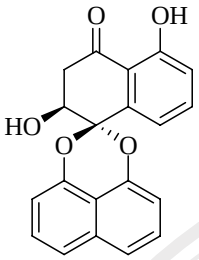
CHAPTER V
APPROVAL OF FUNGI

Preussomerins belong to a class of fungal metabolites previously isolated from dung-colonizing fungi *Preussia isomera*[34-35], *Sporormiella vexans*[36], an unidentified coelomyces (MF 5916)[37] and from an endophytic fungus *Harmonena dematioides*[38]. Altogether, 13 isomers, i.e., preussomerins A-L **[73]-[84]** and 3´-O-demethyl-1-epipreussomerin C **[85]** have been reported. These compounds are known to exhibit antifungal activity[35] and their activity as inhibitor of Ras farnesyl protein transferase, the potential using in cancer chemotherapy, has also been reported[37]. Interestingly, their unique skeleton has attracted the interests of organic chemists and the syntheses of this class of compound have recently appeared in the literature[39-40].

However, there have been two reports on the presence of preussomerins in plants; bipendensin from *Afzelia bipendensis* [41] and palmarumycins JC1 **[70]**, together with JC2 **[71]** and CP_1 **[86]**, from *Jatropha curcas*[25]. Moreover, the latter report confirmed that constituents of *Jatropha curcas* were of plant origin and not of endophytic fungi, since appreciable quantities of the isolated compounds were obtained from the plant extract[25].

Microorganisms are rich source of biologically-active metabolites[42-43], the author found preussomerins and deoxypreussomerins as bioactive chemical constituents of lichen fungus *Microsphaeropsis* spp.BCC 3050. A crude extract of the strain BCC 3050 showed antimycobacterial and antiplasmodial activities. Moreover, three deoxypreussomerins, viz., palmarumycins JC1 **[70],** JC 2 **[71]** and CP_1 **[86]**, have been isolated from a collection of the stems of *Jatropha curcas*. The second and third compounds have antibacterial constituents[43].



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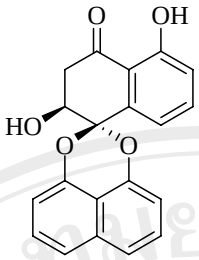

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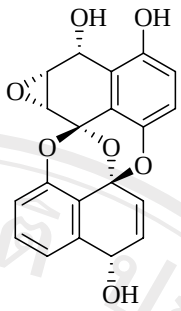

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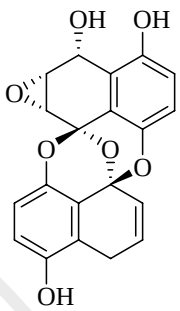

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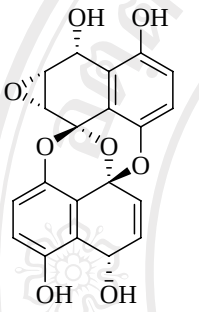

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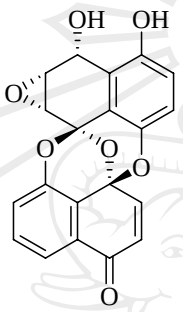

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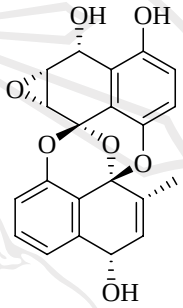

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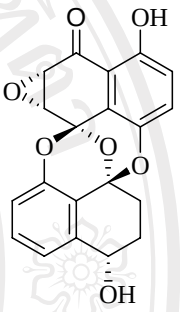

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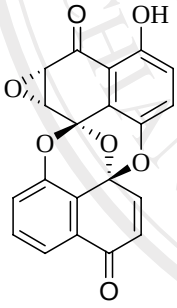

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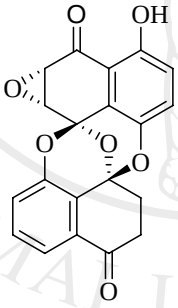

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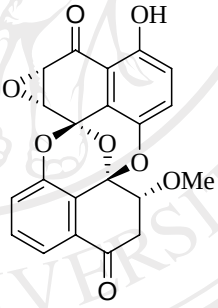

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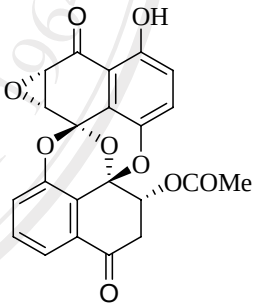

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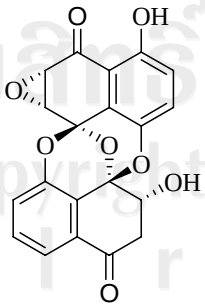

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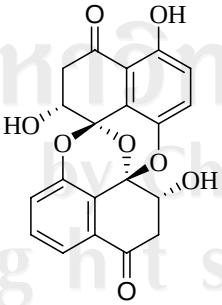

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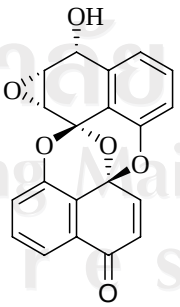

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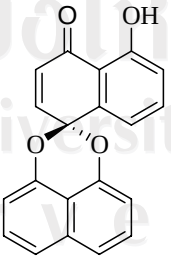

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Palmarumycins JC1 **[70]** and JC2 **[71]** were not found in the extract of freshly-collected fruits; however, they were present in dried fruits extract. Thus, to prove whether the bioactive compounds, palmarumycins JC1 **[70]** and JC2 **[71]**, of *Diospyros ehretioides* fruits are plant or fungal metabolites, following experiments were conducted.

Experiment

5.1 Plant material

The green and fresh and brown and dried fruits of *Diospyros ehretioides* were collected from Kang Tana National Park, Ubon Ratchathani province in September 22, 2004 and were identified by Miss P. Charoenchai, and its voucher specimen (No. BRU 29) was deposited at BIOTEC, Thailand.

5.2 Detection of palmarumycins JC1[70] and JC2[71] in Sephadex LH-20 fractions by ^{1}H-NMR analysis

Fruits of *Diospyros ehretioides* were collected as both green and fresh and brown and dried fruits and divided into two groups: one was freshly milled, macerated with MeOH and evaporated until dryness to give a MeOH extract, while another group was dried fruits (in the same manner as that of the first batch) to give CH_2Cl_2 extract. The MeOH extract and CH_2Cl_2 extract were fractionated by Sephadex LH-20 column chromatography, and fractions were subjected to ^{1}H-NMR analysis for the detection of signal for palmarumycins JC1 **[70]** and JC2 **[71].**

5.3 Isolation of endophytic fungi

Fresh fruits of *Diospyros ehretioides* were cleaned under running tap water and then air-dried. Before surface sterilization, the cleaned fruits were cut into 5 cm-long pieces. Fruit fragments were surface-sterilized as described by Schulz *et al.*[44] with some modifications. Plant fragments were sequentially immersed in 70% EtOH for 1 min, 5% sodium hypochlorite solution for 5 min, and sterile distilled water for 1 min (2 times). The surface-sterilized fruit fragments were cut into small pieces with a sterile blade and placed on sterile water agar plates for further incubation at 30°C. The hyphal tip of endophytic fungus growing out from the plant tissue was cut by a sterile Pasteur pipette and transferred into a sterile potato dextrose agar (PDA) plate. After incubation at 30 °C for 7-14 days, culture purity was determined from colony morphology.

5.4 Isolation of epiphytic fungi

Fungal epiphytes were isolated from dried fruits of *Diospyros ehretioides*. The isolation of epiphytic fungi was the same as that of endophytic fungi except that the cleaned fruits were subjected to fungal isolation without surfaced sterilization.

5.5 Detection of palmarumycins JC1 [70] and JC2 [71] in fungal crude extract by ^{1}H-NMR analysis

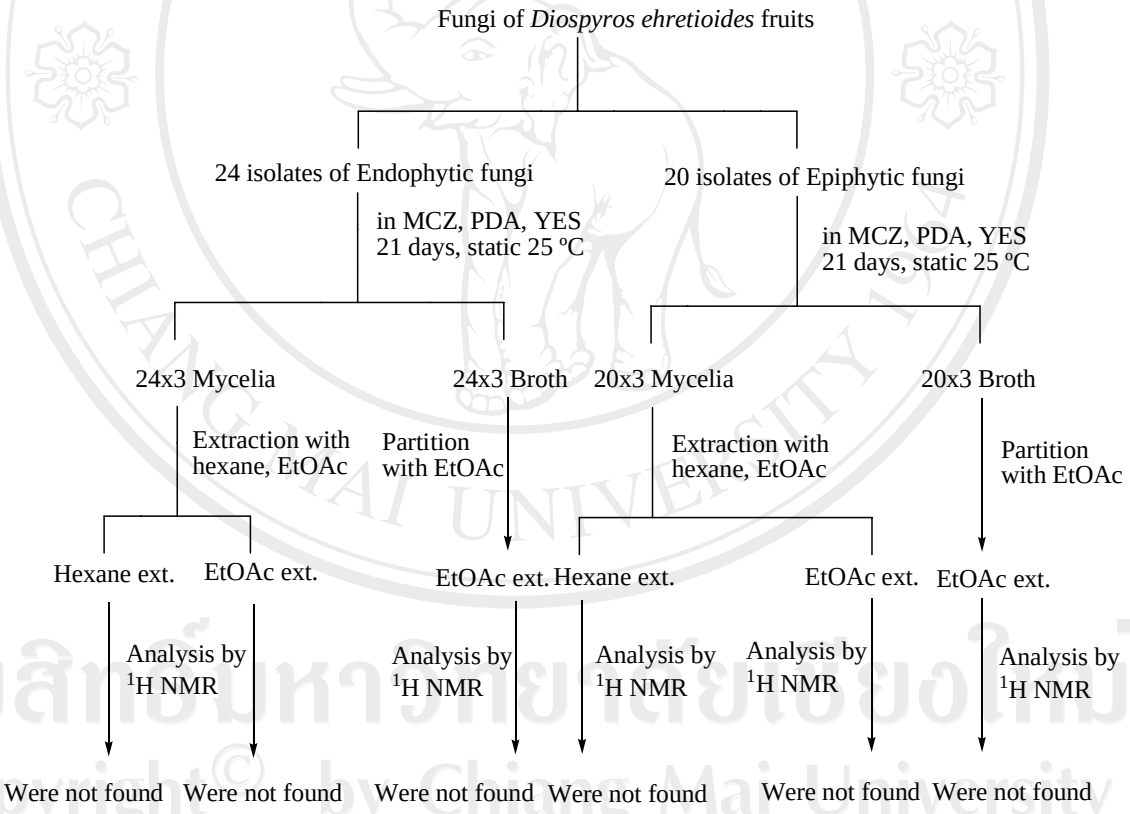
5.5.1 Endophytic fungi

Twenty four isolates of endophytic fungi from *Diospyros ehretioides* fruits were grown in three different culture media including Malt Czapex, potato dextrose, and yeast extract sucrose broths. Fungal culture (250 ml) was filtered to

separate cells and broth. Cells were extracted with hexane and EtOAc, while broth was extracted twice with an equal volume of EtOAc. Both cell and broth extracts were subjected to ^{1}H-NMR analysis (Scheme 6).

5.5.2 Epiphytic fungi

Twenty isolates of epiphytic fungi were cultured in Malt Czapex broth (250 ml). Extraction of cells and broth, as well as ^{1}H-NMR analysis of crude extracts, were carried out in the same manner as that for endophytic fungi (Scheme 6).



Scheme 6. Cultivation of endophytic and epiphytic fungi in three different culture media and detection of palmarumycins JC1 **[70]** and JC2 **[71]** in fungal crude extracts by ^{1}H-NMR analysis

5.6 Results and Discussion

To clarify the origin of palmarumycins JC1 **[70]** and JC2 **[71]**, the author re-collected both fresh and dried *Diospyros ehretioides* fruits. After the extraction process, they were fractionated by Sephadex LH-20 column chromatography, and the fractions were subjected to ^{1}H-NMR analysis for detection of isolated compounds from *Diospyros ehretioides*. As seen in Table 5, palmarumycins were not detected in any fraction of fresh fruit extract, while dimeric naphthoquinones **23** and **72** were found in fraction 2.

As opposed to extract of dried fruit samples, palmarumycin JC1 **[70]** was found in fraction 9, and palmarumycin JC2 **[71]** was observed in fractions 7 and 8 (Table 5).

Table 5. Detection of compounds **23, 70-72** in Sephadex LH-20 fractions by ^{1}H-NMR analysis

Fraction	Fresh fruits				Dried fruits			
	23	**70**	**71**	**72**	**23**	**70**	**71**	**72**
1								
2	+			+				
3								+
4					+			
5					+			
6								
7							+	
8							+	
9						+		
10								
11								

Compound **23** = isodiospyrin

Compound **70** = palmarumycin JC1

Compound **71** = palmarumycin JC2

Compound **72** = isodiospyrol A

Naphthoquinones **23** and **72** were also detected in dried fruit samples (fractions 3-5). It should be noted that during the drying process, there have been fungal colonies growing on fruit surfaces, particularly on the tared fruits. The presence of palmarumycins JC1 **[70]** and JC2 **[71]** in dried fruits of *Diospyros ehretioides*, but not in fresh fruits, proved that palmarumycins (or deoxypreussomerins) are not metabolites of the plant. The isolated palmarumycins may be from fungi associated on dried fruits of *Diospyros ehretioides*. Interestingly, the isolation of palmarumycins from dried fruits could be reproducible; both plant samples collected in years 2003 and 2004 provided the same result, suggesting that the same palmarumycins-producing fungi were associated with this specimen. Therefore, symbiont fungal strains are specific to the plant host (*Diospyros ehretioides*), and they can grow on the fruits during drying process. The author decided to carry out preliminary work on endophytic and epiphytic fungi associated with *Diospyros ehretioides* fruits (collected in 2004). Endophytic fungi were isolated from fresh fruits whilst epiphytic fungi were isolated from dried fruits. As the results, twenty four isolates of endophytic fungi and twenty isolates of epiphytic fungi were obtained. Endophytic fungi were grown in three different culture media including Malt Czapek (MCZ), potato dextrose (PDB), and yeast extract sucrose (YES) broths, while epiphytic fungi were grown in Malt Czapek broth. Mycelia and broth of these fungal cultures were separately extracted with organic solvents. Their extracts were evaporated to dryness under reduced pressure, subsequently subjected to ^{1}H-NMR analysis.

However, palmarumycins were not detected in any crude extract of fungal cells and broth. It is therefore concluded that these fungal isolates could not produce

palmarumycins under laboratory conditions. Should they produce, levels of palmarumycin production may be too low to be detected by the ^{1}H-NMR analysis. It is known that, so far, only a small portion of fungi has been successfully cultured in a laboratory; approximately 100,000 fungal species have been described, and an estimated 1 million species in our planet are still unexplored or could not be cultured under laboratory conditions[45]. Therefore, it is possible that the palmarumycins-producing fungi could not be cultured under our laboratory conditions.

It is worth noticing that palmarumycins have been isolated from three different plant families; Caesalpiniaceae (*Afzelia bipendensis*)[41], Euphorbiaceae (*Jatropha curcas*)[25] and Ebenaceae (*Diospyros ehretioides*). This is rather unusual for the chemotaxonomic point of view, while certain secondary metabolites could be employed as chemotaxonomic markers in particular plant genus[46-47]. Bipendensin was obtained in very small amounts from the sapwood of *Afzelia bipendensis*[41], while appreciable quantities of palmarumycins were isolated from *Jatropha curcas* [25]. In the present work, palmarumycins JC1 **[70]** and JC2 **[71]** were also obtained in good yields (35 mg and 28 mg, respectively, from 3.6 kg of dried fruits). With evidence from the absence of palmarumycins in the extract of freshly-collected sample, while presence in dried fruit extract, together with the chemotaxonomic point of view, the author proposed that palmarumycins are more likely to be fungal metabolites, i.e., endophytes or epiphytes. Krohn and co-workers[48] isolated preussomerins J, K and L from an endophytic fungus associated with the plant, *Atropa belladonna*. Though, in the present work, fungal growth on a natural fruit substrate may give confusion on the metabolite origin, it is a good caution for phytochemists that special attention on plant preparation is needed for phytochemical study. This concern would extend to

traditional herbal plants which are always used in the form of dried plant materials. Interestingly, from time to time, we have learnt that some traditional herbal recipes inconsistently show their remedial benefits, and consequently we always link this inconsistency with levels of plant metabolites that may be different when growing on different geographical areas. However, from the present study, the presence or absence of associated fungi that may be the genuine producer of active metabolites in the plant sample may be taken into account (e.g., deoxypreussomerins in dried fruits of *Diospyros ehretioides*). Further, for the on-going research on the discovery of novel bioactive compounds, this finding suggested a new approach for searching new fungal metabolites on plant materials that may be deliberately infected by fungi or on those with associated fungi grown on the surface (e.g., fruits of *Diospyros ehretioides*).