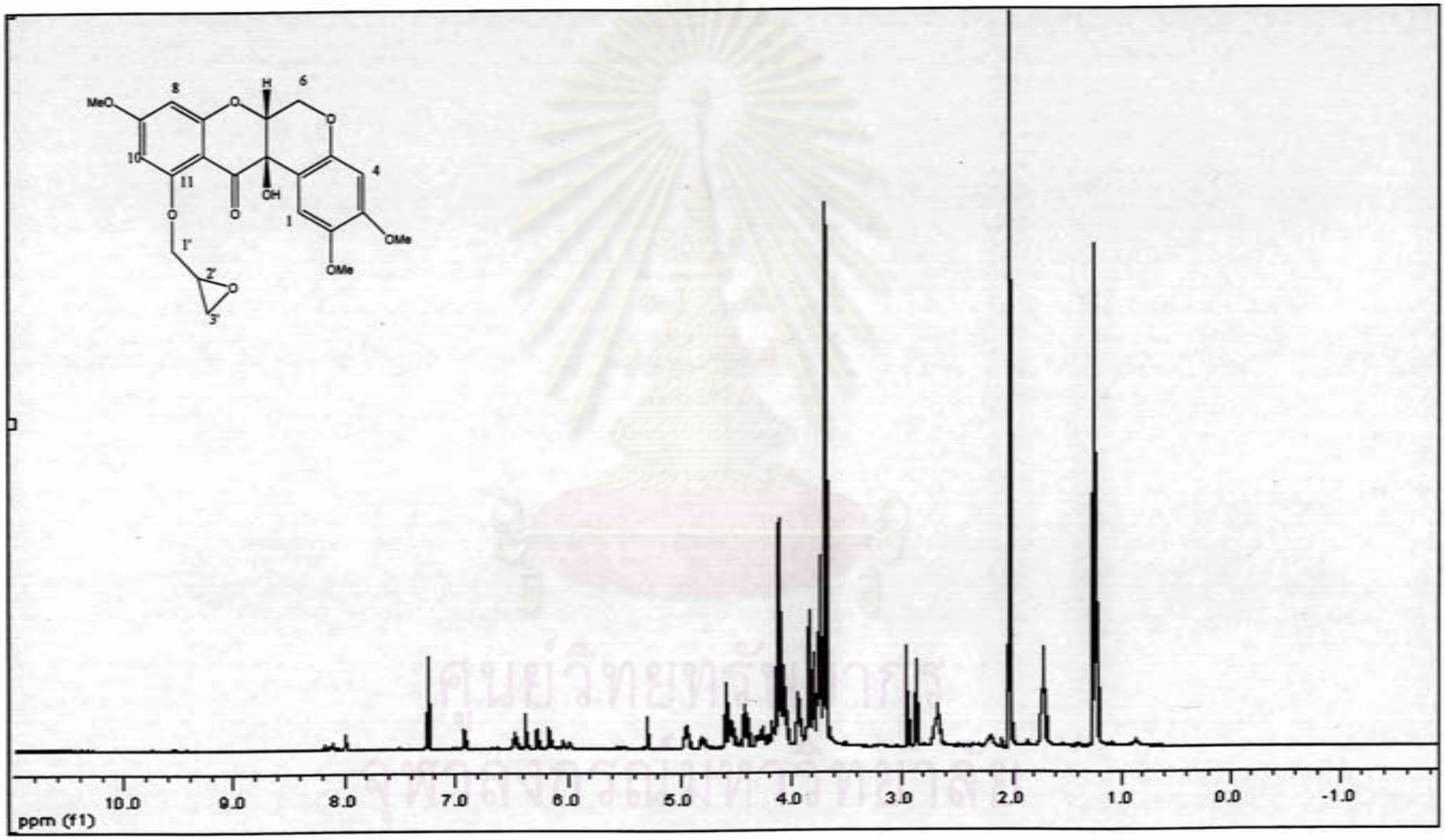
APPENDICES



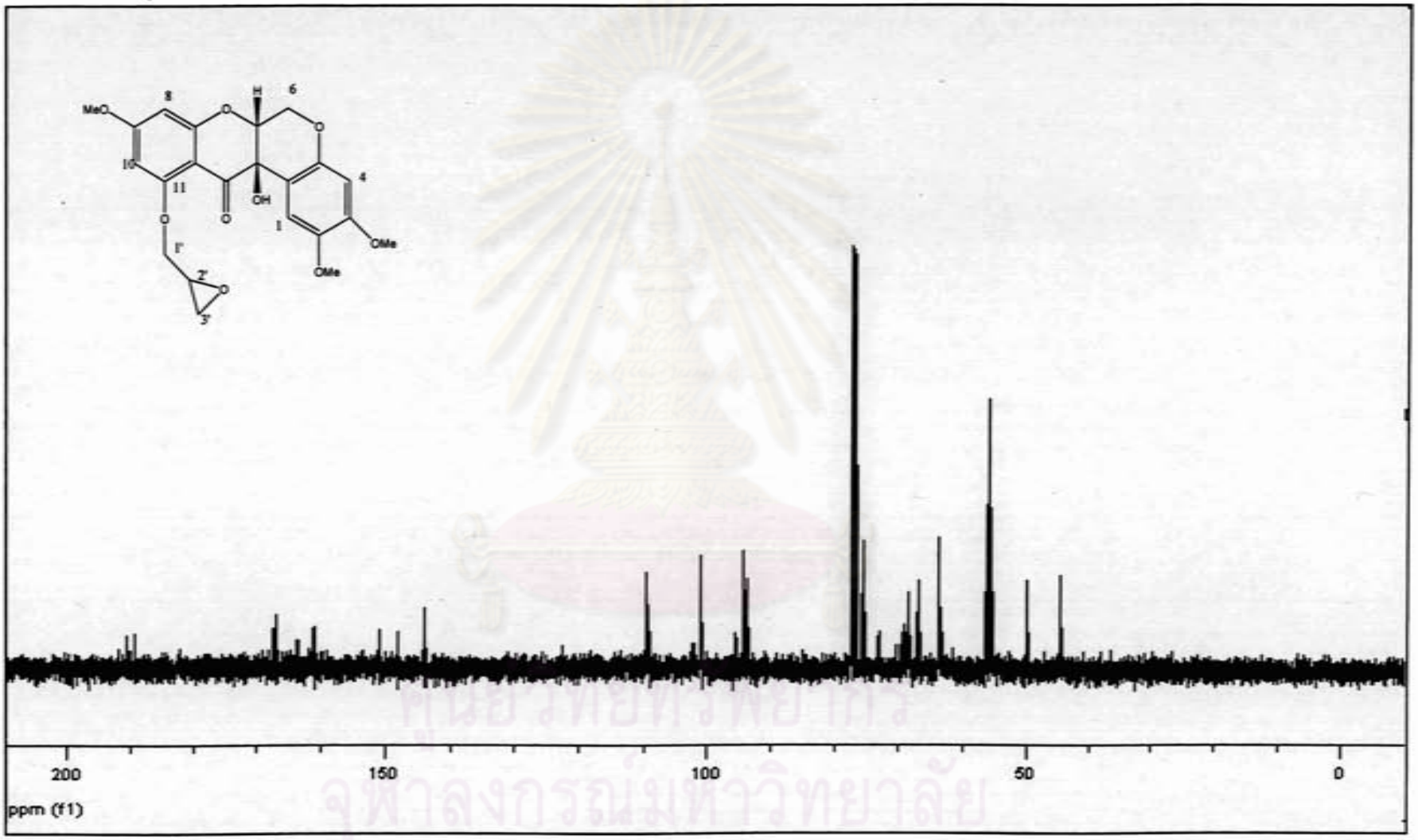
A.1 ^{1}H NMR Spectra of **1**



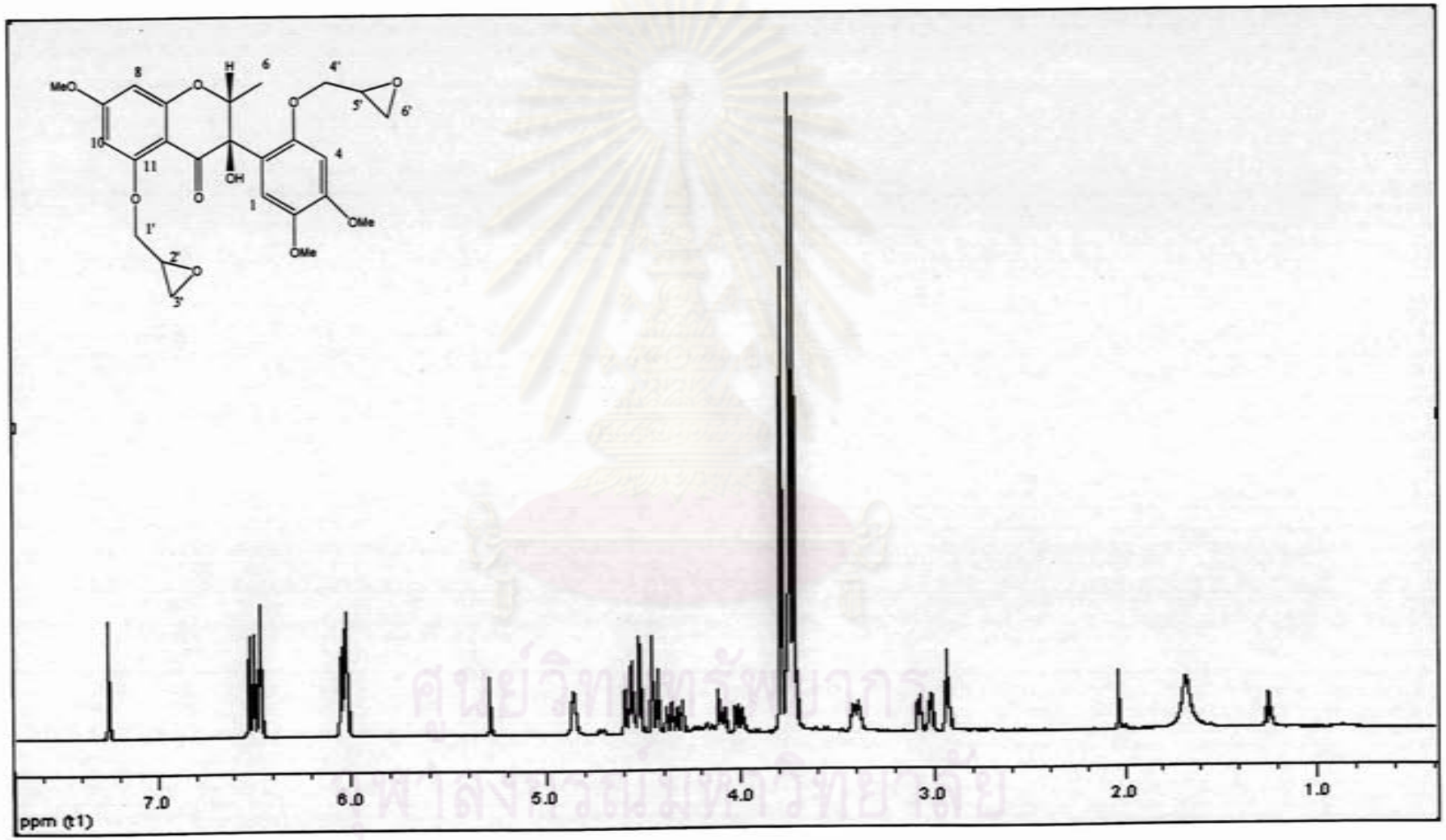
A.2 ^{1}H NMR Spectra of **1**



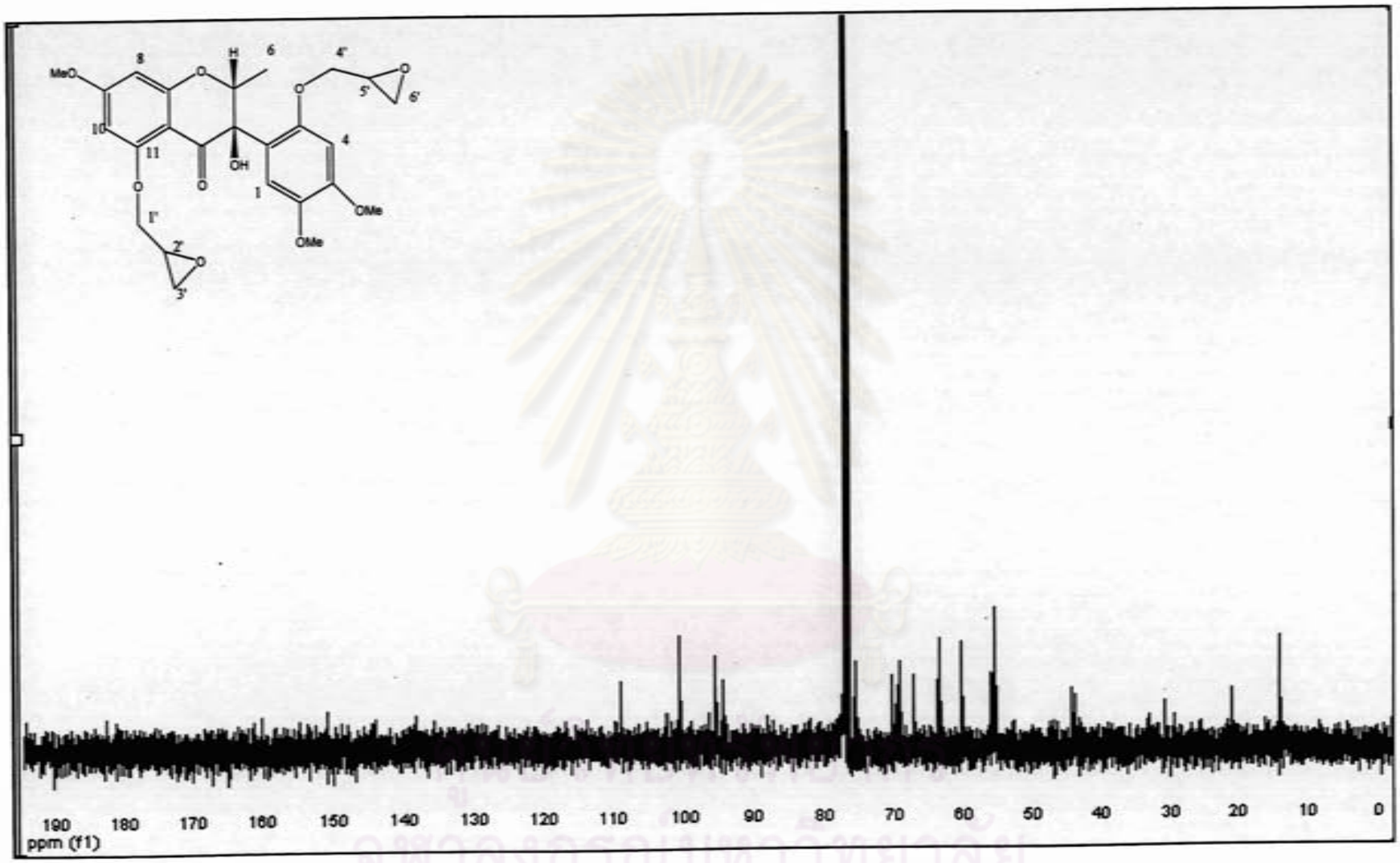
A.3 ^{1}H NMR Spectra of **2**



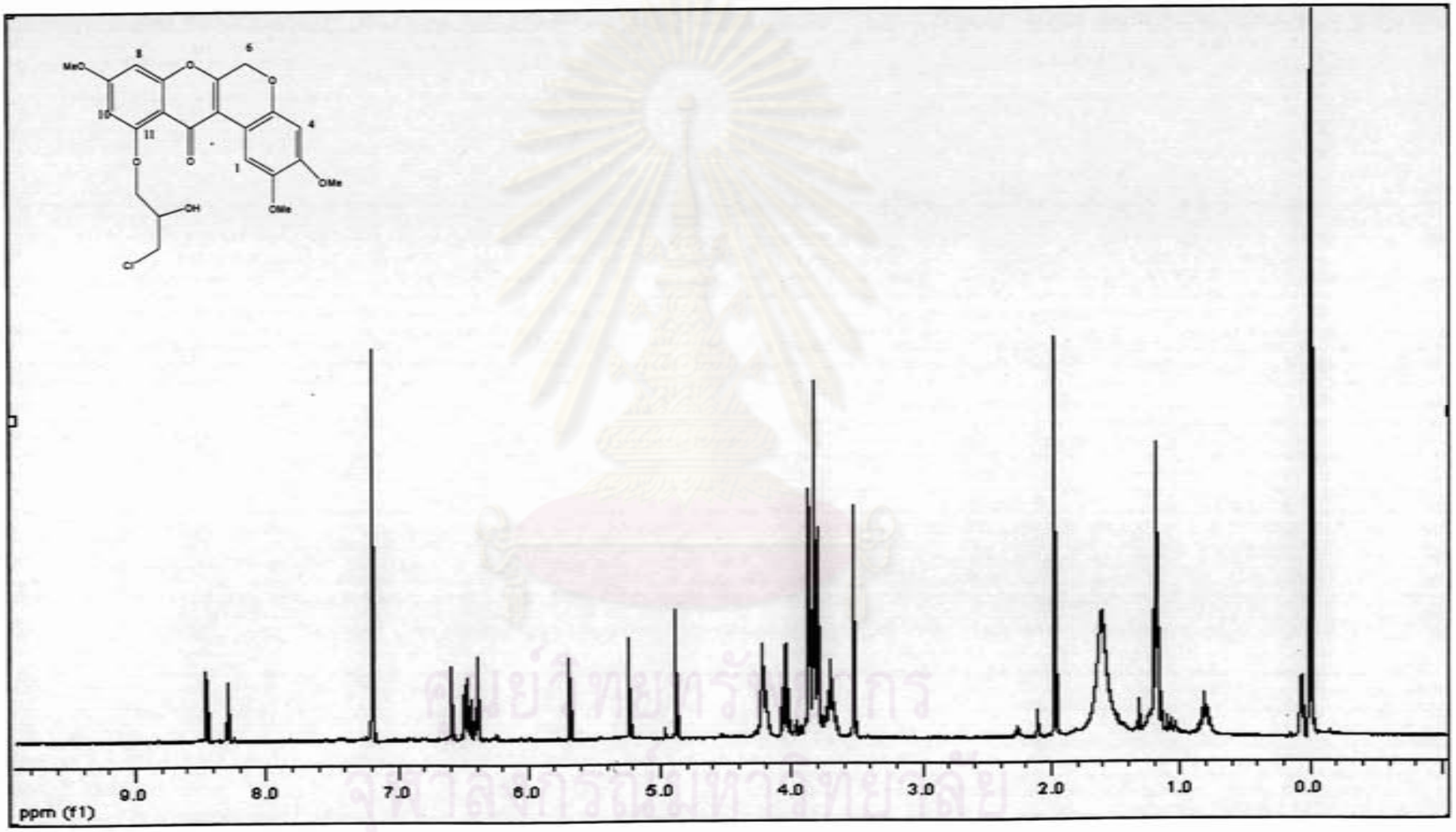
A.4 ^{1}H NMR Spectra of **2**



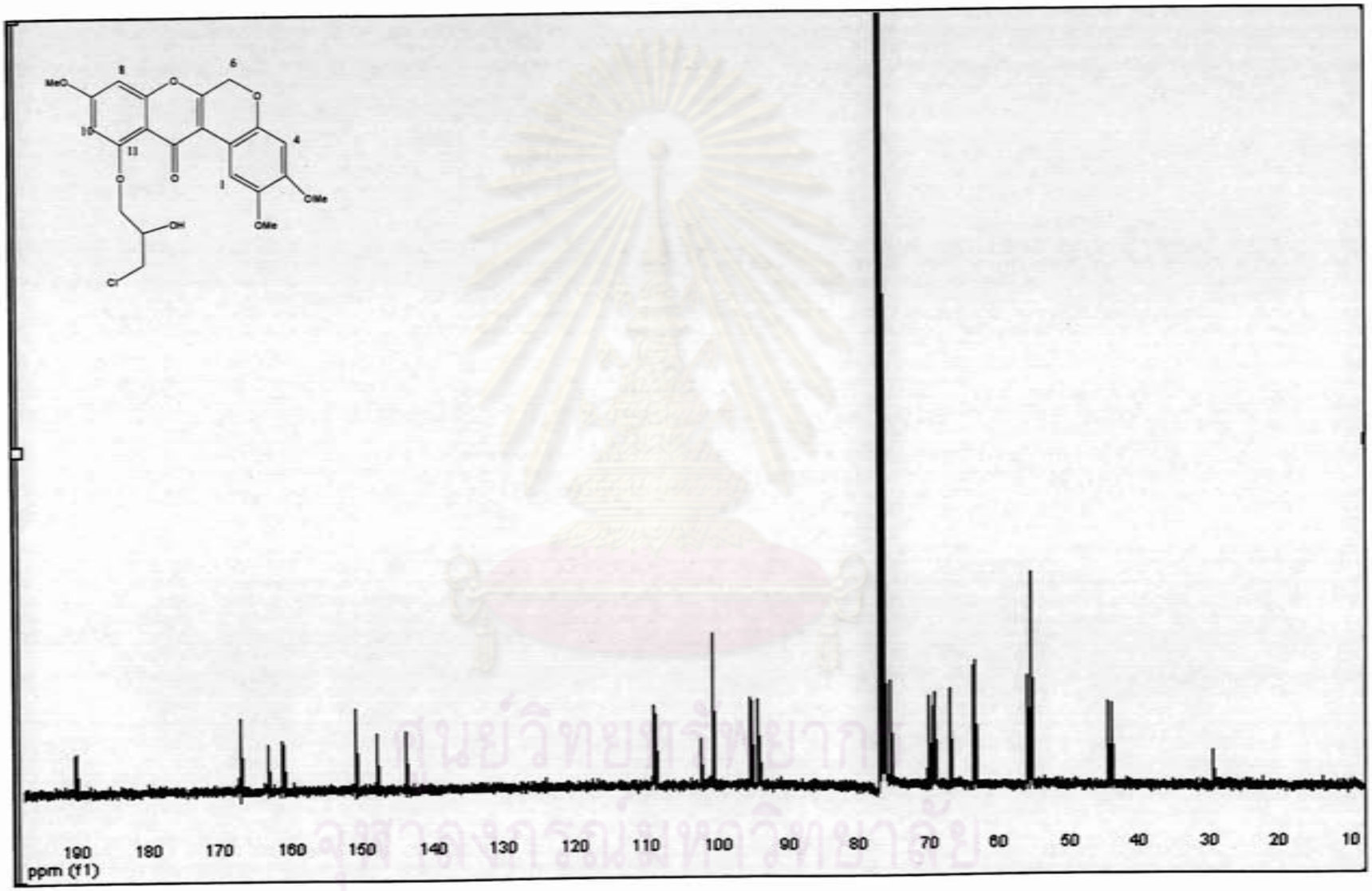
A.5 ^{1}H NMR Spectra of **3**



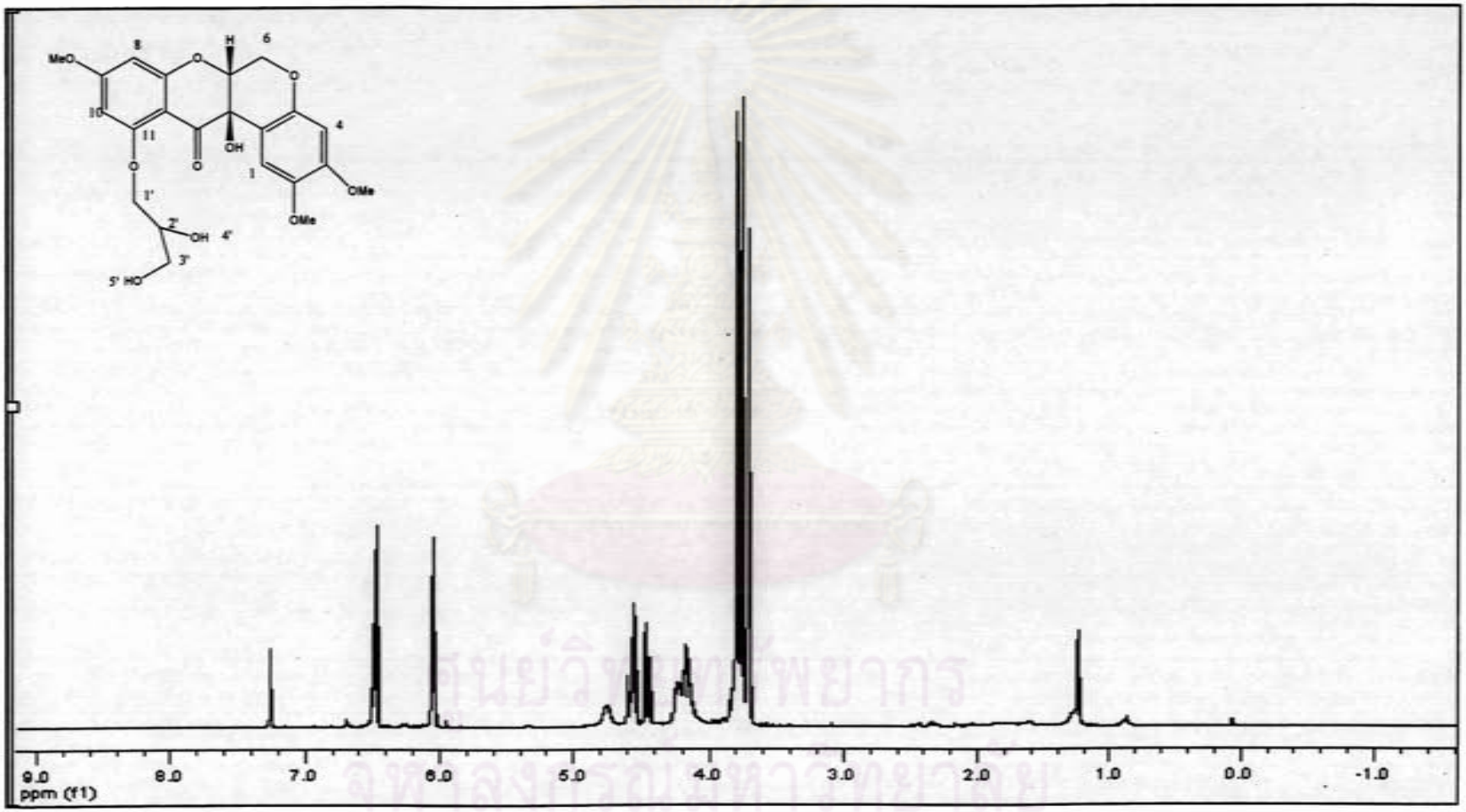
A.6 ^{13}C NMR Spectra of **3**



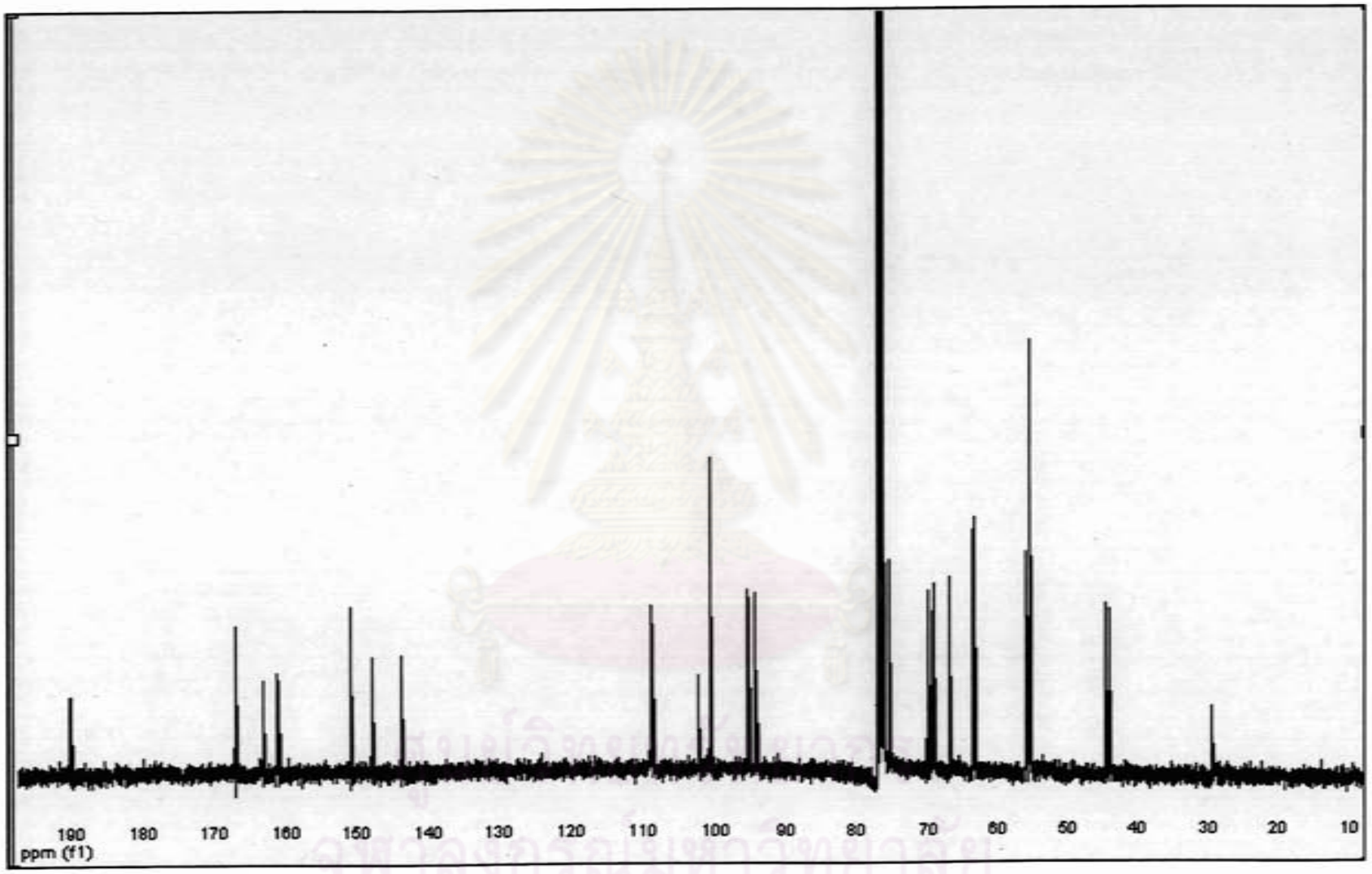
A.7 ^{1}H NMR Spectra of **4**



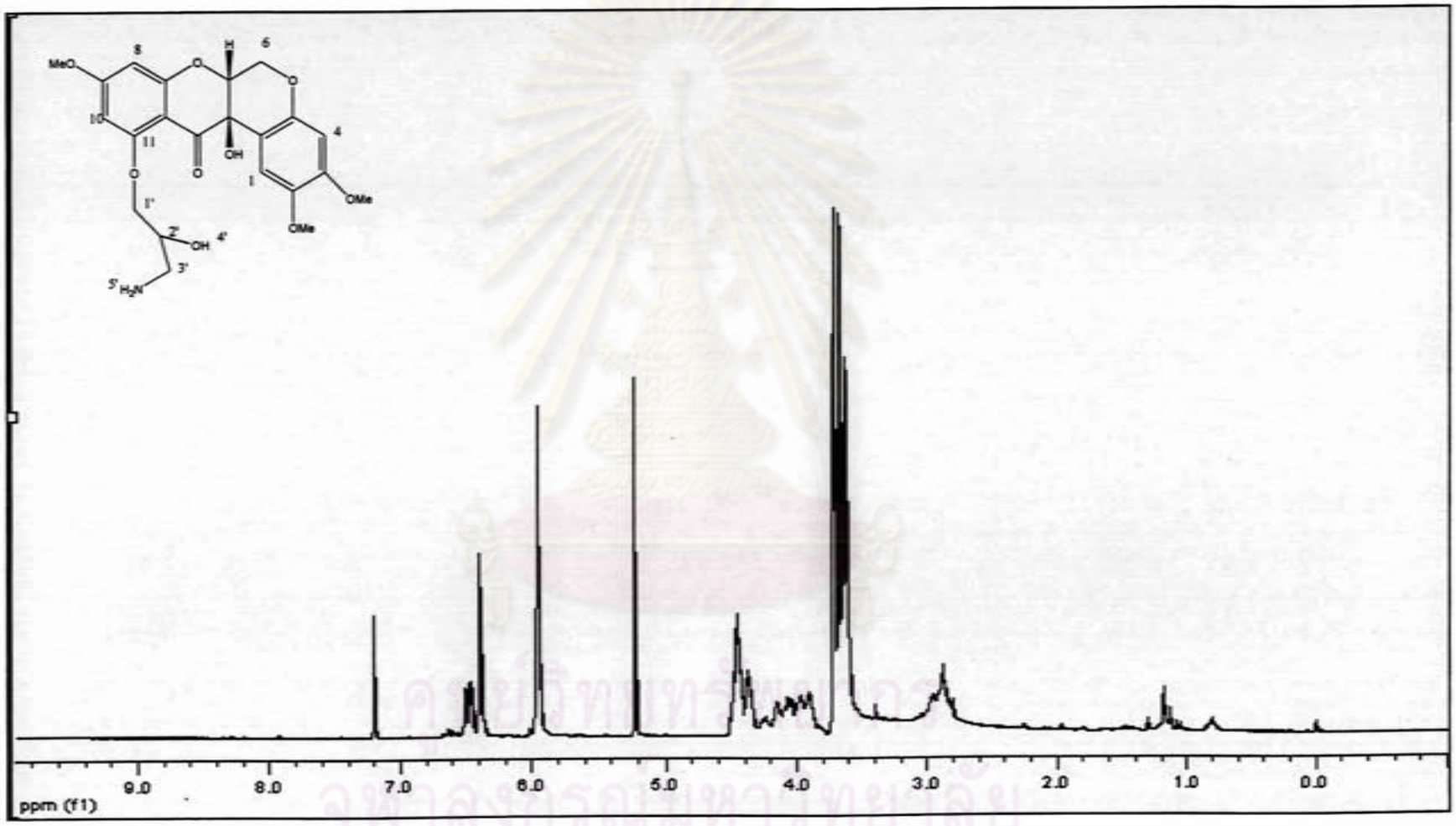
A.8 ^{13}C NMR Spectra of **4**



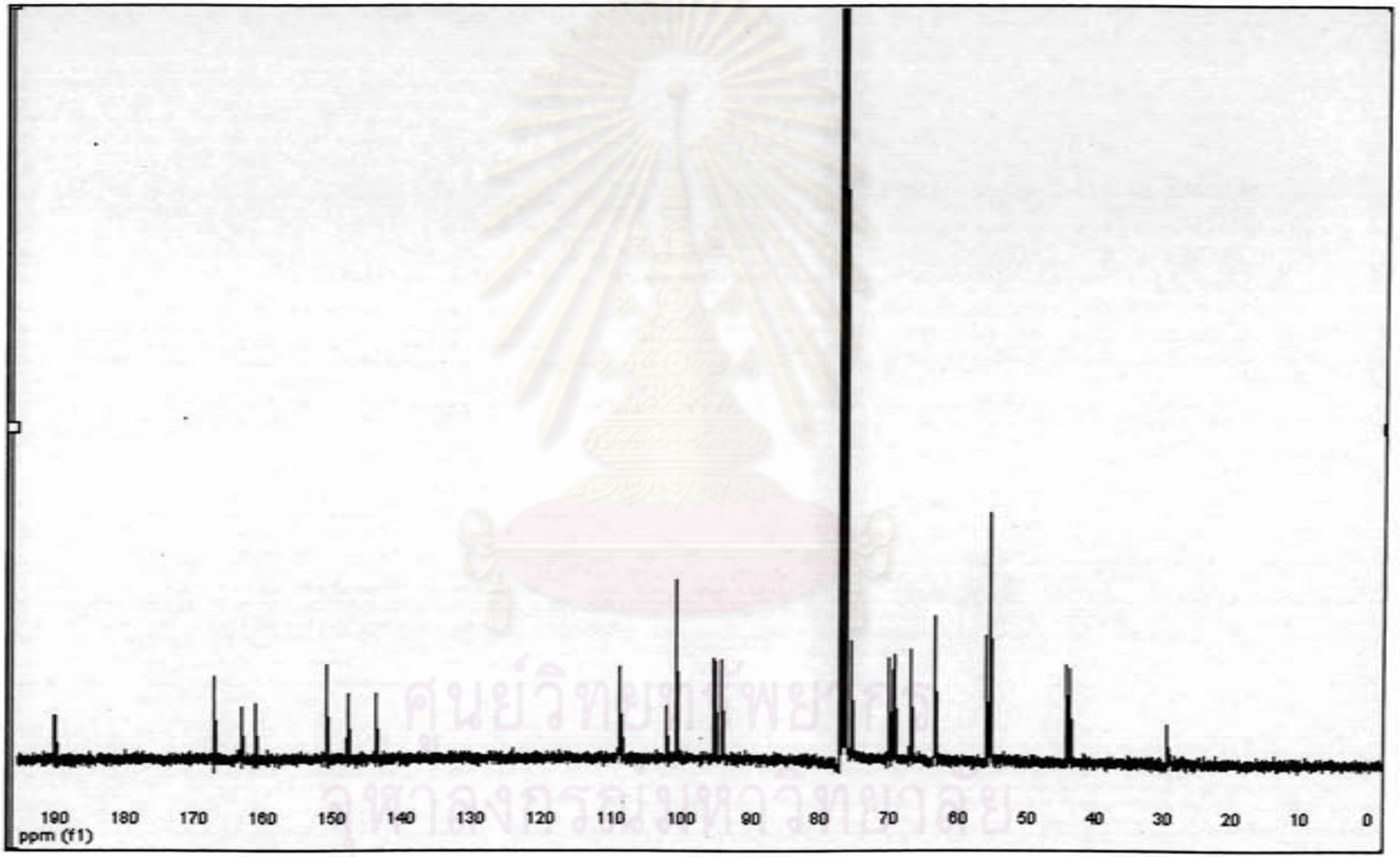
A.9 ^{1}H NMR Spectra of **5**



A.10 ^{13}C NMR Spectra of **5**

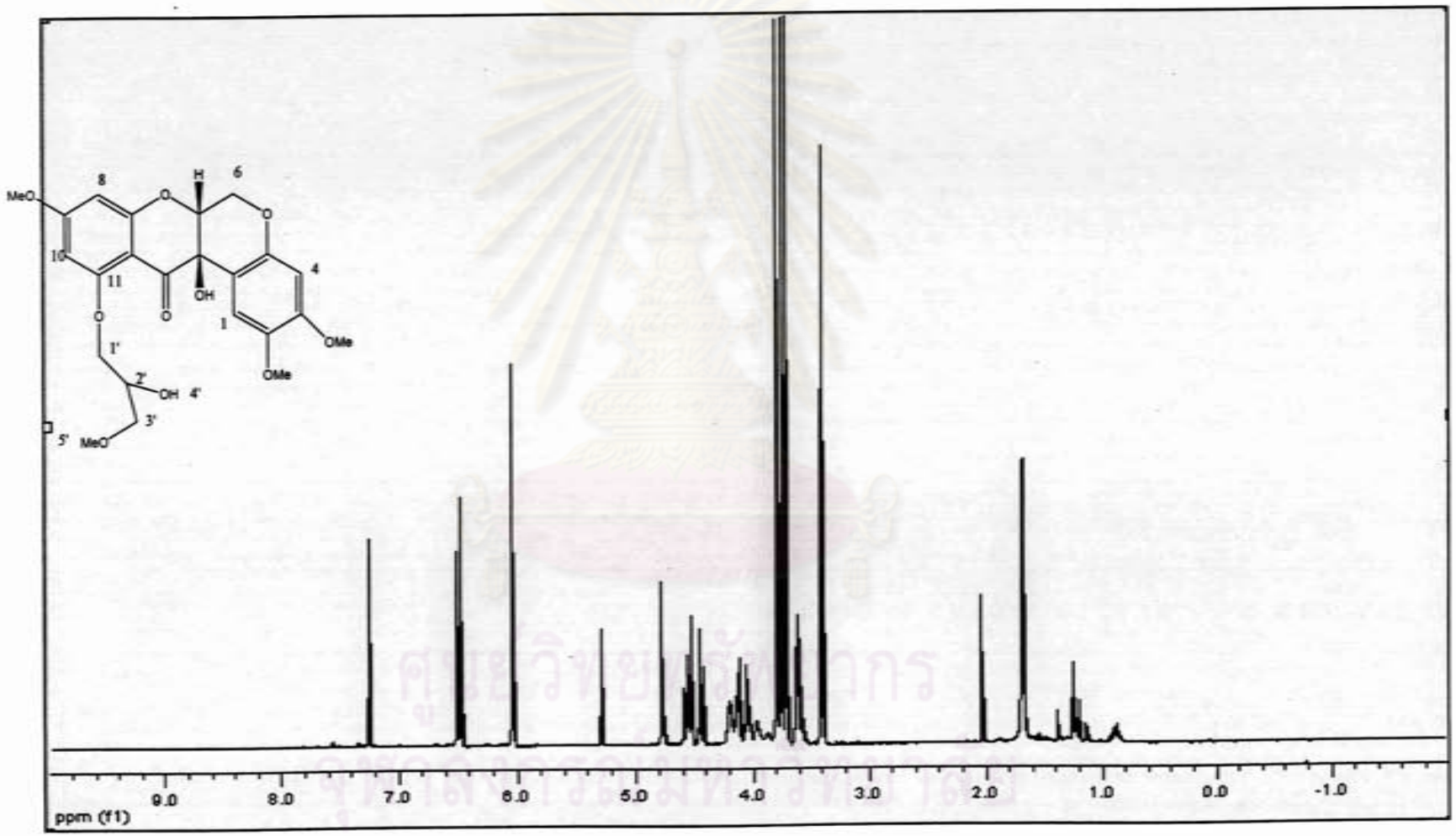


A.11 ^{1}H NMR Spectra of **6**

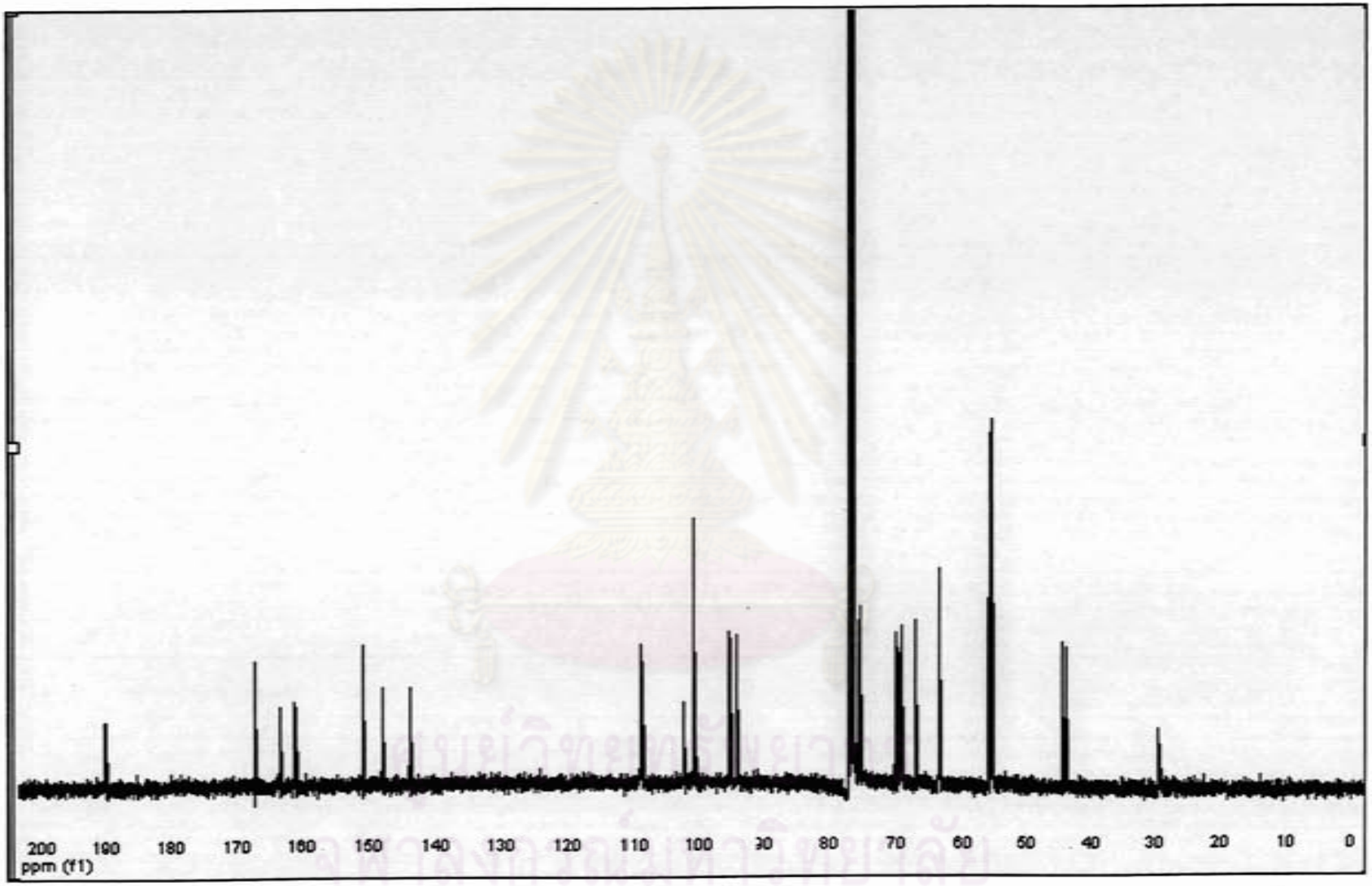


A.12 ^{13}C NMR Spectra of **6**

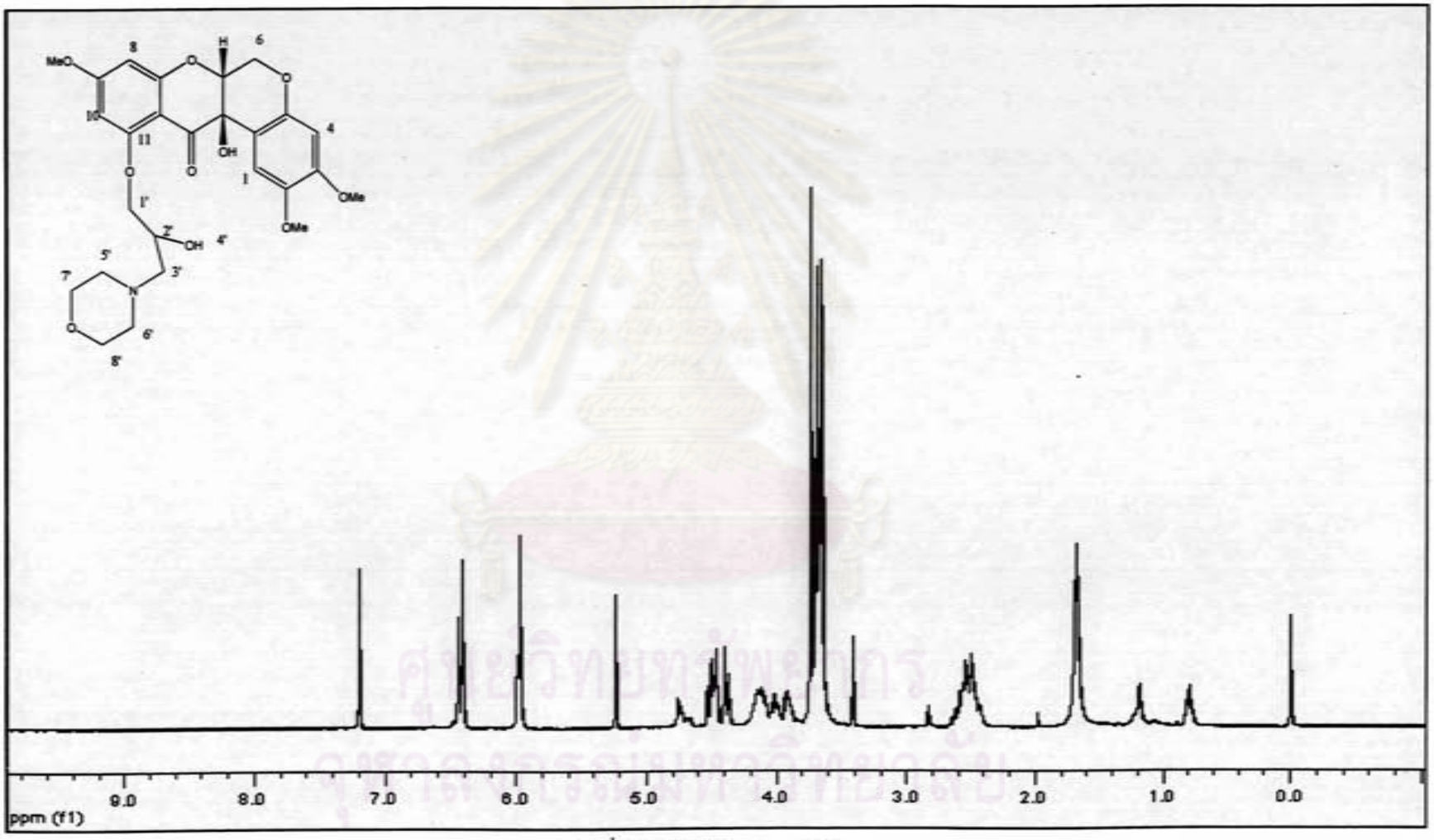
92



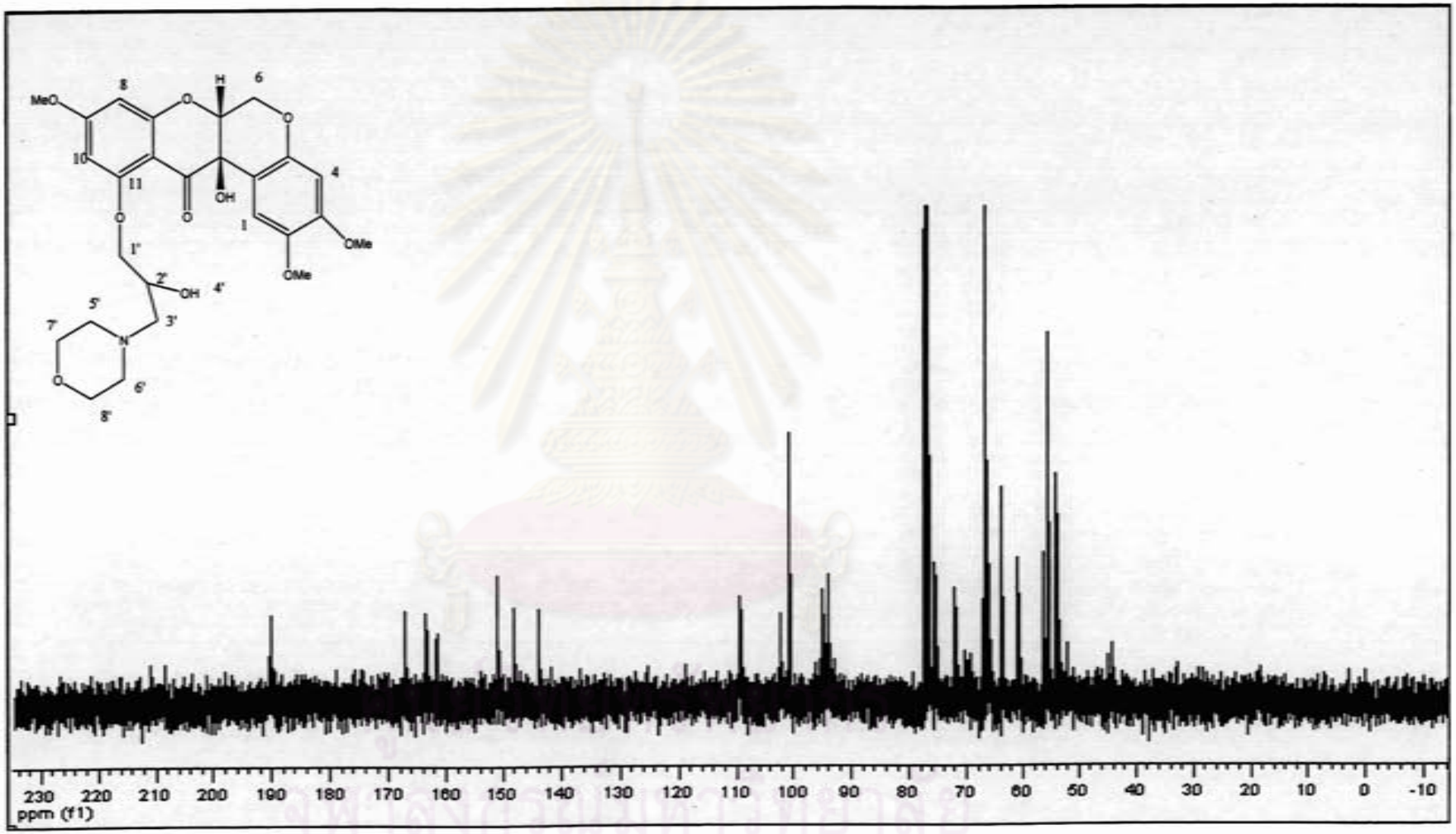
A.13 ^{1}H NMR Spectra of 7



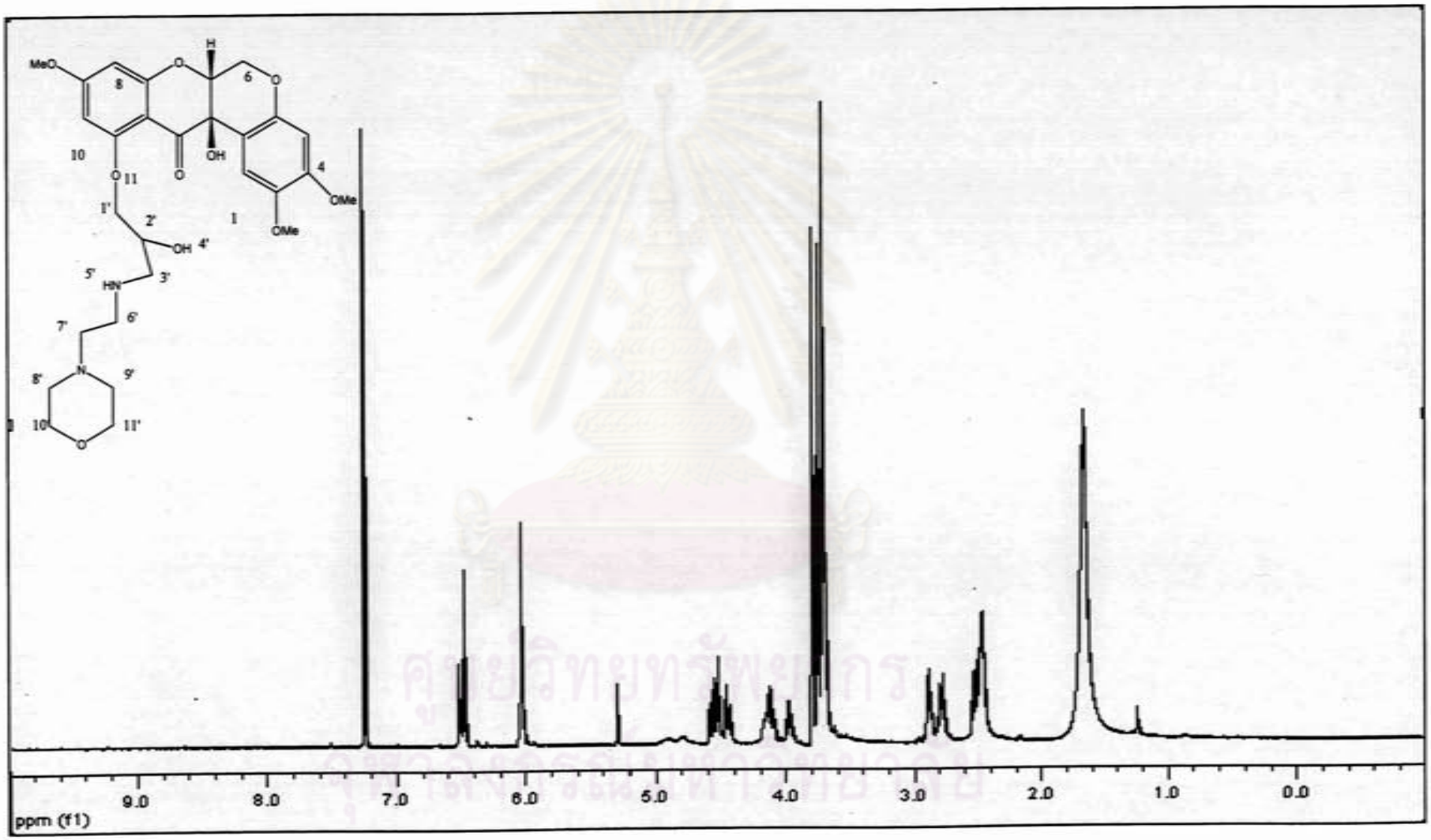
A.14 ^{13}C NMR Spectra of 7



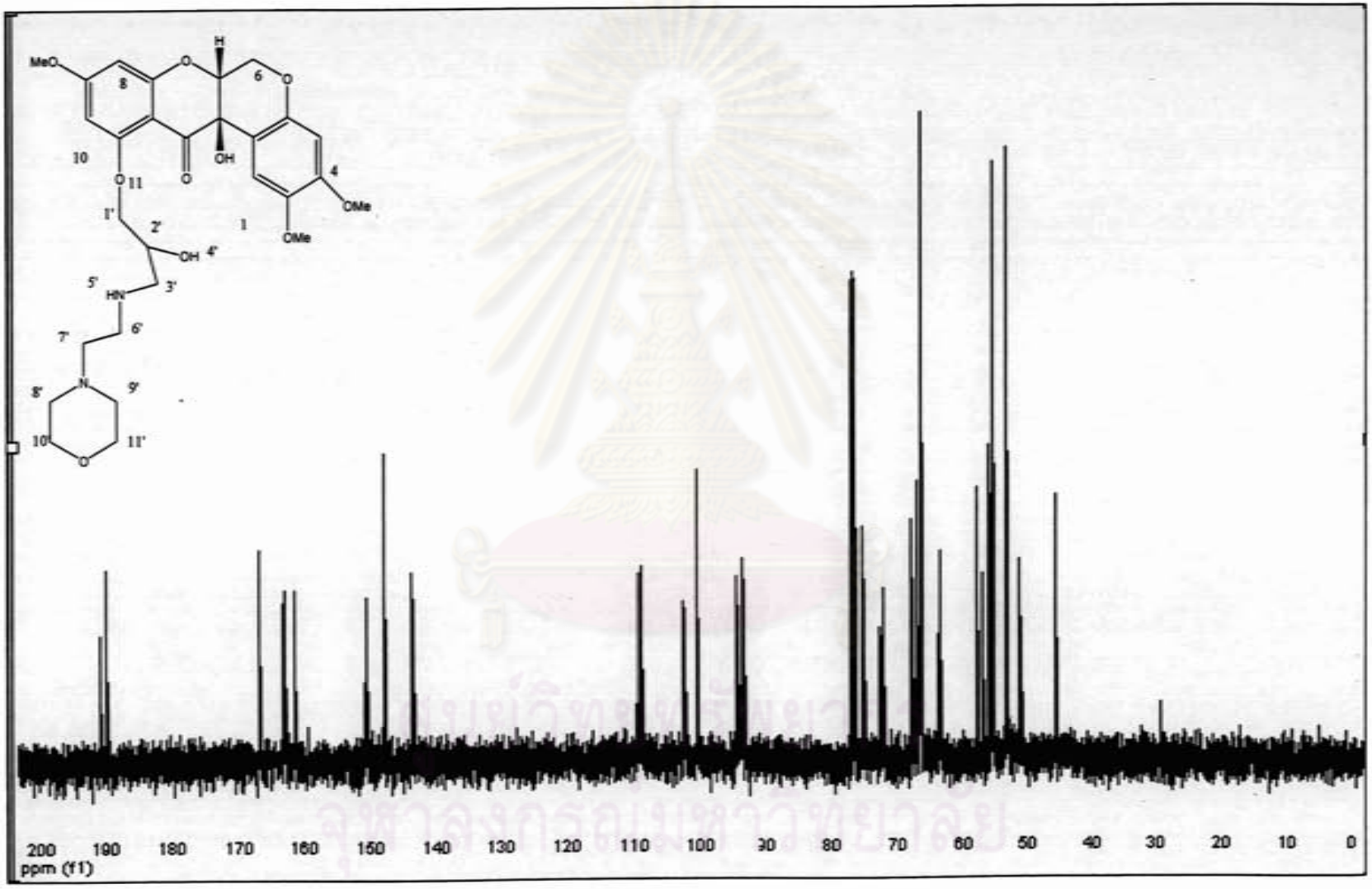
A.15 ^{1}H NMR Spectra of **8**



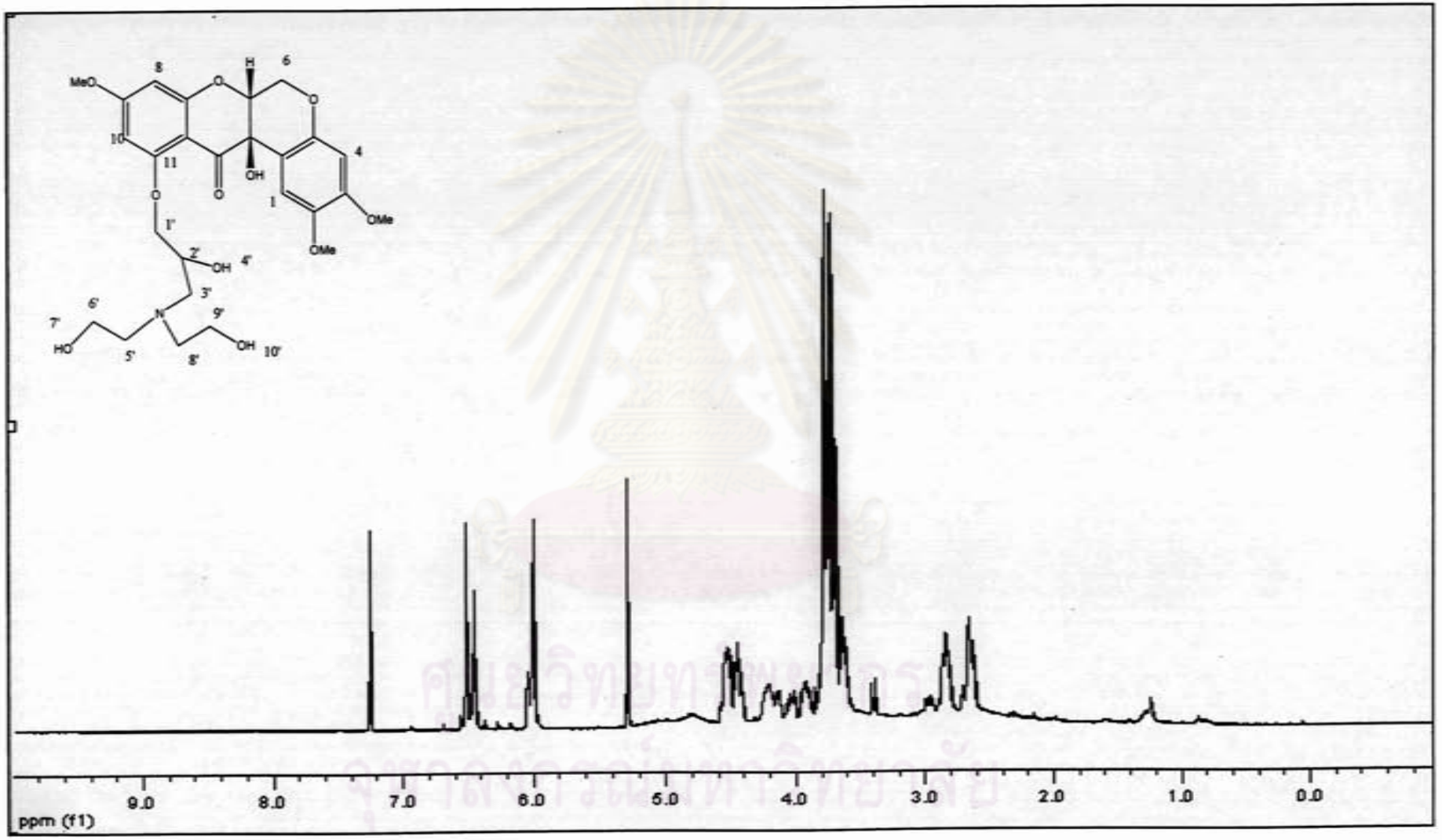
A.16 ^{13}C NMR Spectra of **8**



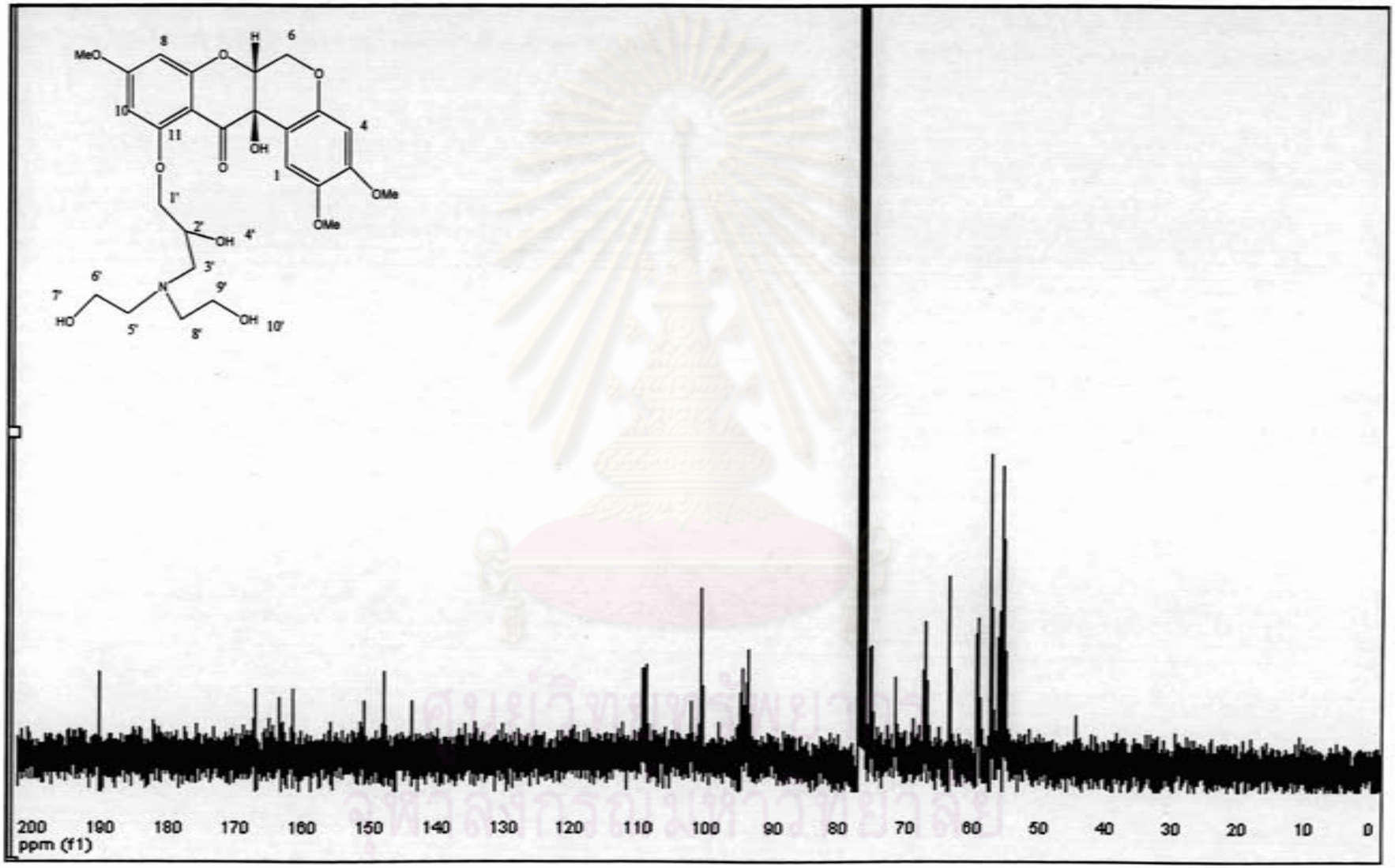
A.17 ^{1}H NMR Spectra of **9**



A.18 ^{13}C NMR Spectra of **9**

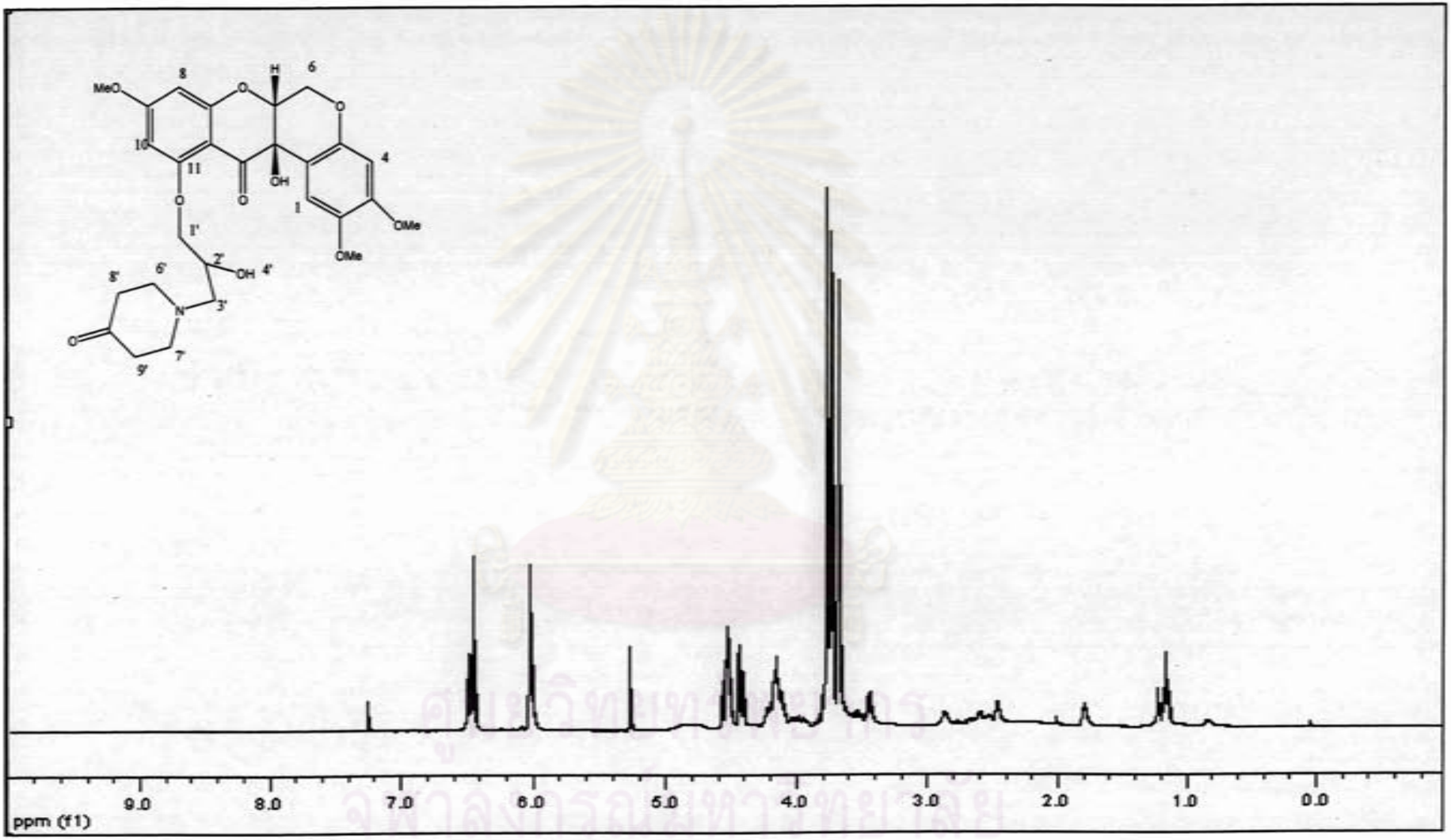


A.19 ^{1}H NMR Spectra of **10**



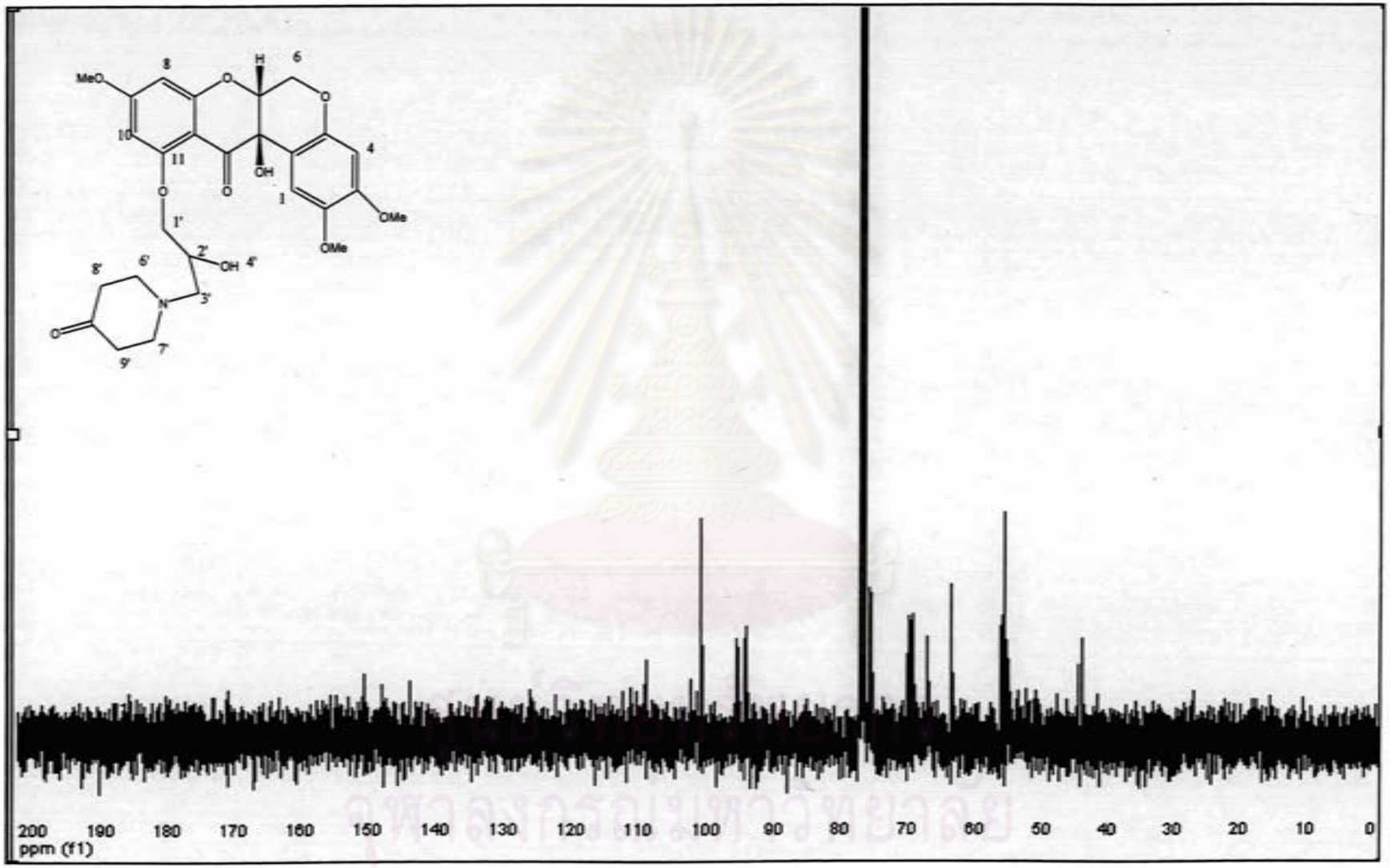
A.20 ^{13}C NMR Spectra of **10**

100

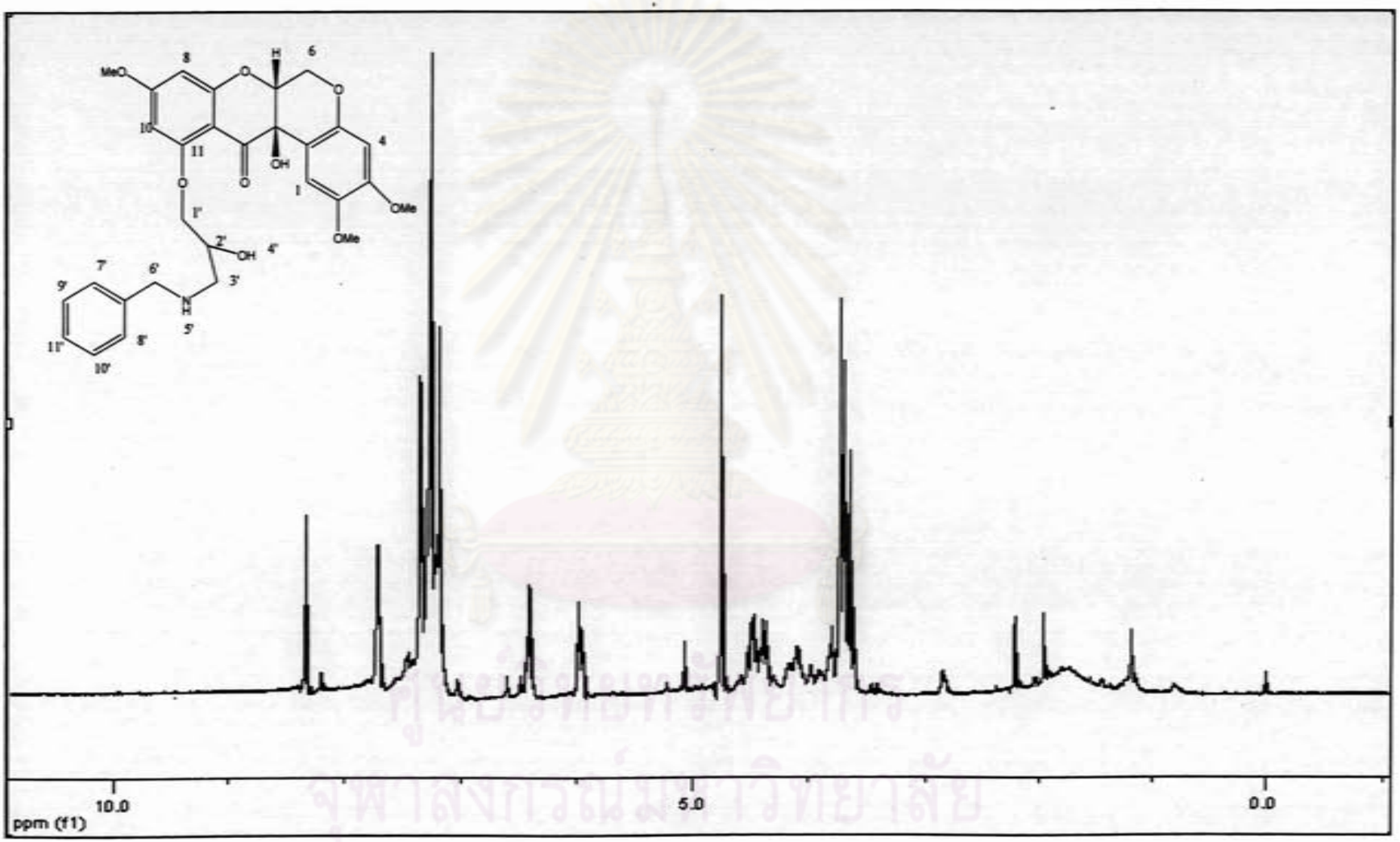


A.21 ^{1}H NMR Spectra of **11**

101

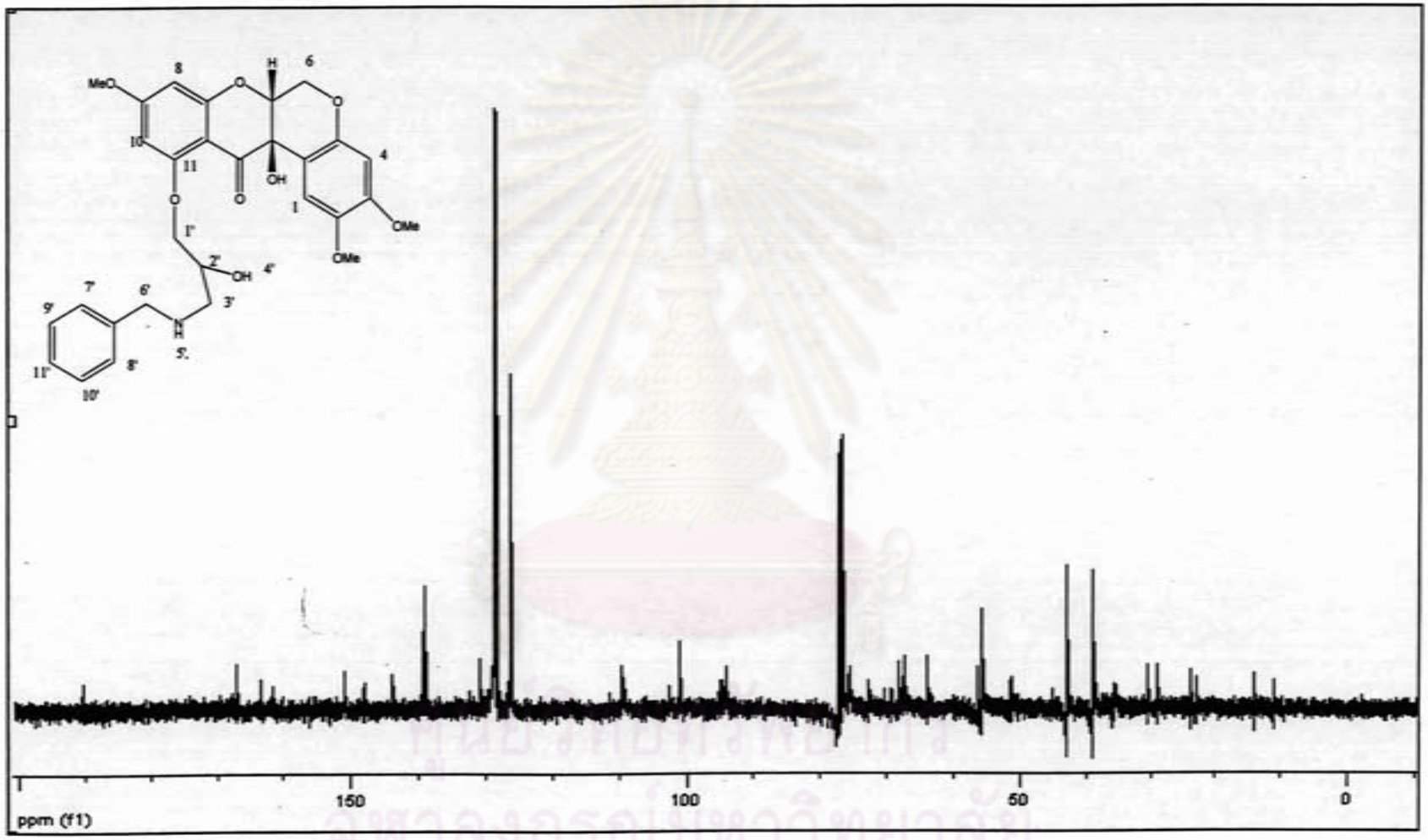


A.22 ^{13}C NMR Spectra of **11**



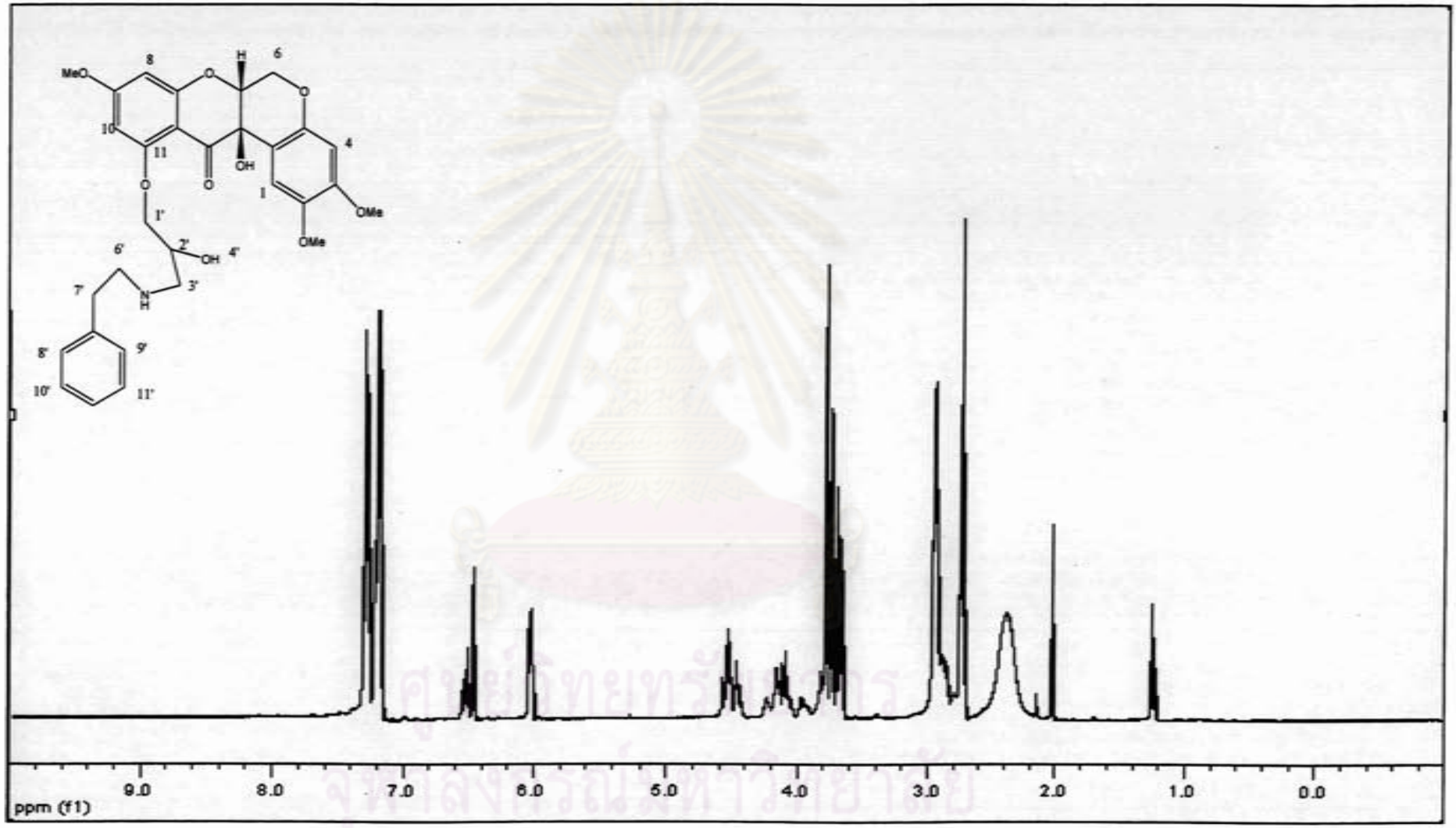
A.23 ^{1}H NMR Spectra of **12**

103

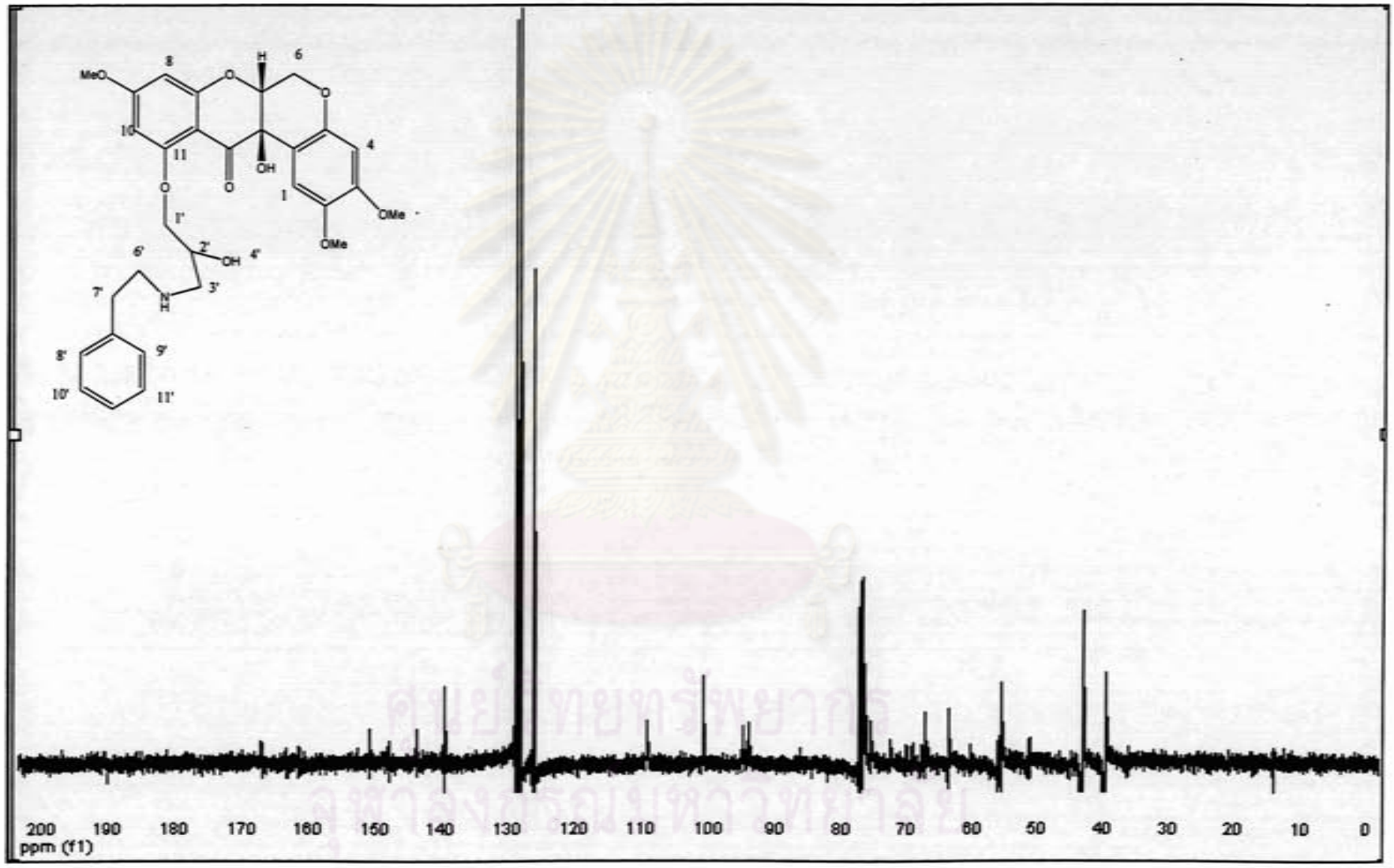


A.24 ^{13}C NMR Spectra of **12**

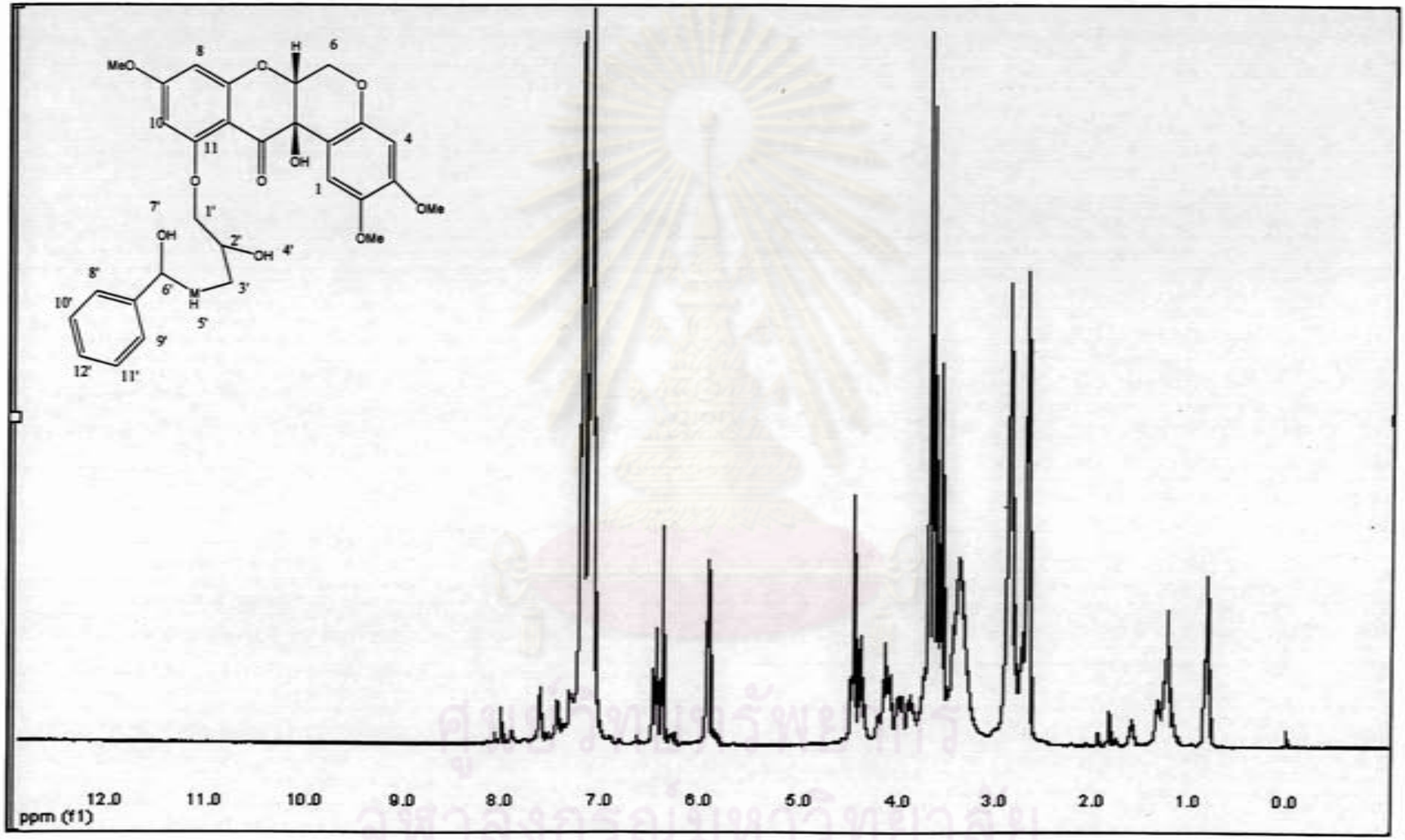
104



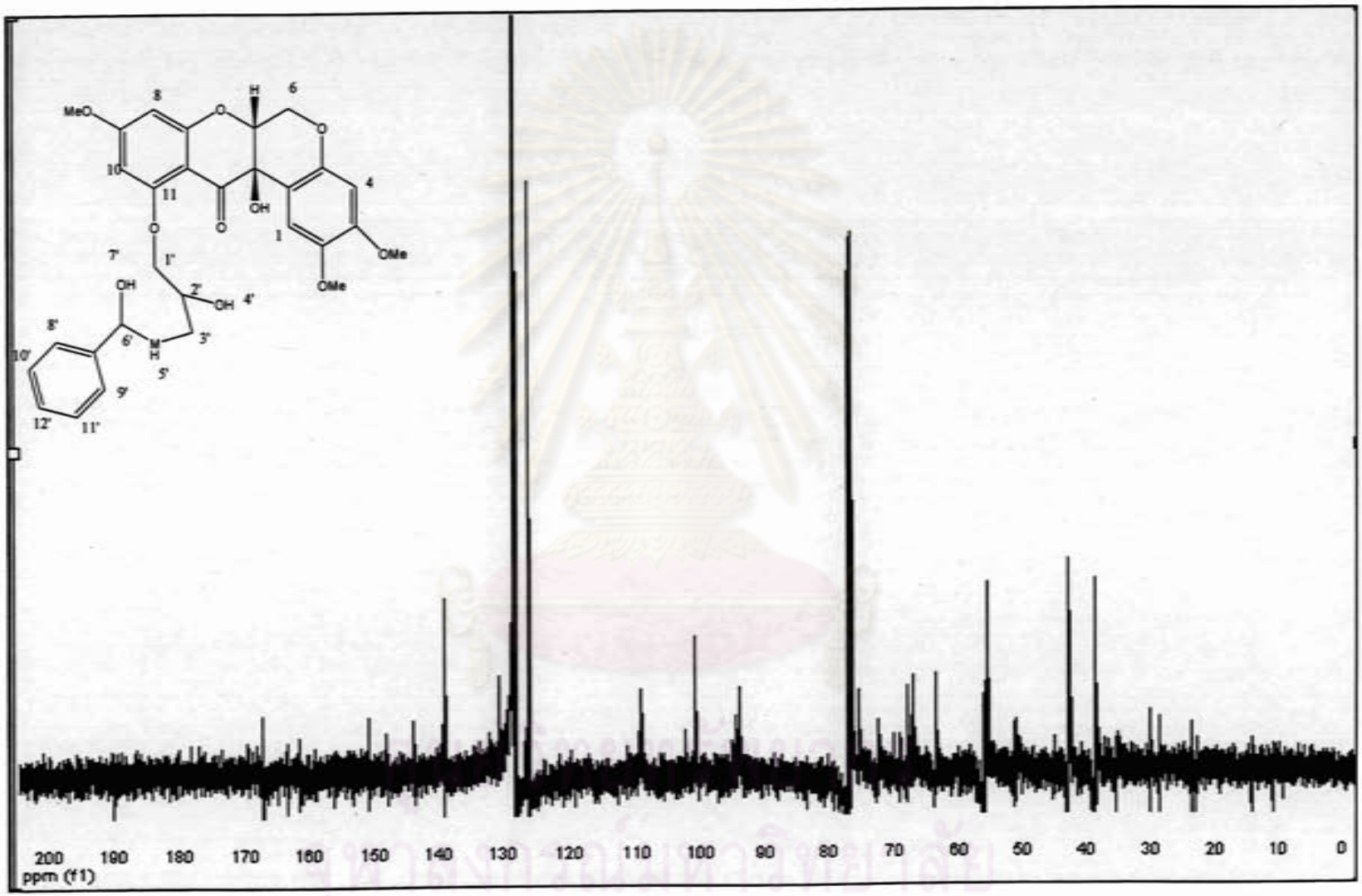
A.25 ^{1}H NMR Spectra of **13**



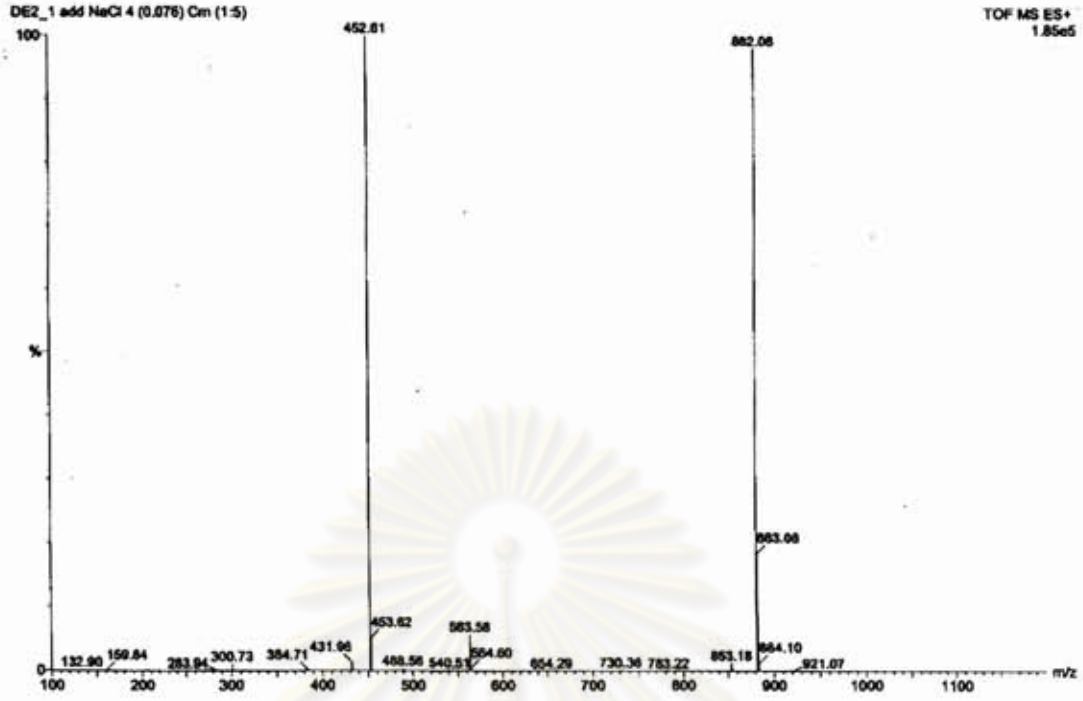
A.26 ^{13}C NMR Spectra of **13**



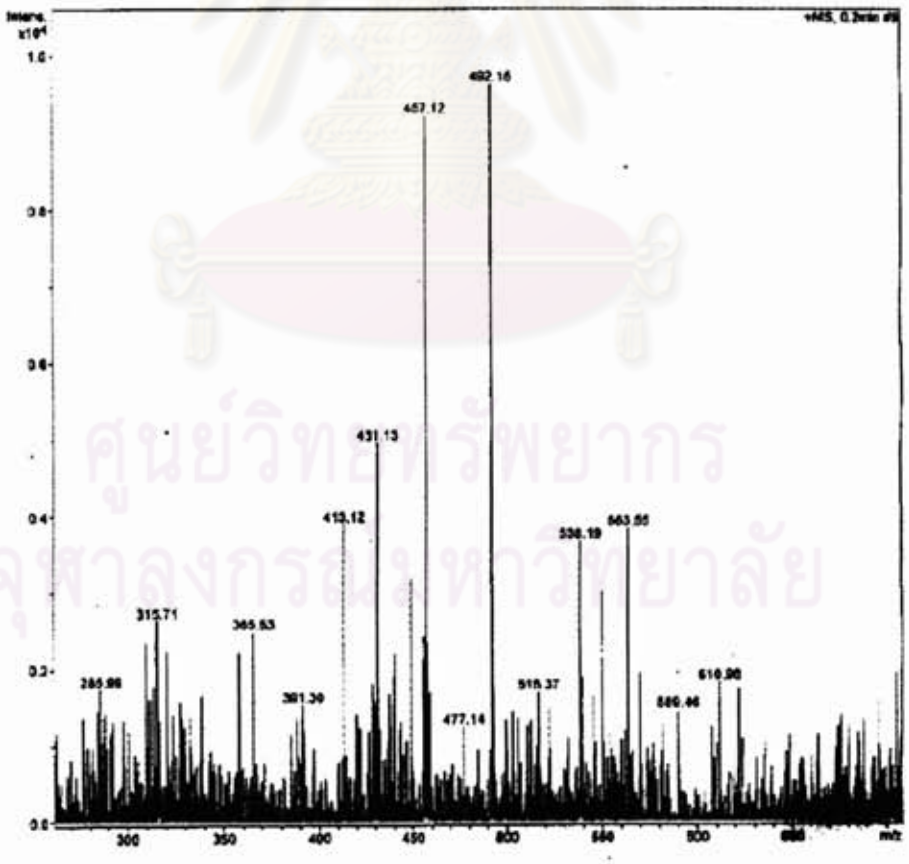
A.27 ^{1}H NMR Spectra of **14**



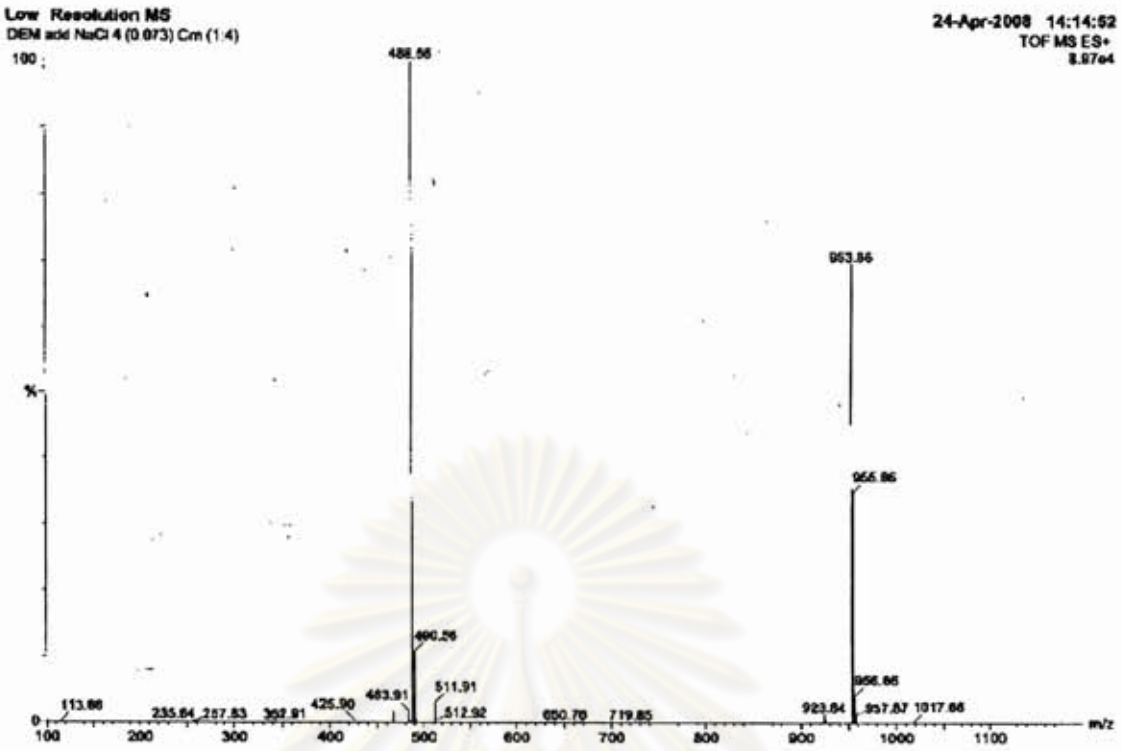
A.28 ^{13}C NMR Spectra of **14**



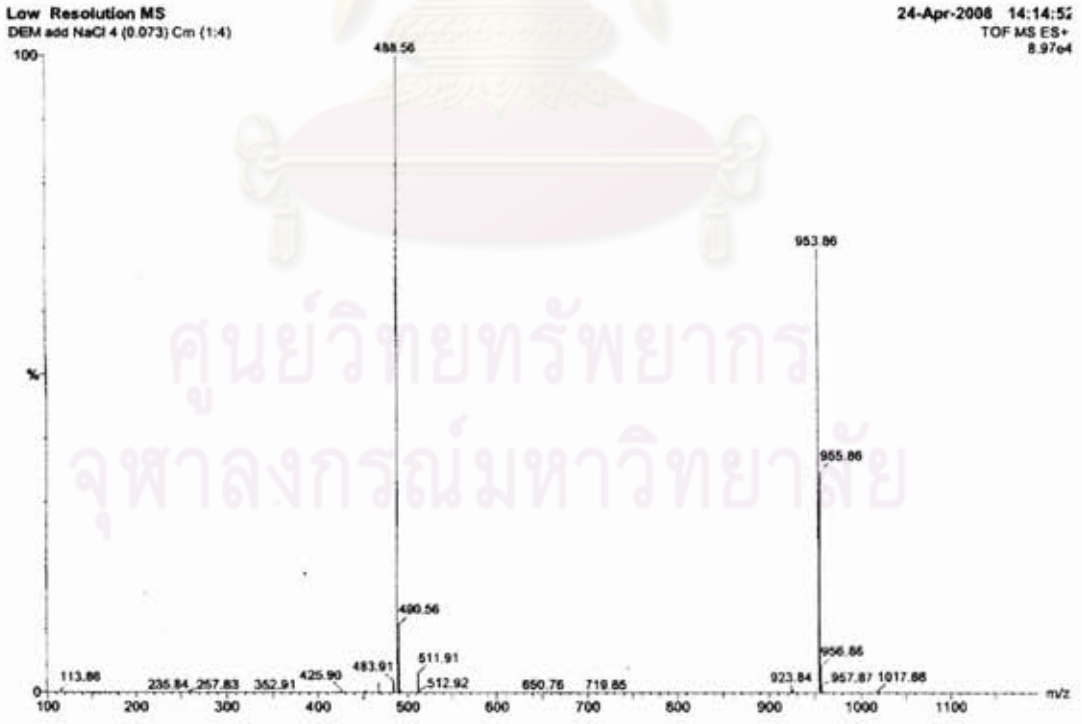
B.1 Mass spectrum of **2**



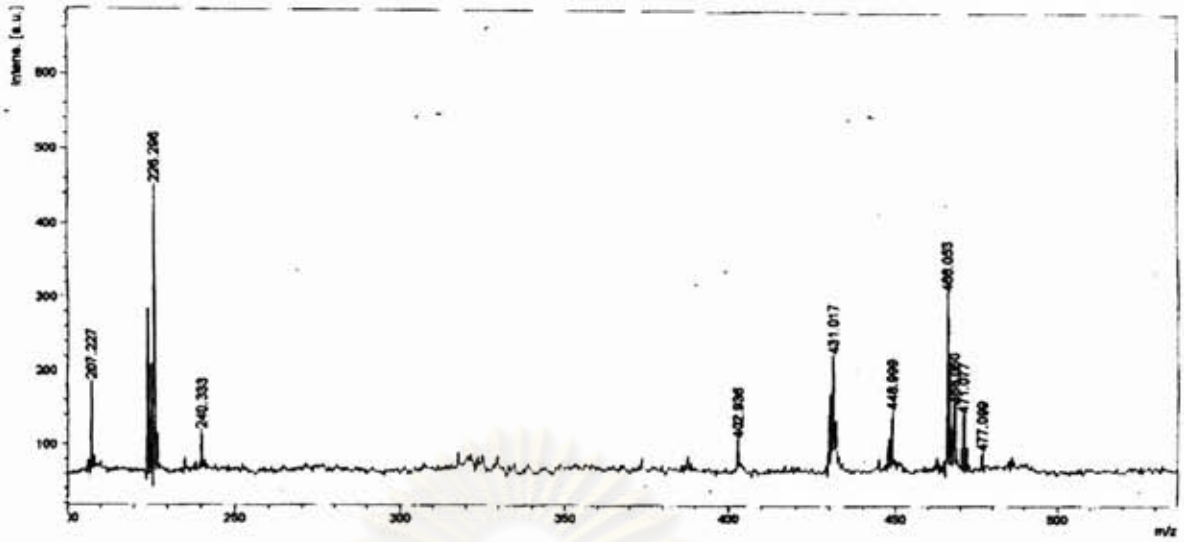
B.2 Mass spectrum of **3**



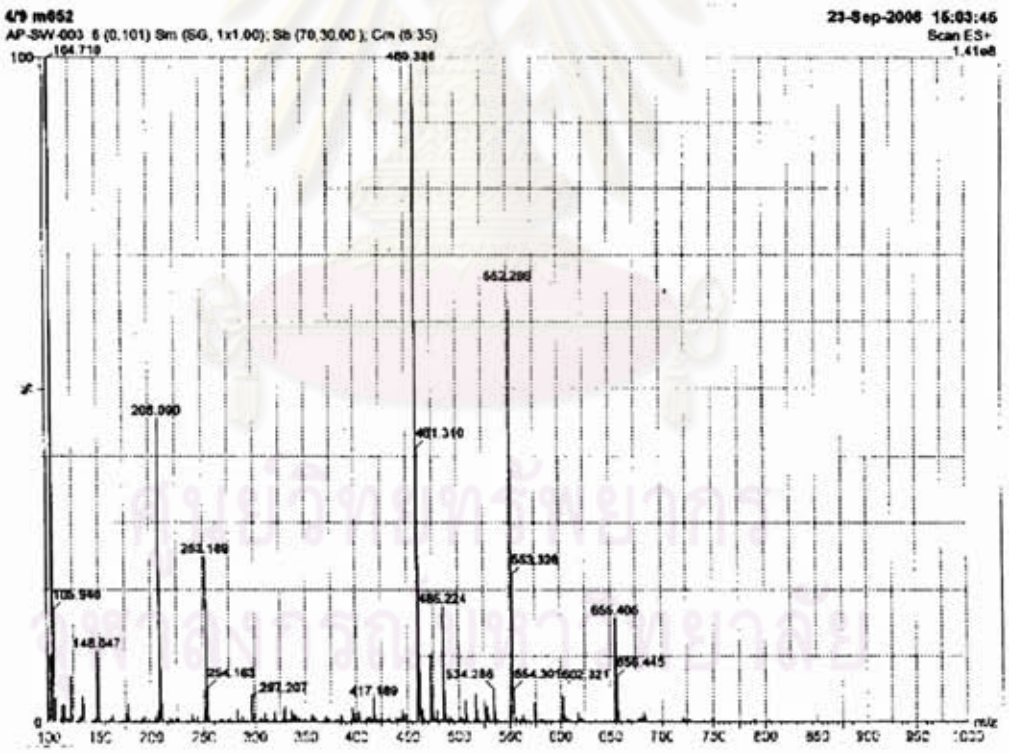
B.3 Mass spectrum of **4**



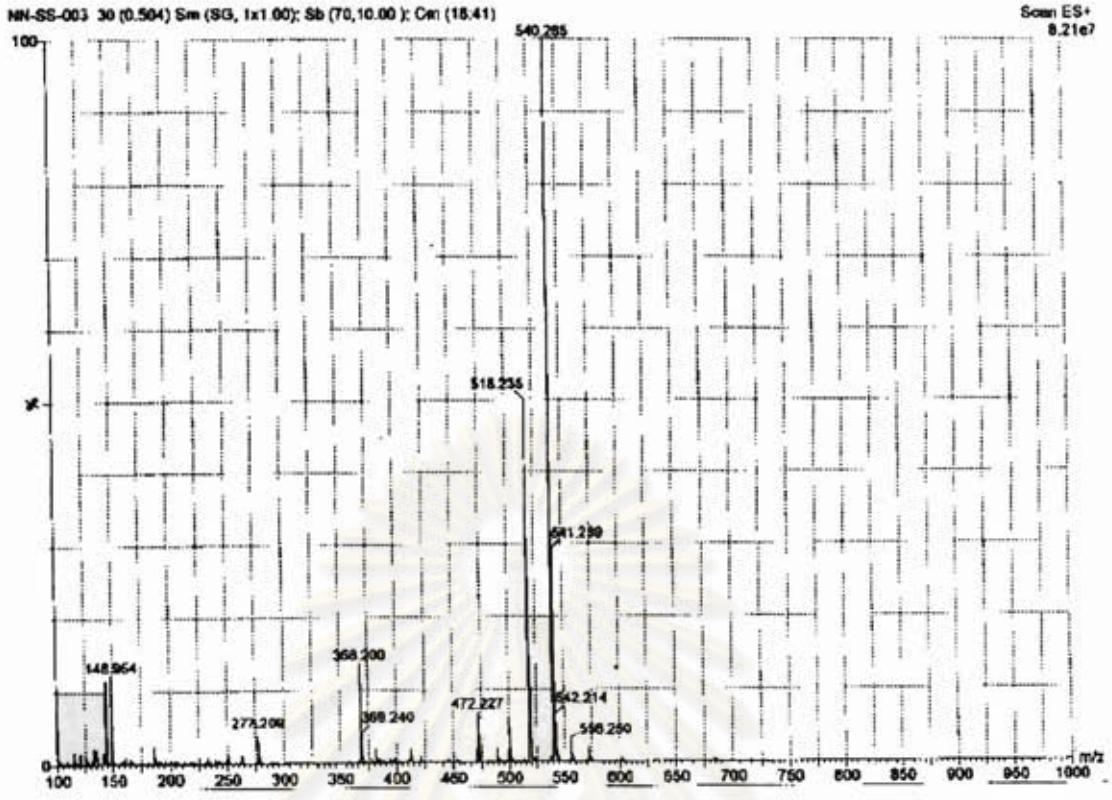
B.3 Mass spectrum of **5**



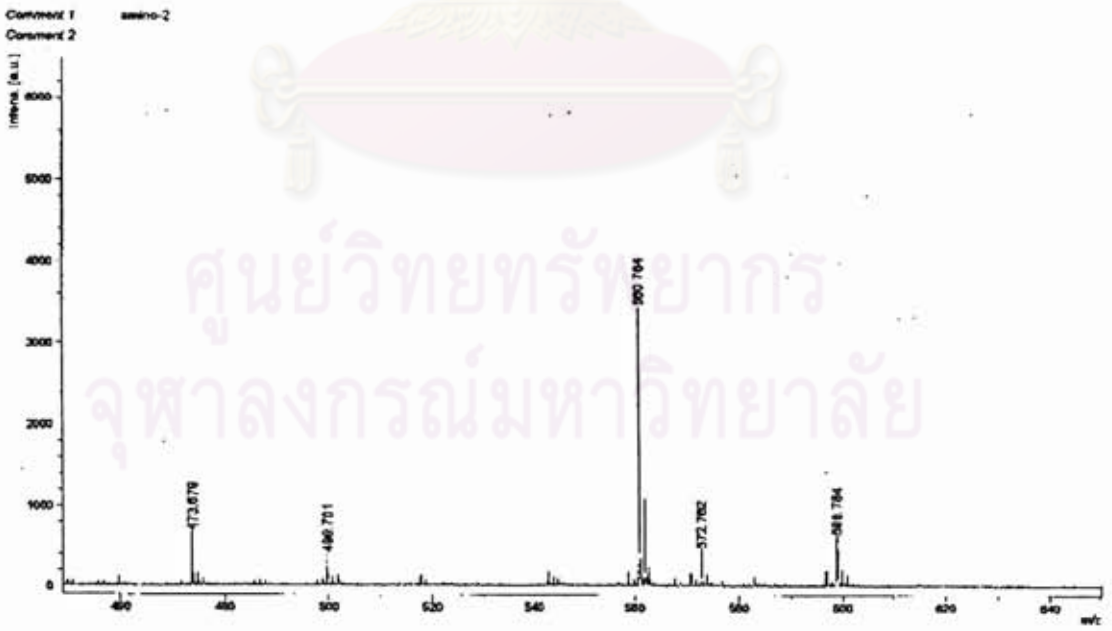
B.5 Mass spectrum of **6**



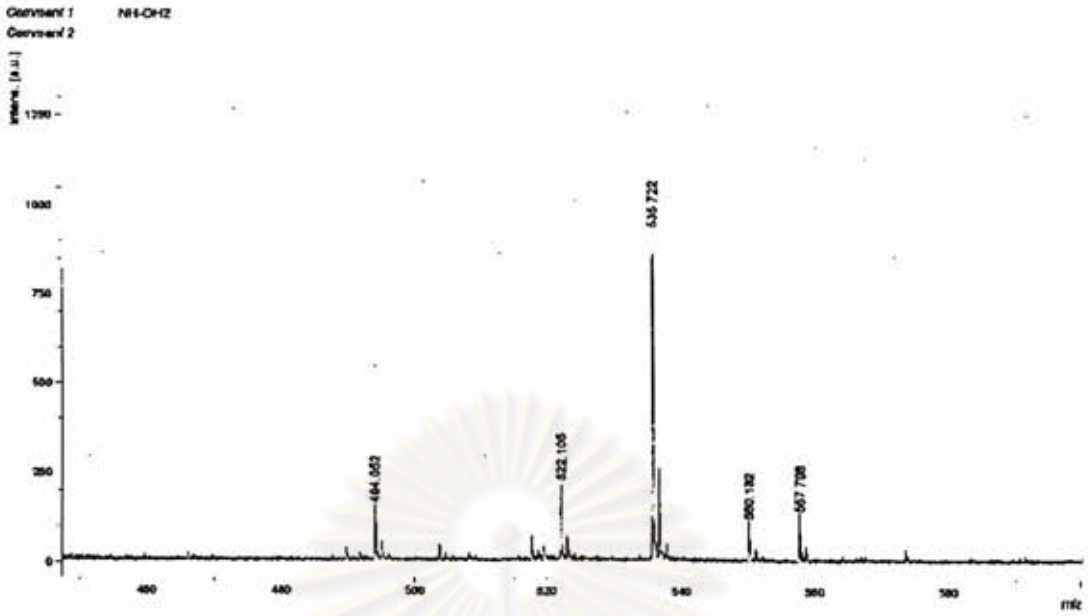
B.6 Mass spectrum of **7**



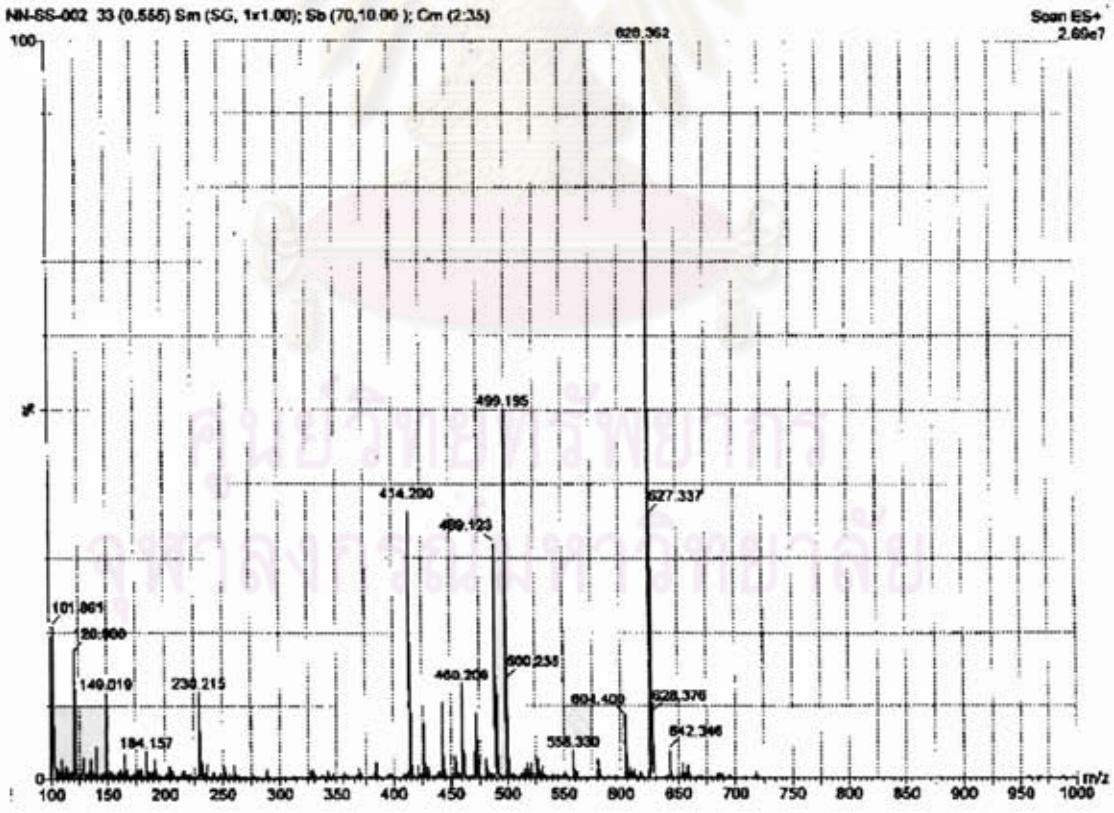
B.7 Mass spectrum of **8**



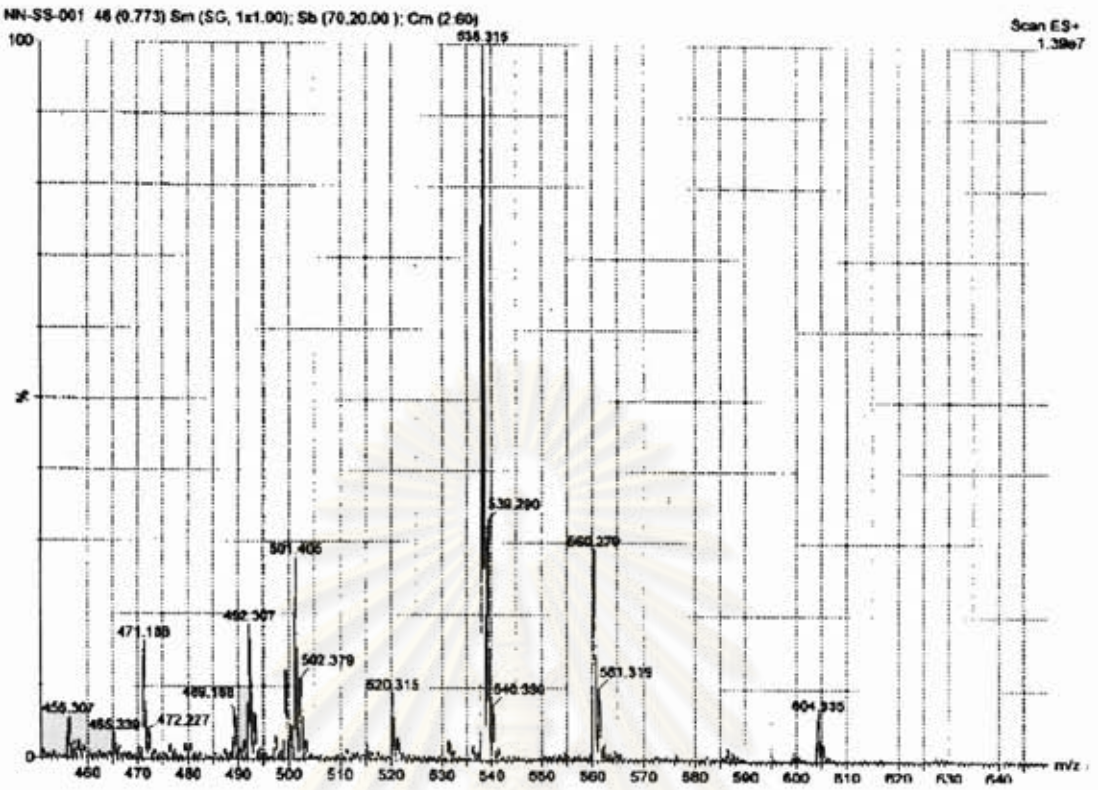
B.8 Mass spectrum of **9**



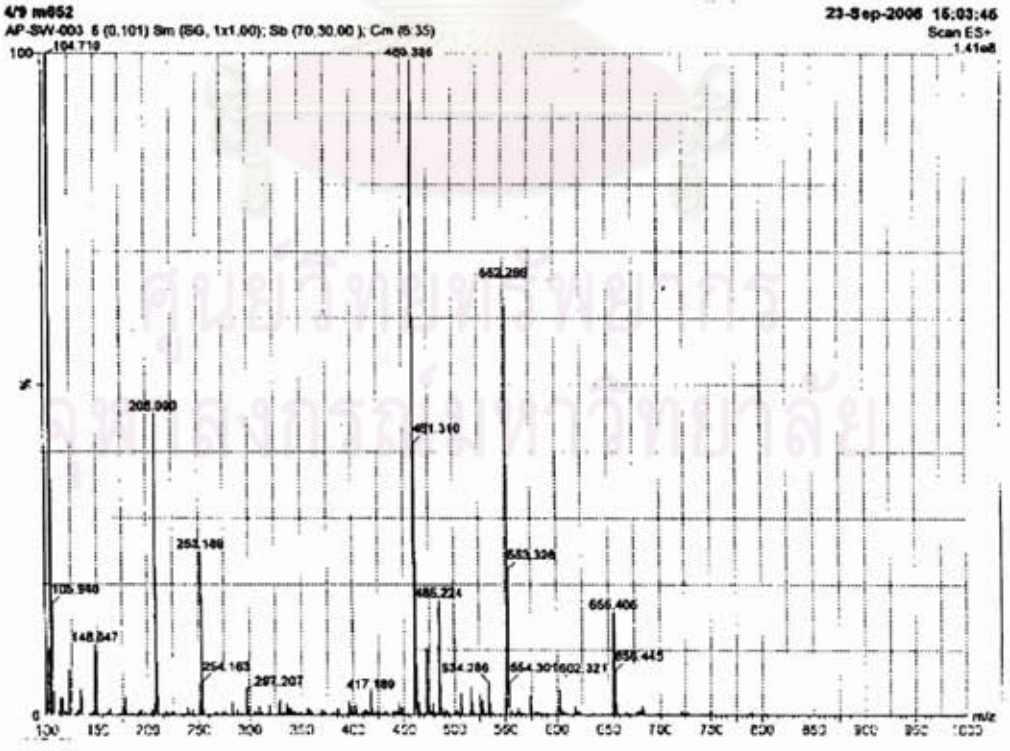
B.9 Mass spectrum of **10**



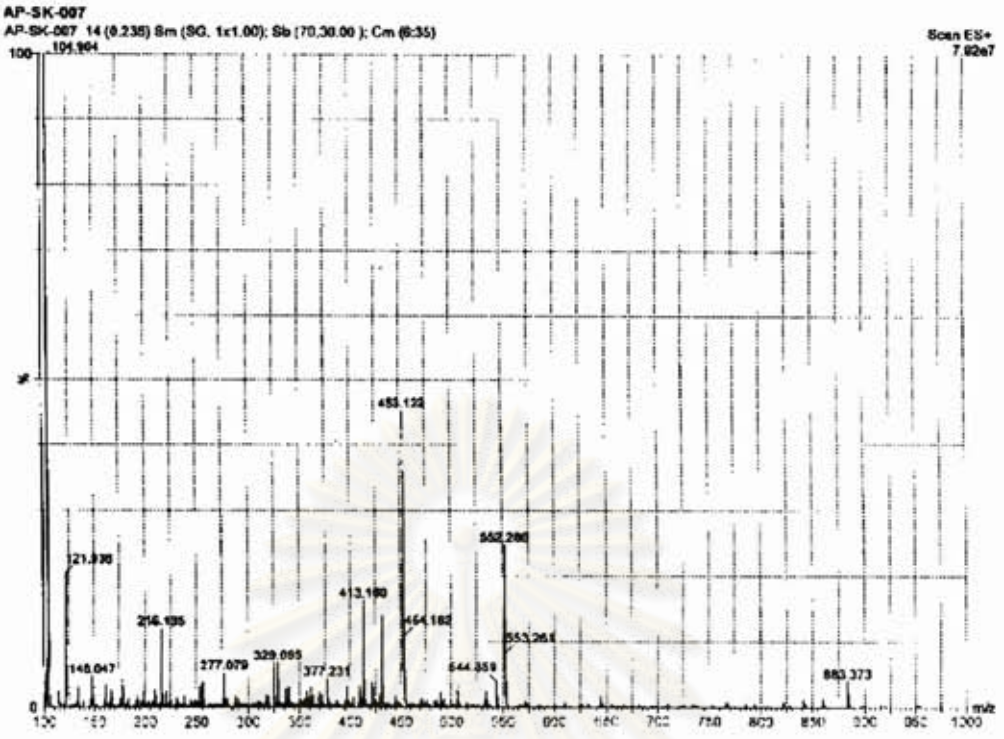
B.10 Mass spectrum of **11**



B.11 Mass spectrum of **12**



B.12 Mass spectrum of **13**



B.13 Mass spectrum of **14**

Supplementary materials

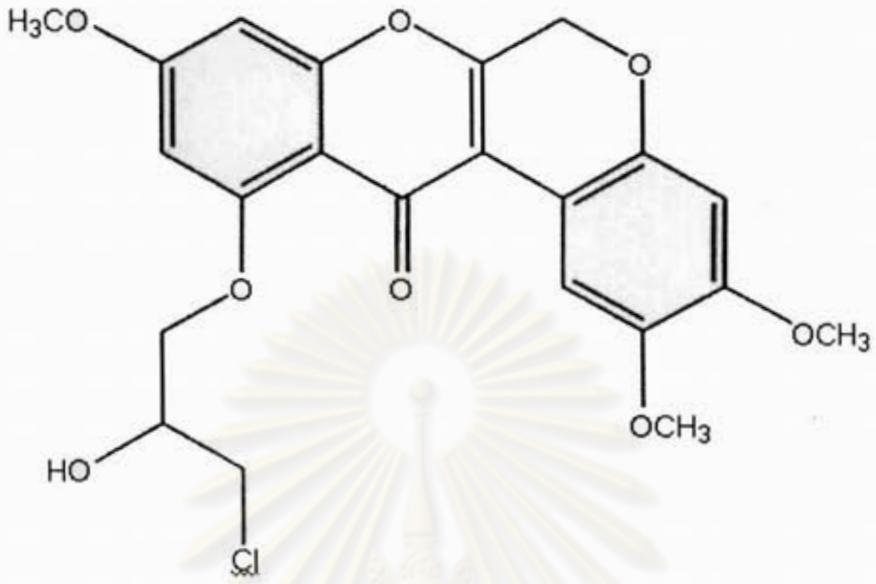


Fig 1 Chemical structure of 11-(3-Chloro-2-hydroxy-propoxy)-2,3,9-trimethoxy-6H-chromeno[3,4-b]chromen-12-one (4)

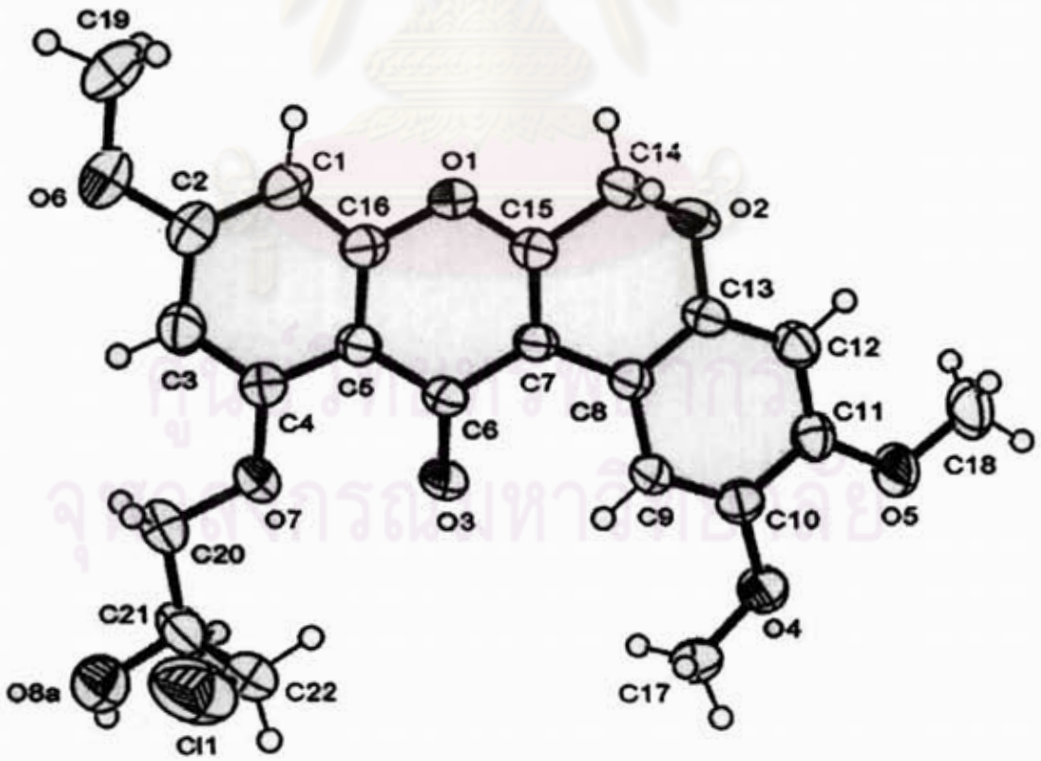


Fig 2 ORTEP view of crystal structure of 4. Thermal ellipsoids are drawn at the 50% probability level.

11-(3-Chloro-2-hydroxy-propoxy)-2, 3, 9-trimethoxy-6H-chromeno[3, 4-b]chromen-12-one (4)

Supranee Sangthong,[a] Thapong Teerawatananond,[b], Chuttree Phurut,[b] Nattaya Ngamrojanavanish,[b] Amon Petson,[b] and Nongnuj Muangsin[b]

Refinements

Refinements were made by full-matrix least squares on all F^2 data using SHELXL97. All non-hydrogen atoms were anisotropically refined, excepted for the oxygen O8B atom which was refined isotropically. The O3 atom is disordered over two positions with site occupancies of 0.88 and 0.12. The hydrogen atoms were positioned geometrically and refined using a riding model, with C—H = 0.93 Å (aromatic), 0.97 Å (CH_2), 0.98 Å (CH_3) and O—H = 0.82 Å. The selected bond lengths and bond angles are given in Table 3. An ORTEP drawing of the co-crystal is illustrated in Fig 3

(4)

Crystal data

$C_{22}H_{20}ClO_8$

Mr = 447.83

Triclinic, P1

Hall symbol: -P 1

a = 7.1534 (4) Å

b = 11.7904 (6) Å

c = 12.7661 (7) Å

α = 76.901 (3)°

β = 86.991 (3)°

γ = 74.455 (3)°

V = 1010.30 (9) Å^3

Z = 2

F_{000} = 466

D_x = 1.472 Mg m^{-3}

Mo Kα radiation

λ = 0.71073 Å

θ = 1.6-27.4°

μ = 0.24 mm^{-1}

T = 293 K

Prism, colourless

0.4 × 0.25 × 0.25 mm

Data collection

Bruker SMART APEX2 diffractometer

Radiation source: Mo

Monochromator: graphite

T = 293 K

Absorption correction: none

14308 measured reflections

4517 independent reflections

Refinement

Refinement on F^2

2879 reflections with $I > 2\sigma(I)$

$R_{int} = 0.027$

$\theta max = 27.4_u$

$\theta min = 1.6_u$

h = −8→9

k = −15→14

l = −16→16

Seconday atom site location: difference Fourier map

Supplement materials

Least-squares matrix: full

$R[F^2 > 2\sigma(F^2)] = 0.073$

$wR(F^2) = 0.244$

S = 1.03

4517 reflections

280 parameters

Primary atom site location: structure-invariant direct methods

Least-squares matrix: full

$R[F^2 > 2\sigma(F^2)] = 0.073$

$wR(F^2) = 0.244$

S = 1.03

4517 reflections

280 parameters

Primary atom site location: structure-invariant direct methods

Special details

Geometry. All e.s.d.'s (except the e.s.d. in the dihedral angle between two l.s. planes) are estimated using the full covariance mat-rix. The cell e.s.d.'s are taken into account individually in the estimation of e.s.d.'s in distances, angles and torsion angles; correlations between e.s.d.'s in cell parameters are only used when they are defined by crystal symmetry. An approximate (isotropic) treatment of cell e.s.d.'s is used for estimating e.s.d.'s involving l.s. planes. Refinement. Refinement of F^2 against ALL reflections. The weighted R-factor wR and goodness of fit S are based on F^2, conventional R-factors R are based on F, with F set to zero for negative F^2. The threshold expression of $F^2 > \sigma(F^2)$ is used only for calculating R-factors(gt) etc. and is not relevant to the choice of reflections for refinement. R-factors based on F^2 are statistically about twice as large as those based on F, and R-factors based on ALL data will be even larger.

Fractional atomic coordinates and isotropic or equivalent isotropic displacement parameters ($Å^2$)

x	y	z	Uiso*/Ueq	Occ. (<1)
Cl1	−0.0092 (2)	0.94025 (12)	0.19402 (15)	0.1046 (6)	
O1	−0.8374 (3)	1.51951 (18)	0.18363 (16)	0.0442 (5)	
O2	−0.9243 (3)	1.73457 (19)	−0.06269 (18)	0.0533 (6)	
O3	−0.6183 (4)	1.28373 (19)	−0.00847 (18)	0.0569 (7)	
O4	−0.5595 (4)	1.5044 (2)	−0.37661 (18)	0.0593 (7)	
O5	−0.7256 (4)	1.7329 (2)	−0.42529 (19)	0.0598 (7)	
O6	−0.7704 (4)	1.2143 (3)	0.50012 (19)	0.0647 (7)	
O7	−0.5507 (4)	1.11003 (19)	0.17029 (19)	0.0569 (7)	
O8A	−0.3015 (5)	0.8004 (3)	0.1812 (3)	0.0630 (12)*	0.721 (7)
O8B	−0.5212 (14)	0.9064 (9)	0.0902 (8)	0.065 (3)*	0.279 (7)
C1	−0.8080 (5)	1.3720 (3)	0.3397 (3)	0.0462 (7)	
H1	−0.8649	1.4325	0.3761	0.055*	
C2	−0.7497 (5)	1.2534 (3)	0.3928 (3)	0.0481 (8)	
C3	−0.6658 (5)	1.1636 (3)	0.3369 (3)	0.0491 (8)	
H3	−0.6284	1.0833	0.3739	0.059*	
C4	−0.6377 (5)	1.1926 (3)	0.2273 (3)	0.0439 (7)	
C5	−0.6986 (4)	1.3150 (2)	0.1675 (2)	0.0373 (6)	
C6	−0.6829 (4)	1.3541 (2)	0.0510 (2)	0.0386 (6)	

C7	−0.7430 (4)	1.4856 (2)	0.0086 (2)	0.0335 (6)
C8	−0.7324 (4)	1.5469 (2)	−0.1050 (2)	0.0349 (6)
C9	−0.6414 (4)	1.4906 (3)	−0.1874 (2)	0.0385 (6)
H9	−0.5791	1.4086	−0.1704	0.046*

Sup-2

Supplementary materials

C10	−0.6421 (5)	1.5538 (3)	H18B	−0.7428	1.8993
C11	−0.7323 (5)	1.6778 (3)	H18C	−0.9431	1.8777
C12	−0.8203 (4)	1.7350 (3)	C19	−0.8713 (8)	1.3027 (4)
H12	−0.8798	1.8174	H19A	−0.8778	1.2648
C13	−0.8203 (4)	1.6705 (3)	H19B	−1.0003	1.3387
C14	−0.8618 (5)	1.6919 (3)	H19C	−0.8033	1.3641
H14A	−0.9637	1.7255	C20	−0.4755 (6)	0.9869 (3)
H14B	−0.7491	1.7195	H20A	−0.5806	0.9514
C15	−0.8108 (4)	1.5571 (3)	H20B	−0.3924	0.9819
C16	−0.7789 (4)	1.3986 (3)	C21	−0.3625 (6)	0.9225 (3)
C17	−0.4441 (6)	1.3836 (3)	H21	−0.4522	0.9363
H17A	−0.3951	1.3604	C22	−0.1986 (8)	0.9707 (4)
H17B	−0.5214	1.3315	H22A	−0.2451	1.0573
H17C	−0.3375	1.3769	H22B	−0.1474	0.9349
C18	−0.8075 (7)	1.8594 (3)	H8OA	−0.3305	0.7659
H18A	−0.7922	1.8867			

Atomic displacement parameters (ก2)

	U^{11}	U^{22}			
Cl1	0.0833 (9)	0.0736 (8)	O4	0.0939 (19)	0.0467 (13)
O1	0.0538 (13)	0.0389 (11)	O5	0.0847 (18)	0.0482 (13)
O2	0.0631 (15)	0.0369 (11)	O6	0.0786 (18)	0.0721 (17)
O3	0.0885 (18)	0.0334 (11)	O7	0.0792 (17)	0.0317 (11)
			C1	0.0509 (18)	0.0529 (19)
			C2	0.0479 (18)	0.057 (2)

C3	0.0484 (18)	0.0448 (17)	0.0452 (13)	−0.0068 (11)	0.0155 (12)	−0.0157 (10)
C4	0.0462 (17)	0.0394 (16)	0.0333 (11)	−0.0099 (12)	0.0076 (11)	−0.0125 (10)
C5	0.0379 (15)	0.0338 (14)	0.0386 (12)	−0.0111 (12)	−0.0031 (11)	−0.0007 (10)
			0.0393 (13)	−0.0204 (14)	0.0069 (11)	−0.0050 (12)
U^{23}	U^{24}	U^{25}	0.0485 (13)	0.0002 (10)	0.0089 (11)	−0.0057 (10)
			0.0401 (16)	−0.0174 (14)	0.0082 (13)	−0.0180 (14)
			0.0408 (17)	−0.0206 (15)	0.0055 (13)	−0.0079 (15)
0.1673 (15)	−0.0262 (6)	0.0239 (8)	−0.0460 (9)			
			0.0498 (19)	−0.0120 (14)	0.0054 (14)	−0.0032 (14)
0.0387 (11)	−0.0068 (9)	0.0104 (9)	−0.0148 (9)			
			0.0436 (17)	−0.0088 (13)	0.0068 (13)	−0.0085 (13)
0.0491 (13)	0.0065 (10)	0.0050 (10)	−0.0121 (10)			
			0.0407 (15)	−0.0090 (11)	0.0051 (11)	−0.0114 (12)

C6	0.0439 (16)	0.0317 (14)	0.0415 (16)	−0.0091 (11)	0.0065 (12)
	−0.0127 (12)				
C7	0.0333 (14)	0.0315 (13)	0.0370 (14)	−0.0074 (10)	0.0043 (11)
		−0.0126 (11)			
C8	0.0334 (14)	0.0335 (14)	0.0388 (15)	−0.0089 (11)	0.0017 (11)
		−0.0105 (12)			
C9	0.0473 (16)	0.0304 (13)	0.0370 (15)	−0.0076 (11)	0.0026 (12)
		−0.0095 (11)			
C10	0.0490 (17)	0.0420 (16)	0.0373 (15)	−0.0122 (13)	0.0014 (12)
		−0.0122 (13)			
C11	0.0490 (17)	0.0406 (16)	0.0365 (15)	−0.0128 (13)	−0.0051 (12)
		−0.0024 (12)			
C12	0.0460 (17)	0.0345 (15)	0.0462 (17)	−0.0045 (12)	−0.0039 (13)
		−0.0053 (13)			
C13	0.0400 (16)	0.0336 (14)	0.0428 (16)	−0.0051 (11)	0.0024 (12)
		−0.0132 (12)			
C14	0.063 (2)	0.0367 (16)	0.0500 (19)	−0.0021 (14)	0.0035 (15)
		−0.0163 (14)			
C15	0.0387 (15)	0.0351 (15)	0.0414 (16)	−0.0065 (11)	0.0035 (12)
		−0.0114 (12)			
C16	0.0385 (15)	0.0407 (16)	0.0397 (15)	−0.0108 (12)	0.0042 (12)
		−0.0136 (13)			
C17	0.079 (3)	0.052 (2)	0.0446 (18)	−0.0135 (17)	0.0154 (16)
		−0.0209 (15)			
C18	0.088 (3)	0.049 (2)	0.052 (2)	−0.0152 (19)	−0.0075 (19)

		0.0071 (17)			
C19	0.102 (3)	0.094 (3)	0.0372 (19)	−0.024 (3)	0.0105 (19)
		−0.019 (2)			
C20	0.066 (2)	0.0372 (17)	0.060 (2)	−0.0096 (15)	0.0025 (17)
		−0.0026 (15)			
C21	0.084 (3)	0.0332 (17)	0.063 (2)	0.0008 (16)	−0.0125 (19)
		−0.0111 (16)			
C22	0.116 (4)	0.0390 (19)	0.078 (3)	0.003 (2)	0.038 (3)
		−0.0081 (19)			

Cl1—C22	1.781 (6)	C2—C3	1.395 (5)
O1—C15	1.339 (4)	C3—C4	1.381 (4)
O1—C16	1.369 (4)	C4—C5	1.433 (4)
O2—C13	1.386 (3)	C5—C16	1.388 (4)
O2—C14	1.404 (4)	C5—C6	1.462 (4)
O3—C6	1.233 (3)	C6—C7	1.472 (4)
O4—C10	1.366 (4)	C7—C15	1.346 (4)
O4—C17	1.415 (4)	C7—C8	1.475 (4)
O5—C11	1.368 (4)	C8—C13	1.395 (4)
O5—C18	1.418 (4)	C8—C9	1.408 (4)
O6—C2	1.356 (4)	C9—C10	1.382 (4)
O6—C19	1.431 (5)	C10—C11	1.400 (4)
O7—C4	1.347 (4)	C11—C12	1.378 (4)
O7—C20	1.431 (4)	C12—C13	1.379 (4)
O8A—C21	1.363 (5)	C14—C15	1.496 (4)
O8B—C21	1.440 (10)	C20—C21	1.489 (5)
C1—C2	1.371 (5)	C21—C22	1.478 (7)
C1—C16	1.389 (4)		

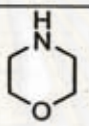
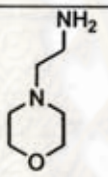
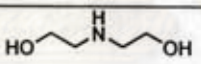
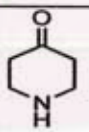
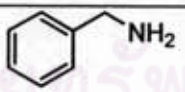
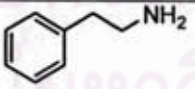
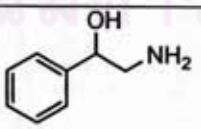
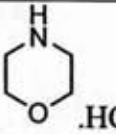
C15—O1—C16	119.1 (2)	C10—C9—C8	121.8 (3)
C13—O2—C14	115.7 (2)	O4—C10—C9	124.8 (3)
C10—O4—C17	118.1 (2)	O4—C10—C11	115.5 (3)
C11—O5—C18	117.8 (3)	C9—C10—C11	119.7 (3)
C2—O6—C19	116.9 (3)	O5—C11—C12	124.9 (3)
C4—O7—C20	119.3 (3)	O5—C11—C10	115.7 (3)
C2—C1—C16	117.5 (3)	C12—C11—C10	119.4 (3)
O6—C2—C1	123.9 (3)	C11—C12—C13	120.2 (3)
O6—C2—C3	115.5 (3)	C12—C13—O2	115.6 (2)

C1—C2—C3	120.6 (3)	C12—C13—C8	122.4 (3)
C4—C3—C2	120.7 (3)	O2—C13—C8	121.9 (3)

Supplementary materials

O7—C4—C3	123.0 (3)	O2—C14—C15	112.4 (3)
O7—C4—C5	115.9 (3)	O1—C15—C7	125.8 (3)
C3—C4—C5	121.0 (3)	O1—C15—C14	111.6 (2)
C16—C5—C4	114.5 (3)	C7—C15—C14	122.6 (3)
C16—C5—C6	120.4 (3)	O1—C16—C5	121.0 (3)
C4—C5—C6	125.1 (3)	O1—C16—C1	113.4 (3)
O3—C6—C5	123.3 (3)	C5—C16—C1	125.6 (3)
O3—C6—C7	121.5 (3)	O7—C20—C21	106.7 (3)
C5—C6—C7	115.2 (2)	O8A—C21—O8B	88.9 (5)
C15—C7—C6	118.3 (3)	O8A—C21—C22	110.3 (3)
C15—C7—C8	116.4 (2)	O8B—C21—C22	128.5 (5)
C6—C7—C8	125.2 (2)	O8A—C21—C20	113.9 (3)
C13—C8—C9	116.4 (3)	O8B—C21—C20	98.8 (5)
C13—C8—C7	118.3 (2)	C22—C21—C20	114.0 (3)
C9—C8—C7	125.3 (2)	C21—C22—Cl1	112.0 (3)

Calculation of %Topoisomerase II inhibition

Compounds	R	% top II Inhibition
Doxorubicin	-	-
Etoposide	-	85
1	-	87
2	-	91
3	-	85
4	Cl	80
5	OH	56
6	NH_2	83
7	OMe	80
8		97
9		75
10		89
11		78
12		84
13		91
14		81
15	 .HCl	84

%Topoisomerase II inhtbition of Etoposide

Topoisomerase II cause relaxation 48%, we assumed that it leads to relaxation 100%
Therefore, Etoposide cause relaxation 15%, we assumed that it leads to relaxation
(100% x 15%)/ 48% = 13.25%

%TopoisomeraseII inhibition = 100- %relaxation
Consequently Etoposide results in topoisomerase II inhibition (100-13.25) = 68.75%

- Calculation for 6-deoxyclitoriacetal derivatives as followed like this.

VITA

Supranee Sangtong was born on January 25, 1984 in Khonkaen, Thailand. She received Bachelor Degree of Science in Chemistry from Khonkaen University in 2006. Since 2007, she has become a student in Master degree of Chulalongkorn University studying in Program of Biotechnology, Faculty of Science, Chulalongkorn University.