UNIVERSITI TEKNOLOGI MARA

ELECTRONIC CIRCULAR DICHROISM STUDIES OF PENTACYCLIC OXINDOLE ALKALOIDS AND FLAVONOIDS FROM MALAYSIAN *UNCARIA LONGIFLORA* VAR. *PTEROPODA* (MIQ.) RIDSD.

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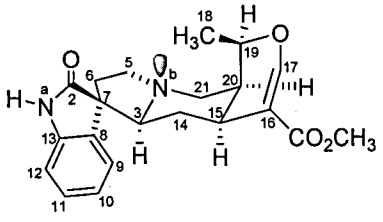
ABSTRACT

This study was performed with the aim of establishing the absolute configuration of six new chiral constituents comprising five pentacyclic oxindole alkaloids and a flavonoid isolated from *Uncaria longiflora* var. *pteropoda*. The absolute configurations of the new chiral compounds were established by comparing the cotton effects (CEs) of the experimental ECD spectra to the simulated ECD values, as well as to the ECD spectra of six known related compounds in hand. The known alkaloids were isopteropodine, pteropodine, Uncarine F and speciophylline while the known flavonoids were *epi*-catechin and *epi*-afzelechin. The POAs rauniticine-*allo*-oxindole B **ULS1** and rauniticinic-*allo* acid B **ULS2** were both established to have the 3*S*, 7*R*, 15*S*, 19*R*, 20*S* stereochemistry. The absolute configuration of three other new alkaloids were also determined: 2-oxosecologanine **ULL5** possess a 4*S*, 8*R*, 9*S* configuration while isoformosaninol **ULL6** and formosaninol **ULL7** exhibited a 3*S*, 7*S*, 15*S*, 16*R*, 17*R*, 19*R*, 20*R* and a 3*S*, 7*R*, 15*S*, 16*R*, 17*R*, 19*R*, 20*R* stereochemistry, respectively. The new flavonoid, uncariechin **ULL14** was established to possess a 2*R*, 3*R* configuration.



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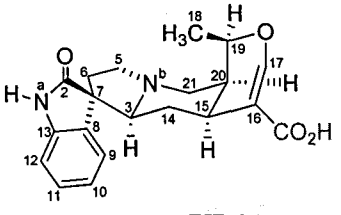

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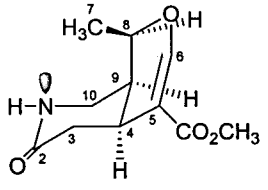

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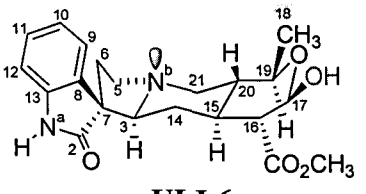

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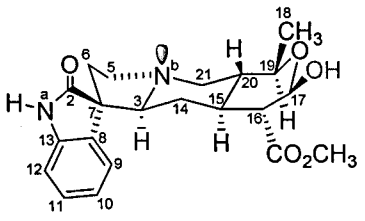

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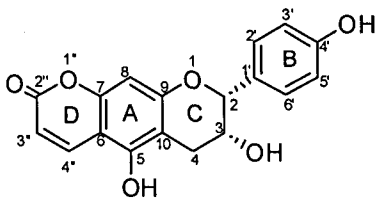

ULL14

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CHAPTER ONE

INTRODUCTION

1.1 The Genus *Uncaria*

The genus *Uncaria* Havil. belongs to the Rubiaceae family, subfamily *Cinchonoideae*, and it is placed in the tribe *Cinchoneae* under the subtribe *Mitragyninae*. It is a vine or shrub with characteristic peduncles that appear as curved hooks on the side shoots (Heitzman *et al.*, 2005). This genus, comprising some 34 species is distributed mainly in tropical regions, including Southeast Asia, Africa and South America. Cultivation varies based on the species and region. Of these, about 14 species of *Uncaria* are available in Malaysia.

Most of the species in the genus are known for their medicinal properties. Throughout the literatures, there are various uses described for different species of *Uncaria*. The Chinese medical plant, *U. rhynchophylla* is used to treat infantile fevers and nervous disorders of children, dizziness in adults, motes in vision and bilious disorders while another Chinese medicinal plant *U. hirsuta* is reported to be used to treat hypertension, colds, headaches, infantile convulsions and high fever Li (1978). In Malaysia, the more common representatives of *Uncaria* are *U. gambir* along with *U. acida* and *U. longiflora*. *U. gambir* are reported to have have anti-bacterial, anti-fungal and other properties that justify their uses in the treatment of burns and external callous ulcer and 'sakit kudis', which causes scurf or dry military scabs or crust formed on the skin [(Gimlette and Burkill (1930), Soepardi (1964)]. The fresh leaves and the young shoots of *U. gambir* are used to cure diarrhoea and dysentery, and as a gargle for sore throats. For *U. acida* and *U. longiflora* var. *pteropoda*, the leaves are

rubbed on the body to relieve pain from rheumatism, while their juice is used for thrush and mixed with iron-rust for framboesia (Burkill, 1935).

In South America, for at least 2000 years, among many Peruvian tribes, especially the Ashaninka, *U. tomentosa*, also known as *una de gato* (cat's claw), has been deeply believed to possess magical healing power, and has been extensively used for the treatment of asthma, cancer, cirrhosis, fevers, gastritis, diabetes, rheumatism, dysentery, inflammation of urinary tract, and many other diseases (Keplinger *et al.*, 1999; Aguilar *et al.*, 2002; Heitzman *et al.*, 2005). Recently, medical preparations from this plant have become very popular in Europe and America, particularly as an anticancer remedy. In a majority of the studies, the high biological activity of cat's claw is attributed to unique tetracyclic (TOAs) and pentacyclic oxindole alkaloids (POAs) which may exist in many stereoisomeric forms (Phillipson *et al.*, 1978; Laus, 1978; Seki *et al.*, 1993; Laus and Keplinger, 1994). However, since TOAs and POAs appear to be antagonistic in some studies, mixtures of these alkaloids are less suitable for medicinal use (Reinhard, 1999). Due to a wide spectrum of the plant activity, it is nevertheless believed that there is a synergistic participation of other chemical compounds in the healing process (Reinhard, 1999). Figure 1.1 shows some marketed products containing *Uncaria* sold all over the world, including in Peru, Mexico, Brazil and Malaysia (Manjakani pills and Pil Binari).

Various active principles have also been found to be present in the South East Asian *Uncaria*. A number of them have been cultivated for their chemical principles; *U. gambir* for catechin and *U. elliptica* for rutin while isolation of pentacyclic and tetracyclic oxindole alkaloids have been well-documented (Phillipson and Hemingway, 1973; Kam *et al.*, 1992; Laus, 2004; Heitzman *et al.*, 2005).

1.2 Problem Statement

Among the species in the *Uncaria* genus, one particularly complex species is *Uncaria longiflora* (Poir.) Merr. which comprises at least three major entities, namely *Uncaria pteropoda* Miq., *Uncaria longiflora* (Poir.) Merr. *sensu stricto* and *Uncaria havilandiana* S. Moore (Ridsdale, 1978). The species represents a rather well-studied group from the viewpoint of its alkaloidal content reported to be present in the flowers, shoots, leaves, barks, stems or hooks, and roots (Yeoh *et al.*, 1968; Phillipson and Hemingway, 1973; Phillipson *et al.*, 1975; Kam *et al.*, 1992; Heitzman *et al.*, 2005). Although Ridsdale's revision has helped resolve the problems related to the identification of the individual species, *Uncaria longiflora* nevertheless remains taxonomically a problematic species, since not much of the published work on it specified the species variations. The latest published work that clearly mutualises the *pteropoda* and *longiflora* variations was done by Kam and co-workers in 1992 (Kam *et al.*, 1992). Their research had nevertheless only afforded two stereoisomeric pentacyclic oxindole alkaloids, isopteropodine and pteropodine from the leaves extract of *Uncaria longiflora* var. *pteropoda* (Miq.) which was in accordance with previous reports by Yeoh and colleagues in 1966.

In our recent phytochemical studies on *Uncaria longiflora* var. *pteropoda* (Miq.) we have obtained the two stereoisomeric POAs reported earlier which have been widely reported to possess many biological activities. POAs are monoterpene alkaloids containing an oxindole nucleus which consist of five asymmetric carbons atoms that can exist in thirty-two forms of stereoisomers (Shamma *et al.*, 1967; Cordell, 1981). Although the relative configuration of the chiral compounds has been determined by NMR studies, the absolute configuration of new compounds has not been established. Due to this complexity of its chiral centres, and the lack of recent

circular dichroism studies on this type of alkaloids, it is of interest to establish the absolute configuration of these collection of alkaloid, especially the expected new chiral compounds by comparison of experimental and simulated electronic circular dichroism studies (ECD) spectra using time dependence density functional theory (TDDFT).

1.3 Objectives of the Study

In view of the lack of study on circular dichroism on pentacyclic oxindole alkaloids and the need to establish absolute configuration of six new chiral compounds isolated from of *Uncaria longiflora* var. *pteropoda* (Miq.), the objectives of this study are as follows:

1. To obtain experimental ECD spectra of twelve chiral compounds isolated from *Uncaria longiflora* var. *pteropoda* comprising nine oxindole alkaloids and three flavonoids.
2. To obtain simulated ECD spectra of nine pentacyclic oxindole alkaloids (four known and five new) using time dependence density functional theory (TDDFT) and ab initio methods.
3. To establish the absolute configuration of six new chiral compounds comprising four pentacyclic oxindole alkaloids, a novel oxindole alkaloid and a flavonoid based on comparison of experimental and simulated CD spectra.

CHAPTER TWO

LITERATURE REVIEW

Malaysia's rainforest, being part of the world's tropical rainforest biome, is considered one of the most evolved and diverse rainforests in the world, as it is believed to be untouched by the Ice Age. Among the families of plants growing in Malaysia rainforest, the family of Fabaceae (previously known as Leguminosae) contained the most number of species, followed by the plants of the Rubiaceae (Soepadmo, 1999). *Uncaria longiflora* var. *pteropoda* (Miq.) is a species within the genus *Uncaria* of the Rubiaceae family (Burkill, 1935). In this chapter, the botanical features, medicinal properties, phytochemistry and bioactivity of the genus of *Uncaria* are given. A detailed description of the species of *Uncaria longiflora* var. *pteropoda* is also included. In addition, the biogenetic pathways of the prevalent pentacyclic oxindole alkaloids and flavonoids found in the genus of *Uncaria* species, as well as the determination of the configuration of stereochemistry in natural products are also briefly elaborated.

2.1 The Genus *Uncaria*

Uncaria is a genus belonging to the family Rubiaceae. The genus has undergone many revisions in terms of intratribal classifications (Ridsdale, 1978; Andersson and Persson, 1991; Robbrecht, 1993) and the latest taxonomic rearrangement of *Uncaria* is shown as follows;

Family: Rubiaceae
Subfamily: *Cinchonoideae*
Tribe: *Cinchoneae*
Subtribe: *Mitragyninae*
Genus: *Uncaria*

The genus *Uncaria*, containing some 34 species, is distributed mainly in tropical regions, including Southeast Asia, Africa and Southeast America. Their cultivation varies based on the species and the region. All of the species are similar in appearance, and are very easily recognizable. They are lianas with monopodial main shoots, and more or less horizontally patent, plagiotropic lateral shoots of limited growth; thus, the lianas climb by their divergent spreading shoots. Their leaves are more or less coriaceous. Some of the flowers are in globose heads and peduncle, whilst some of the species have a peduncle without flowers, and they are converted into stout hooks. The *Uncaria*'s flowers have a cylindrical calyx with five-lobed. The stamen of the flower is short at the corolla mouth. The ovary is fusiform with long and slender style. The seeds are numerous, minute and winged at each end (Ridsdale, 1978).

Of the 34 known species of *Uncaria*, about 14 species are available in Malaysia, among which include *U. cordata* (Lour.) Merr., *U. borneensis* Havil., *U. attenuata* Korth., *U. barbata* Merr., *U. canescens* Korth., *U. kunstleri* King., *U. acida* (Hunt.) Roxb., *U. elliptica* R.Br. *ex* G. Don, *U. longiflora* (Poir.) Merr., *U. gambir* (Hunt). Roxb., *U. callophylla* Bl. *ex* Korth., *U. lanosa* Wall., *U. roxburghiana* Korth., and *U. homomalla* Miq. (Ridsdale, 1978). The most popular among the local *Uncaria* species is *Uncaria gambir* Roxb., known as '*gambir*' or "kait-kait". They climb by grappling thus contributing to their names, such as "kait-kait" or "kekait" in the Malay language, which means hook. Figure 2.1 shows some representative *Uncaria* species.

U. rhynchophylla

U. tomentosa

U. guianensis

U. lanosa var. *ferruginea*

U. lanosa var. *appendiculata*

U. gambir

U. sinensis

U. hirsuta

Figure 2.1 Some representative *Uncaria* Species

2.2 Medicinal Properties of the Genus *Uncaria*

In South America, for at least a quarter of a century, *U. tomentosa*, known as *una de gato* (cat's claw), has been deeply believed to possess magical healing powers by the Ashaninka people in the Peruvian rainforest. The Ashaninka priests exclusively use this plant to influence the communication between the physical and spiritual dimensions of human beings (Keplinger *et al.*, 1999). The therapeutic uses of *U. tomentosa* come from its bark or root bark aqueous extract. This extract decoction is used for treatment of a wide range of health problems including abscesses, allergies, arthritis, asthma, cancer, chemotherapy side effect, contraception, disease prevention, fevers, gastric ulcers, haemorrhages, inflammations, menstrual irregularity, recovery from child birth, rheumatism, skin impurities, urinary tract inflammation, AIDS, weakness, viral infections and wounds (Jones, 1995; Keplinger *et al.,* 1999; Akesson *et al.,* 2003; Heitzman *et al.,* 2005; Prado *et al.*, 2007).

In Asia, the medicinal values of the *Uncaria* plants are equally significant. *Uncaria rhynchophylla* has been used as a crude drug in oriental medicines. Li (1978) recorded that *U. rhynchophylla* is used to treat infantile fevers and nervous disorders of children, dizziness in adults and bilious disorders. This species of *Uncaria* is also one of the main ingredients in Chinese drug "Gou-teng" (Japanese known as "Chotoko"). "Gou-teng" is used as a sedative, antispasmodic, analgesic, anticonvulsive, hypotensive, antiepileptic and antiviral (Yano *et al.,* 1991; Lee *et al.,* 1999a). The infusion of *U. rhynchophylla* and *U. sinensis* is known as "Kampo", a broad term for traditional Japanese herbal medicine. It is used to relieve hypertension and its associated symptoms, such as headaches and dizziness (Yano *et al.,* 1991; Watanabe *et al.*, 2003). Another Chinese medicinal plant *U. hirsuta* is also reported to

be used in "Gou-teng" (Wu and Chan, 1994), as well as to treat primary hypertension when mixed with other plant medicines (Chang *et al.*, 1989).

Figure 2.2 Gou-teng and Kampo containing *Uncaria* species

Another Asian *Uncaria* plant, *Uncaria gambir* or "gambier" is widely used as a tanning material and a mordant in dyeing. It is employed medicinally as an astringent. An infusion of the fresh leaves and young shoots is taken for diarrhea and dysentery, and is used as a gargle for sore throat and as a mouth freshener. Dried components of this plant prepared from an aqueous extract of leaves and twigs are applied to burns and scurf. In the past, *U. gambir* was chewed with betel leaves and some believe it causes the reddening of the mouth of betel chewers. In Borneo, the poultice of the plant is applied externally for lumbago and sciatica. Interestingly, this species of *Uncaria* is also used as an ingredient in many ancient Chinese herbal recipes. In Indonesia, it is used as an ingredient in the traditional formulation called 'jamu', which is popularly consumed by Malaysians and Indonesians for overall well-being (Burkill, 1935). Examples of the medicinal uses of other species belonging to the genus are tabulated in Table 2.1 (Burkill, 1935; Watkin *et. al.*, 1986; Keplinger *et al.*, 1999; Heitzman *et al.*, 2005).

TABLE 2.1
Medicinal Uses of Some *Uncaria* Species

Species	Medicinal Uses
U. guianensis (Aubl.)	Dysentry, cancer, arthritis, diabetes, inflammation, immune system stimulant, contraceptive, irritable bowel syndrome, colitis and Crohn's disease.
U. hirsute Havil.	Primary hypertension
U. macrophylla Wall.	Original component in Chinese Medicine as Gou-teng: sedative, antispasmodic, analgesic, anticonvulsive, hypotensive, antiepileptic and antiviral
U. rhynchophylla (Miq.) Jacks.	Therapeutic: to relieve hypertension, infantile convulsion and other illnesses. As Gou-teng: sedative, antispasmodic, analgesic, anticonvulsive, hypotensive, antiepileptic and antiviral
U. sinesis (Oliv.)	Fever, nervous disorders, spasmolytic, analgesic and hypertension
U. tomentosa (Willd.) DC.	Abscesses, allergies, arthritis, asthma, cancer, chemotherapy side effect, contraception, disease prevention, fevers, gastric ulcers, haemorrhages, inflammations, menstrual irregularity, recovery from child birth, rheumatism, skin impurities, urinary tract inflammation, weakness, viral infections and wounds
U. gambir (Hunt.) Roxb.	Relieve pain, rheumatism, framboesia
U. acida (Hunt.) Roxb.	Relieve pain
U. lanosa var. *ferrea* Wall.	Decoction for cleaning wounds and ulcers, infusion for intestines inflammation
U. lanosa var. *glabrata* (Bl.) Ridsd.	Remedy for food poisoning
U. longiflora var. *pteropoda* (Miq.) Ridsd	Relieve pain of rheumatism, framboesia
U. cordata var. *cordata* (Lour.) Merr.	Cure ulcers

2.3 The Species *Uncaria longiflora var. pteropoda*

Uncaria longiflora var. *pteropoda* is a variety or subspecies of *Uncaria longiflora* (Poir.) Merr. From the revision made by Ridsdale in 1978, this plant is a synonym to *Uncaria pteropoda* Miq., *Uruparia pteropoda* O.K. and *Uncaria laevifolia* Elm. The taxonomic classification of *Uncaria longiflora* var. *pteropoda* is shown as follows:

Family: Rubiaceae
Subfamily: *Cinchonoideae*
Tribe: *Cinchoneae*
Subtribe: *Mitragyninae*
Genus: *Uncaria*
Species: *Uncaria longiflora*
Variety: *pteropoda*
Botanical name: *Uncaria longiflora* var. *pteropoda*

Uncaria longiflora var. *pteropoda* is a woody, strong shrubby climber with monopodial main shoots, and more or less horizontally patent, plagiotropic lateral shoots of limited growth thus the lianas climb (by means of hooks) by their divergent spreading shoots. Its leaves are oblong-ovate, 7.5-10 cm (3-4') long, petiole conspicuously winged, acuminate, entire and smooth. The flowers are in globose heads and peduncle where they are converted into stout hooks. The flowers have elliptic or boat-shaped calyx with five-lobed, usually pallid to light brown. The stamen of the flower is short at the corolla mouth. The ovary is fusiform with long and slender style. The seeds are numerous, minute and winged at each end (Ridsdale, 1978). This species can be found in tropical regions, including Peninsular Malaysia, Sumatra, Borneo and Philippines. In Malaysia, this plant is known as *kekait darat* or *kekait besi*. Traditionally, the leaves of this species variety of *Uncaria* are rubbed on the body to relieve pain from rheumatism, while its juice is used for thrush and mixed with iron-rust for framboesia (Burkill, 1935).

Figure 2.3 *Uncaria longiflora* var. *pteropoda*

2.4 Phytochemistry of the Genus *Uncaria*

The *Uncaria* genus has been instrumental in the discovery of medicinal natural products since the earliest phytochemical report of *Uncaria* was published in 1928. The paradigm of this genus continued particularly due to their alkaloidal content, principally due to the work of Phillipson and co-workers (Phillipson and Hemingway, 1973a,b; Phillipson and Hemingway, 1975; Phillipson *et al.*, 1978; Ponglux *et al.*, 1980; Tantivatana *et al.*, 1980; Phillipson and Supavita, 1981; Phillipson and Supavita, 1983) and others (Yeoh *et al.*, 1965; Merlini *et al.*, 1967; Chan, 1968; Merlini *et al.*, 1972; Goh and Junan, 1985; Kam *et al.*, 1991; Liu and Feng, 1992). Today, over 151 compounds including alkaloids, terpenes, quinovic acid glycosides, flavonoids and coumarins have been isolated and identified from this genus, and the most recognized compound is the alkaloid mitraphylline, which has been identified in 20 out of the 34 species. However, a major consideration of the comparison of identified components in *Uncaria* species is that the chemical composition can vary based on its geographical and seasonal collections (Phillipson and Hemingway, 1973; Kam *et al.*, 1992), which may be explained by the existence of ecotypes and chemotypes (Laus, 2004).

Among the species, the most widely explored is the Peruvian *Uncaria tomentosa* (cat's claw), which has yielded over 50 different compounds, and 35 of which been identified in only a couple of other species (Laus *et al.*, 1997; Kitajima *et al.*, 2000; Falkiewicz and Lukasiak, 2001; Laus, 2004; Heitzman *et al.*, 2005). Other species that also have yielded a significant number of compounds include *U. elliptica* (44 compounds), *U. attenuata* (34 compounds) and *U. rhynchophylla* (33 compounds) (Heitzman *et al.*, 2005).

2.4.1 Alkaloids

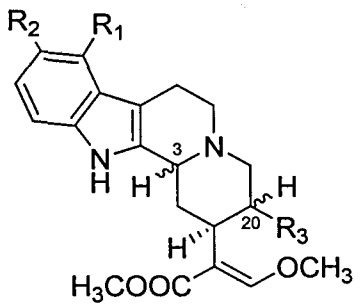
Alkaloids are secondary metabolites that contain basic nitrogen atoms. One major group which is usually highly complex in structure is the indole alkaloids derived from the amino acid tryptophan. This group of alkaloids, and its related oxindole alkaloids, are among the compounds that have been identified the most in *Uncaria* species. They have been isolated from various parts of *Uncaria* plants including bark, flower, hooks, stems, leaves and roots. Some of them are prevalent within the genus.

a. Indole Alkaloids

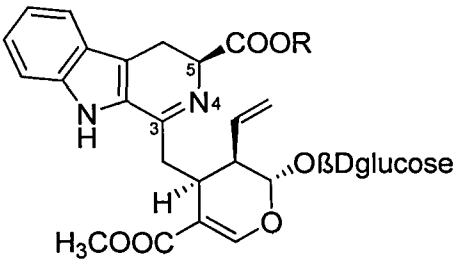
The indole alkaloids found in *Uncaria* species are mostly the yohimbinoid- or heteroyohimbinoid-type, particularly the tetracyclic and pentacyclic group. They are monoterpene alkaloids containing an indole nucleus that consists of a number of asymmetric carbons atoms thus can exist in number forms of stereoisomers (Cordell, 1981). Besides these, simple and other new skeleton of indole alkaloids have also been isolated from the genus.

i. Tetracyclic Indole Alkaloids

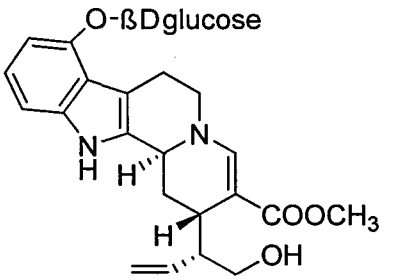
Throughout the literature, a total of 15 tetracyclic indole alkaloids have been identified from the genus of *Uncaria*. Among the tetracyclic indole alkaloids isolated from this genus, the alkaloid dihydrocorynantheine (**1**) and hirsutine (**2**) were found to be the most prominent, since they have been identified in eight and nine species, respectively (Table 2.2). The structures of the 15 tetracyclic indole alkaloids are shown below.



1 R_1=R_2=H, R_3=Et, 3α-H, 20β-H
2 R_1=R_2=H, R_3=Et, 3β-H, 20β-H
3 R_1=H, R_2=OH, R_3=Et, 3α-H, 20β-H
4 R_1=OH, R_2=H, R_3= CH=CH_2, 3α-H, 20β-H
5 R_1=R_2=H, R_3= CH=CH_2, 3β-H, 20β-H
6 R_1=OH, R_2=H, R_3=Et, 3α-H, 20β-H
7 R_1=R_2=H, R_3= CH=CH_2, 3α-H, 20β-H
8 R_1=R_2=H, R_3= CH=CH_2, 3β-H, 20α-H



9 R=H
10 R=



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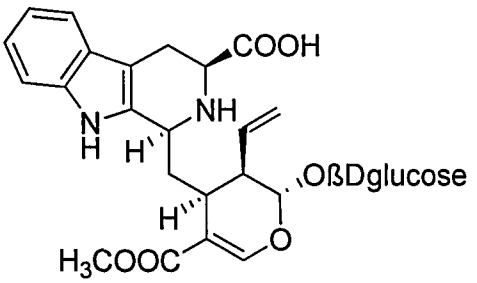

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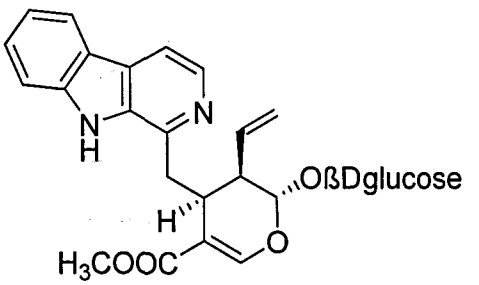

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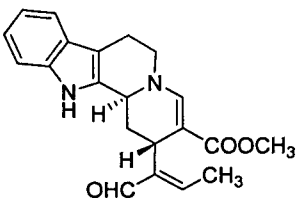
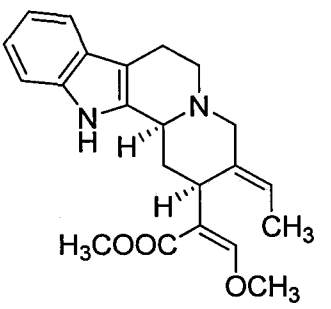


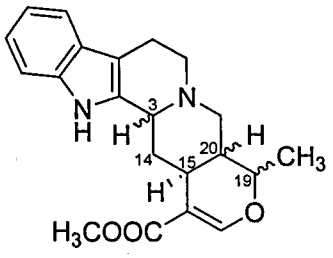
14



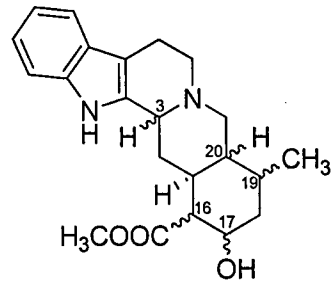
15

ii. Pentacyclic Indole Alkaloids

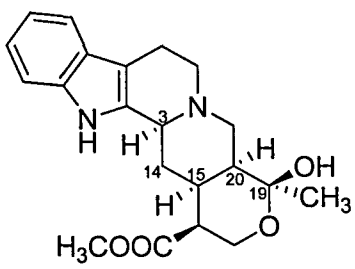
Among the 28 pentacyclic indole alkaloids isolated from the *Uncaria* genus, 3-isoajmalicine (**19**) is the most prevalent with its occurrence in nine species. The structures of the 28 pentacyclic indole alkaloids are shown below. Table 2.3 gives the occurence of other pentacyclic indole alkaloids in the genus *Uncaria*.



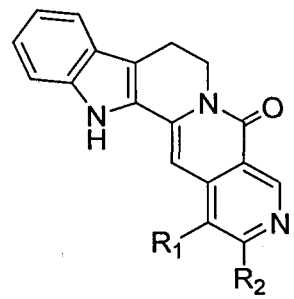
16 3α-H, 19α-CH_3, 20α-H
17 3α-H, 19β-CH_3, 20α-H
18 3β-H, 19β-CH_3, 20α-H
19 3β-H, 19α-CH_3, 20β-H
20 3β-H, 19β-CH_3, 20β-H
21 3α-H, 19α-CH_3, 20β-H
22 3β-H, 19β-CH_3, 20α-H
23 3α-H, 19β-CH_3, 20β-H
24 3α-H, 19β-CH_3, 20α-H, 14α-OH
25 3β-H, 19β-CH_3, 20α-H, 14β-OH
26 3β-H, 19α-CH_3, 20α-H



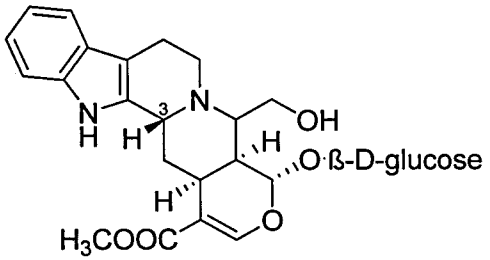
27 3β-H, 20β-H, 16α-C, 17α-OH
28 3α-H, 20α-H, 16α-C, 17α-OH
29 3β-H, 20β-H, 16α-C, 17β-OH
30 3α-H, 20β-H, 16α-C, 17α-OH
31 3α-H, 20α-H, 16β-C, 17α-OH
32 3α-H, 20β-H, 16α-C, 17β-OH



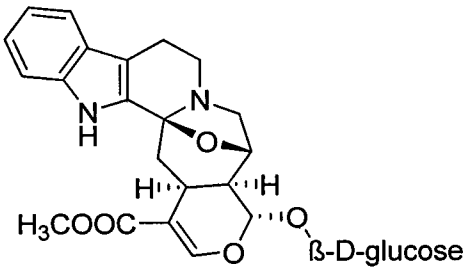
33



34 R_1=CH=CH_2, R_2=H
35 R_1=CH(OH)CH_3, R_2=H
36 R_1=H, R_2=CH_3



37



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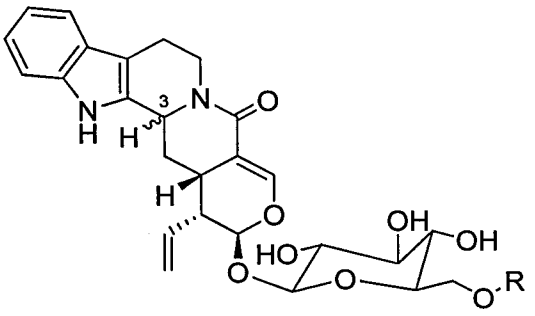

39 R=(*E*)-feruloyl, 3β-H
40 R=H, 3β-H
41 R=H, 3α-H

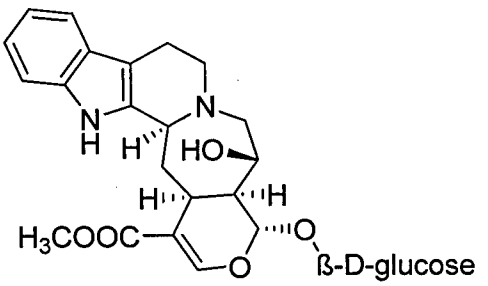

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TABLE 2.2 Tetracyclic Indole Alkaloids Isolated from *Uncaria* Species

Alkaloids	Species from which Alkaloids isolated	References
5α–Carboxystrictosidine (**9**)	*U. tomentosa*	Aquino *et al.*, 1991; Kitajima *et al.*, 2000
Corynantheine (**7**)	*U. rhynchophylla*	Phillipson *et al.*, 1978; Laus and Teppner, 1996
epiallo–Corynantheine (**8**)	*U. attenuata*	Phillipson *et al.*, 1978
3,4–Dehydro–5–carboxystrictosidine (**12**)	*U. tomentosa*	Kitajima *et al.*, 2000
Dihydrocorynantheine (**1**)	*U. africana, U. attenuata, U. callophylla, U. cordata, U. elliptica, U. guianensis, U. longiflora* var. *longiflora, U. nervosa, U. rhynchophylla*	Phillipson *et al.*, 1978; Goh and Junan, 1985; Kam *et al.*, 1992
Gambireine (**4**)	*U. callophylla, U. longiflora* var. *longiflora*	Kam *et al.*, 1991, 1992
Gambirine (**6**)	*U. callophyla, U. elliptica, U. longiflora* var. *longiflora*	Phillipson *et al.*, 1978; Goh and Junan, 1985; Kam *et al.*, 1991, 1992
Glabratine (**11**)	*U. lanosa, U. longiflora* var. *longiflora*	Arbain *et al.*, 1992, 1993
Geissoschizine methyl ether (**15**)	*U. rhynchophylla*	Phillipson *et al.*, 1978
Hirsutaside A (**10**)	*U. hirsuta*	Xin *et al.*, 2008
Hirsuteine (**5**)	*U. attenuata, U. guianensis, U. nervosa, U. rhynchophylla, U. sinensis, U. tomentosa*	Phillipson *et al.*, 1978; Laus and Teppner, 1996
Hirsutine (**2**)	*U. attenuata, U. guianensis, U. kunstleri, U. nervosa, U. rhynchophylla, U. sessilifructus, U. sinensis, U. tomentosa*	Phillipson *et al.*, 1978; Laus and Teppner, 1996
Hirsutine *N*-oxide	*U. tomentosa*	Phillipson *et al.*, 1978
Isogambirine (**3**)	*U. callophylla*	Kam *et al.*, 1992
Lyaloside (**13**)	*U. tomentosa*	Kitajima *et al.*, 2000
Vallesiachotamine (**14**)	*U. rhynchophylla, U. tomentosa*	Aimi *et al.*, 1982

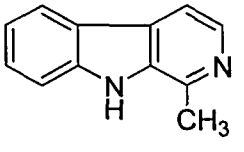
TABLE 2.3 Pentacyclic Indole Alkaloids Isolated from *Uncaria* Species

Alkaloids	Species from which Alkaloids isolated	References
Angustidine (**36**)	*U. homomalla, U. rhynchophylla*	Phillipson *et al.*, 1978
Agustine (**34**)	*U. bernaysii, U. elliptica, U. guiainensis, U. homomalla, U. rhynchophylla*	Phillipson *et al.*, 1978
Agustoline (**35**)	*U. guianensis, U. homomalla, U. rhynchophylla*	Phillipson *et al.*, 1978
Ajmalicine (**21**)	*U. africana, U. elliptica, U. orientalis*	Phillipson and Supavita, 1981, 1983
19-*epi*-Ajmalicine (**23**)	*U. africana, U. elliptica*	Tantivatana *et al.*, 1980; Phillipson and Supavita, 1981
Akuammigine (**26**)	*U. attenuata, U. bernaysii, U. elliptica, U. lanosa*	Phillipson *et al.*, 1978
Alloyohimbine (**28**)	*U. borneensis*	Kam *et al.*, 1991
Cadambine (**38**)	*U. sinensis*	Endo *et al.*, 1983
Diangoutengjian (**33**)	*U. yunnanensis*	Tao *et al.*, 2001
3α-Dihydrocadambine (**42**)	*U. sinensis*	Endo *et al.*, 1983; Aisaka *et al.*, 1985; Shimada *et al.*, 1999
14β-Hydroxy-3-isorauniticine (**18**)	*U. elliptica*	Phillipson and Supavita, 1983
14β-Hydroxyl-3-isorauniticine (**25**)	*U. elliptica*	Phillipson and Supavita, 1983
14α-Hydroxyrauniticine (**24**)	*U. attenuata, U. lanosa*	Herath *et al.*, 1979; Ponglux *et al.*, 1980; Ponglux *et al.*, 1990
3-Isoajmalicine (**19**)	*U. acida, U. africana, U. attenuata, U. elliptica, U. hirsuta, U. homomalla, U. orientalis, U. sessilifructus, U. sterrophylla,*	Phillipson *et al.*, 1978; Wurm *et al.*, 1998
19-*Epi*-3-isoajmalicine (**20**)	*U. attenuata, U. elliptica, U. attenuata, U. orientalis, U. sessilifructus*	Phillipson *et al.*, 1978; Tantivatana *et al.*, 1980; Phillipson and Supavita, 1981; Kam *et al.*, 1992;
3β-Isodihydrocadabine (**37**)	*U. sinensis*	Endo *et al.*, 1983; Aisaka *et al.*, 1985; Shimada *et al.*, 1999
Isorauniticine (**22**)	*U. elliptica*	Tantivatana *et al.*, 1980; Phillipson and Supavita, 1981, 1983
Pseudoyohimbine (**27**)	*U. bernaysii, U. borneensis*	Goh and Junan, 1985; Kam *et al.*, 1992
Rauniticine (**17**)	*U. lanosa, U. attenuata, U. elliptica*	Ponglux and Supavita, 1980; Phillipson and Supavita, 1981, 1983; Arbain *et al.*, 1998

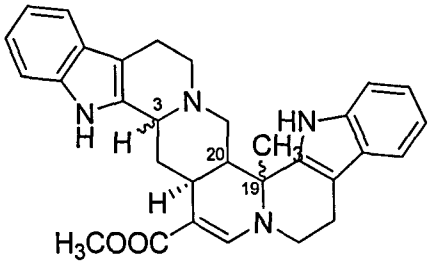
Alkaloids	Species from which Alkaloids isolated	References
Rhynchophine (**39**)	*U. rhynchophylla*	Aimi *et al.*, 1982
Strictosamide (**41**)	*U. rhynchophylla*	Aimi *et al.*, 1982
Tetrahydroalstonine (**16**)	*U. africana, U. attenuata, U. bernaysii, U. elliptica*	Phillipson *et al.*, 1978; Ponglux and Supavita, 1980; Phillipson and Supavita, 1981
Tetrahydroalstonine *N*-oxide	*U. elliptica*	Phillipson and Supavita, 1981
Vincoside lactam (**40**)	*U. rhynchophylla*	Aimi *et al.*, 1982
Yohimbine (**30**)	*U. callophylla*	Kam *et al.*, 1992
α-Yohimbine (**31**)	*U. callophylla*	Kam *et al.*, 1992
β-Yohimbine (**32**)	*U. callophylla*	Kam *et al.*, 1992
3-*Epi*-β-yohimbine (**29**)	*U. borneensis, U. cordata*	Kam *et al.*, 1991

iii. Other Indole Alkaloids

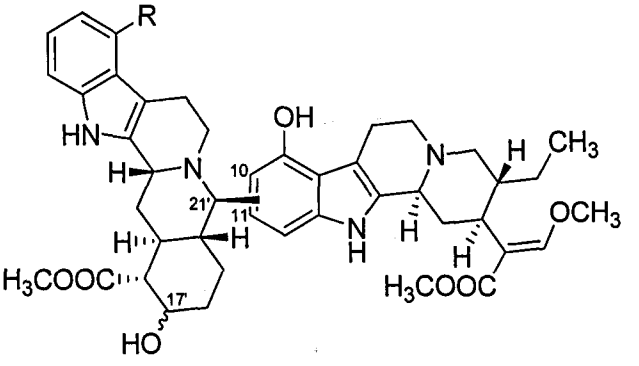
The simplest indole alkaloid that is present in *Uncaria* species is harmane (**43**). It has been isolated from various parts of *U. acida*, *U. attenuata*, *U. barbata*, *U. borneensis*, *U. callophylla*, *U. canescens*, *U. elliptica*, *U. lanosa*, *U. nervosa* and *U. orientalis* (Phillipson *et al.*, 1978). The bis indole alkaloids, callophylline (**46**), callophylline A (**47**) and callophylline B (**48**) were isolated from the leaves of Malaysian *U. callophylla* as new alkaloids (Kam *et al.*, 1991). Callophylline and callophylline B possess gambirine and pseudoyohimbine skeletons, whereas callophylline A contains the 3-*epi*-β-yohimbine skeleton. *U. elliptica*, another species of *Uncaria*, also yielded two new bis indole alkaloids identified as roxburghine X (**44**) and D (**45**) (Herath *et al.*, 1979). The roxburghines has been claimed to be unique to the genus in that each molecule is derived from two molecules of tryptamine and one molecule of secologanin.



43



44 (stereochemistry unknown)
45 3β-H, 19α-CH_3, 20β-H



46 10-21' bond, R=H, 17'α-OH
47 10-21' bond, R=H, 17'β-OH
48 11-21' bond, R=OH, 17'α-OH

b. Oxindole Alkaloids

Oxindole alkaloids are a monoterpene group of alkaloids that exhibits an oxindole moiety (N-C=O) in ring B (refer Fig. 2.3). These alkaloids are typically found to co-occur which their corresponding corynantheoid or ajmalicinoid analogues (Cordell, 1981). The occurrence of this group of alkaloids in the *Uncaria* genus may be derived from the transition of heteroyohimbines (indole alkaloids) *via* indolenine intermediates brought about by differences in cell pH values (Phillipson and Supavita, 1983). Most of the identified oxindole alkaloids in the *Uncaria* genus exhibit tetracyclic and pentacyclic oxindole skeletons.

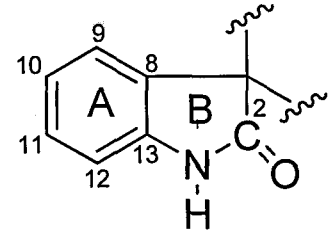
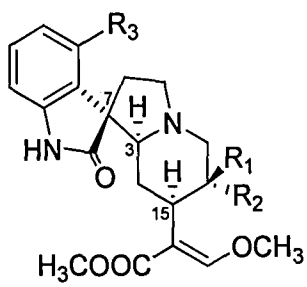


Figure 2.4 Basic Structure of an Oxindole Alkaloid

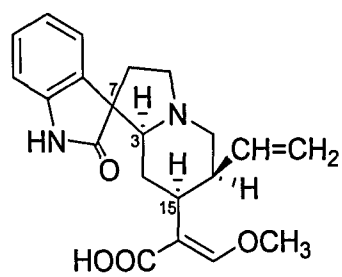
i. Tetracyclic Oxindole Alkaloids (TOAs)

The tetracyclic oxindole alkaloid, rhynchophylline (**49**) was the earliest alkaloid found in *Uncaria* species of *rhynchophylla* (Kondo *et al.*, 1928). Rhynchophylline and its close stereoisomer, isorhynchophylline (**55**), are the prevalent TOAs within the *Uncaria* genus and has been identified in 18 species (refer to Table 2.4). Their *N*-oxides were found from 10 species of *Uncaria,* as reported by Phillipson and co-workers in 1978. They suggest that the discovery of these *N*-oxides may be due to the further oxidation of the oxindole alkaloids to non-basic products (i.e. hydroxylamines, oximes). They further suggested that these compounds may play

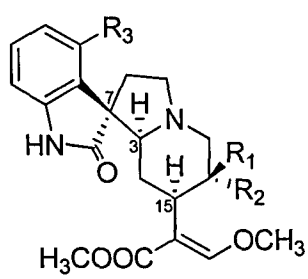
some part in the interconversion of the isomers, or that the reversible oxidation of the tertiary base to the *N*-oxides is essential to some plant metabolic processes. Fifteen years later, the 16-carboxyderivatives of these two TOAs were isolated from *U. sinensis* (Liu and Feng, 1993). Other TOAs found from this genus include corynoxine B (**50**), corynoxeine (**51**), isorotundifoline (**52**), 18,19-dehydrocorynoxinic acid (**53**), 18,19-dehydrocorynoxinic acid B (**54**), corynoxine (**56**), isocorynoxeine (**57**) and rotundifoline (**58**). Their structures are shown below while their existence in Uncaria species is shown in Table 2.4.



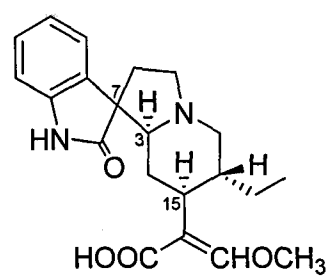
49 R_1=H, R_2=Et, R_3=H
50 R_1=Et, R_2=H, R_3=H
51 R_1=H, R_2= CH=CH_2, R_3=H
52 R_1=H, R_2=Et, R_3=OH



53 C7-A (Oxindole C=O below C/D plane)
54 C7-B (Oxindole C=O above C/D plane)



55 R_1=H, R_2=Et, R_3=H
56 R_1=Et, R_2=H, R_3=H
57 R_1=H, R_2= CH=CH_2, R_3=H
58 R_1=H, R_2=Et, R_3=OH



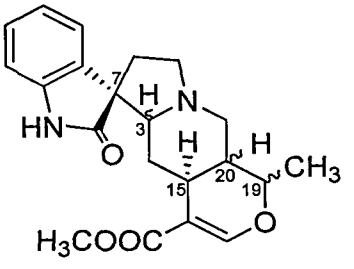
59 C7-A (Oxindole C=O below C/D plane)
60 C7-B (Oxindole C=O above C/D plane)

TABLE 2.4 Tetracyclic Oxindole Alkaloids Isolated from *Uncaria* Species

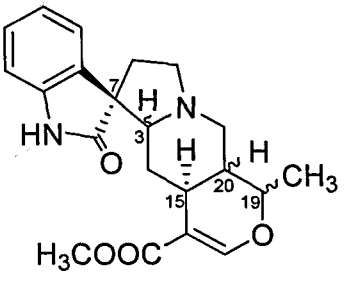
Alkaloid	Species from which alkaloid isolated	References
Corynoxeine (**51**)	*U. acida, U. attenuata, U. borneensis, U. longiflora* var. *longiflora, U. rhynchophylla, U. sinensis*	Kam *et al.*, 1992; Laus and Teppner, 1996
Corynoxine (**56**)	*U. attenuata, U. cordata, U. kunstleri, U. macrophylla, U. sessillifructus, U. sterrophylla*	Phillipson *et al.*, 1978; Lee *et al.*, 2000
Corynoxine B (**50**)	*U. attenuata, U. cordata, U. kunstleri, U. macrophylla, U. sessillifructus, U. sterrophylla*	Phillipson *et al.*, 1978; Lee *et al.*, 2000
18,19-Dehydrocorynoxinic acid (**53**)	*U. rhynchophylla*	Yuan *et al.*, 2008
18,19-Dehydrocorynoxinic acid B (**54**)	*U. rhynchophylla*	Yuan *et al.*, 2008
Isocorynoxeine (**57**)	*U. attenuata, U. borneensis, U. lanosa, U.longiflora* var. *longiflora, U. rhynchophylla, U. sinensis*	Phillipson *et al.*, 1978; Laus and Teppner, 1996
Isorhynchophylline (**55**)	*U. acida, U. africana, U. attenuata, U. bernaysii, U. borneensis, U. callophylla, U. cordata, U. elliptica, U. guianensis, U. kunstleri, U. longiflora var. longiflora, U. macrophylla, U. rhynchophylla, U. sessilifructus, U. sinensis, U. sterrophylla, U. talbotii, U. tomentosa*	Phillipson *et al.*, 1978; Wagner *et al.*, 1985; Ponglux *et al.*, 1990; Kam *et al.*, 1992; Laus and Teppner, 1996; Sakakibara *et al.*, 1998
Isorhynchophyllic acid (**59**)	*U. sinensis*	Liu and Feng, 1993
Isorhynchophylline *N*-oxide	*U. acida, U. africana, U. attenuata, U. bernaysii, U. guianensis, U. kunstleri, U. longiflora* var. *longiflora, U. macrophylla, U. rhynchophylla, U. tomentosa*	Phillipson *et al.*, 1978
Isorotundifoline (**52**)	*U. attenuata, U. callophylla, U. elliptica, U. tomentosa*	Phillipson *et al.*, 1978
Rhynchophyllic acid (**60**)	*U. sinensis*	Liu and Feng, 1993
Rhynchoplylline (**49**)	*U. acida, U. africana, U. attenuata, U. bernaysii, U. borneensis, U. callophylla, U. cordata, U. elliptica, U. guianensis, U. kunstleri, U. longiflora* var. *longiflora, U. macrophylla, U. rhynchophylla, U. sessilifructus, U. sinensis, U. sterrophylla, U. talbotii, U. tomentosa*	Phillipson *et al.*, 1978; Wagner *et al.*, 1985; Ponglux *et al.*, 1990; Kam *et al.*, 1992; Laus and Teppner, 1996; Sakakibara *et al.*, 1998
Rhynchophylline *N*-oxide	*U. acida, U. africana, U. attenuata, U. bernaysii, U. guianensis, U. kunstleri, U. longiflora* var. *longiflora, U. macrophylla, U. rhynchophylla, U. tomentosa*	Phillipson *et al.*, 1978
Rotundifoline (**58**)	*U. attenuata, U. callophylla, U. elliptica, U. tomentosa*	Phillipson *et al.*, 1978; Kam *et al.*, 1992

ii. Pentacyclic Oxindole Alkaloids (POAs)

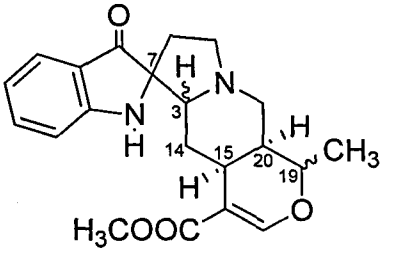
Numerous pentacyclic oxindole alkaloids have been identified from the genus *Uncaria*. The most recognized alkaloid in *Uncaria* is mitraphylline (**61**), which has been identified in 20 out of 34 species (Heitzman *et al.*, 2005). Other known compounds include formosanine (**62**), pteropodine (**63**), uncarine F (**64**), isomitraphylline (**65**), speciophylline (**67**), isopteropodine (**68**), isoformosanine (**69**), 3-iso-rauniticine pseudoindoxyl (**71**), isopteropodic acid (**73**), pteropodic acid (**74**) and mitraphyllic acid (**75**) (Phillipson and Hemingway, 1975; Phillipson *et al.*, 1978; Phillipson and Supavita, 1983; Liu and Feng, 1993; Tanahashi *et al.*, 1997). Rauniticine oxindole A (**66**), akuammigine pseudoindoxyl (**72**) and rauniticine pseudoindoxyl (**70**) were isolated as new natural products from the genus (Phillipson and Supavita, 1983). The structures of these alkaloids are shown in below while their existence in *Uncaria* species is shown in Table 2.5.



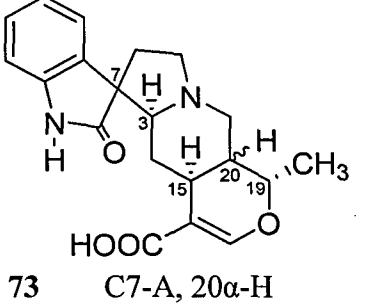
61 3α-H, 19α-CH_3, 20β-H
62 3α-H, 19β-CH_3, 20β-H
63 3α-H, 19α-CH_3, 20α-H
64 3β-H, 19α-CH_3, 20α-H



65 3α-H, 19α-CH_3, 20β-H
66 3α-H, 19β-CH_3, 20α-H
67 3β-H, 19α-CH_3, 20α-H
68 3α-H, 19α-CH_3, 20α-H
69 3α-H, 19β-CH_3, 20β-H



70 3α-H, 19β-CH_3
71 3β-H, 19β-CH_3
72 3β-H, 19α-CH_3



73 C7-A, 20α-H
74 C7-B, 20α-H
75 C7-B, 20β-H

Figure 2.7 Structures of tetracyclic oxindole alkaloids from Uncaria

TABLE 2.5 Pentacyclic Oxindole Alkaloids Isolated from *Uncaria* Species

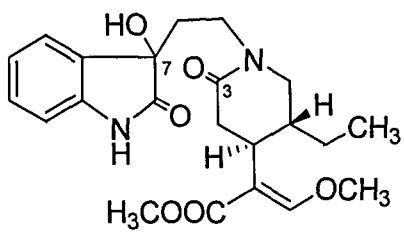
Alkaloids	Species from which alkaloids isolated	References
Akuammigine pseudoindoxyl (**72**)	*U. elliptica*	Phillipson and Supavita, 1983
Formosanine (Uncarine B) (**62**)	*U. attenuata, U. elliptica, U. gambir, U. hirsuta, U. laevigata, U. orientalis, U. sessilifructus*	Phillipson *et al.*, 1978; Herath *et al.*, 1978; Tantivatana *et al.*, 1980; Wu and Chan, 1994;
Isoformosanine (Uncarine A) (**69**)	*U. attenuata, U. cordata, U. gambir, U. hirsuta, U. laevigata, U. orientalis, U. sessilifructus*	Phillipson *et al.*, 1978; Wu and Chan, 1994
Isomitraphylline (**65**)	*U. africana, U. bernaysii, U. callophylla, U. elliptica, U. guianensis, U. hirsuta, U. homomalla, U. laevigata, U. lancifolia, U. lanosa, U. longiflora* var. *longiflora, U. orientalis, U. perrottetii, U. scandens, U. sessilifructus, U. sterrophylla, U. tomentosa, U. veluntina*	Phillipson *et al.*, 1978; Tantivatana *et al.*, 1979; Wagner *et al.*, 1985; Diyabalanage *et al.*, 1997a,b
Isomitraphylline *N*-oxide	*U. attenuata, U. guianensis, U. hirsuta, U. homomalla, U. laevigata, U. lanosa, U. longiflora* var. *longiflora, U. orientalis, U. perrottetii, U. scandens, U. sessilifructus*	Phillipson *et al.*, 1978
Isopteropodic acid (**73**)	*U. sinensis*	Liu and Feng, 1993
Isopteropodine (Uncarine E) (**68**)	*U. bernaysii, U. donisii, U. guianensis, U. homomalla, U. laevigata, U. lanosa, U. longiflora* var. *longiflora, U. longiflora* var. *pteropoda, U. orientalis, U. roxburghiana, U. scandens, U. sinensis, U. sterrophylla, U. tomentosa, U. veluntina*	Phillipson *et al.*, 1978; Tantivatana *et al.*, 1979; Wagner *et al.*, 1985; Kam *et al.*, 1991; Arbain *et al.*, 1993; Tanahashi *et al.*, 1997; Aquino *et al.*, 1997; Lee *et al.*, 1999a, b
Isopteropodine *N*-oxide	*U. bernaysii, U. donisii, U. homomalla, U. lanosa, U. longiflora* var. *longiflora, U. orientalis, U. roxburghiana, U. scandens, U. sinensis, U. veluntina*	Phillipson *et al.*, 1978
3-Isorauniticine pseudoindoxyl (**71**)	*U. elliptica*	Phillipson and Supavita, 1983
Mitraphyllic acid (**75**)	*U. sinensis*	Liu and Feng, 1993
Mitraphylline (**61**)	*U. africana., U. attenuata, U. bernaysii, U. callophylla, U. elliptica, U. gambir, U. guianensis, U. hirsuta, U. homomalla, U. laevigata, U. lancifolia, U. lanosa, U. longiflora* var. *longiflora, U. orientalis, U. perrottetii, U. scandens, U. sessilifructus, U. sterrophylla, U. tomentosa, U. veluntina,*	Phillipson *et al.*, 1978; Herath *et al.*, 1978; Tantivatana *et al.*, 1979, 1980; Wagner *et al.*, 1985

Alkaloids	Species from which alkaloids isolated	References
Mitraphylline *N*-oxide	*U. africana, U. attenuata, U. guianensis, U. hirsuta, U. homomalla, U. laevigata, U. lancifolia, U. lanosa, U. longiflora* var. *longiflora, U. orientalis, U. perrottetii, U. scandens, U. sessilifructus*	Phillipson *et al.*, 1978
Pteropodine (Uncarine C) (**63**)	*U. bernaysii, U. donisii, U. guianensis, U. homomalla, U. lanosa, U. longiflora* var. *longiflora, U. longiflora var. pteropoda, U. orientalis, U. perrottetii, U. roxburghiana, U. scandens, U. sinensis, U. sterrophylla, U. tomentosa, U. veluntina*	Phillipson *et al.*, 1978; Tantivatana *et al.*, 1979; Wagner *et al.*, 1985; Kam *et al.*, 1991; Arbain *et al.*, 1993; Tanahashi *et al.*, 1997; Aquino *et al.*, 1997; Lee *et al.*, 1999a, b;
Pteropodic acid (**74**)	*U. sinensis*	Liu and Feng, 1993
Pteropodine *N*-oxide	*U. bernaysii, U. donisii, U. homomalla, U. lanosa, U. longiflora* var. *longiflora, U. orientalis, U. roxburghiana, U. scandens, U. sinensis, U. veluntina*	Phillipson *et al.*, 1978
Speciophylline (Uncarine D) (**67**)	*U. attenuata, U. bernaysii, U. donisii, U. guianensis, U. homomalla, U. laevigata, U. lanosa, U. longiflora* var. *longiflora, U. orientalis, U. perottetii, U. roxburghiana, U. scandens, U. sinensis, U. sterrophylla, U. tomentosa, U. veluntina*	Phillipson *et al.*, 1978; Arbain *et al.*, 1993; Tanahashi *et al.*, 1997
Speciophylline *N*-oxide	*U. bernaysii, U. donisii, U. homomalla, U. lanosa, U. longiflora* var. *longiflora, U. orientalis, U. roxburghiana, U. scandens, U. sinensis, U. sterrophylla, U. veluntina*	Phillipson *et al.*, 1978
Uncarine F (**64**)	*U. bernaysii, U. donisii, U. homomalla, U. lanosa, U. longiflora* var. *longiflora, U. orientalis, U. perrottetii, U. roxburghiana, U. scandens, U. sessilifructus, U. sinensis, U. sterrophylla, U. veluntina*	Phillipson *et al.*, 1978; Tanahashi *et al.*, 1997
Uncarine F *N*-oxides	*U. bernaysii, U. donisii, U. homomalla, U. lanosa, U. longiflora var. longiflora, U. orientalis, U. roxburghiana, U. sinensis, U. tomentosa, U. veluntina*	Phillipson *et al.*, 1978; Tanahashi *et al.*, 1997
Rauniticine oxindole A (**66**)	*U. elliptica*	Phillipson and Supavita, 1983
Rauniticine pseudoindoxyl (**70**)	*U. elliptica*	Phillipson and Supavita, 1983

(Extracted from Heitzman *et al.*, 2005 and Laus, 2004)

iii. Other Oxindole Alkaloids

Two new types of oxindole alkaloids deduced as 3-oxo-7-hydroxy-3,7-secorhynchophylline (**76**) and salacin (**77**) were isolated from a methanolic extract of stem bark and hook of the Thai medicinal plant *U. salaccenis* (synonym *U. attenuata*) by Ponglux and co-workers in 1990. The former was reported as a diastereomeric mixture. Seven years later, two novel D-seco oxindole alkaloids named Us-7 (**78**) and Us-8 (**79**) were isolated from the same *Uncaria* species by the same research group (Aimi *et al.*, 1997).



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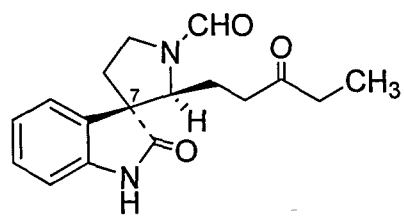

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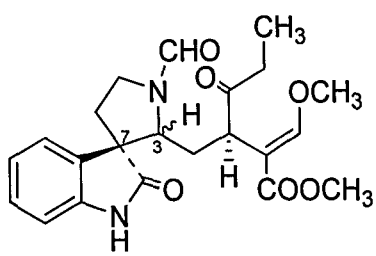
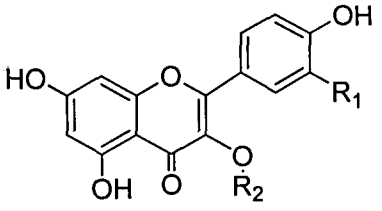


78 3β-H
79 3α-H

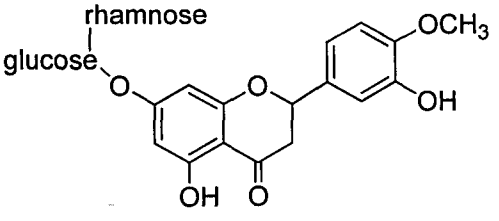
2.4.2 Flavonoids

To date, compared to other classes of natural products mentioned above, flavonoids have not been widely reported from the *Uncaria* genus. The most common representative is *epi*-catechin (**134**) which has been identified from four species, including *U. macrophylla*, *U. elliptica*, *U. rhynchophylla* and *U. tomentosa* (Zhu *et al.*, 1997; Wirth and Wagner, 1997; Yang *et al.*, 2000). Catechin (**133**), a stereoisomer of *epi*-catechin was isolated from four kinds of *Uncaria gambir* extract from West Sumatra, Indonesia by Anggraini and co-workers (2011). Other flavonoids including kaempferol, quercetin (**127**), quercitrin (**129**), and the glycosides of flavonol including afzelin (**130**), hyperoside, isoquercitrin, rutin (**128**), manghaslin and neohesperidin (**135**) have been isolated from *U. hirsuta* (Wu and Chan, 1994). Rutin was also isolated from *U. elliptica* (Law and Das, 1987), while the flavonol glucoside hyperin (**131**) and trifolin (**132**) have been reported from the leaves (Aimi *et al.*, 1982) and branches (Han *et al.*, 2000), as well as the stems (Jeoung *et al.*, 2002) of *U. rhynchophylla.* Wirth and Wagner (1997) reported the isolation of cinchonain Ia (**136**) and cinchonain Ib (**137**) from the bark of *U. tomentosa* collected in Peru.

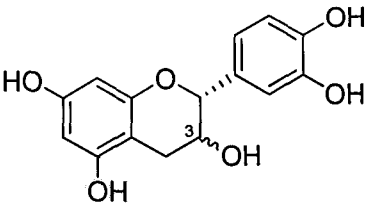
Several dimeric flavonoids have also been isolated from *Uncaria* species. A study on *U. gambir* by Nonaka and Nishioka (1980) has resulted in the isolation of novel chalcan-flavan dimmers, namely, gambiriin A1 (**138**), A2 (**139**), A3 (unknown stereochemistry), B1 (**140**), B2 (**141**), and B3 (suggested structure **142**), along with a proanthocyanidin dimer, gambiriin C (**143**).



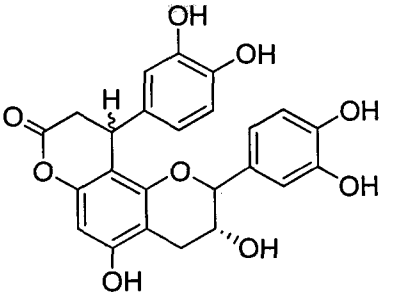
127 R_1=OH, R_2=H
128 R_1=OH, R_2=rutinose
129 R_1=OH, R_2=rhamnose
130 R_1=H, R_2=rhamnose
131 R_1=OH, R_2=galactose
132 R_1=H, R_2=galactose



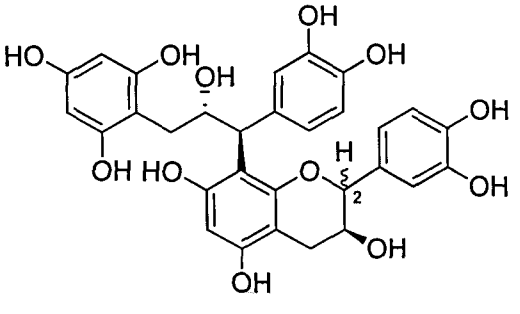
135



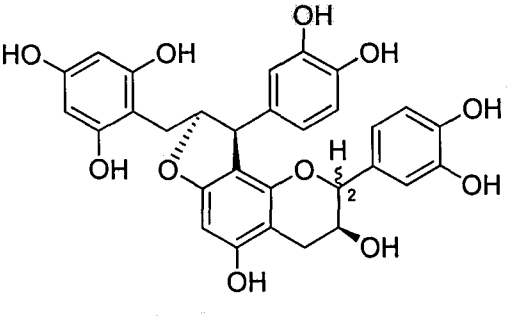
133 3β-OH
134 3α-OH



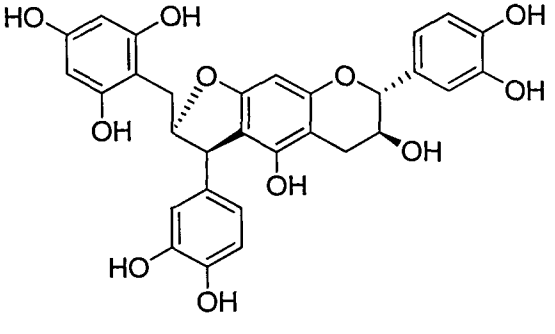
136 α-H
137 β-H



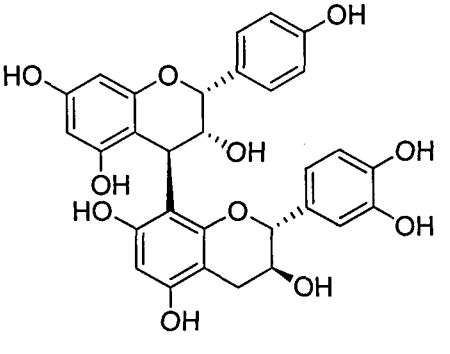
138 2β-H
139 2α-H



140 2β-H
141 2α-H



142

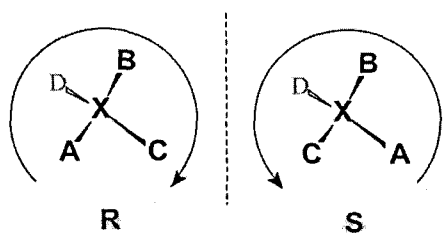


143

2.5 Determination of Configuration of Stereochemicals in Natural Products

The term "stereochemical" is derived from the Greek word *stereos* meaning solid, referring to a chemical in three dimensions. All of the macromolecules, and indeed most of the small ones, present in living organisms are chiral. Chirality, as an inherent property of organic compounds which are products of biological processes, was first described by Louis Pasteur, as stated by Lamzin *et al.* (1995). Hence, essentially all of their interactions show stereospecificity. Enantiomeric pairs that show absolutely equivalent physicochemical properties in a symmetric environment can behave remarkably differently in the biological world, depending on their handedness. Since many compounds isolated from *Uncaria* are chiral molecules, especially the oxindole alkaloids, it is essential to briefly discuss the techniques applied for the determination of the relative and absolute configuration of these molecules.

A widely used nomenclature called the *R/S* system defines a chiral carbon atom as being *R* (derived from the Latin *rectus* for right) or *S* (*sinister* for left). The carbon is viewed from the direction opposite to the substituent atom of lowest atomic mass (i.e. D; lowest priority). *R/S* enantiomers are defined on the basis of the chiral center. If the atomic mass of the remaining three substituents decreases in a clockwise direction, the configuration of the chiral carbon is *R*, otherwise it is *S* as shown below (Lamzin *et al.*, 1995).



The determination of the architecture of any molecule is not complete until its configuration is known, since configuration (relative or absolute) is an integral part of a structure. Among the techniques that are applied for the determination of configuration of this chiral/ stereochemical molecules include X-ray crystallography and spectroscopic methods including Nuclear Magnetic Resonance (NMR), Electronic Circular Dichroism (ECD), Raman Optical Activity (ROA) and Vibrational Circular Dichroism (VCD), as well as chemical correlation method.

NMR characterization is useful for a molecule which features more than one chiral centre to obtain the relative stereochemistry of the molecule. Two approaches are applied; by observing chemical shifts and by determining coupling constants of the protons and carbons. The chemical shifts would differ as the geometry may place certain protons or carbons in shielding/ deshielding portions of functional groups. The coupling constant is necessary in order to determine the relationships between the neighboring protons or carbons, as well as their proximity. These approaches, in combination with the 2D NMR correlation experiments especially NOESY and ROESY, are usually favorable in natural product research due to the challenges in skill, techniques and knowledge applied. However, knowledge of the 3D structure of the molecule *via* a construction of a molecular model is often helpful in obtaining a visual image of the molecule.

NMR spectroscopy can also be used in the determination of absolute configuration of chiral molecules. The methods involved are the Mosher's method and chiral liquid crystal NMR (Kusumi *et al.*, 1991). Both of these methods basically depend on converting the enantiomers into diastereomers, either by covalent chemical bonding or by complexing in some fashion, with another, usually enantiomerically pure chiral auxiliary. Once the enantiomers have been converted into diastereomers,

their ratio can be determined by NMR or chromatographic methods. This may include NMR in a chiral solvent or with a chiral complexing agent, or by chromatography of unmodified enantiomers on a chiral stationary phase. However, all of these methods are less favorable in natural products chemistry since the Mosher's method requires derivatization or intermolecular interactions, whilst the chiral liquid crystal NMR method generally requires a larger sample size (40 to 50 mg) and complex experimental procedures (Flack and Bernardinelli, 2008).

X-Ray crystallography is the most widely method used for revealing the complete, three-dimensional (3D) structure of the atoms in a molecule. It is still considered to be the most reliable technique for the determination of absolute configuration of a chiral molecule, since it can provide information on the optical enantiomer(s) present in the crystal. However, a central problem in this technique is the lack of a lens system for focusing X-rays, which is also known as the phase problem (Lamzin *et al.*, 1995). Moreover, this technique requires a single crystal of suitable size adaptable for X-ray diffraction. This is not always convenient since it is not easy to grow a single crystal of a pure chiral compound, especially if it not very stable (Lamzin *et al.*, 1995).

Another chiroptical technique in identifying the configuration of a molecule in biologically more realistic conditions (liquid, oil or solution form) is by using electronic circular dichroism (ECD) spectroscopy. ECD is a technique on the characterization of chiral molecule by circular dichroism (CD) spectroscopy in the UV/Vis region (from 163 nm to 1100 nm) of left and right circularly polarized light by conventional detection in transmission, or more sensitively in emission. ECD is a very sensitive diagnostic tool for determining the absolute configuration and conformation, and also for monitoring intermolecular interactions where chiral

systems are involved (Eliel *et al.*, 1994; Berova *et al.*, 2000). The strength and advantages of ECD over other chiroptical methods include its high sensitivity ($\Delta A/A \sim 10^2$, while for vibrational circular dichroism $\Delta A/A \sim 10^4$ to 10^6), and the need for much smaller sample amounts (less than 1 mg, or even a few micrograms). Nevertheless, ECD has some inherent weaknesses and limitations stemming from the low signal resolution and the diffulties in assigning the signals to specific chromophoric sites without resorting to excited-state quantum-mechanical calculations (Berova *et al.*, 2007). However, ECD undoubtedly remains as one of the most broadly used chiroptical techniques for solving various stereochemical and analytical problems since the spectra of enantiomers can be simulated using *ab initio* calculations. Among such quantum computations of ECD spectra are those based on density functional theory (DFT) and gauge-invariant atomic orbitals (GIAO). These techniques have been used to uncover errors in the assignment of absolute configuration by other methods including X-ray crystallography (Berova *et al.*, 2007).

Apart from ECD, Raman optical activity (ROA) and vibrational circular dichroism (VCD) are two other chiroptical techniques which can be used in identifying the configuration of a molecule in a solution form. The basic principle of ROA is that there is interference between light waves scattered by the polarizability and optical activity tensors of a chiral molecule, which leads to a difference between the intensities of the right- and left-handed circularly polarized scattered beams. The spectrum of intensity differences recorded over a range of wavenumbers reveals information about chiral centers in the sample molecule. ROA can be observed in a number of forms, depending on the polarization of the incident and the scattered light. For instance, in the scattered circular polarization (SCP) experiment, the incident light is linearly polarized and differences in circular polarization of the scattered light are

measured. In the dual circular polarization (DCP) method, both the incident and the scattered lights are circularly polarized, either in phase (DCPI) or out of phase (DCPII). Due to its sensitivity to chirality, ROA is a useful probe of biomolecular structure and behavior in aqueous solution (He *et al.*, 2011). However, this technique is not widely recognized due to the limited availability of instrumentation and quantum chemistry software. It is also less effective than VCD due to its limitation in determining the absolute configuration in small chiral molecule. In addition, interpreting the spectra of ROA is very time consuming since the spectra is more extensive and complex compared to that of VCD. ROA, nevertheless, is complementary to VCD and it is especially useful in the 50–1600 cm^{-1} spectral region and considered to be the technique of choice for determining optical activity for proton energies less than 600 cm^{-1} (He *et al.*, 2011).

Apart of those methods, another classical method for the determination of the absolute configuration of a chiral compound relatively is by chemical correlation. This method applied comparison of optical rotation ($[\alpha]_D$), and/or CD spectrum of reference compound with that of known absolute configuration (Harada *et al.*, 1997). Although this method is frequently used, a careful selection of reference compound is necessary for a reliable analysis.

Based on the limitations of each of the method above, it is essential to use a combination of those stated methods in determining the absolute stereochemistry of a chiral molecule. This is especially important in natural product research when dealing with a molecule bearing several chiral centres on a fused ring system such as the pentacyclic oxindole alkaloids.

2.5.1 General Principles of Circular Dichroism Spectroscopy

Plane or linearly polarized light can be viewed as the vector sum of left and right circularly polarized light of equal amplitude and phase. When an optically active medium is traversed by plane polarized light in the wavelength range in which the chromophore of an optically active molecule absorbs, the plane of polarization is rotated at an angle, α, and the optically active matter absorbs the left and right hand circularly polarized light differently. The resulting light is therefore elliptically polarised and the medium exhibits circular dichroism (CD) (Velluz *et al.*, 1965). CD is measured as a quantity called mean residue ellipticity (curve in degrees-cm^2/dmol) in the function of wavelength (λ in nm). Optically active compounds possessing a chromophore will exhibit a maximum or a minimum, or both, in absorption band in the region being investigated. These phenomena are known as Cotton effects (CEs). The CEs, which provides much structural information, is characterized by its position, magnitude, sign (negative or positive) and shape of the curve. Figure 2.5 illustrates the physical principles of circular dichroism.

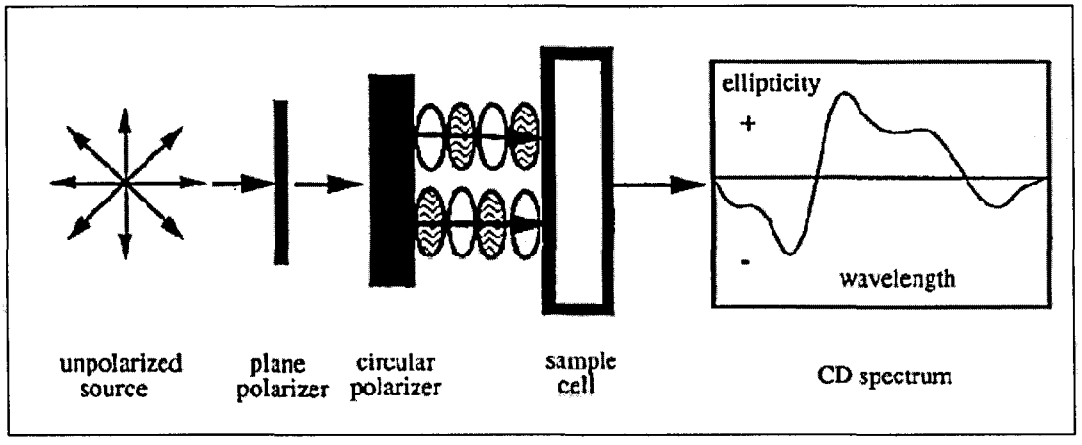


Figure 2.5 Physical Principles of Circular Dichroism

2.5.2 Absolute Configuration of Pentacyclic Oxindole Alkaloids

All of the alkaloids isolated from the *U. longiflora* var. *pteropoda* stems extract were identified as heteroyohimbine-type oxindole alkaloids, also called pentacyclic oxindole alkaloids (POAs). The general NMR features of this type of alkaloid can lead to the determination of relative configuration of stereoisomeric compounds which will help with the establishment of the structure and stereochemistry at the chiral centres.

The general structure and atom numbering of heteroyohimbine-type oxindole alkaloids or POAs is represented by Figure 2.6 (Shamma *et al.*, 1967; Seki *et al.*, 1993). Theoretically, this type of alkaloid can exist in the form of thirty-two stereoisomers due to the presence of five asymmetric centres (C-3, C-7, C-15, C-19 and C-20). However, biosynthetically, the configuration of the asymmetric centre at the C-15 position is fixed in *S* form restricting the number of isomers to be sixteen (Cordell, 1974; Stöckigt, 1980; Beke *et al.*, 2001). These isomers were classified by Shamma and colleagues in 1967 into four groups, namely, *normal-*, *pseudo-*, *allo-*, and *epiallo*-types, then tabulated by Seki *et al.* in 1993 (Table 2.4) although the *pseudo*-type isomers have not been found in nature due to the serious steric hindrance between the oxindole moiety and the underside of ring D. Therefore, the number of these isomers that need to be considered in practice is limited to twelve.

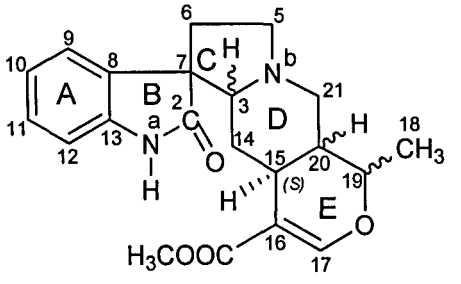


Figure 2.6 General Structure of the Heteroyohimbine-Type Oxindole Alkaloids

TABLE 2.4
Stereoisomers of the Heteroyohimbine-Type Oxindole Alkaloids

Type	Configuration of C3-H	Configuration of C20-H	D/E ring relationship	Configuration of C19-H	Configuration of C7	Alkaloid
normal	*S (α)*	*R (β)*	*trans*	*S (β)*	*S*	Isomitraphylline
					R	Mitraphylline
				R (α)	*S*	Uncarine A (isoformosanine)
					R	Uncarine B (formosanine)
epiallo	*R (β)*	*S (α)*	*cis*	*S (β)*	*S*	Uncarine D (speciophylline)
					R	Uncarine F
				R (α)	*S*	Rauniticine-*epiallo*-oxindole A
					R	Rauniticine-*epiallo*-oxindole B
allo	*S (α)*	*S (α)*	*cis*	*S (β)*	*S*	Uncarine E (isopteropodine)
					R	Uncarine C (pteropodine)
				R (α)	*S*	Rauniticine-*allo*-oxindole A
					R	Rauniticine-*allo*-oxindole B
pseudo	*R (β)*	*R (β)*	*trans*	*S (β)*	*S*	-
					R	-
				R (α)	*S*	-
					R	-

(Seki *et al.*, 1993)

The assignment of configuration and group to the POAs has been described by Shamma *et al.* (1966). However, in *S* biosynthetically, the configuration of the stereochemistry at C-15 position of the structure is fixed form (Cordell, 1974; Stöckigt, 1980). A year later, Beecham and co-workers (1968) studied the stereochemistry of ring D and E of the POAs formosanine, isoformosanine, pteropodine, isopteropodine, speciophylline and uncarine F by means of NMR spectra, experimental ECD and equilibrium reactions. They also suggested that the experimental ECD spectra can be used to confirm the assignment of the stereochemistry at C-7 of the structure. In 1993, eight natural POAs, isolated from *Uncaria* species, were analyzed by means of ^{1}H, ^{13}C NMR along with 2D NMR correlation experiments (Seki *et al.*, 1993). Eight years later, these POAs were studied by ^{15}N NMR spectroscopy (Muhammad *et al.*, 2001). More recently, the characterization of these POAs by solid-state NMR techniques using ^{13}C and ^{15}N CPMAS NMR has been reported by Paradowska and co-workers (2008). They also provide theoretical calculations of shielding constants using the density functional theory (DFT) and gauge-invariant atomic orbitals (GIAO) approach. However, to date, the only POAs whose absolute stereochemistry have been determined by single crystal X-ray crystallography were pteropodine and isopteropodine (Muhammad *et al.*, 2001), and mitraphylline and speciophylline (Laus and Wurst, 2003).

CHAPTER THREE

RESEARCH EXPERIMENTAL

3.1 General Instrumentation and Computational Method

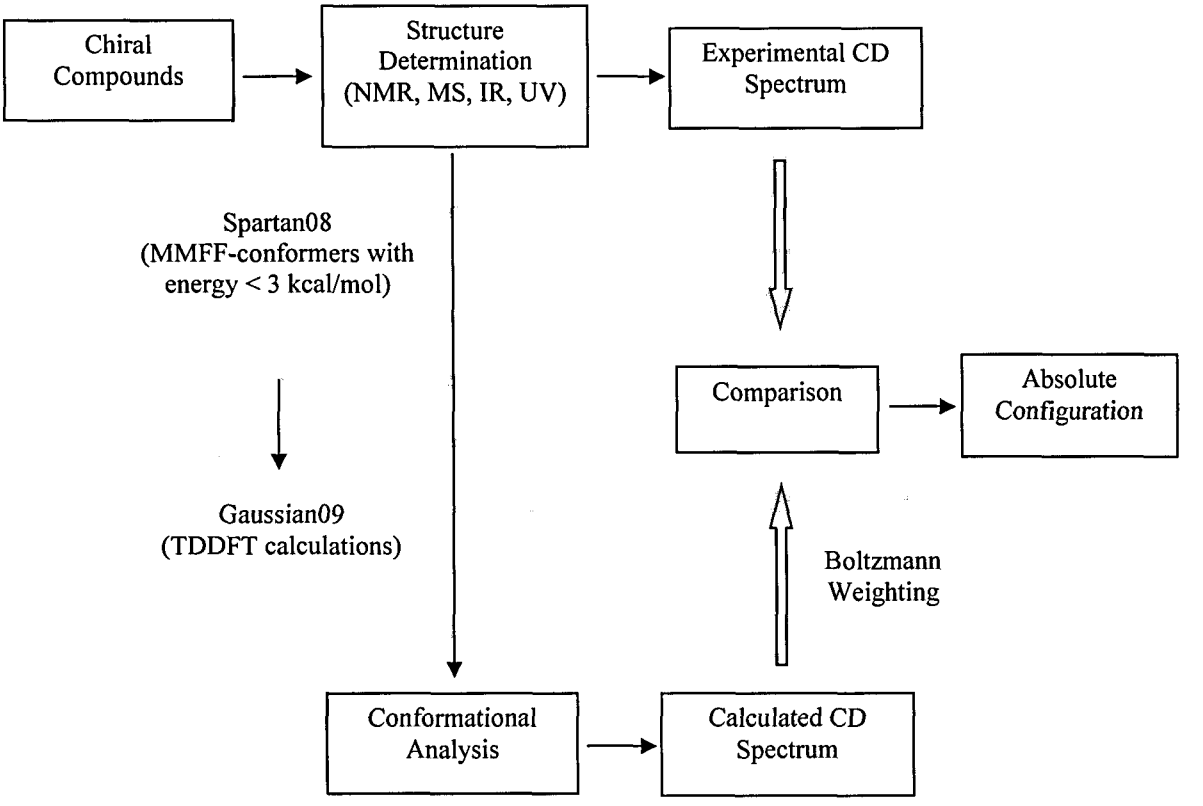
The ECD spectra were obtained on an Applied Photophysics Chirascan CD spectrometer using a 5 mm cell and acetonitrile was used as the solvent. For the conformational search, as well as the ECD, the absolute configurations of the new compounds were chosen. The conformational search and geometry optimization were carried out at the molecular mechanics level of theory employing MMFF force field incorporated in Spartan08 (Wavefunction, Irvine, CA) software package (Ding *et al.*, 2010). The selected conformers exhibiting a cutoff energy under 3 kcal/mol were further geometry optimized at the modest B3LYP/6-31G (d,f) level of theory and calculated using time dependent density functional theory (TDDFT) at 298K in the gas phase at the B3LYP/6-31G (d, f) level built to Gaussian09 software (Frisch *et al.*, 2010) to simulate the ECD spectra. The calculated ECD spectra were Boltzmann weighted and were shifted ~ -15 nm, while the UV wavelengths were scaled down by a factor of 2.5×10^{-5}.

CHAPTER FOUR

ELECTRONIC CIRCULAR DICHROISM (ECD) STUDIES

4.1 Determination of Absolute Configuration of Chiral Compounds

As described in Section 2.5, several techniques have been applied for the determination of configuration of chiral molecules. These include X-ray crystallography and spectroscopic methods such as Nuclear Magnetic Resonance (NMR), Electronic Circular Dichroism (ECD), Raman Optical Activity (ROA) and Vibrational Circular Dichroism (VCD), as well as chemical correlation method. Since all of the new compounds isolated from the plant are chiral compounds, it is of interest to confirm their absolute configurations adapting the ECD studies. The ECD technique was chosen due to its sensitive diagnostic tool for determining the absolute configuration and conformation, and for monitoring intermolecular interactions where chiral systems are involved (Eliel *et al.*, 1994; Berova *et al.*, 2000), as well as in consideration of limited quantities of the new compounds in hand. The scheme for determining the absolute configuration of the compounds by theoretical calculation of CD spectrum and comparison with the experimental CD spectrum is shown in Scheme 4.1. Systematic conformational search was carried out using molecular mechanics force field (MMFF) followed by theoretical calculation using time dependence density functional theory (TDDFT) of stable conformers. This computational method was chosen in the present study due to the applicability of its principles to any molecule, and its ability to produce high accuracy in ECD prediction (Berova *et al.*, 2007).



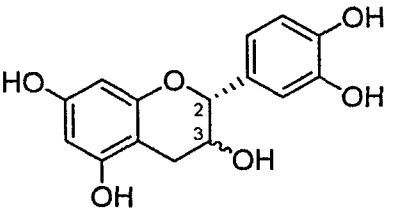
Scheme 4.1 The Scheme for Determining the Absolute Configuration of the Compounds by Theoretical Calculation of CD Spectrum and Comparison with the Experimental CD Spectrum.

In this study, the absolute configurations of a new flavanoid (a flavan-3-ol) (**ULL14**) and five new POAs (**ULS1**, **ULS2**, **ULL5**, **ULL6** and **ULL7**) were established mainly by comparison of their Cotton effects (CEs) observed in the experimental and simulated ECD spectra, as well as by comparison with the published data for the flavonoids (Friedrich and Galensa, 2002; Slade *et al.*, 2005) and for the alkaloids (Beecham *et al.*, 1968). ECD calculations have been increasingly used for the interpretation of CD spectra (Bringmann *et al.*, 2009). However, Zaugg *et al.* (2011) reported that the calculated and experimental spectra are comparable, except for a moderate wavelength shift due to minor difference between calculated and solution conformers of the flexible molecules. In addition, differences might arise

from either inaccurate excited-state calculations, or from incorrectly predicted geometries and relative energies of the various conformers obtained, possibly related to the use of the B3LYP function (Schwabe and Grimme, 2007).

4.2 ECD Spectra of Flavan-3-ols

According to Slade *et al.* (2005), flavan-3-ols are characterized by two phenyl chromophores whose UV absorption bands are at *ca.* 280 and 240 nm, giving fingerprint ECD Cotton effects at the respective wavelengths. The flavan-3-ols have two stereocenters, and therefore four possible diastereomers, namely (2*R*,3*S*)-*trans*, (2*S*,3*R*)-*trans*, (2*R*,3*R*)-*cis* and (2*S*,3*S*)-*cis* are possible. The structure of *epi*-catechin (3α-OH) and *epi*-afzalechin (3β-OH) which are common representatives of natural flavan-3-ols are shown below.



4.2.1 Experimental ECD spectra

The experimental UV and ECD spectra were first run for the two isolated known flavan-3-ols, (-)-*epi*-catechin and (-)-*epi*-afzalechin to observe the above effects, and were subsequently compared to the ECD spectrum of uncariechin (Figure 4.1 - 4.2). As shown in Figure 4.2, the experimental ECD spectra of (-)-*epi*-catechin and (-)-*epi*-afzalechin showed negative CEs at *ca.* 280 nm (-9.4 and -6.5, respectively) and *ca.* 240 nm (-1.9 and -2.0, respectively) consistent with a 2*R*,3*R*-*cis* configuration as reported by Slade *et al.* (2005). Thus, the absolute configuration of both of these known flavonoids were clearly established as (-)-2*R*,3*R*-*epi*-catechin and (-)-2*R*,3*R*-*epi*-afzalechin.

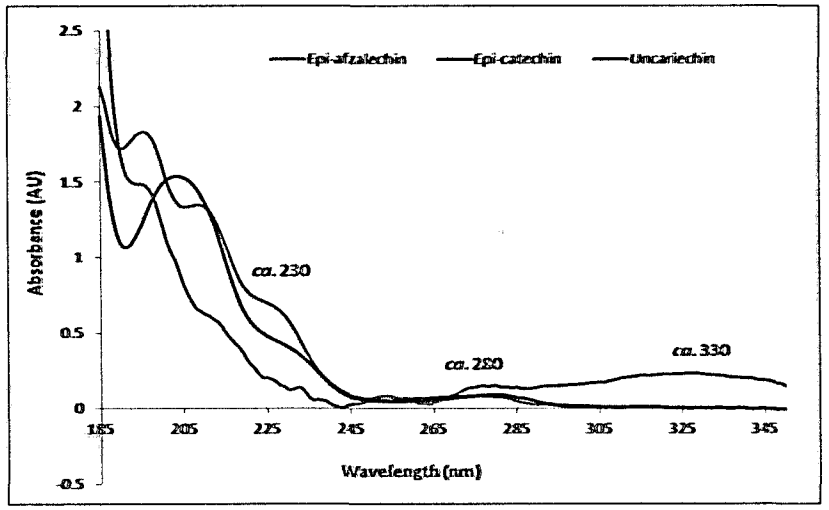


Figure 4.1 Overlaid Experimental UV Spectra of (-)-*Epi*-catechin, (-)-*Epi*-afzalechin and Uncariechin

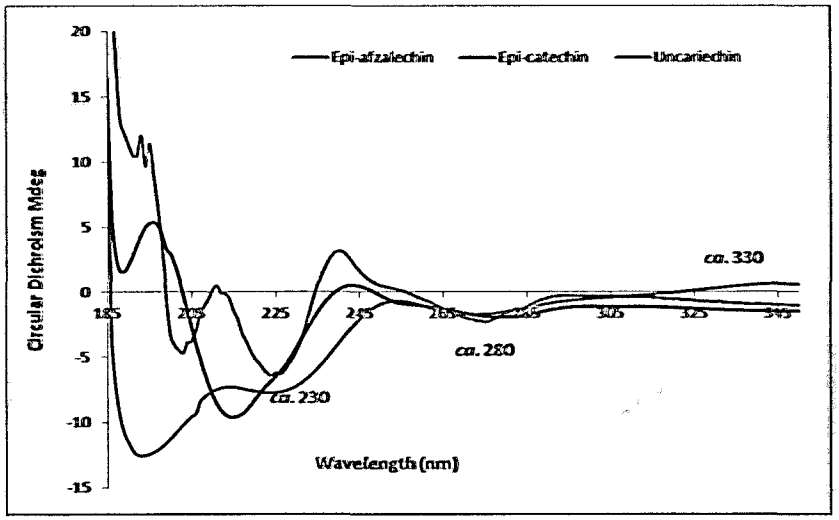
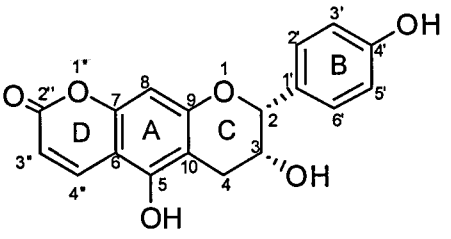


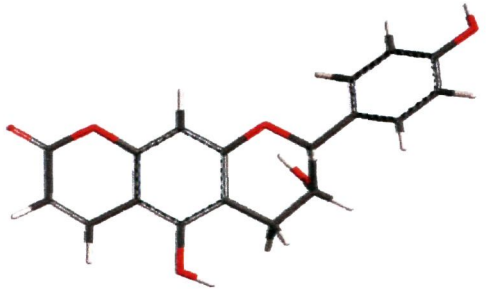
Figure 4.2 Overlaid Experimental ECD Spectra of (-)-*Epi*-catechin, (-)-*Epi*-afzalechin and Uncariechin

4.2.2 Simulated ECD data for Uncariechin

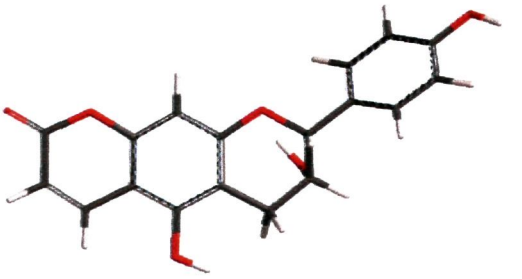
For uncariechin (structure shown below), the proton NMR experiments established its relative configuration as having a *cis* relationship between ring B and the 3-OH group which would support two possible diastereomers, a (2*R*,3*R*) or a (2*S*,3*S*) configurations. However Nanjo *et al.* (1996) reported a positive optical rotation for a (2*S*,3*S*) isomer, and a negative optical rotation value for a (2*R*,3*R*) isomer. Based on this, a configuration of (2*R*,3*R*) is suggested for uncariechin for which a negative optical rotation (-312.4) was recorded.



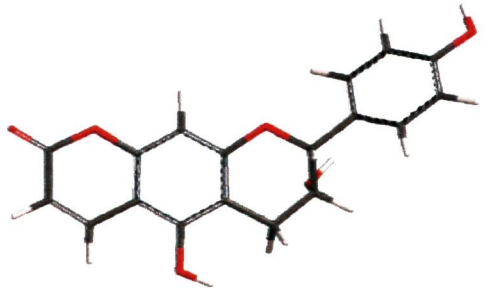
To confirm the absolute configuration at C-2 and C-3 for uncariechin as (2*R*,3*R*), a systematic conformational search of the (2*R*,3*R*) isomer was carried out on Spartan08 program (as described in Section 3.1). This generated 12 conformers from which 8 conformers, **ULL14a** (34.2%), **ULL14b** (15.2%), **ULL14c** (14.0%), **ULL14d** (13.4%), **ULL14e** (8.8%), **ULL14f** (6.3%), **ULL14g** (5.3%) and **ULL14h** (2.7%) were below an energy cutoff of 3 kcal/mol (Figure 4.3). The calculated ECD spectra for the selected (2*R*,3*R*) diastereoisomers were submitted to Gaussian for ab initio calculations. The UV (Figure 4.4) and ECD spectra (Figure 4.5) for all of the conformers were Boltzmann weighted (BW) and plotted against the experimental spectra. Figure 4.5 shows that the experimental ECD spectra was comparable with ECD calculated for its conformers which showed negative CEs at 280 nm (-7.1) and 240 nm (-3.5) consistent with a 2*R*,3*R*-*cis* configuration. Another positive CE at 330 nm (+1.1) in the experimental spectrum was likely due to a $\pi \rightarrow \pi^*$ transitions in the extended π-system of the α-pyranone moiety (Dastan *et al.*, 2012). Since uncariechin presumably originates from (-)-2*R*,3*R*-*epi*-afzalechin, a close match in their ECD spectra would be a better indicator of the similarity in conformation between the two molecules although the presence of any substituent on the benzene ring A may change the helicity of the molecule and may result in different CEs (Snatzke, 1965; Slade *et al.*, 2005). As shown earlier in Figure 4.2, the ECD spectral pattern of uncariechin matches that of (-)-*epi*-afzalechin well, hence confirming its configuration to be *cis*(2*R*,3*R*) leading to the establishment of the stereochemistry as (-)-2*R*,3*R* uncariechin.



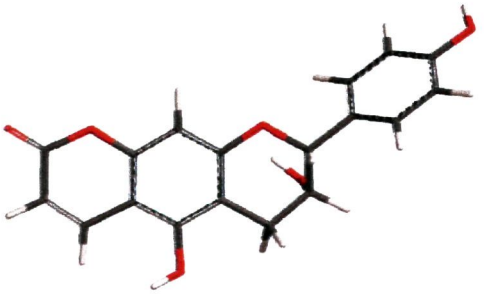
ULL14a (0.00 kcal/mol)



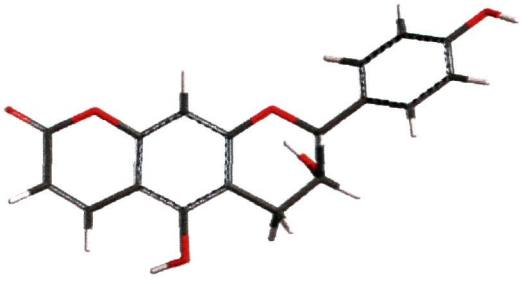
ULL14b (0.48 kcal/mol)



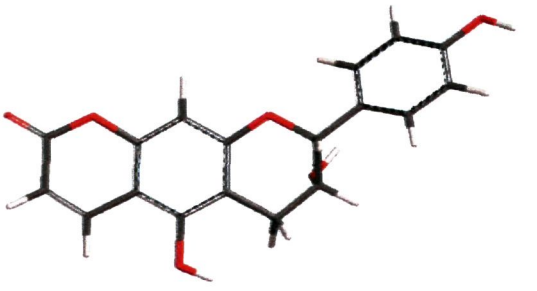
ULL14c (0.53 kcal/mol)



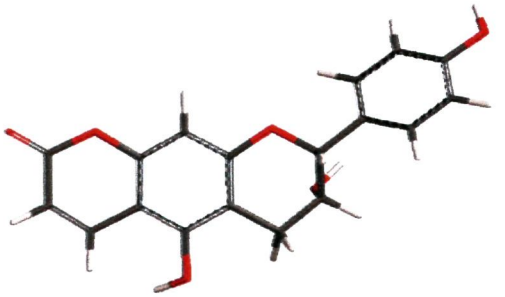
ULL14d (0.55 kcal/mol)



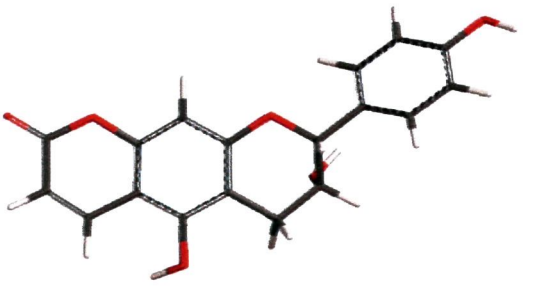
ULL14e (0.80 kcal/mol)



ULL14f (1.00 kcal/mol)



ULL14g (1.11 kcal/mol)



ULL14h (1.50 kcal/mol)

Figure 4.3 Optimized Structures and Relative Energies of ULL14 Conformers of 2*R*,3*R* Possessing Energy Cutoff of 3 kcal/mol Used for ECD Calculations

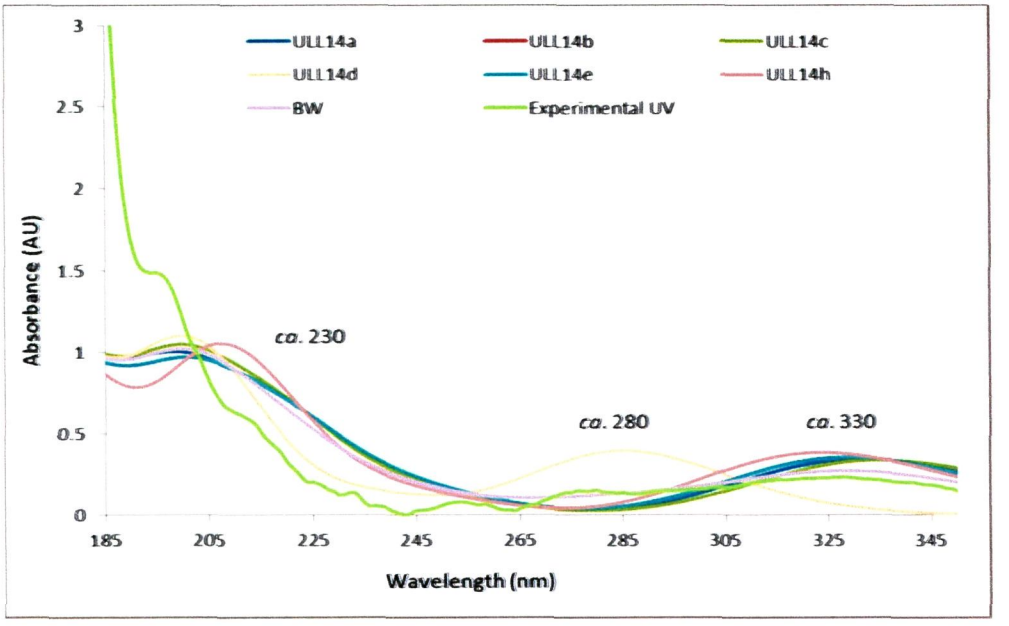


Figure 4.4 Overlaid Experimental and Calculated UV Spectra of ULL14 (Uncariechin)

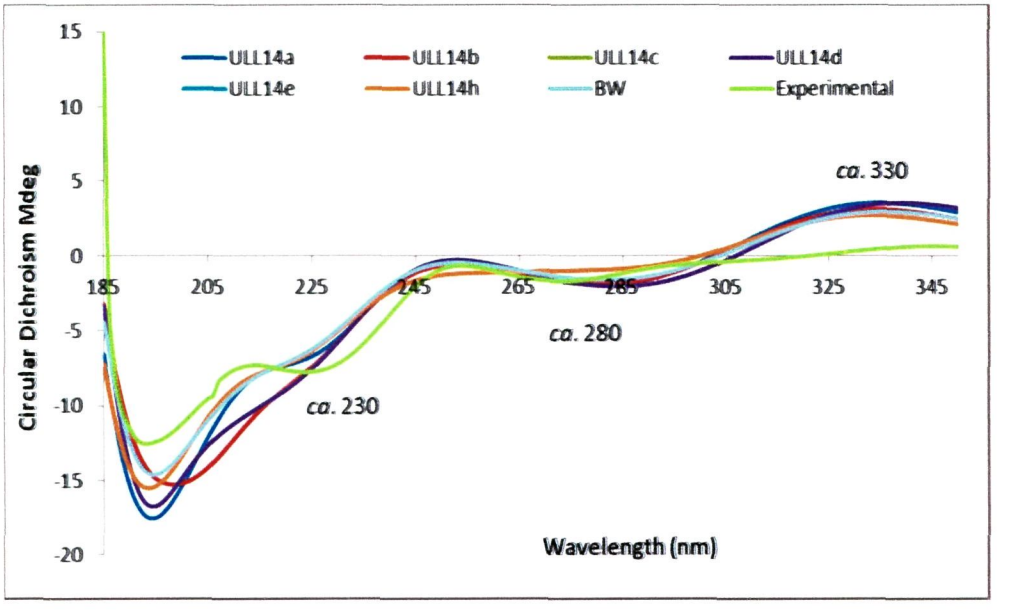


Figure 4.5 Overlaid Experimental and Calculated ECD Spectra of ULL14 (Uncariechin)

4.3 ECD Spectra of Alkaloids

Through intensive literature search, the only CD study found of the stereochemistry for the POAs was done by Beecham and co-workers in 1968 (Beecham *et al.*, 1968). However, they only studied the stereochemistry of ring D and E of the POAs, including, formosanine, isoformosanine, pteropodine, isopteropodine,

speciophylline and uncarine F by means of experimental ECD. They suggested that the experimental ECD spectra can be used to establish the assignment of the stereochemistry at C-3 and C-7 of the structure by observing the bands at 252 and 290 nm, respectively. The sign of the 290 nm band reflects the orientation of the oxindole carbonyl group above (positive sign) or below (negative sign) the plane. To confirm the proposed correlations, experimental ECD for the known POAs in hand were run and compared with their values.

In this study, the UV absorbance for all of the known POAs in hand (isopteropodine **ULS3/ULL1**, pteropodine **ULS4/ULL2**, uncarine F **ULS6/ULL3** and speciophylline **ULS7**) (Figure 4.6) were at *ca.* 205, 253 and 283 nm for a $\pi \rightarrow \pi^*$ and an $n \rightarrow \pi^*$ transitions of the oxindole chromophore and at *ca.* 220 and 238 nm for the transition of an α,β-unsaturated carboxylic acid or an α,β-unsaturated carboxylic acid methyl ester system (Figure 4.7) resulting in CEs at *ca.* 205, 220, 238, 253 and 283 nm (Figure 4.8).

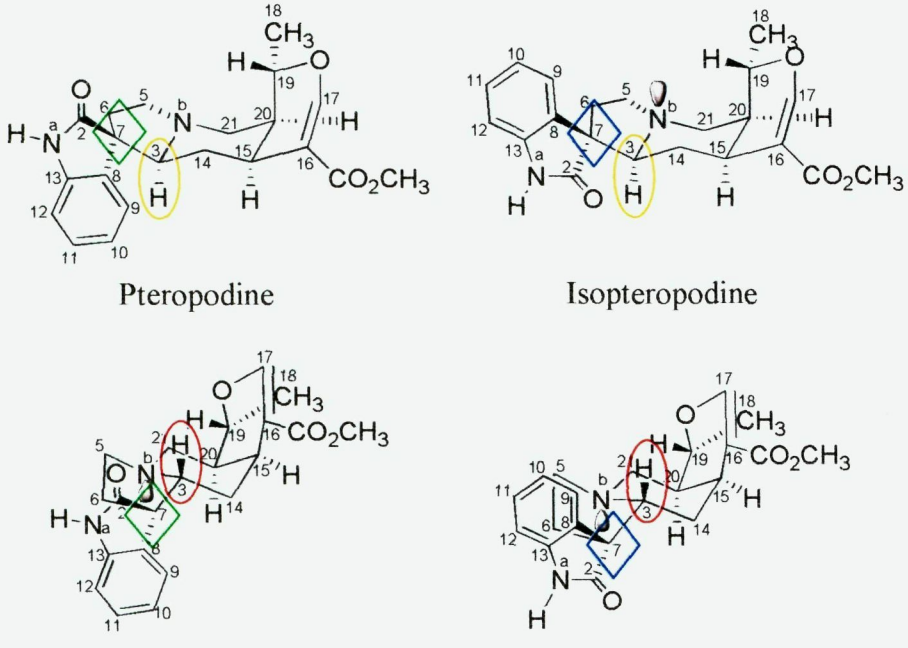


Figure 4.6 Structures of the Known POAs

4.3.1 Experimental ECD for Known POAs

Table 4.1 gives the CE values obtained from the experimental ECD of the known POAs below, along with the reported values by Beecham *et al.* (1968). As stated previously, the stereochemistry of the C-15 of the POAs is biosynthetically fixed as 15*S* (Cordell, 1974). However, the correlation of CEs with stereochemistry at other centres may be dependent on their configuration at C-7 and C-3. Inspection on the ECD spectra of pteropodine and isopteropodine (with 3*R* configuration), and uncarine F and speciophylline (with 3*S* configuration) as shown in Table 4.1 revealed that the change at the orientation at the *spiro* centre C-7 causes an inversion of CE at 283 and 205 nm in both series of isomers. The change in orientation at C-3 between pteropodine/isopteropodine and uncarine F/speciophylline causes an inversion of CE at 253 nm from negative to positive, respectively. This is true as these chromophores are due to the transitions of a $\pi \rightarrow \pi^*$ and an $n \rightarrow \pi^*$ of the oxindole chromophore, which are located near the stereogenic centres C-7 and C-3 (see Figure 4.6). This observation, thus agreed with the study done by Beecham and co-workers (1968), since the ECD spectra patterns were found to be comparable with their work and enable the distinction between the 3*S*, 3*R* and 7*R*, 7*S* isomers.

TABLE 4.1 Observed Cotton Effects of Known POAs (in 0.05 M in ACN)

POAs/ λ_{max}	205	220	218[a]	238	240[a]	253	252[a]	283	290[a]
Isopteropodine **ULS3/ULL1** (3*S*, 7*S*,19*S*, 20*S*)	+4.5	+0.5	+2	-0.4	-1.5	-2.8	-21	-0.4	-3
Pteropodine **ULS4/ULL2** (3*S*, 7*R*,19*S*, 20*S*)	-3.1	-1.5	-11	+0.8	+5	-2.7	-15	+0.6	+3
Uncarine F **ULS6/ULL3** (3*R*, 7*R*,19*S*, 20*S*)	+0.6	+7.5	-5 (↑)	-1.2	-8	+4.0	+15	+1.1	+4
Speciophylline **ULS7** (3*R*, 7*S*,19*S*, 20*S*)	+3.4	+0.5	+11	-0.6	-5	+2.2	+6	-0.1	-2

[a]Beecham *et al.* (1968) (0.001 M in MeOH, spectra λ_{max} (nm) scale=210-310)

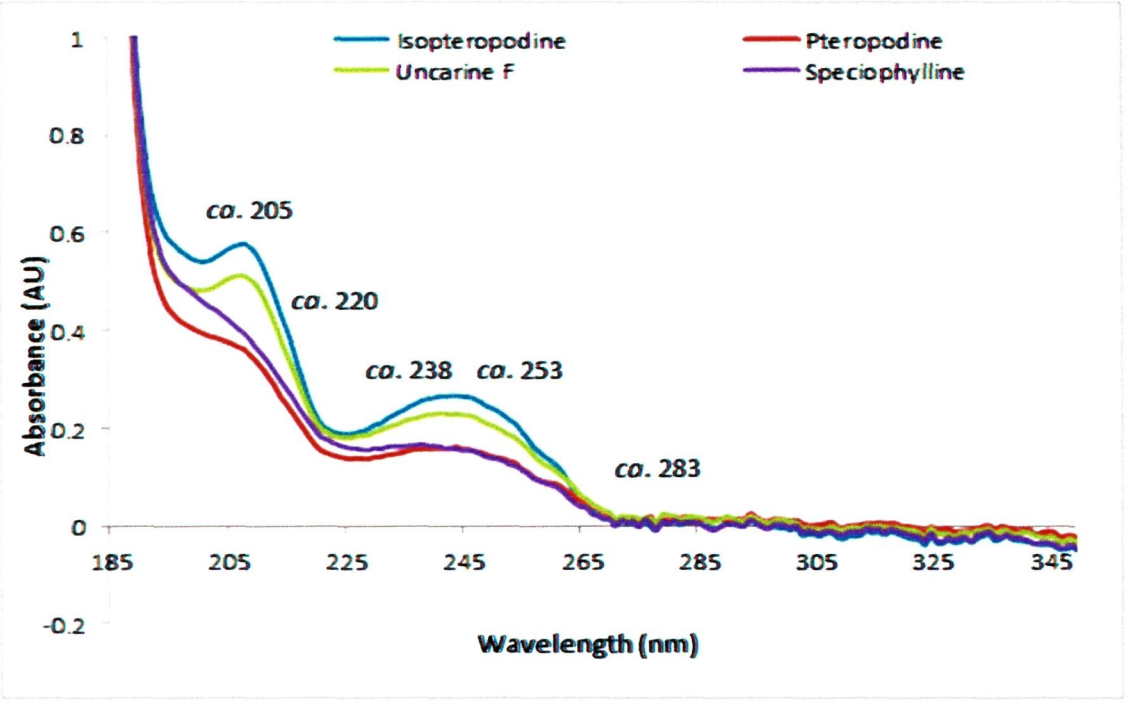


Figure 4.7 Overlaid Experimental UV Spectra of Known POAs

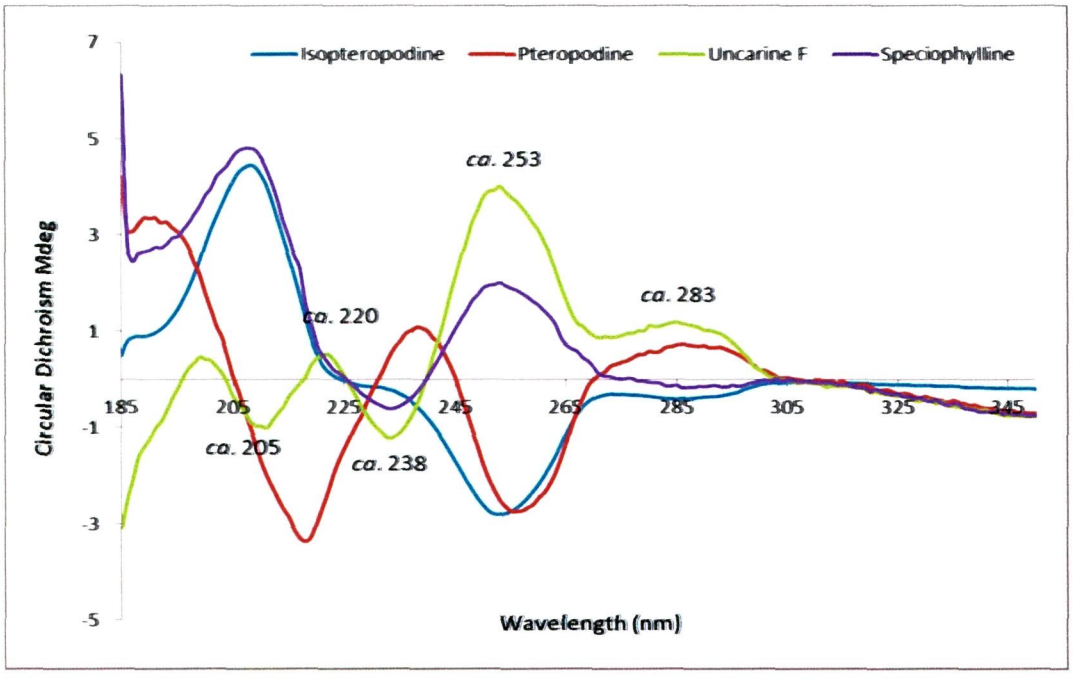
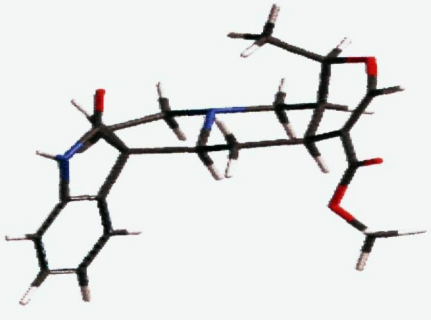


Figure 4.8 Overlaid Experimental ECD Spectra of Known POAs

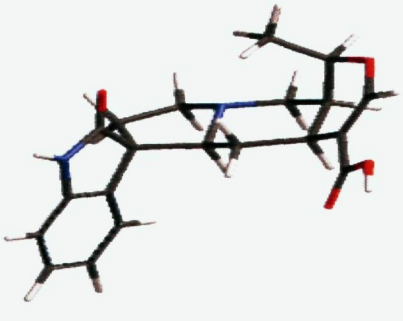
4.3.2. Simulated ECD for Known POAs

4.3.2.1 Rauniticine-allo-oxindole B ULS1 and Rauniticinic-allo acid B ULS2

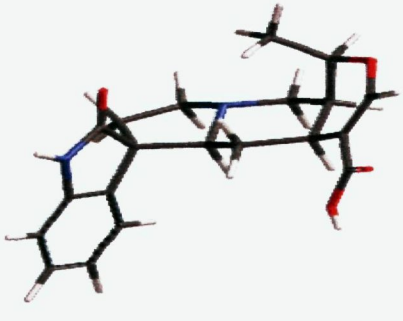
The structure elucidation of the new oxindole alkaloids **ULS1** and **ULS2** using NMR techniques led to the determination of their stereochemistry as 3*S*, 7*R*, 19*R*, 20*S*. For the prediction of absolute configuration by simulated method, a systematic conformational search of the isomers carried out on Spartan08 program generated a single conformer for **ULS1**, **ULS1a** (100%) and 3 conformers for **ULS2** out of which 2 conformers, **ULS2a** (80.8%) and **ULS2b** (19.2%) were under an energy cutoff of 3 kcal/mol (Figure 4.9). However, the ECD calculation for **ULS1** conformer did not generate any data. Thus, an approximation of its calculated UV and ECD was made with **ULS2** since the two alkaloids only differ at the 16-carboxy moiety, where the former possesses an ester, while the latter possesses an acid moiety.



ULS1a (0.00 kcal/mol)



ULS2a (0.00 kcal/mol)



ULS2b (0.85 kcal/mol)

Figure 4.9 Optimized Structures and Relative Energies of ULS1 and ULS2 Conformers of 3*S*, 7*R*, 15*S*, 19*R*, 20*S* Possessing Energy Cutoff of 3 kcal/mol Used for ECD Calculations

The calculated UV and ECD spectra for the selected diastereoisomers of **ULS2** were Boltzmann weighted (BW) and compared to its experimental ECD spectrum (Figure 4.10 and Figure 4.11). The CEs which represent the *spiro* centre C-7 and C-3 at *ca.* 253 and 205 nm comparable with the experimental ECD except for the CE at 283 nm which should be giving positive sign gave a negative sign in the calculated. However, significant differences between the calculated and experimental spectra of **ULS2** was observed might be likely resulted from an overestimation of the UV absorbance in the calculations, or from minor conformational differences in solution and gas (Dastan *et al.*, 2012).

The experimental ECD spectral data pattern for the new POAs (**ULS1** and **ULS2**) were compared to those of the known POAs, all showing negative/positive CEs at the respective wavelengths (Table 4.1, Figure 4.12). The absolute configuration of 7*R* for the *spiro* C-7 was confirmed by the positive CE at *ca.* 285 (+0.1) for **ULS1** similar to those of pteropodine (+0.7) which also possesses 7*R* configuration (Figure 4.13, Figure 4.14). Although the sign of the CE of **ULS2** at that wavelength was -0.5, the curve was approaching a positive value. These two alkaloids are the epimers of pteropodine at the C-19 position, where both **ULS1** and **ULS2** possess the 19*R* configuration, while pteropodine possesses the 19*S* configuration thus establishing the absolute stereochemistry of these two compounds to have the 3*S*, 7*R*, 19*R*, 20*S* configurations. These findings allow the placement of **ULS1** and **ULS2** into the same group of alkaloids as pteropodine and isopteropodine, namely the *allo*-type isomers.

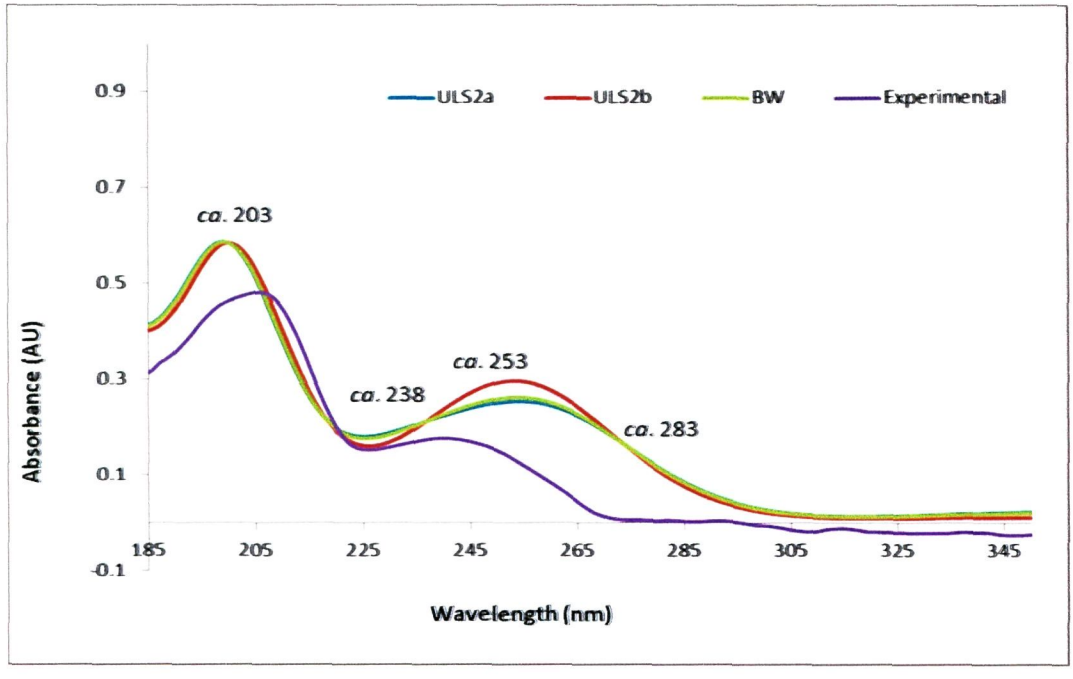


Figure 4.10 Overlaid Experimental and Calculated UV Spectra of ULS2

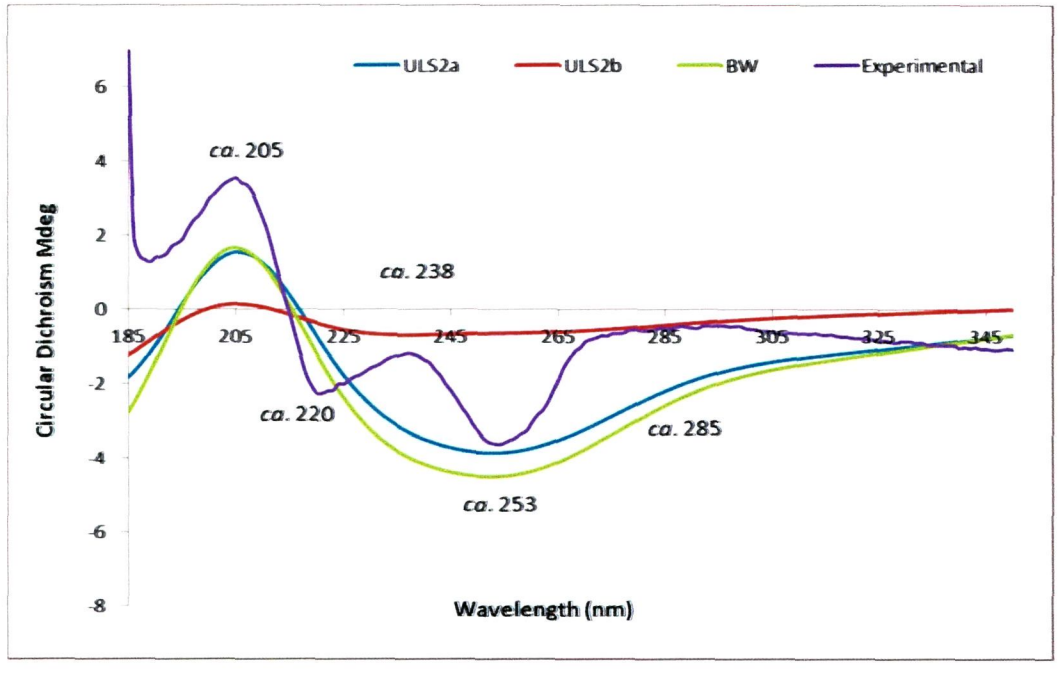


Figure 4.11 Overlaid Experimental and Calculated ECD Spectra of ULS2

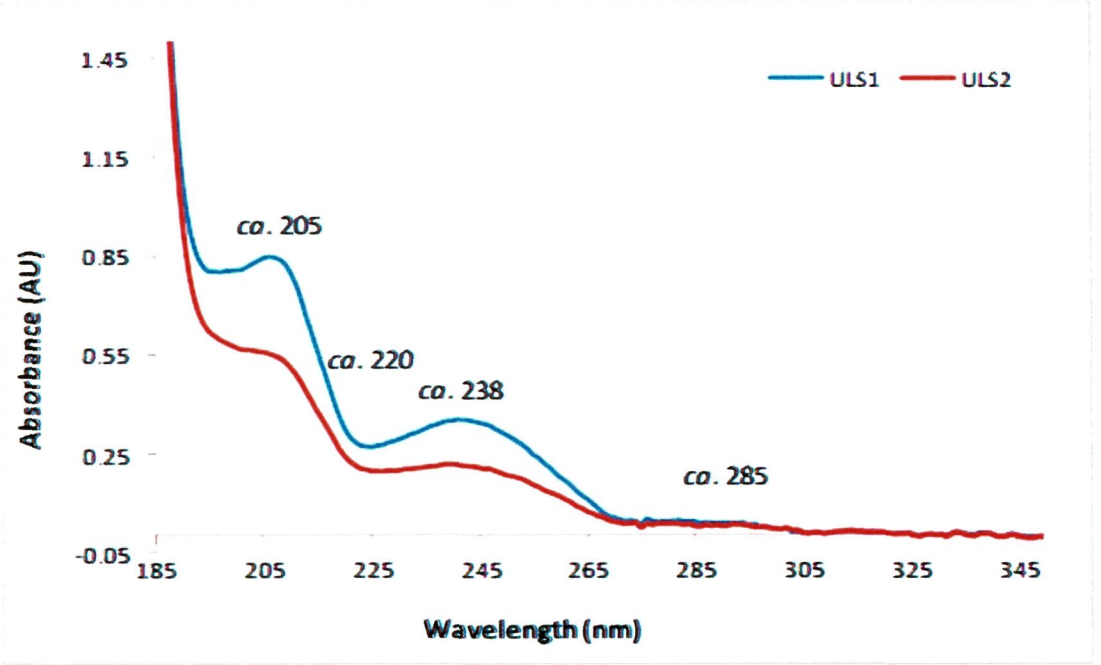


Figure 4.12 Overlaid Experimental UV Spectra of ULS2 and ULS1

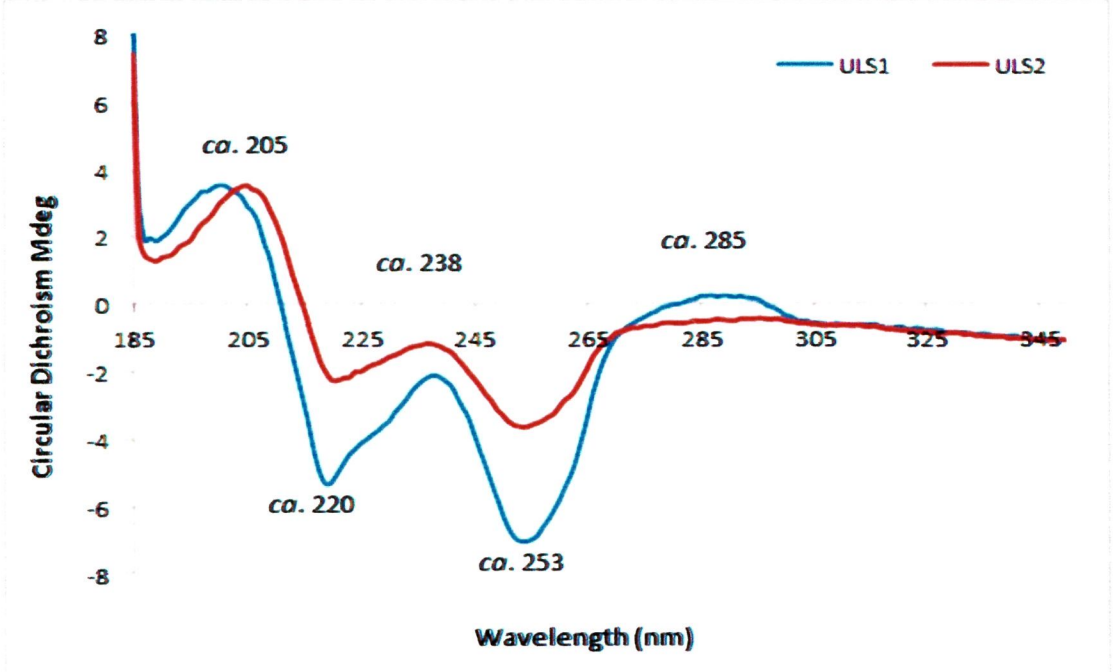


Figure 4.13 Overlaid Experimental ECD Spectra of ULS2 and ULS1

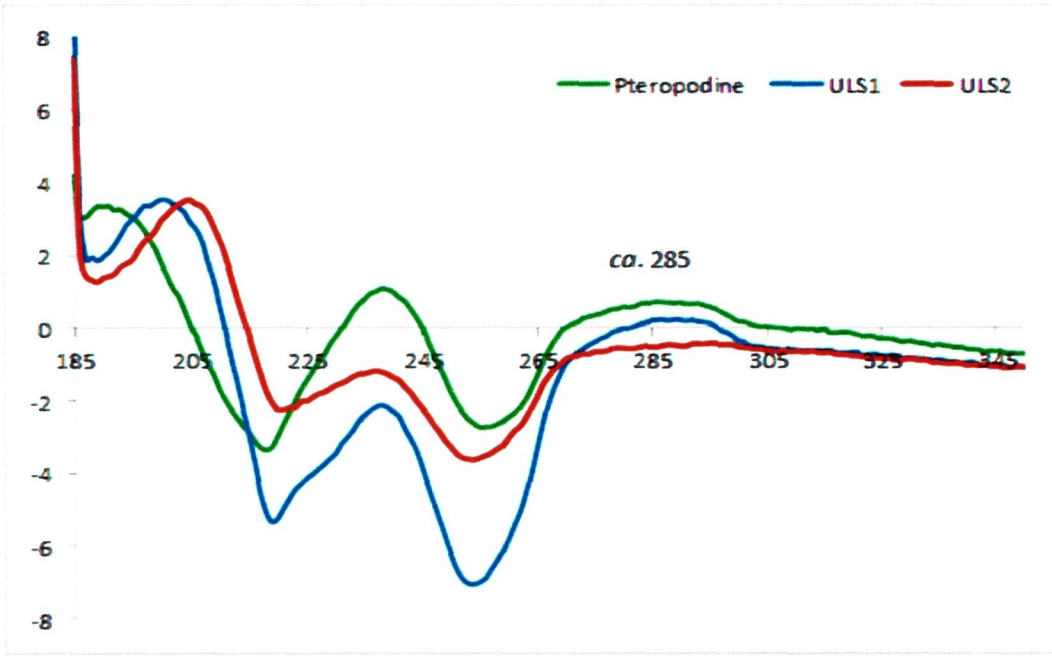
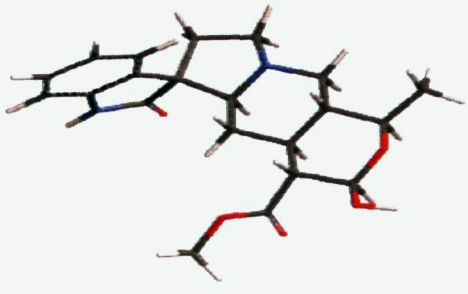


Figure 4.14 Overlaid Experimental ECD Spectra of ULS1, ULS2 and Pteropodine

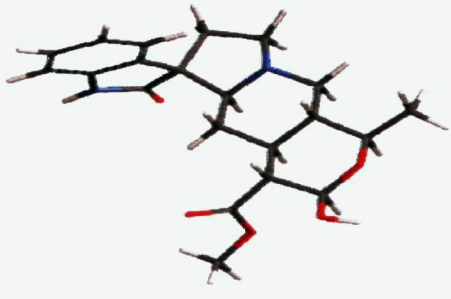
4.3.2.2 Isoformosaninol ULL6 and Formosaninol ULL7

The structure elucidation of the new oxindole alkaloids **ULL6** and **ULL7** using NMR techniques led to the stereochemistry of 3*S*, 7*S*, 16*R*, 17*R*, 19*R*, 20*R* for the former and 3*S*, 7*R*, 16*R*, 17*R*, 19*R*, 20*R* for the latter. The two new stereogenic centres of C-16 and C-17 are introduced by the hydration of the double bond between the two carbons. A systematic conformational search of the isomers carried out on Spartan08 program generated four conformers for **ULL6** from which three conformers, **ULL6a** (96.0%), **ULL6b** (2.9%) and **ULL6c** (1.2%), and four conformers for **ULL7** from which two conformers **ULL7a** (98.9%) and **ULL7b** (0.4%) were below an energy cutoff of 3 kcal/mol (Figure 4.15). The calculated UV and ECD spectra for the selected diastereoisomers were Boltzmann weighted (BW) and compared with the experimental ECD spectrum of **ULL6** and **ULL7** (Figure 4.16 - Figure 4.18). A close match for the weighted ECD spectrum and the experimental ECD of both **ULL6** and **ULL7** confirmed their absolute configuration and led to the

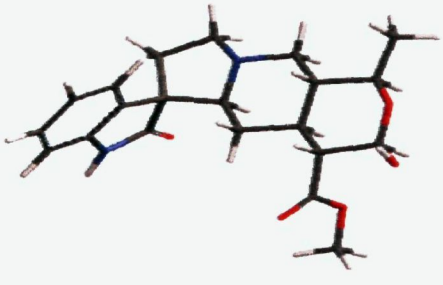
establishment of their stereochemistry as 3*S*, 7*S*, 16*R*, 17*R*, 19*R*, 20*R* and 3*S*, 7*R*, 16*R*, 17*R*, 19*R*, 20*R*, respectively, belongs to the *normal*-type POA. **ULL6** and **ULL7** are epimers at the *spiro* C-7 position, where the former possesses a 7*S* configuration while the latter possesses a 7*R* configuration. This configuration can be seen from the CEs at *ca.* 285 nm where a negative sign (-0.63) was observed for **ULL6** and a positive sign (+0.01) was observed for **ULL7** (Figure 4.17 and Figure 4.19, respectively). However, the CE at *ca.* 205 for these *normal*-type POAs seemed to be independent of the C-7 stereogenic centre, consistently producing a negative CE for both ECD spectra.



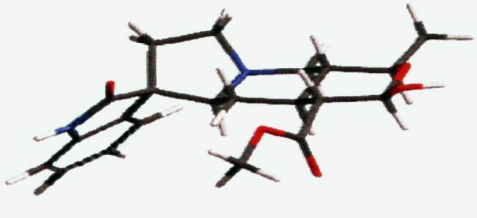
ULL6a (0.0 kcal/mol)



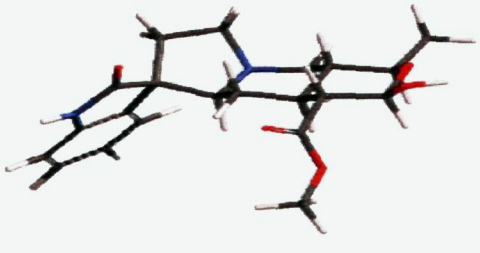
ULL6b (2.08 kcal/mol)



ULL6c (2.62 kcal/mol)



ULL7a (0.0 kcal/mol)



ULL7b (2.92 kcal/mol)

Figure 4.15 Optimized Structures and Relative Energies of ULL6 Conformers of 3*S*, 7*S*, 15*S*, 16*R*, 17*R*, 19*R*, 20*R* and of ULL7 Conformers of 3*S*, 7*R*, 15*S*, 16*R*, 17*R*, 19*R*, 20*R* Possessing Energy Cutoff Under 3 kcal/mol Used for ECD Calculations

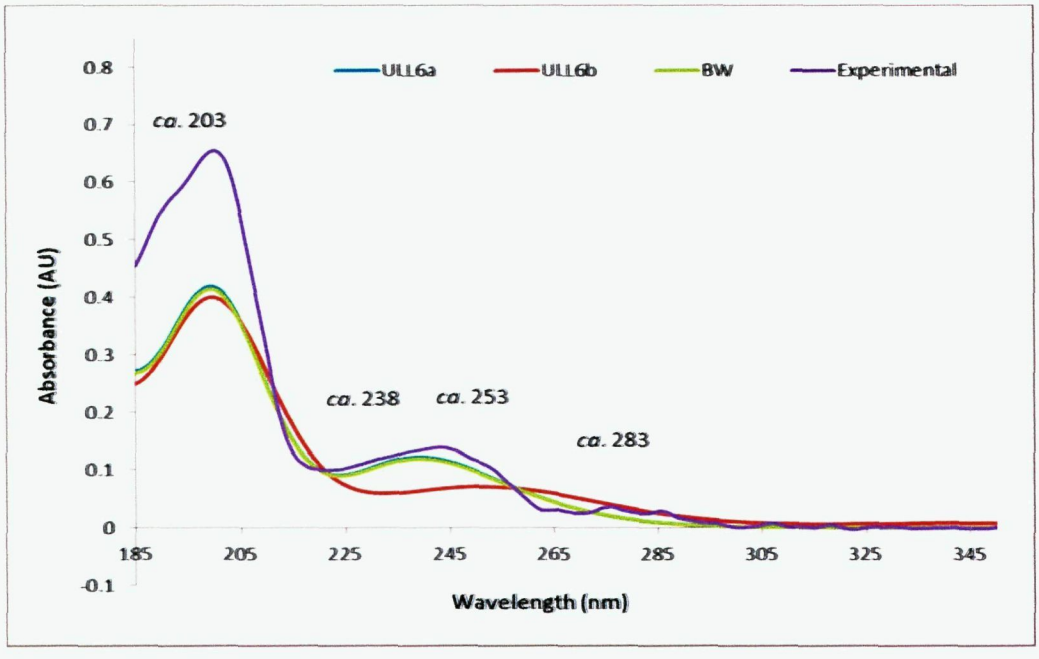


Figure 4.16 Overlaid Experimental and Calculated UV Spectra of ULL6

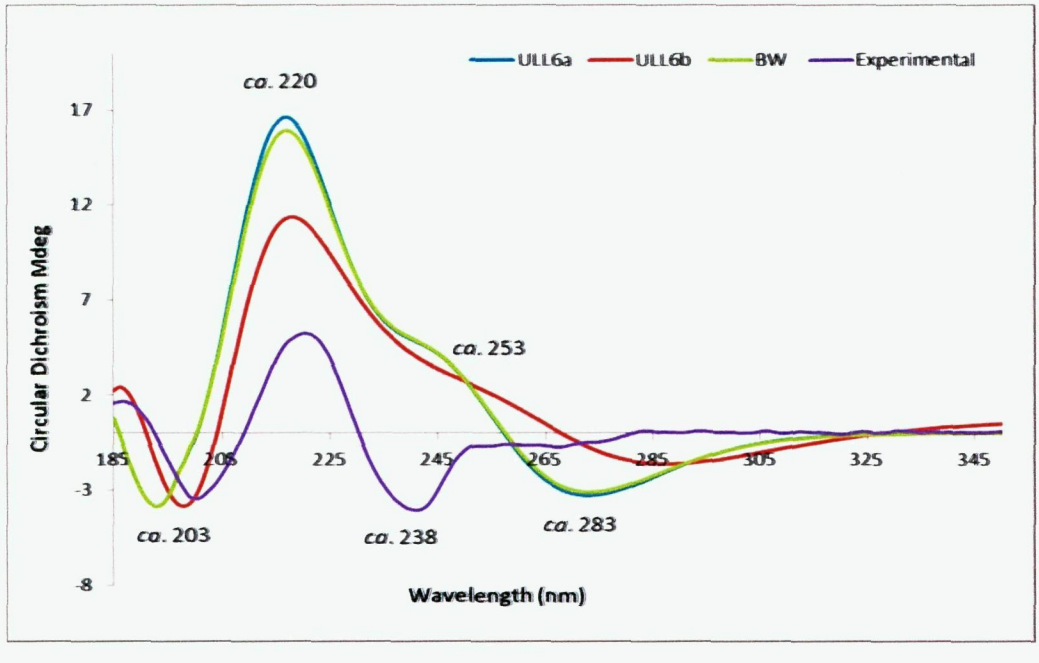


Figure 4.17 Overlaid Experimental and Calculated ECD Spectra of ULL6

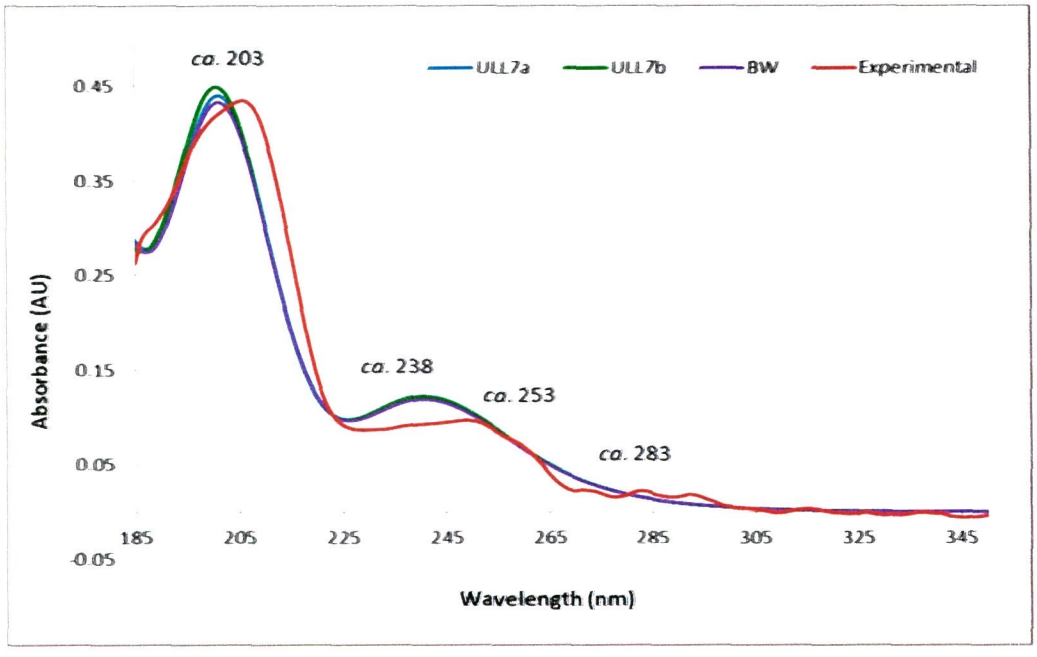


Figure 4.18 Overlaid Experimental and Calculated UV Spectra of ULL7

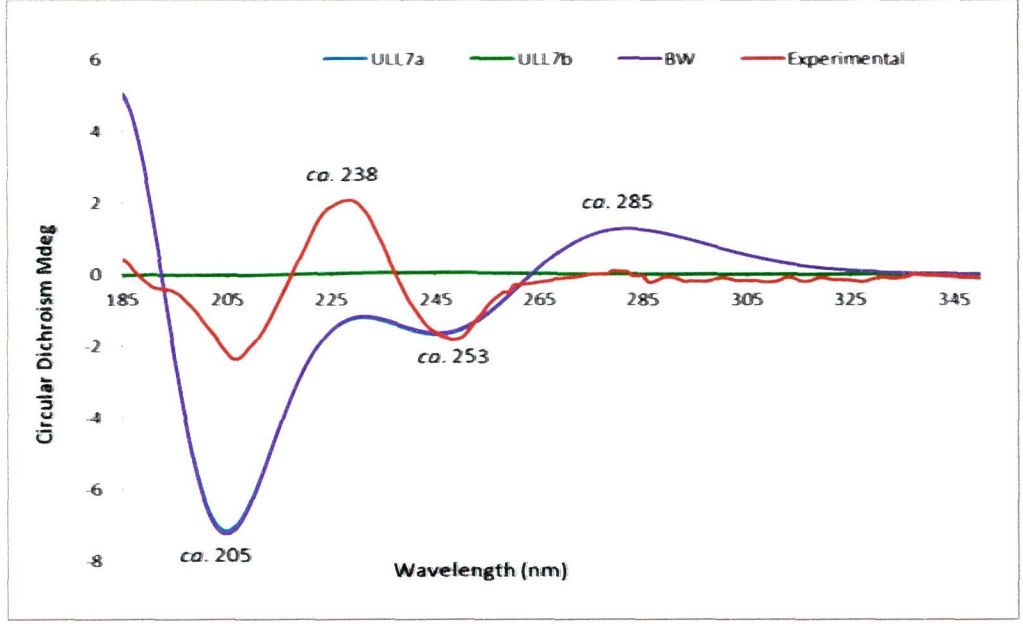
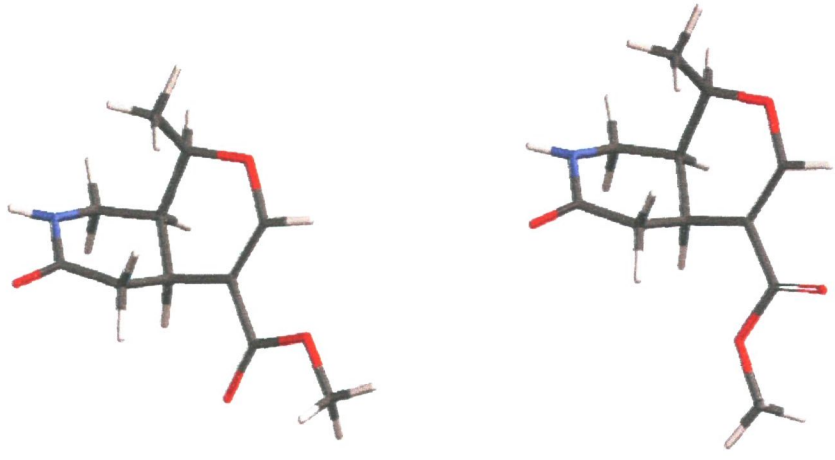


Figure 4.19 Overlaid Experimental and Calculated ECD Spectra of ULL7

4.3.2.3 Oxosecologanine ULL5

The structure elucidation of the new **ULL5** using NMR techniques led to the stereochemistry of 8*R*, 9*S*. A systematic conformational search of this isomer carried out on Spartan08 program generated two conformers, **ULL5a** (69.5%) and **ULL5b** (30.5%) both of which were below an energy cutoff of 3 kcal/mol (Figure 4.20). The calculated UV and ECD spectra for the selected 4*S*, 8*R*, 9*S* diastereoisomers were Boltzmann weighted (BW) and compared to the experimental ECD spectrum of 2-oxosecologanine (Figure 4.21 and Figure 4.22). It can be suggested that the CE at *ca.* 203 nm may be due to an n → π* transition of the N_b lone pair, while the CEs at *ca.* 220 nm and *ca.* 238 nm are caused by a π → π* transition of the α,β-unsaturated carboxylic acid methyl ester system. A close match for the Boltzmann-weighted ECD spectrum and the experimental ECD spectrum allows the establishment of the **ULL5** stereochemistry as 4*S*, 8*R*, 9*S*.



ULL5a (0.00 kcal/mol) **ULL5b** (0.49 kcal/mol)

Figure 4.20 Optimized Structures and Relative Energies of ULL5 Conformers of 4*S*, 8*R*, 9*S* Possessing Energy Cutoff of 3 kcal/mol Used for ECD Calculations

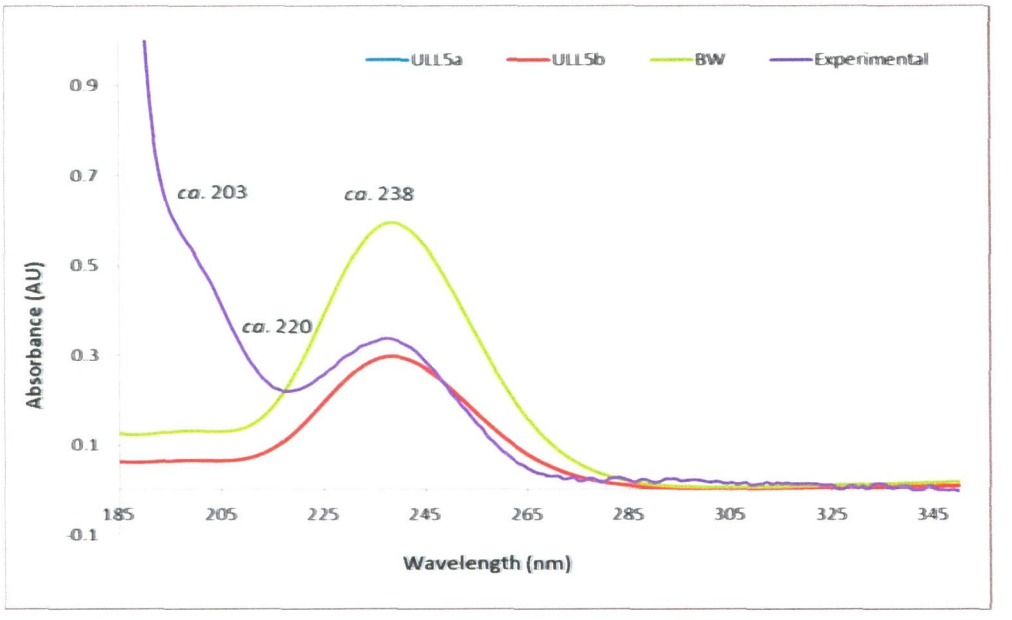


Figure 4.21 Overlaid Experimental and Calculated UV Spectra of ULL5

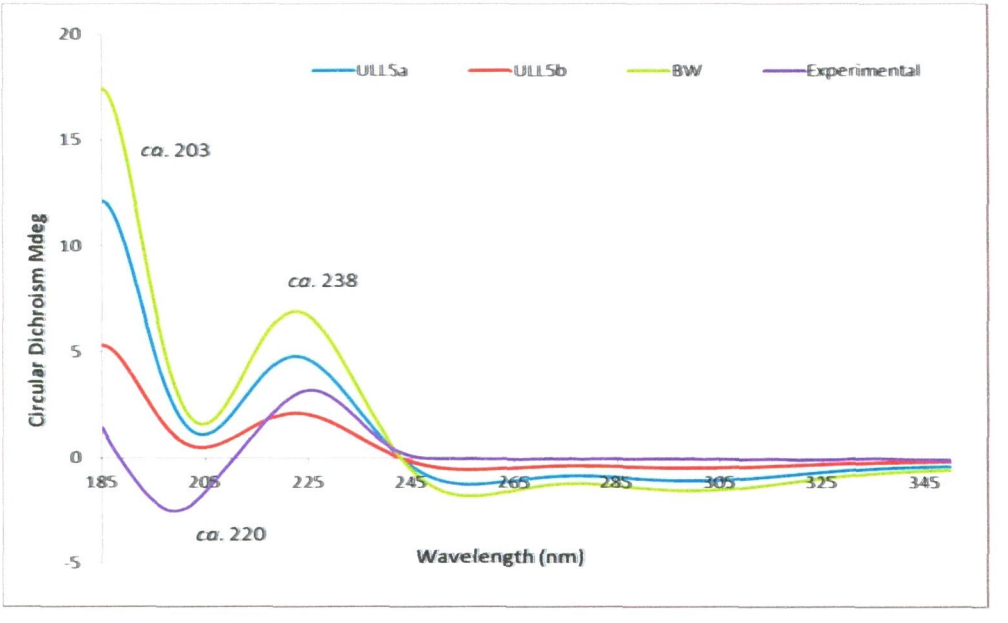


Figure 4.22 Overlaid Experimental and Calculated ECD Spectra of ULL5

In summary, the absolute configurations of the POAs were established by comparing the CEs of the experimental ECD spectra to the calculated ECD values, as well as to the ECD spectra of the known POAs in hand. The study has found that the stereochemistry of the stereogenic centres, C-3 and C-7 correlated well with the sign

of the CEs at *ca.* 205, 253 and 283 nm for the *allo*- and *epiallo*-type POA. However, for the *normal*-type POA, the respective stereogenic centres only correlated with the CEs at *ca.* 253 and 283 nm. This is true as these chromophores are due to the transitions of a $\pi \rightarrow \pi^*$ and an $n \rightarrow \pi^*$ of the oxindole chromophore, which location are near to the stereogenic centre C-7 and C-3 (see Figure 4.6). This observation, thus agreed with the study done by Beecham and co-workers (1968), since the ECD spectra patterns were found to be comparable with their work and enable the distinction between the 3*S*, 3*R* and 7*R*, 7*S* isomers. In addition, positive and negative CEs at *ca.* 220 and 238 nm caused by a $\pi \rightarrow \pi^*$ transitions of the α,β-unsaturated carboxylic acid methyl ester systems could not be directly correlated to the stereochemistry at any of the remaining stereogenic centres.

Small shifts in wavelengths were observed between experimental and calculated ECD spectra arising from minor conformational differences in solution and gas state. However, some of the calculated ECD spectra are significantly different from the experimental ECD spectra which might have been caused by compatibility problems in converting the Spartan file of the molecule into the pdb format required for the Gaussian calculations, as explained earlier. In addition, the problems might also arise from the process of conformational analysis, as well as from the geometry optimization calculation for the drawn POA structures. It is anticipated that the problems can be overcome by varying the factors in this process. It should also be noted that slight impurities (especially of other chiral molecules) in the sample might also affect the experimental ECD of samples. ECD has also been reported to have some inherent weaknesses and limitations stemming from the low signal resolution and the difficulties in assigning the signals to specific chromophoric sites without resorting to excited-state quantum-mechanical calculations (Berova *et al.*, 2007).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

This study was performed with the aim of establishing the absolute configuration of six new chiral constituents comprising five pentacyclic oxindole alkaloids and one flavonoid isolated from *Uncaria longiflora* var. *pteropoda*. The absolute configurations of the new chiral compounds were established by comparing the cotton effects (CEs) of the experimental ECD spectra to the simulated ECD values, as well as to the ECD spectra of five known related compounds in hand. The known alkaloids were isopteropodine, pteropodine and Uncarine F while the known flavonoids were *epi*-catechin and *epi*-afzelechin.

The POAs rauniticine-*allo*-oxindole B **ULS1** and rauniticinic-*allo* acid B **ULS2** were both established to have the 3*S*, 7*R*, 15*S*, 19*R*, 20*S* stereochemistry. The absolute configuration of three other new alkaloids were also determined: 2-oxosecologanine **ULL5** possess a 4*S*, 8*R*, 9*S* configuration while isoformosaninol **ULL6** and formosaninol **ULL7** exhibited a 3*S*, 7*S*, 15*S*, 16*R*, 17*R*, 19*R*, 20*R* and a 3*S*, 7*R*, 15*S*, 16*R*, 17*R*, 19*R*, 20*R* stereochemistry, respectively. The new flavonoid, uncariechin **ULL14** was established to possess a 2*R*, 3*R* configurations.

It is recommended that the compounds isolated in this study be used to build an MS database for LC-MS dereplication studies of other plants in the genus in order to quickly identify new phytochemicals. Finally, experimental CD spectra of POAs isolated from other *Uncaria* species or pure standards should be also carried out in order to ensure that the results obtained in this study are reproducible.

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APPENDIX

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Flavan-3-ols from the leaves of Malaysian *Uncaria longiflora* var. pteropoda (Miq.) Ridsd

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ABSTRACT

A novel flavonoid, (−)-2*R*,3*R*-3,5,4′-trihydoxyflavan-[6,7:5″,6″]-2″-pyranone, named uncariechin (**1**), was isolated from the methanolic extract of the leaves of *Uncaria longiflora* var. *pteropoda* (Miq.) Ridsd. along with the known (−)-*epi*afzelechin (**2**) and (−)-*epi*catechin (**3**), methyl 4-hydroxybenzoate and 4-hydroxybenzaldehyde, four pentacyclic oxindole alkaloids, isopteropodine, pteropodine, uncarine F and isopteropodic acid, previously found in the stems, and two coumarins, scopoletin and 3,4-dihydroxy-7-methoxycoumarin. Structures of the compounds were elucidated by 1D and 2D NMR, FTIR, UV, MS, and experimental as well as calculated electronic circular dichroism (ECD) data. Compounds **2** and **3** were evaluated for their neurotoxic and neuroprotective properties against differentiated SH-SY5Y neuroblastoma cell lines using the MTS assay. Compounds **2** and **3** did not show any neurotoxic effects but showed strong protective potential against hydrogen peroxide-induced neurotoxicity with maximum cell viability at a concentration of 1 μM.

1. Introduction

We have previously reported the biological activity of the genus *Uncaria*, a genus belonging to the Rubiaceae family (Ahmad et al., 2011). The genus comprises thirty-four species, which are mainly shrubby woody climbers distributed in tropical regions, including Southeast Asia, Africa and Southeast America (Risdale, 1978). *U. longiflora* var. *pteropoda* is one of the fourteen species found in Malaysia (Risdale, 1978). Our previous work on the woody stem extracts of this plant led to the isolation of two new hetero-yohimbine-type oxindole alkaloids, namely, rauniticine-*allo*-oxindole B and rauniticinic-*allo* acid B along with five of their stereoisomers (Salim et al., 2011). We have also reported on the chemotaxonomic significance of the pentacyclic oxindole alkaloids in the species (Salim and Ahmad, 2011). In this paper, we report the isolation and characterization of a novel flavonoid (−)-2*R*,3*R*-3,5,4′-trihydoxyflavan-[6,7:5″,6″]-2″-pyranone, named uncariechin (**1**), along with (−)-*epi*afzelechin (**2**) and (-)-*epi*catechin (**3**) from the methanol extract of the leaves as well as methyl 4-hydroxybenzoate and 4-hydroxybenzaldehyde, four pentacyclic oxindole alkaloids, isopteropodine, pteropodine, uncarine F and isopteropodic acid previously found in the stems (Salim and Ahmad, 2011) and two coumarins, scopoletin and 3,4-dihydroxy-7-methoxycoumarin (Abu-Eittah and El-Tawil, 1985) by spectroscopic techniques including FTIR, UV and 1D and 2D NMR spectroscopy, MS, electronic circular dichroism (ECD) measurements and calculations. This is the first time the flavonoid composition of this plant has been described. In view of the potential of catechins as therapeutic cytoprotective agents for the treatment of neurodegenerative and other diseases (Mandel and Youdim, 2004), we investigated the neurotoxic and neuroprotection properties of the flavan-3-ols isolated against differentiated SH-SY5Y neuroblastoma cell line.

2. Results and discussion

Compound **1** was obtained from the MeOH extract of the leaves of *U. longiflora* var. *pteropoda* as pale yellow crystals (m.p. 249–250 °C). It was observed on TLC as a bluish fluorescent spot under UV light (365 nm) and on UV measurement it showed absorption maxima at 276, 248, 212 and 195 nm suggesting the presence of a flavan moiety (Merken and Beecher, 2000). HRESI-MS indicated a molecular formula of $C_{18}H_{14}O_6$ and twelve degrees

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of unsaturation. The IR spectrum showed characteristic absorption bands for free hydroxyl groups (3435 cm^{-1}), conjugated lactone carbonyl stretching (1685 cm^{-1}), a cyclic ether group (1522 cm^{-1}) and methylene group bending (1457 cm^{-1}).

The aromatic region of the ^1H NMR spectrum of compound **1** showed a pair of *ortho*-coupled proton resonances at δ 7.44 (2H, d, J = 8.4 Hz, H-2′, H-6′) and at δ 6.88 (2H, d, J = 8.7 Hz, H-3′, H-5′) for a *para*-substituted ring B of a flavonoid-type compound. A hydroxyl group was placed at C-4′. These assignments were supported by the COSY spectrum which showed correlations between the H-2′ and H-3′ and between H-5′ and H-6′ resonances as well as between the H-6′ and H-2 resonances of ring C. For ring C, the COSY spectrum showed coupling between resonances at δ 5.17 (H-2), δ 4.38 (H-3), and δ 2.99–2.94 (2H-4). The chemical shift of H-3 indicated the presence of a hydroxyl group at this position establishing the presence of a flavan-3-ol. The 2H-4 proton resonances showed correlations in the HMBC spectrum with the C-5 (δ 114.78), C-9 (δ 160.15) and C-10 (δ 103.89) resonances. A singlet at δ 6.42 indicated a single proton on ring A. HMBC correlations between this resonance and the C-9 and C-10 resonances indicated this proton was at C-8 (δ 94.45). The H-8 resonance also showed HMBC correlations with the oxygenated C-7 resonance at (154.81) and a resonance at δ 102.08 which was ascribed to C-6.

Three carbon resonances remained to be assigned, a lactone carbonyl resonance (δ 160.55) and two alkene resonance (δ 138.64 and δ 109.71). The corresponding ^1H NMR resonances occurred at δ 8.06 (d, J = 9.6 Hz, H-4″) and δ 6.07 (d, J = 9.6 Hz, H-3″). The HMBC spectrum was used to determine whether a linear or angular pyranocoumarin was present. ^3J correlations were seen between the H-4″ resonance and C-5, C-7 and C-2″ resonances. The molecular formula indicated the need for a fused ring and a pyranone ring was indicated. In the NOESY experiment run in DMSO, a correlation was observed between the H-4″ and the 5′-OH (δ 6.03) proton resonances also confirming that the attachment was linear.

The relative configuration of compound **1** was established using a NOESY experiment and a model. The H-2 proton resonance showed correlations in the NOESY spectrum with H-3 and one of H-4 proton resonances, allowing for the placement of ring B and the 3-OH on the same face of the molecule. A $J_{2,3}$ coupling constant of <1 Hz further confirmed a *cis* relationship for H-2 and H-3 in compound **1** (Friedrich and Galensa, 2002). The flavan-3-ols have two stereocenters and therefore four possible diastereomers, (2*R*,3*S*)-*trans*, (2*S*,3*R*)-*trans*, (2*R*,3*R*)-*cis* and (2*S*,3*S*)-*cis* are possible. A *cis* relationship between ring B and the 3-OH group would support a (2*R*,3*R*) or a (2*S*,3*S*) configuration (Friedrich and Galensa, 2002).

Firstly, the absolute configuration of the known flavan-3-ols (−)-*epi*afzalechin (**2**) and (−)-*epi*catechin (**3**) were confirmed. As for **1**, a $J_{2,3}$ coupling constant of <1 Hz suggested a *cis* configuration for both **2** and **3** for a (2*R*,3*R*) or a (2*S*,3*S*) conformation. The measured optical rotation for compounds **2** and **3** were −151 and −125, respectively, supporting a (2*R*,3*R*) configuration (Nanjo et al., 1996). According to Slade et al. (2005), flavan-3-ols are characterized by two phenyl chromophores whose UV absorption bands are between 200 and 240 and between 260 and 280 nm giving fingerprint ECD Cotton effects at the respective wavelengths. A 2*R*,3*R*-*cis* configuration will show two negative Cotton effects at these wavelengths. To confirm this, experimental ECD analyses for **2** and **3** were carried out in which two negative Cotton effects at *ca.* 220 nm and *ca.* 270 nm were observed, consistent with a 2*R*,3*R*-*cis* configuration of the compounds. Thus, the absolute configuration of these compounds were established as (−)-2*R*,3*R*-*epi*catechin and (−)-2*R*,3*R*-*epi*afzalechin.

To determine the absolute configuration of **1** and investigate the effect of the pyranone moiety on its CD spectrum, both experimental and calculated ECD analyses were carried out. The latter was done *via* a systematic conformational search of the (2*R*,3*R*) isomer with the Spartan08 program using molecular mechanics force field (MMFF) calculations. This generated 12 conformers from which 8 conformers were under an energy cut off of 3 kcal/mol. An ECD analysis was then calculated for each of these conformers using time dependent density functional theory (TDDFT) at the B3LYP/6-31G (d, f) level built to Gaussian09 software (Ding et al., 2010). The calculated ECD spectra were Boltzmann weighted (BW) and compared to the experimental ECD spectrum of **1**. As shown in Fig. 1, the BW-ECD spectrum for the 2*R*,3*R* isomer of **1** showed a negative Cotton effect at *ca.* 220 and a small but distinct negative CE at *ca.* 270 nm. In addition, the calculated ECD spectrum also showed a positive CE at *ca.* 325 nm likely due to a $\pi \rightarrow \pi^*$ transition in the extended π-system of pyranone moiety (Dastan et al., 2012). The two negative CEs observed indicated that the presence of the pyranone moiety fused to ring A of **1** did not affect the helicity of the molecule which was found to conform to Snatzke's helicity rule (Snatzke and Ho, 1971) as reported by Slade et al. (2005). A good match was found for the weighted ECD spectrum and the experimental ECD spectrum of compound **1** (Fig. 1) confirming a (2*R*,3*R*) absolute configuration leading to the establishment of compound **1** as (−)2*R*,3*R*-uncariechin. The structure of compound **1** is given in Fig. 2.

In view of the reported cytoprotective activities of catechins (Mandel and Youdim, 2004) the neurotoxic and neuroprotective potential of the flavan-3-ols isolated in this study was evaluated. However, due to a limited amount of compound **1**, only compounds **2** and **3** were tested. The effect of pre-incubation with compounds **2** and **3** on the production of reactive oxygen species (ROS) by differentiated human neuroblastoma SH-SY5Y cell-line in the presence or absence of oxidative stress (H_2O_2) was evaluated at a concentration range of 1 nM–1 mM. Our data showed that both compounds **2** and **3** produced no neurotoxic

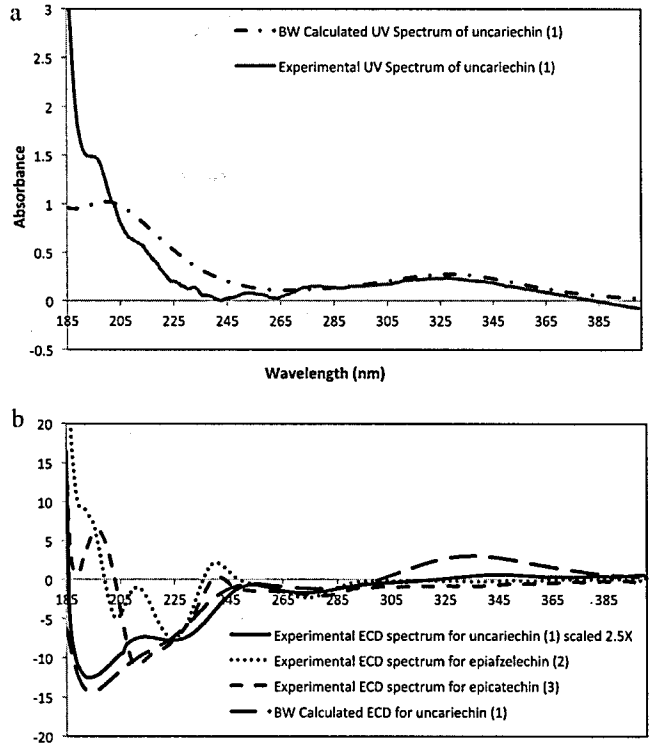

Fig. 1. (a) Boltzmann weighted (BW) calculated UV and experimental UV spectra for compound 1 (b) comparison of Boltzmann weighted calculated ECD and experimental ECD spectra for compounds 1, 2 and 3.

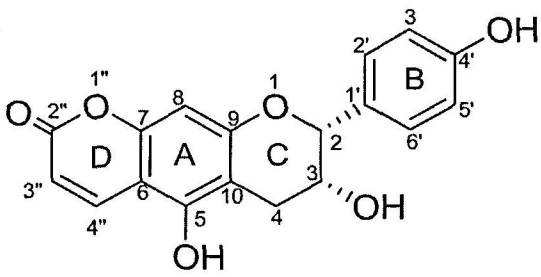

Fig. 2. Structure of compound 1.

effect on neuron phenotypic cells (differentiated SH-SY5Y; Fig. 3) with a surprising increase in cell viabilty values exceeding 100% (negative control) at all concentrations suggesting the compounds' ability to proliferate the cells. We therefore investigated whether compounds **2** and **3** can exert a neuroprotective effect on the neuron phenotypic cells, SH-SY5Y. Hydrogen peroxide-induced neurotoxicity was used as a positive control (Godkar et al., 2006). Incubation with compounds for 2 h followed by exposure to 230 μM H_2O_2 showed an increase in cell viability to 75–88% and 77–85%, respectively, compared to a 52% cell viability for the positive control (Fig. 4). Compounds **2** and **3** exhibited the highest cell viability of 88% and 85%, respectively, at a concentration of 1 μM with compound **2** displaying stronger neuroprotective potential than compound **3**. These findings are particularly relevant, since H_2O_2 is quantitatively the most important of the peroxides generated in brain cells (Dringen et al., 2005) and its intracellular accumulation can induce oxidative stress leading to neuronal apoptosis (Chandra et al., 2000).

In earlier studies, it has been reported that pre-treatment with *epi*catechin and 3-*O*-methyl-*epi*catechin attenuated neurotoxicity induced by oxidized low-density lipoprotein (oxLDL) in mouse-derived striatal neurons (Schroeter et al., 2001). Pre-treatment with the compounds at a concentration of 30 μM prior to oxLDL administration led to cell viability of 90–93% as compared with 43% cell viability following treatment with oxLDL alone. In a related study, *epi*gallocatechin-3-gallate (EGCG) conferred protection against 6-OHDA-induced human neuroblastoma SH-SY5Y cell damage (Levites et al., 2002) where pretreatment for 15 min with the compound (0.1–10 μM) conferred significant protection against 6-OHDA neurotoxicity (38% cell viability at 50 μM). The authors also reported that EGCG showed maximal cell survival at 1 μM (93%) with no effect up to 10 μM and a gradual decrease in cell viability at higher concentrations. Their results are in good agreement with our data for compound **3** (*epi*catechin) as shown in Fig. 4 where the highest cell viability was also observed at 1 μM

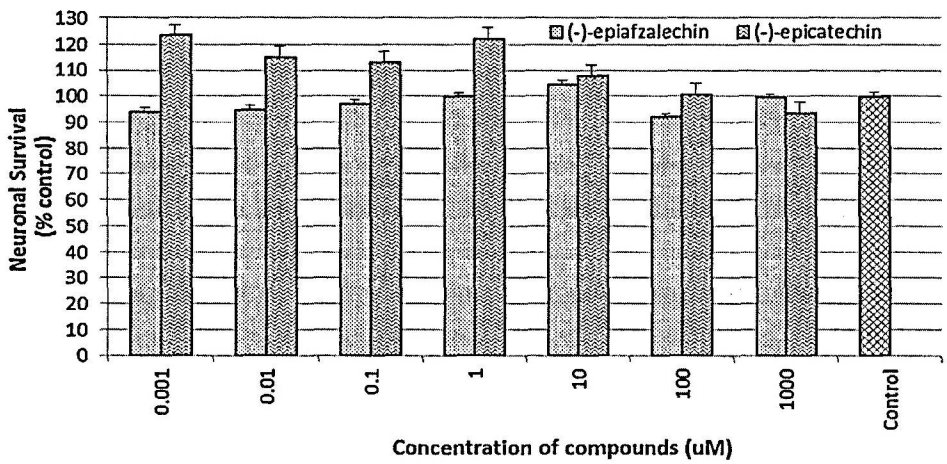

Fig. 3. Neurotoxic properties of (−)-*epi*afzalechin and (−)-*epi*catechin against differentiated human neuroblastoma SH-SY5Y cell viability, assessed by MTS assay after 24 h exposure at 37 °C. Data is presented as mean ± SEM ($n = 6$).

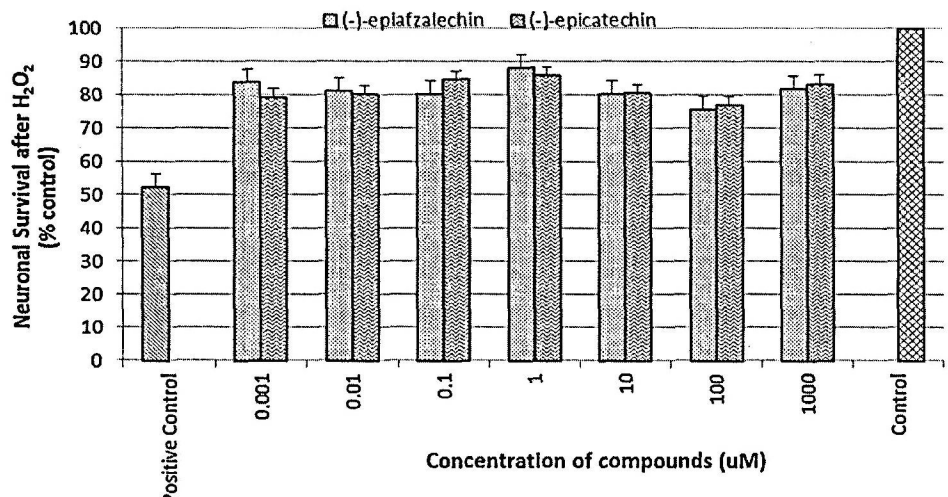

Fig. 4. Neuroprotective properties of (−)-*epi*afzalechin and (−)-*epi*catechin against H_2O_2-induced neurotoxicity on differentiated human neuroblastoma SH-SY5Y as assessed by MTS assay after 24 h of incubation at 37 °C 5% CO_2. Data is presented as mean ± SEM ($n = 6$).

with a gradual decrease in cell viability at higher concentrations up to 100 μM. A similar trend was observed for compound **2** (*epi*afzelechin) for which, to the best of our knowledge, neurotoxic and neuroprotective potential have not been reported.

3. Experimental

3.1. General

TLC and PTLC were performed using pre-coated aluminium-backed supported silica gel 60 F_{254} (0.2 mm thickness) and glass supported silica gel 60 F_{254} (1.0 mm thickness), respectively. Flavonoids were detected on TLC stained with aluminium chloride ($AlCl_3$) reagent in which a positive result was indicated by the observation of yellow spots visualized under UV light at 365 nm. Column chromatography was carried out using silica gel 60, 70–230 mesh ASTM (Merk 7734) whereas radial chromatography was carried out using glass plates with Merck's silica gel Kieselgel 60 PF_{254} Merk Art 7749. Mass spectra were measured on an Agilent Technologies 6520 QTOF LC/MS equipped with a dual-ESI source and an Agilent Technologies LC system 1200 series, where the experiment for compounds **1–3** were run on negative mode, while the other compounds were run on positive mode. The ultraviolet (UV) spectra were obtained in methanol on a Shimadzu UV–vis 160i. The infrared (IR) data was recorded on a PerkinElmer model FT-IR spectrometer as KBr disks. Optical rotations were measured on a JASCO P1020 digital polarimeter. Melting points were determined using X-4 melting-point apparatus with microscope JM628 digital thermometer. The ECD spectrum for compounds **1**, **2** and **3** were obtained on an Applied Photophysics Chirascan CD spectrometer using a 5 mm cell and acetonitrile as the solvent. ^{1}H- and ^{13}C-NMR data for compound **1** were obtained in acetone-d_6 on Bruker 300 Ultrashield NMR spectrometer measured at 300 and 75 MHz, respectively.

3.2. Plant materials

Uncaria longiflora var. *pteropoda stems and leaves* were collected from Hutan Simpan Bangi, Selangor, Malaysia. The leaves and the stems of the plant were separated and the voucher specimens (HTBP 1336) were deposited at the Herbarium of Taman Botani Putrajaya, Malaysia.

3.3. Extraction and isolation of compounds

The leaves of *U. longiflora* var. *pteropoda* were cut into small pieces, air-dried and ground into a fine powder. The finely ground plant material was weighed, and extracted exhaustively with methanol at room temperature for 72 h. The solvent was removed under reduced pressure to yield 550 g of crude extract which was successively triturated to afford 67 g, 72 g, 28 g and 362 g of hexane, chloroform, ethyl acetate and methanol extracts, respectively. The methanol extract (362 g) was then subjected to liquid–liquid partitioning between MeOH and Et_2O to remove the excess tannins. The dissolved portion was filtered through cotton wool and the solvent was evaporated to dryness using a rotary evaporator leaving 120 g of dark solid. Fractionation of the solid with vacuum liquid chromatography (VLC) using solvents of increasing polarity (DCM, EtOAc and MeOH) yielded five fractions (100 ml volumes).

Based on the TLC profiles, fractions 3 and 4 were found to contain a high density of flavonoids and were subsequently combined and subjected to further fractionation *via* column chromatography using DCM and MeOH with gradient elution to afford 11 fractions (F1-11) which was collected based on bands observed on the column. F7 to F9 were pooled and subjected to preparative thin layer chromatography (PTLC) using a solvent system of DCM:EtOAc (3:2) resulting in the isolation of pure compound **1** (17 mg). F10 afforded compound **2** (44 mg) upon purification with PTLC using a solvent system $CHCl_3$:MeOH (5:1). Employment of a different solvent system [$CHCl_3$:MeOH (33:7)] on the same fraction successfully yielded a small amount of 3,4-dihydroxy-7-methoxycoumarin (4 mg). Similarly, compound **3** (100 mg) was purified from F11 by PTLC using $CHCl_3$: MeOH (62:13) as a solvent system. Fraction 2 was found to contain two distinct spots on TLC with UV visualisation at short wave length (254 nm) and upon PTLC development with solvent system Hexane:EtOAc (4:1) afforded methyl 4-hydroxybenzoate (8 mg) and 4-hydroxybenzaldehyde (4 mg).

The other five compounds were isolated from the chloroform extract (72 g) which was subjected to acid-base extraction to afford a crude alkaloid mixture (53 g). This mixture was chromatographed using VLC with increasing solvent polarity using hexane, DCM, EtOAc and MeOH to give nine fractions (100 ml volumes) of which those with the same TLC profiles were combined. Isopteropodine (3552 mg), pteropodine (2481 mg) and scopoletin (21 mg) were purified with column chromatography using solvent system Hexane:EtOAc (7:3) from pooled fractions F2 and F3. Preparative TLC of F5 using solvent system DCM:EtOAc (7:3) yielded uncarine F (43 mg) while centrifugal PTLC on F6 using solvent system $CHCl_3$:MeOH (20:1) led to the isolation of isopteropodic acid (55 mg). All known compounds were characterized by NMR spectroscopy and comparison with literature (Seki et al., 1993; Pouchert and Behnke, 1993; Prasad et al., 2000).

3.4. Characterization of compounds 1–3

The characterization of compound **1** is given below.

3.4.1. (−)-(2R,3R)-uncariechin (1)

Pale yellow amorphous solid, mp 249–250 °C. $[\alpha]D^{20}$ −312.42° (MeOH, *c*0.015); MS *m/z* = 325.0724 $[M-H]^+$, (calcd: $[M]^+$ 326.0719)) $C_{18}H_{14}O_6$; UV (MeOH) λ_{max} nm: 276, 248, 216; IR (KBr) υ_{max} cm^{-1}: 3435, 3239, 1685, 1620, 1602, 1522, 1457; ^{1}H NMR (Acetone-*D*, 300 MHz) δ ppm: 8.06 (1H, *d*, *J* = 9.6 Hz, H-4″), 7.44 (2H, *d*, *J* = 8.4 Hz, H-2′, H-6′), 6.88 (2H, *d*, *J* = 8.7 Hz, H-3′, H-5′), 6.42 (1H, *s*, H-8), 6.07 (1H, *d*, *J* = 9.6 Hz, H-3″), 5.17 (1H, *s*, H-), 4.38 (1H, *m*, H-3), 3.00 (3H, *br s*, 4′-OH, 5-OH, 3-OH), 2.99 (1H, *dd*, *J* = 3.0, 15 Hz, H-4α), 2.94 (1H, *dd*, *J* =3.0, 15 Hz, H-4β); ^{13}C NMR (Acetone-*D*, 75 MHz) δ ppm: 160.55 (C-2″), 160.15 (C-9), 157.07 (C-4′), 154.81 (C-5), 152.16 (C-7), 138.64 (C-4″), 129.70 (C-1′), 128.17 (C-2′), 128.17 (C-6′), 114.78 (C-3′), 114.78 (C-5′), 109.71 (C-3″), 103.89 (C-10), 102.08 (C-6), 94.45 (C-8), 79.46 (C-2), 64.97 (C-3), 28.43 (C-4).

3.5. Computational method

TDDFT calculations were carried out at 298 K in the gas phase with Gaussian 09 (Frisch et al., 2010). For the conformational search as well as the ECD, the absolute configuration of **1** (2*R*,3*R*) was chosen. The conformational search and geometry optimization were carried out at the molecular mechanics level of theory employing MMFF force field incorporated in Spartan08 (Wavefunction, Irvine, CA) software package. The conformers were selected and further geometry optimized at the modest B3LYP/6-31G (d,f) level of theory and TDDFT at the B3LYP/6–31(d,f) level of the theory basis set employed to simulate the ECD spectrum. The predicted wavelengths were used without any scaling. The adequacy of B3LYP/6-31(d,f) to optimize the geometry and to calculate the ECD spectra of flavan-3-ols similar to compound **1** have been demonstrated previously (Ding et al., 2010).

3.6. Cell line and culture conditions

The human neuroblastoma cell line, SH-SY5Y was acquired from Dr. Carol Sanfeliu (Department of Pharmacology and Toxicology, Institute of Biological Research, Barcelona, Spain). The SH-SY5Y cell line was originally established from a bone marrow biopsy of a neuroblastoma patient and is a third successive subclone of the parent cell lines SK-N-SH (Hana et al., 2010). Original studies by Pahlman et al. (1984) reported that SH-SY5Y possess neuron-like properties, including neurite outgrowth, and morphological changes, and have been extensively used as an *in vitro* model for CNS. Neuroblastoma (SH-SY5Y) cells were adapted to grow in 1:1 of Minimum Essential Medium Eagle (EMEM) (Sigma, USA). Nutrient mixture F12-Ham (Sigma, USA) supplemented with 1% non-essential amino acids (PAA Laboratories Gmbh, Austria), 1% L-glutamine (Sigma, USA), 1% 50 μg/ml gentamicin (PAA Laboratories Gmbh, Austria), and 10% fetal bovine serum (PAA Laboratories Gmbh, Austria). The cells were maintained in 5% CO_2 incubator (Contherm Scientific Ltd, New Zealand) at 37 °C with 95% humidity.

3.7. Differentiation of cell line by retinoic acid

The SH-SY5Y cells were allowed to achieve 80–100% confluency in a tissue culture flask with an estimated number of 10^6 cell/ml. Approximately 2×10^4 cells/ml were seeded onto a 96-well plate and incubated for 24 h. The cell were then induced to differentiate to become neuronal-phenotypic cells by adding 10 μM retinoic acid (RA) [Sigma, USA] and further incubated in a humidified atmosphere containing CO_2 at 37 °C. The media was changed after three days with fresh RA and cells were ready to be used on the 6th day.

3.8. Neurotoxic and neuroprotective assay

Neurotoxicity tests were performed by incubating cultured cells (1×10^4cells/ml) with ranges of test compound concentrations (1 nM–1 mM) overnight in a humidified atmosphere containing 5% CO_2 at 37 °C. The results were assessed by the MTS assay on the next day. For the neuroprotection assay, the cultures were incubated with a serial dilution of compounds at final concentrations ranging from 1 nM to 1 mM for 2 h. Cells were subsequently exposed to 230 μM hydrogen peroxide (H_2O_2, 30%, MERCK, Germany, which caused 52% of cell viability) before being treated with test compounds. The cultures were further incubated for 24 h, and then cell viability was again determined by the MTS assay. Results were representative of at least three independent experiments, and expressed as percentage of the value observed without any treatment (negative control). Cells treated with H_2O_2 served as a positive control.

3.9. Statistical analysis

Each experiment was carried out at least in triplicate. Data were reported as mean ± standard error (SE) of six replicate readings. The significance of differences among different groups was determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test using GraphPad PRISM Version 5.0 whereby positive significance was indicated as asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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