

Tyrosinase and 5 α -Reductase Inhibitory Components from *Artocarpus incisus* Tree

清水, 邦義
九州大学大学院農学研究院

<https://doi.org/10.15017/14831>

出版情報：九州大学農学部演習林報告. 82, pp.21-63, 2001-03-28. 九州大学農学部附属演習林
バージョン：
権利関係：



Original Article

Tyrosinase and 5 α -Reductase Inhibitory Components from *Artocarpus incisus* Tree*

Kuniyoshi Shimizu**

Abstract

The inhibitory components on tyrosinase and 5 α -reductase from Papua New Guinean (PNG) and Thai woody plants were investigated.

First, the inhibitory components of *Artocarpus incisus* heartwood which showed the strongest inhibitory activity in 23 PNG wood species, their structure-activity relationships and inhibitory mechanism were evaluated. Tyrosinase inhibitory activity-guided fractionation led to the isolation of seven active compounds including two new compounds, 3,2',4'-trihydroxy-6'',6''-dimethyl-pyrano(3'',2'':4,5)- *trans*- stilbene (artocarbene) and 6-(3''-methyl-1''-butenyl)-5,7,2',4'-tetrahydroxyflavone (isoartocarpesin). The structure-activity relationships suggested that specific natural or synthesized compounds having 4-substituted resorcinol skeleton had potent tyrosinase inhibitory ability. Kinetic studies have indicated that specific compounds having 4-substituted resorcinol skeleton exhibit competitive inhibition of the oxidation of DL-DOPA by mushroom tyrosinase.

Second, 5 α -reductase inhibitory components from PNG and Thai plants were investigated respectively, and their structure-activity relationships were discussed. The methanol extract of heartwood of *A. incisus* showed potent 5 α -reductase inhibitory activity. Chlorophorin and artocarpin showed more potent inhibitory effects (IC_{50} =37 μ M and 85 μ M, respectively) than did α -linolenic acid, which is known as a naturally occurring potent inhibitor. The inhibitory effects of 17 samples prepared from Thai plants on 5 α -reductase activity were examined. The acetone extract of *A. incisus* leaves showed potent 5 α -reductase inhibitory activity. Fractionation guided by 5 α -reductase inhibitory test led to the isolation of 2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone (IC_{50} =38 μ M) and a novel geranylated chalcone, 3'-geranyl-2',3,4,4'-tetrahydroxychalcone (IC_{50} =104 μ M) from the acetone extract of *A. incisus* leaves. Structure-activity relationship suggested that the presence of an isoprene-derived substituent (prenyl and geranyl) would enhance 5 α -reductase inhibitory effects.

Key words : *Artocarpus incisus*, Moraceae, heartwood, artocarbene, tyrosinase inhibitor, melanin biosynthesis inhibitor, isoartocarpesin, 4-substituted resorcinols, structure-activity relationship, 5 α -reductase inhibitor, leaves, 3'-geranyl-2',3,4,4'-tetrahydroxychalcone

* *Artocarpus incisus* 樹木からのチロシナーゼ及び5 α -リダクターゼ阻害成分

** Laboratory of Forest Biology and Biochemistry, Division of Forest Bioscience, Department of Forestry and Forest Products Science, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581 九州大学大学院農学研究院森林資源科学部門森林機能開発学講座森林生物化学研究室

Contents

1. Preface
2. Tyrosinase Inhibitors
 - 2.1. Introduction
 - 2.2. The Inhibitory Components from *Artocarpus incisus* on Melanin Biosynthesis
 - 2.2.1. Experimental
 - 2.2.1.1. Sample woods
 - 2.2.1.2. Extraction of heartwood meals of 23 PNG species
 - 2.2.1.3. Extraction and separation of extractives of *A. incisus*
 - 2.2.1.4. Isolated compounds from *A. incisus*
 - 2.2.1.5. Determination of tyrosinase inhibitory activity
 - 2.2.1.6. Inhibitory effect on melanogenesis using cultured B16 melanoma cells
 - 2.2.1.7. Inhibitory effect on melanogenesis using brown guinea pig
 - 2.2.2. Results and Discussion
 - 2.2.3. Summary
 - 2.3. Inhibition of Tyrosinase by Flavonoids, Stilbenes and Related 4-Substituted Resorcinols: Structure-Activity Relationships
 - 2.3.1. Experimental
 - 2.3.1.1. Materials
 - 2.3.1.2. Enzyme assays
 - 2.3.2. Results and Discussion
 - 2.3.3. Summary
3. 5 α -Reductase Inhibitors
 - 3.1. Introduction
 - 3.2. The 5 α -Reductase Inhibitory Components from Papua New Guinean Woods
 - 3.2.1. Experimental
 - 3.2.1.1. Materials
 - 3.2.1.2. Sample woods
 - 3.2.1.3. Isolated compounds from *A. incisus*
 - 3.2.1.4. Preparation of liver microsomes
 - 3.2.1.5. 5 α -Reductase assay
 - 3.2.2. Results and Discussion
 - 3.2.3. Summary
 - 3.3. The 5 α -Reductase Inhibitory Components from Thailand Plants
 - 3.3.1. Experimental
 - 3.3.1.1. Sample plants
 - 3.3.1.2. 5 α -Reductase assay
 - 3.3.1.3. Extraction of samples
 - 3.3.1.4. Extraction and separation of extractives of *A. incisus*

- 3.3.1.5. Isolated compounds from *A. incisus*
- 3.3.2. Results and Discussion
- 3.3.3. Summary
- 3.4. Structure-Activity Relationships
- 3.4.1. Experimental
- 3.4.2. Results and Discussion
- 3.4.3. Summary
- 4. Conclusion
- 5. References
- 6. Acknowledgement

1. Preface

Woods as found in tree and bushes was of primary importance to ancient humans in their struggle to control their environment. Subsequent evolution through Bronze and Iron Ages up to our present technologically advanced society has hardly diminished the importance of wood. Today, its role as a source of paper products, furniture, building materials, and fuel is still of major significance.

Wood consists of a mixture of polymers, often referred to as lignocellulose. The cellulose microfibrils consist of an immensely strong, linear polymer of glucose. They are associated with smaller, more complex polymers composed of various sugars called hemicelluloses. These polysaccharides are embedded in an amorphous phenylpropane polymer, lignin, creating a remarkably strong composite structure, the lignocellulosic cell wall.

Wood also contains materials that are largely extraneous to this lignocellulosic cell wall. These extracellular substances can range from less than 1 to about 35% of the dry weight of the wood, but the usual range is 2-10%. Among these components are the mineral constituents, salts of calcium, potassium, sodium, and other metals, particularly those present in the soil where the tree is growing. Some of the extraneous components of wood are too insoluble to be extracted by inert solvents and remain to give extractive-free wood its color; very often these are high-molecular-weight polyphenolics. Most of the extraneous components, however, can be removed with neutral, inert organic solvents or water to yield the *extractives*, a mixture of naturally occurring organic compounds.

Woody tissue is capable of synthesizing a wide range of natural products, some of great complexity, sometimes in considerable quantity, and at times, in relatively pure form. Some of them, such as simple carbohydrates, phytosterols, nonalkaloidal nitrogenous compounds, and simple aliphatic, alicyclic, and aromatic compounds, are lignocellulosic precursors or are otherwise involved in the intermediary metabolism of the living cell. Among the more interesting materials, however, are the wide array of compounds that woody tissue has developed to protect itself from animals, insects, fungi, bacteria, and other decay-causing microorganisms.

The products and chemical intermediates derived from these natural products are from renew-

able resources, for example woody plants etc., with consequent advantages over competitive materials derived from petrochemicals. The significance of these compounds, however, does not stop here, for they also have a considerable effect on the utilization of wood. The odor, color, and decay resistance of wood are a function of the extractives.

It is worth noting that almost half of all prescription drugs contain substances of natural origin. Even aspirin was first derived in part from willow bark. Current ethnobotanical studies, especially in the tropics, are of considerable importance for the identification of pharmaceutically effective, native remedies before the tropical forests are decimated, and native herbal practices are replaced by "modern" medicinal practice.

The investigation of bioactive natural products has, in recent years, assumed a greater sense of urgency in response to the expanding human population and its subsequent demands for food, good health, and increasing areas of land on which to live.

Pigmentation is one of most obvious phenotypical characteristics in the natural world. Of the pigments, melanin is one of the most widely distributed pigments found in bacteria, fungi, plants and animals. Melanins are heterogeneous polyphenol-like biopolymers with a complex structure and color varying from yellow to black (Prota, 1988). Their biosynthesis can be observed by someone who leaves the surface of a cut apple, potato or banana exposed to air (Pawelek *et al.*, 1982). The initial reaction in the pathway of melanin formation from tyrosine through the enzymatic oxidation of a monophenol (L-tyrosine) and/or *o*-diphenol [L- β -(3,4-dihydroxyphenyl)alanine: L-DOPA] to its corresponding *o*-quinone is catalyzed by a copper-containing enzyme called tyrosinase (EC 1.14.18.1) (Sánchez-Ferrer *et al.*, 1995), as shown in Fig. 1. Tyrosinase, also known as polyphenol oxidase, is widely distributed in the phylogenetic scale. It is responsible for not only melanization in animals but also browning in plants. The latter case is considered to be deleterious to the color quality of plant-derived foods and beverages. Tyrosinase

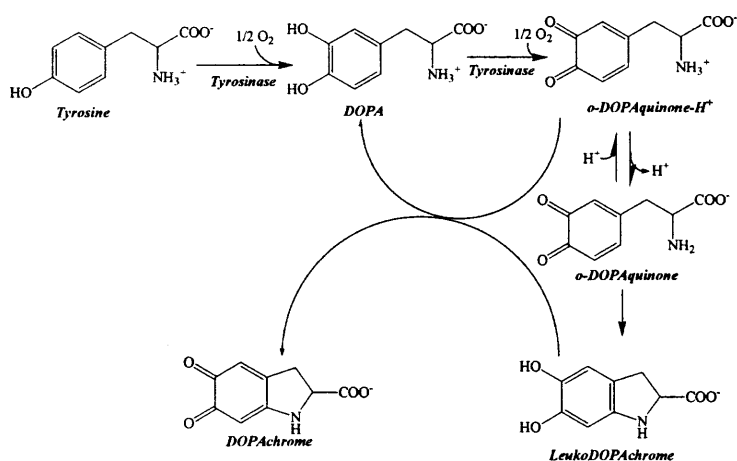


Fig.1 The reaction sequence of the melanin biosynthesis pathway from tyrosine to dopachrome (Garcla-Canovas *et al.*, 1982).

catalyzes the oxidation of phenolic substrates to *o*-quinones, which are then polymerized to brown, red, or black pigments. This unfavorable darkening from enzymatic oxidation generally results in a loss of nutritional value and has been of great concern (Friedman *et al.*, 1996). In addition, tyrosinase inhibitors have become increasingly important in cosmetic (Maeda *et al.*, 1991) and medicinal products in relation to hyperpigmentation. Hence, tyrosinase inhibitors should have broad applications.

Virilization in mammals is mediated by two steroid hormones, testosterone and dihydrotestosterone. 5 α -Reductase catalyzes the NADPH-dependent reduction of a variety of steroids containing 3-keto, $\Delta^{4,5}$ double bond, testosterone to dihydrotestosterone (Fig. 2). Both hormones bind to a typical steroid hormone receptor, the androgen receptor, and activate genes containing androgen-responsive DNA sequences. However, the conversion of testosterone to dihydrotestosterone amplifies the androgenic signal: since dihydrotestosterone cannot be aromatized to estrogen, its effect remains purely androgenic. In addition, dihydrotestosterone binds to the human androgen receptor with greater affinity than testosterone, and the receptor-dihydrotestosterone complex appears to be more stable *in vitro* under conditions that promote transformation of receptor-androgen complexes to the DNA-binding state (Grino *et al.*, 1990). The circulating level of dihydrotestosterone in plasma is 1/10 of the testosterone levels; nevertheless, dihydrotestosterone plays a major role in the development of secondary sex characteristics. Benign prostatic hyperplasia, prostate cancer and certain diseases of the skin may be dependent upon dihydrotestosterone. Acne, idiopathic female hirsutism and male pattern baldness have all been linked to increased 5 α -reductase activity in the areas of the skin effected (Price, 1975; Darley, 1984; Shweikert *et al.*, 1974; Brooks, 1986; Tenover, 1991), suggesting that 5 α -reductase inhibitors may also be useful in the treatment of these common afflictions.

In this work, we discussed the inhibitory components on tyrosinase and 5 α -reductase from tropical woody plants and their structure-activity relationships.

In chapter 2, the components having anti-tyrosinase activity and melanin synthesis inhibitory effects in the heartwood extracts of Papua New Guinean (PNG) wood species were examined by both *in vivo* and *in vitro* experiments. Additionally, structure-activity relationships were discussed from the viewpoints of the 4-substituted resorcinol skeleton.

In chapter 3, the components having 5 α -reductase inhibitory activity in the extracts of PNG and Thai plants were examined and their structure-activity relationships were discussed.

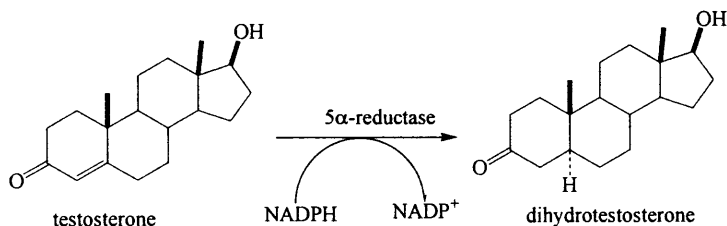


Fig.2 5 α -Reductase catalysed conversion of testosterone to dihydrotestosterone.

2. Tyrosinase Inhibitors

2.1. Introduction

Tropical forest area occupies 40–50 % of the all forest area in the world. The Papua New Guinea (PNG) mainland and surrounding islands possess an extraordinarily rich flora and great diversity of vegetation types that parallel the diverse physiography of lands. A high proportion of the land area has a forest cover, most of it evergreen rain forest. The great diversity of tropical woods appears in qualitative and quantitative diversity of extractives of the woods from a chemical point of view of the wood components. Recently, we have reported an anti-fungal component, angolensin in PNG rosewood (Pilotti *et al.*, 1995). Thus, the tropical woods will give us a great chance to find bioactive components.

As discussed in Preface, melanin biosynthesis inhibitory compounds are useful not only for the material used in cosmetics as skin-whitening agents but also as the remedy for disturbances in pigmentation. The color of mammalian skin and hair is determined by a number of factors. The most important factor is the degree and distribution of melanin pigmentation. Tyrosinase (phenol oxidase) is known to be a key enzyme for melanin biosynthesis (Sánchez-Ferrer *et al.*, 1995) in plants, microorganism and mammalian cells. Therefore, many tyrosinase inhibitors have been reported (Koiso, 1990) and tested as cosmetics and pharmaceuticals to prevent overproduction of melanin in epidermal layers. For example, kojic acid (Cabanès *et al.*, 1994) and arbutin (Maeda *et al.*, 1996) have been utilized as cosmetic agents. Also, tyrosinase is one of the most important key enzymes in the insect molting process (Brunet, 1980) and investigating its inhibitors may be important in finding alternative insect control agents. Furthermore, melanin formation is considered to be deleterious to the color quality of plant-derived food. This broadens the possible use of tyrosinase inhibitors as food additives, in addition to insect control agents and whitening agents. In this chapter, we have focused on the possibility of using tyrosinase inhibitors as whitening agents for cosmetic products. We report here the inhibitory components of *Artocarpus incisus*, which showed the strongest inhibitory activity in 23 PNG wood species and discusses structure-activity relationships and inhibitory mechanism.

2.2. The Inhibitory Components from *Artocarpus incisus* on Melanin Biosynthesis

2.2.1. Experimental

2.2.1.1. Sample woods

The meals of 23 PNG wood species (*Albizia falcataria*, *Alstonia scholaris*, *Amoora* sp., *Anthocephalus chinensis*, *Artocarpus incisus*, *Buchanania* sp., *Calophyllum* sp., *Cananga odorata*, *Canarium indicum*, *Canarium oleosum*, *Dracontomelon dao*, *Dysoxylum peltigrewianum*, *Eucalyptus deglupta*, *Garcinia latissima*, *Hibiscus ellipticifolius*, *Intsia bijuga*, *Neonuclea acuminata*, *Octomeles sumatrana*, *Palaquium galactoxylum*, *Pterocarpus indicus*, *Terminalia* sp., *Toona surenii* and *Xanthophyllum papuanum*) were obtained from PNG Forest Research Institute. A large amount of wood blocks of *A. incisus* was obtained from Okinawa prefecture.

All voucher specimens are preserved at the herbarium of Department of Forest Products, Kyushu University in Japan.

2.2.1.2. Extraction of heartwood meals of 23 PNG species

The heartwood portion was chipped and milled to pass a # 40 screen. The milled heartwood was Soxhlet-extracted with methanol for 10 h.

2.2.1.3. Extraction and separation of extractives of *A. incisus*

Air-dried milled heartwood of *A. incisus* (37 kg) was extracted for 10 days with Et₂O at room temperature and the extract concentrated to dryness. The dry Et₂O extract (330 g) was crystallized successively from Et₂O / hexane and MeOH. The yellow solid deposited (240 g) was collected and recrystallized from MeOH / H₂O yielding compound **1**. The mother liquor (89 g) was separated repeatedly by column chromatography (CC) and medium pressure liquid chromatography (MPLC) on silica gel using ethyl acetate-hexane gradient as eluent. Tyrosinase inhibitory fraction (200mg, TLC, silica gel, ethyl acetate/hexane, 1:1, R_f 0.05-0.4) was obtained. Compound **2** (22 mg), **3** (10 mg), **4** (22 mg), **5** (12 mg), **6** (12 mg), **7** (6 mg), **8** (30 mg) and **9** (13 mg) were isolated from the inhibitory fraction by preparative HPLC (Inertsil PREP-ODS: 20 mm i.d. $\times$ 250 mm) using H₂O-CH₃CN.

2.2.1.4. Isolated compounds from *A. incisus*

All nuclear magnetic resonance (NMR) experiments were performed at 400 MHz. Sample was dissolved in acetone-*d*₆, CD₃OD or DMSO-*d*₆ and chemical shifts were referred to internal TMS for ¹H NMR and to deuterated solvents for ¹³C NMR.

Compound **1** (Sato *et al.*, 1996), **3** (Krohn *et al.*, 1986), **5** (Takasugi *et al.*, 1978), **7** (Mabry *et al.*, 1970a) and **8** (Sato *et al.*, 1996): MS and NMR muched well with published data.

Compound **2**, (+)-dihydromorin, yellow powder; [α]_D²⁰+63.5° (c 0.10, MeOH). CD[θ]_D²⁰ (MeOH, nm): +4905 (260), -9437 (294), +4822 (326). FAB-HR-MS *m/z* : 305.0727 ([M+H]⁺, calcd. for C₁₅H₁₃O₇ : 305.0662). NMR muched well with published data (Schultz *et al.*, 1995).

Compound **4**, (+)-norartocarpanone, pale yellow; [α]_D²⁰+14.5° (c 0.12, MeOH). CD[θ]_D²⁰ (MeOH, nm): +1015 (256), -4355 (292), +592 (330). MS and NMR were identical to published data (Deshpande *et al.*, 1975).

Compound **6**, Artocarbene, yellow powder. ¹H NMR (acetone-*d*₆): δ 1.39 (6H, s, H-6"), 5.60 (1H, *d*, J = 9.76 Hz, H-5"), 6.38 (1H, *dd*, J_{5',6'} = 8.54 Hz, J_{3',5'} = 2.44 Hz, H-5'), 6.44 (1H, *d*, J = 2.44 Hz, H-3'), 6.47 (1H, *d*, J = 1.46 Hz, H-6), 6.60 (1H, *d*, J = 1.46 Hz, H-2), 6.66 (1H, *d*, J = 9.76 Hz, H-4"), 6.89 (1H, *d*, J = 16.36 Hz, H-8'), 7.33 (1H, *d*, J = 16.36 Hz, H-7'), 7.39 (1H, *d*, J = 8.55 Hz, H-6'). ¹³C NMR (400MHz, acetone-*d*₆): δ 28 (C-6"(-Me)), 77 (C-6"), 104 (C-3'), 106 (C-6), 107 (C-2), 108 (C-5'), 109 (C-4), 117 (C-1'), 118 (C-4"), 125 (C-7'), 126 (C-8'), 128 (C-6'), 129 (C-5"), 141 (C-1), 154 (C-3), 155 (C-

5), 157 (C-2'), 160 (C-4'). Positive ion FAB-MS: m/z 311.

Compound **9**, isoartocarpesin, yellow powder; FAB-MS: 355 ($[M+H]^+$). 1H NMR (acetone- d_6): δ 1.04 (6H, *d*, $J = 6.83$ Hz, 4'', 5''-H), 2.39 (1H, *m*, 3''-H), 6.51 (1H, *dd*, $J = 8.55$, 2.45 Hz, 5'-H), 6.54 (1H, *s*, 8-H), 6.56 (1H, *d*, $J = 2.44$ Hz, 3'-H), 6.58 (1H, *dd*, $J = 0.78$, 16.12 Hz, 1''-H), 6.72 (1H, *dd*, $J = 7.08$, 16.12 Hz, 2''-H), 7.03 (1H, *s*, 3-H), 7.77 (1H, *d*, $J = 8.55$ Hz, 6'-H), 14.01 (1H, *s*, 5-OH). ^{13}C NMR (acetone- d_6): δ 23.61 (4'', 5''-C), 34.38 (3''-C), 94.63 (8-C), 104.78 (3'-C), 105.48 (10-C), 108.91 (3-C), 109.60 (5'-C), 109.88 (6-C), 111.17 (1'-C), 117.75 (1''-C), 131.36 (6'-C), 142.40 (2''-C), 157.00 (9-C), 159.72 (2'-C), 161.19 (5-C), 162.65 (7-C), 162.99 (4'-C), 163.09 (2-C), 184.14 (4-C).

2.2.1.5. Determination of tyrosinase inhibitory activity

Tyrosinase inhibitory activity was assayed by the dopachrome method (Hearing, 1987) with modification. Zero point one ml of mushroom tyrosinase solution (1250 U/ml, Wako pure chemical industries, Ltd.), 2.0 ml of L-tyrosine (0.1 %), 0.7 ml of McIlvain buffer (pH 6.8) and 0.2 ml of DMSO with or without sample, were mixed, incubated at 37 °C for 20 minutes, and the absorbance at 475 nm was measured before and after incubation. The relative tyrosinase activity (%) added with each sample was calculated as follows: Tyrosinase activity (%) = $(B-A) / (D-C) \times 100$. A and B represent the absorbance in the presence of sample before and after incubation, respectively. C and D represent the absorbance without sample before and after incubation, respectively. Kojic acid (Tokyo Kasei Kogyo Co., Ltd.) was used as a positive standard.

2.2.1.6. Inhibitory effect on melanogenesis using cultured B16 melanoma cells

Inhibitory effect on melanogenesis using cultured B16 melanoma cells was examined by the method previously reported (Tomita *et al.*, 1990) with modification. Mouse B16 melanoma cells (2.0×10^4 cells/ml) were subcultured in Eagle's minimum essential medium. After 2 days incubation (37 °C, 5% CO₂), the sample dissolved in DMSO (0.5%, 25 μ l) was added to the cultured medium to result in the sample concentration of 100 ppm. The control was added with DMSO without sample. Kojic acid was used as a positive standard. After the incubation was continued for 4 days, the cell suspension was centrifuged at 2000 rpm for 5 minutes. Then the color and volume of resulting cells were compared with control. The whitening degree of the cells was observed with naked eyes.

2.2.1.7. Inhibitory effect on melanogenesis using brown guinea pig

Inhibitory effect on melanogenesis *in vivo* using brown guinea pig was examined by the method previously reported (Imokawa *et al.*, 1986) with modification. Three animals per group were used. The back of brown guinea pig was shaved clean by hair clipper. The back was irradiated with 1 J/cm² (2 mW/cm² $\times$ 500 sec) from UV-B lamp (Toshiba FL40S · BLB) at six times for two weeks, resulting in pigment formation. Then after a week, the back (1 $\times$ 1.5 cm) was applied 12.5 μ l of sample dissolved in DMSO (2%), once a day for 3 weeks. These experiments have been performed twice. The color of resulting back skin was compared with control

(DMSO). The blanching effects were evaluated by colorimeter as brightness, estimated as follow: ΔL (% , brightness) = L (21 days) - L (1 day). The brightness data were analyzed with student's t-test. Differences of $P < 0.05$ were regarded as significant.

2.2.2. Results and Discussion

The methanol extract of *A. incisus* showed the strongest tyrosinase inhibitory activity at the concentration of 10 ppm in 23 species (Table 1). In spite of the crudeness, its activity was almost equivalent to kojic acid known as a potent tyrosinase inhibitor (Cabanés *et al.*, 1994). Therefore, *A. incisus* was selected for the further investigation.

Melanin biosynthesis inhibitory effect of the methanol extract of *A. incisus* was examined by using mouse B16 melanoma cells. The methanol extract of *A. incisus* showed potent inhibitory activity and significant color whitening compared to control. Its activity was almost equivalent to kojic acid used as a positive control. The cytotoxicity of the extract to the cells was not observed at a concentration of 100 ppm (Fig. 3).

Table 1 The effect of methanol extracts from heartwoods of PNG trees on tyrosinase activity.
(Sample concentration: 10 ppm, substrate: L-tyrosine)

Samples	Relative activity(%)
<i>Albizia falcataria</i>	178
<i>Alstonia scholaris</i>	102
<i>Amoora</i> sp.	98
<i>Anthocephalus chinensis</i>	82
<i>Artocarpus incisus</i>	20
<i>Buchanania</i> sp.	118
<i>Calophyllum</i> sp.	84
<i>Cananga adorata</i>	87
<i>Canarium indicum</i>	100
<i>Canarium oleosum</i>	72
<i>Dracontomelon dao</i>	118
<i>Dyosyllum peltigrewianum</i>	68
<i>Eucalyptus deglupta</i>	122
<i>Garcinia latissima</i>	114
<i>Hibiscus ellipticifolius</i>	110
<i>Intsia bijuga</i>	90
<i>Neonuclea acuminata</i>	115
<i>Octomeles smatrana</i>	68
<i>Palaquium galactoxylum</i>	113
<i>Pterocarpus indicus</i>	82
<i>Terminalia</i> sp.	88
<i>Toona surenii</i>	103
<i>Xanthophyllum papuanum</i>	87
Kojic acid	19
DMSO (Control)	100

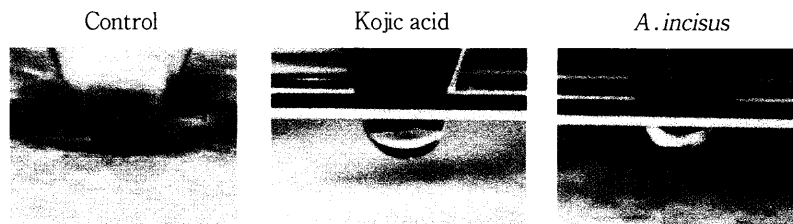


Fig.3 Inhibitory effect of methanol extracts of *A. incisus* and kojic acid on melanin synthesis of B16 melanoma cells at the sample concentration of 100 ppm.

The color of resulting cells was compared with control by naked eyes.

The volume of resulting cells with sample (kojic acid or *A. incisus*) was equivalent to control.

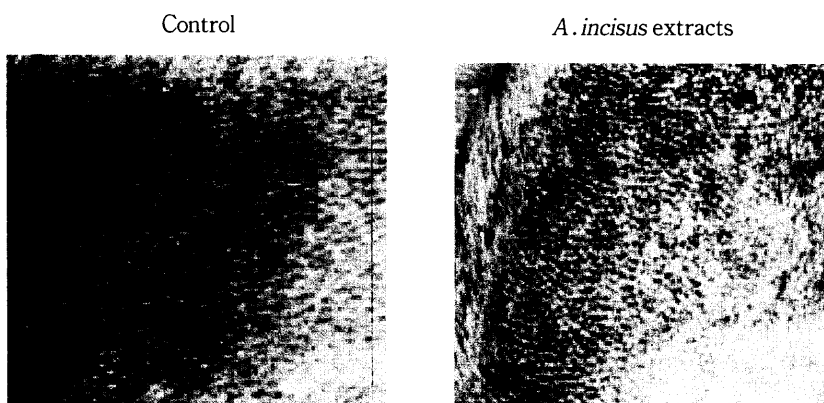


Fig.4 Effect of mother liquor from *A. incisus* on pigmentation induced on the back of brown guinea pigs at the sample concentration of 2% (after 3 weeks).

The application of mother liquor ($\Delta L: 6.9 \pm 1.1\%$) caused lighter color than control ($\Delta L: 4.5 \pm 1.0\%$) ($n=3, P<0.05$).

Melanin biosynthesis inhibitory effect of mother liquor which was obtained by crystallization of ether extracts from *A. incisus* was examined by using brown guinea pig. The back of brown guinea pig was shaved, irradiated with UV-B, leading to form the pigment. The time course of skin color change was observed while applying sample solution at the concentration of 2% to the back, compared with that of control. After 21 days, ΔL was $4.5 \pm 1.0\%$ (mean $\pm$ SD) for the control group and $6.9 \pm 1.1\%$ for the mother liquor group. The application of mother liquor to the skin for three weeks caused higher color lightening than control ($P < 0.05$) (Fig. 4). Neither

inflammation nor red spots on the back skin was observed. These results suggest that extracts of *A. incisus* have high color lightening ability.

To isolate tyrosinase inhibitory components, a large scale extraction was performed. Tyrosinase inhibitory activity-guided fractionation led to the isolation of compound 1 ~ 9 (Fig. 5), although compound 1 and 7 showed no tyrosinase inhibitory activity at the concentration of 10 ppm. Compound 1, 3, 5, 7 and 8 were characterized by their FAB-MS, ^1H and ^{13}C NMR spectra by comparison with data in literature, and identified as artocarpin (Sato *et al.*, 1996),

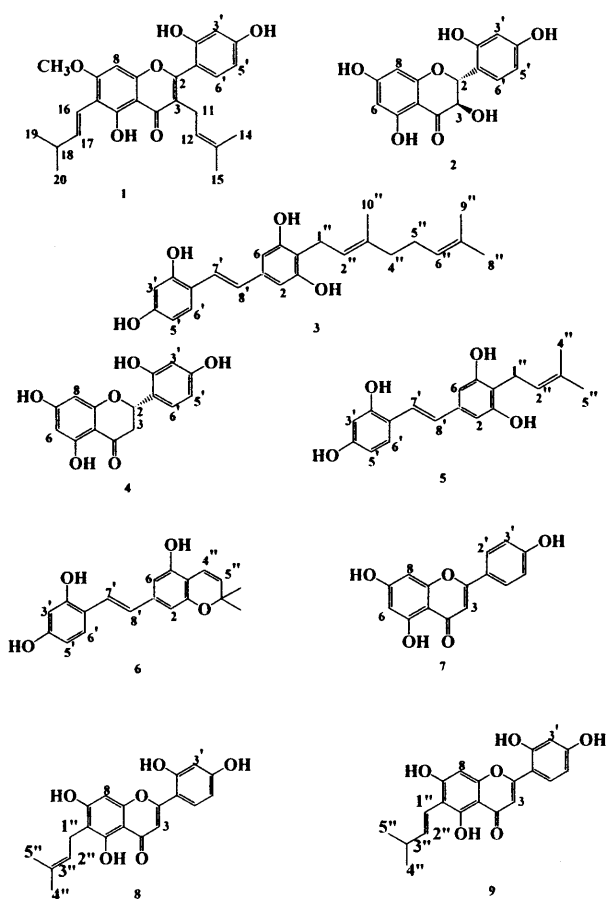


Fig.5 The chemical structures of compound 1-9.

chlorophorin (Krohn *et al.*, 1986), 4-prenyloxyresveratrol (Takasugi *et al.*, 1978), apigenin (Mabry *et al.*, 1970a) and artocarpesin (Sato *et al.*, 1996), respectively.

Compound **2** was characterized as (+)-dihydromorin by comparison with data in literature (Schultz *et al.*, 1995). The CD spectrum $\{[\theta]^{20}(\text{MeOH}, \text{nm}): +4905(260), -9437(294), +4822(326)\}$ was characteristic of 2*R*, 3*R*-dihydroflavonol (Kasai *et al.*, 1988). Therefore, compound **2** was identified as (2*R*, 3*R*)-5,7,2',4'-tetrahydroxydihydroflavonol.

Compound **4** was characterized as (+)-norartocarpanone by comparison with published data (Deshpande *et al.*, 1975). The CD spectrum $\{[\theta]^{20}(\text{MeOH}, \text{nm}): +1015(256), -4355(292), +592(330)\}$ suggested the configuration at C-2 to be *S* (Gaeffield, 1970). Therefore, compound **4** was identified as (2*S*)-5,7,2',4'-tetrahydroxyflavanone.

Compound **6** was identified as follows. Compound **6** was obtained as a yellow powder. The ^{13}C NMR spectrum of **6** revealed 19 carbon atoms (DEPT: 8×C, 9×CH and 2×Me). The FAB mass spectral data ($[\text{M}+\text{H}]^+=311$) together with the ^{13}C NMR data suggested the molecular formula to be $\text{C}_{19}\text{H}_{18}\text{O}_4$. The ^1H NMR spectrum of **6** suggested the presence of a *trans* disubstituted double bond flanked by quaternary carbons based on the presence of two doublet olefinic proton resonances at δ 7.33 ($J=16.36$ Hz) and δ 6.89 ($J=16.36$ Hz). Two one-proton doublets at δ 6.60 ($J=1.46$ Hz) and 6.47 ($J=1.46$ Hz) in the ^1H NMR spectrum suggested that the A ring had a trisubstitution pattern. Three aromatic protons on the B ring revealed a clear ABX system [δ 6.38 (*dd*, $J=8.54, 2.44$ Hz), δ 6.44 (*d*, $J=2.44$ Hz) and δ 7.39 (*d*, $J=8.55$ Hz)] typical of substitutions at the 2'-and 4'-positions on the B ring. The presence of a dimethylchromene ring [δ 5.60, 6.66 (*d*, $J=9.76$ Hz) assigned to the *cis*-olefinic protons and 1.29 (2×Me)] was indicated from the ^1H NMR spectrum. ^1H - ^1H COSY relationships permitted the assignment of aromatic proton chemical shifts and also supported the substitution pattern. HMQC data were used to assign carbon resonances to their attached protons. In the HMBC spectrum, the olefinic carbon at δ 126 (C-8') was correlated with the aromatic protons at δ 6.60 (H-2) and δ 6.47 (H-6). Moreover, the aromatic proton at δ 7.39 (H-6') caused a cross-peak with the olefinic carbon at δ 125, identified as C-7'. In the NOESY spectrum, NOE enhancement between 6''(-Me) protons at δ 1.39 and the olefinic proton at δ 5.60 identified the latter as H-5''. Full proof of the skeleton of **6** was obtained by a combination of HMBC, DEPT, ^1H - ^1H COSY, NOESY and HMQC experiments. Therefore, **6** was elucidated as a novel 3,2',4'-trihydroxy-6'',6''-dimethyl-pyrano(3'',2'':4,5)-*trans*-stilbene, which the author gives the trivial name "artocar-bene".

Stilbene with dimethylchromene ring is rare structure in plant. To the best of our knowledge, this is the first report of the occurrence of a stilbene with dimethylchromene ring in the Moraceae. Similar structural type had been found in the Leguminosae (Garcia *et al.*, 1986). Stilbenes are found in many plant genera, and some are considered to be phytoalexins (Brinker *et al.*, 1991). They exhibit a variety of biological and pharmacological activities including protein tyrosine kinase (PTK) and protein kinase C (PKC) inhibitory effects (Jayatilake *et al.*, 1994).

Compound **9** was identified as follow. The ^{13}C NMR spectrum revealed 20 carbon atoms (DEPT: 10×C, 8×CH and 2×Me). The FAB mass spectral data ($[\text{M}+\text{H}]^+=355$) together with

the ^{13}C NMR data suggested the molecular formula to be $\text{C}_{20}\text{H}_{18}\text{O}_6$. The ^1H NMR spectrum showed one set of 3-methyl-1-butenyl signals at δ 1.04 (6H, *d*, J = 6.83 Hz, $\text{Me} \times 2$), 2.39 (1H, *m*, $^{\text{H}}\text{>C}<$), 6.58 (1H, *dd*, J = 0.78, 16.12 Hz, $=\text{CH}-\text{Ph}$), and 6.72 (1H, *dd*, J = 7.08, 16.12 Hz, $\text{CH}=\text{}$) together with one chelated phenolic signal at δ 14.01 (1H, *s*, C-5 OH). The ABC type coupled aromatic proton signals appeared at δ 6.51 (1H, *dd*, J = 2.45, 8.55 Hz), 6.56 (1H, *d*, J = 2.44 Hz) and 7.77 (1H, *d*, J = 8.55) were reasonably assigned to the H-5', H-3', and H-6' of a 2', 4'-dioxxygenated flavone, respectively. The other two aromatic singlets at δ 7.03 and 6.54 were assigned as the H-3 and H-6 or H-8 signals of a flavone as in artocarpetin B (Chung *et al.*, 1995). The ^{13}C NMR spectrum provided signals due to a 3-methyl-1-butenyl moiety: δ 23.61 ($\text{CH}_3 \times 2$), 34.38 ($^{\text{H}}\text{>C}<$), 117.75, 142.40 ($-\text{CH}=\text{CH}-$) (Hano *et al.*, 1990). COLOC data were analyzed in order to clarify the location of the 3-methyl-1-butenyl group and to assign all NMR signals. H-1" showed correlation with C-5 (δ 161.19) and C-7 (δ 162.65). In addition, δ 6.54 (H-8) was correlated to the carbons of C-7 (δ 162.65), C-9 (δ 157.00) and C-10 (δ 105.48). These spectral data indicated that the 3-methyl-1-butenyl group should be substituted at C-6. $^1\text{H}-^1\text{H}$ COSY relationships permitted the assignment of aromatic proton chemical shifts and also supported the substitution pattern. $^{13}\text{C}-^1\text{H}$ COSY data were used to assign carbon resonances to their attached protons. Full proof of the skeleton of **9** was obtained by a combination of COLOC, DEPT, $^1\text{H}-^1\text{H}$ COSY and $^{13}\text{C}-^1\text{H}$ COSY experiments. Therefore, **9** was elucidated as a novel 6-(3"-methyl-1"-butenyl)-5,7,2',4'-tetrahydroxyflavone, which the author gives the trivial name 'isoartocarpesin'.

The genus *Artocarpus* consists of 50 or more species of evergreen and deciduous trees belonging to the Moraceae. The heartwood of *Artocarpus* species is a rich source of flavones with isoprenoid substituents. In particular, artocarpin was most readily available because it crystallized out of the hexane extract of some timber samples (Harborne *et al.*, 1975). Also, *A. incisus* is grown for breadfruit. Studies of flavonoids in *Artocarpus* species have shown the presence of 3-methyl-1-butenyl substituted flavones, for example compound **1** (artocarpin), **4** ((+)-norartocarpin), **8** (artocarpesin) and cycloartocarpin (Venkataraman, 1972). Compared to the prenylated flavones, fewer 3-methyl-1-butenylated flavones such as a novel compound **9** have been reported in the Moraceae.

The concentration of 50 % inhibition (IC_{50}) of each sample on tyrosinase activity was compared with kojic acid (Table 2). Compound **1** (artocarpin) and **7** (apigenin) did not show potent tyrosinase inhibitory activity. The others showed potent tyrosinase inhibitory activity. Especially compound **3** (chlorophorin), **4** ((+)-norartocarpinone), **5** (4-prenyloxyresveratrol) and **6** (artocarpene) showed much higher tyrosinase inhibitory activity than kojic acid.

This is the first report of melanin synthesis inhibitory effects of *A. incisus* heartwood extracts. *A. incisus* extracts apparently inhibited not only tyrosinase activity but also melanin biosynthesis of cultured B-16 melanoma cells without any cytotoxicity. Furthermore, the extracts showed distinct whitening of back of brown guinea pig without skin irritation. Thus, the potentiality of *A. incisus* extracts both as material of a useful skin whitening agent and as a remedy for the disturbances in pigmentation is evident. The use in cosmetics and drugs is possible, if safety is

Table 2 The comparison of IC₅₀ of each compound on tyrosinase activity.

Compound	IC ₅₀ (μ M)
1	>228
2	25
3	0.26
4	1.76
5	0.66
6	2.45
7	>185
8	13.5
9	21.1
Kojic acid	8.66

guaranteed. In this study, we isolated seven potent tyrosinase inhibitory components including two new compounds. These compounds were (+)-dihydromorin, chlorophorin, (+)-norartocarpanone, 4-prenyloxyresveratrol, artocarpene, artocarpesin and isoartocarpesin. These compounds were likely the cause for melanin biosynthesis inhibitory effects.

2.2.3. Summary

The inhibitory effects of methanol extracts of heartwood of 23 PNG wood species on tyrosinase activity were examined. The extract of *A. incisus* showed the strongest tyrosinase inhibitory activity which was equivalent to kojic acid. The extract apparently inhibited melanin biosynthesis of cultured B16 melanoma cells without any cytotoxicity and back of brown guinea pig without skin irritation. Thus, the potentiality of the extracts of heartwood of *A. incisus* both as material of a useful skin whitening agent and as a remedy for the disturbances in pigmentation is evident. Tyrosinase inhibitory activity-guided fractionation led to the isolation of seven active compounds including two new compounds which have been characterized as 3,2',4'-trihydroxy-6'',6''-dimethyl-pyrano(3'',2'':4,5)-*trans*-stilbene, named artocarpene and 6-(3''-methyl-1''-butenyl)-5,7,2',4'-tetrahydroxyflavone, named isoartocarpesin. Other active compounds were (+)-dihydromorin, chlorophorin, (+)-norartocarpanone, 4-prenyloxyresveratrol, artocarpene and artocarpesin. These compounds likely caused melanin biosynthesis inhibitory effects.

2.3. Inhibition of Tyrosinase by Flavonoids, Stilbenes and Related 4-Substituted Resorcinols: Structure-activity relationships

2.3.1. Experimental

2.3.1.1. Materials

The compounds [(-)-pinocembrin (2) (Bick *et al.*, 1972), (+)-aromadendrin (4) (Janes *et al.*, 1960), ($\pm$)-fustin (5) (Imamura *et al.*, 1967), ($\pm$)-taxifolin (6) (Kondo, 1951), (+)-

dihydromyricetin (**2**) (Miller *et al.*, 1979), chrysin (**11**) (Harborne *et al.*, 1982), kaempferol (**13**) (Schultz *et al.*, 1995), quercetin (**14**) (Imamura *et al.*, 1967), myricetin (**15**) (Mabry *et al.*, 1970b), pinosylvin (**21**) (Schultz *et al.*, 1992), oxyresveratrol (**22**) (Malan *et al.*, 1988) and bis(2,4-dihydroxyphenyl)methane (**34**) (Kim *et al.*, 1993) were provided by the Laboratory of Wood Chemistry, Department of Forest Products, Faculty of Agriculture, Kyushu University in Japan, and their purities and identification had been confirmed by comparison with references. The following reagents were purchased: [($\pm$)-flavanone (**1**), flavone (**10**), 2,4-dihydroxybenzaldehyde (**26**), 2,4-dihydroxy-N-(2-hydroxyethyl)benzamide (**29**), 2,4-dihydroxybenzophenone (**30**), 4-hexylresorcinol (**38**) and 4-dodecylresorcinol (**39**) from Aldrich Chem. Co.], [($\pm$)-naringenin (**3**) and morin (**17**) from Sigma Chem. Co.], [2,4-dihydroxyacetophenone (**27**), 2,4-dihydroxybenzoic acid (**28**), resorcinol (**32**), L-tyrosine and DL- β -(3,4-dihydroxyphenyl) alanine (DL-DOPA) from Wako Pure Chemical Industries, Ltd.], [4-(2-pyridylazo) resorcinol (**31**), 4-(2-thiazolylazo) resorcinol (**33**) from Dojindo Laboratories] and [4-chlororesorcinol (**35**), 4-ethylresorcinol (**40**) from Tokyo Kasei Kogyo Co., Ltd.]. The reagents (+)-dihydromorin (**8**), (+)-norartocarpanone (**9**), apigenin (**12**), artocarpin (**16**), artocarpesin (**18**), isoartocarpesin (**19**), 4-prenyloxyresveratrol (**23**), chlorophorin (**24**), artocarbene (**25**) (Chapter 2) and (-)-angolensin (**20**) (Pilotti *et al.*, 1995) were isolated previously.

4-Methylresorcinol (**36**), 4-(phenylmethyl) resorcinol (**37**) and 4-propylresorcinol (**41**) were prepared by reduction of **26**, **30** and 2', 4'-dihydroxypropiophenone (Aldrich Chem. Co.) with NaBCH₃CN (Aldrich Chem. Co.), respectively. EIMS, m/z: 36(M⁺: 124), 37(M⁺: 200), 41(M⁺: 152).

2.3.1.2. Enzyme assays

Mushroom tyrosinase [EC 1. 14. 18. 1] activity was determined by using L-tyrosine or DL-DOPA as the substrate. L-Tyrosine oxidation assay was done as described in chapter 2.2.. DL-DOPA oxidation assay of 0.1 ml of mushroom tyrosinase solution (625 U/ml, Wako Pure Chemical Industries, Ltd.), 0.7 ml of DL-DOPA buffer solution (2.0 mM), 0.1 ml of McIlvain buffer (pH 6.8) and 0.1 ml of DMSO with or without sample were mixed and incubated at 25 °C. A control reaction was conducted without the test sample. The absorbance was measured at 475 nm before and after incubation. The percentage of inhibition of tyrosinase was calculated as follows: tyrosinase inhibition (%) = (A-B) / A $\times$ 100, where A represents the difference in the absorbance of the control sample between the incubation time of 0.35 and 0.45 min, and B represents the difference in the absorbance of the test sample between the incubation time of 0.35 and 0.45 min. The results were from the three concurrent readings and each S.D. was usually within 2% of the mean. Kojic acid (Tokyo Kasei Kogyo Co., Ltd.) was used as a positive standard.

2.3.2. Results and Discussion

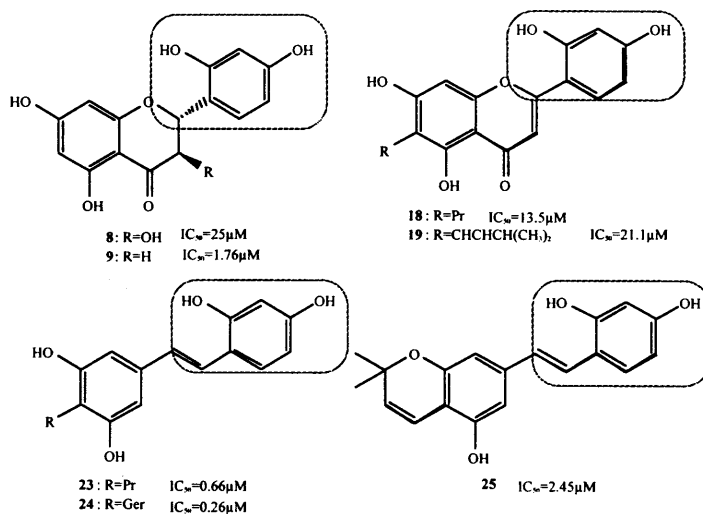
To study structure-activity relationships, several flavonoids and stilbenes were tested for their inhibitory activity on tyrosinase (substrate: L-tyrosine) by measuring the concentration required

Table 3 Inhibitory activity of flavonoids and stilbenes oil tyrosinase (substrate: L-tyrosine).

No	name	R3	R5	R6	R7	R2'	R3'	R4'	R5'	(C2, C3)	IC ₅₀ (μM)
1	(±)-flavanone	H	H	H	H	H	H	H	H	2S,2R	>200
2	(-)-pinocembrin	H	OH	H	OH	H	H	H	H	2S	>200
3	(±)-naringenin	H	OH	H	OH	H	H	OH	H	2S,2R	>200
4	(+)-aromadendrin	OH	OH	H	OH	H	H	OH	H	(2R,3R)	lag time decrease ^a
5	(±)-fustin	OH	H	H	OH	H	OH	OH	H	(2R,3R), (2S,3S)	lag time decrease ^a
6	(±)-taxifolin	OH	OH	H	OH	H	OH	OH	H	(2R,3R), (2S,3S)	lag time decrease ^a
7	(+)-dihydromyricetin	OH	OH	H	OH	H	OH	OH	OH	(2R,3R)	lag time decrease ^a
8	(+)-dihydromorin	OH	OH	H	OH	OH	H	OH	H	(2R,3R)	25 ^b
9	(+)-norartocarpanone	H	OH	H	OH	OH	H	OH	H	2S	1.76 ^b
10	flavone	H	H	H	H	H	H	H	H		>200
11	chrysin	H	OH	H	OH	H	H	H	H		>200
12	apigenin	H	OH	H	OH	H	H	OH	H		>185 ^b
13	kaempferol	OH	OH	H	OH	H	H	OH	H		103
14	quercetin	OH	OH	H	OH	H	OH	OH	H		lag time decrease ^a
15	myricetin	OH	OH	H	OH	H	OH	OH	OH		lag time decrease ^a
16	artocarpin	Pr ^d	OH	CHCHCH(CH ₃) ₂	OCH ₃	OH	H	OH	H		>228 ^b
17	morin	OH	OH	H	OH	OH	H	OH	H		>330
18	artocarpesin	H	OH	Pr ^d	OH	OH	H	OH	H		13.5 ^b
19	isoartocarpesin	H	OH	CHCHCH(CH ₃) ₂	OH	OH	H	OH	H		21.1 ^b
20	(-)-angolensin										>200
		R3	R4	R5		R2'	R4'				
21	pinosylvin	OH	H	OH		H	H				>46
22	oxyresveratrol	OH	H	OH		OH	OH				0.98
23	4-prenyloxyresveratrol	OH	Pr ^d	OH		OH	OH				0.66 ^b
24	chlorophorin	OH	Ger ^c	OH		OH	OH				0.26 ^b
25	artocarbene ^c										2.45 ^b

^a Means promotion effect which could act as cofactor like diphenol (Sánchez-Ferrer *et al.*, 1995).

^b Obtained from Table 2. ^c See Fig. 8. ^d Pr^d prenyl. ^cGer:geranyl.

Fig. 6 The chemical structures and IC₅₀ of active components from *A. incisus*.

The boxed part: 4-substituted resorcinol skeleton.

Pr: prenyl.

Ger: geranyl.

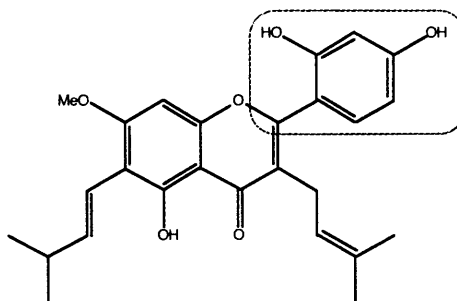


Fig. 7 The chemical structure of **16**.
The boxed part: 4-substituted resorcinol skeleton.

to 50% inhibition of this enzyme activity (IC_{50}). The nine compounds (**8**, **9**, **12**, **16**, **18**, **19**, **23**, **24** and **25**) isolated from *A. incisus* were examined for their inhibitory activity to mushroom tyrosinase in chapter 2.2.. Among these compounds, seven compounds, without **12** and **16**, exhibited potent tyrosinase inhibitory activity [Table 3, Kojic acid (positive standard, substrate: L-tyrosine): $IC_{50}=8.66 \mu M$]. Interestingly, the tyrosinase inhibitory compounds from *A. incisus* had 4-substituted resorcinol as a common skeleton (Fig. 6). This brief structure-activity relationship could mean that the 4-substituted resorcinol skeleton is important for revealing the tyrosinase inhibitory activity. In addition, it should be noted that artocarpin (**16**) did not show inhibitory activity, in spite of having 4-substituted resorcinol skeleton at ring B (Fig. 7). Therefore, to clarify which substructure is important to reveal the tyrosinase inhibitory effect, further structure-activity relationships were examined in detail. The test compounds were flavonoids and stilbenes isolated from various plants, synthesized or commercially available. The results were summarized in Table 3 and Fig. 8.

Among five stilbenes (**21**-**25**), four (**22**-**25**) having 4-substituted resorcinol skeleton showed potent tyrosinase inhibitory activities, but one (**21**) did not. These results can be explained by the fact that hydroxylation of **21**, resulting in **22**, increases its inhibitory activity dramatically. Also, the addition of isoprenyl chain (prenyl or geranyl) to the stilbenes having 4-substituted resorcinol skeleton slightly increased their inhibitory activities (**22**: $IC_{50} = 0.98 \mu M \rightarrow$ **23**: $IC_{50} = 0.66 \mu M \rightarrow$ **24**: $IC_{50} = 0.26 \mu M$). Resveratrol (3,4',5-trihydroxystilbene), 3,5-dihydroxy-4'-methoxystilbene, 3,4'-dimethoxy-5-hydroxystilbene, trimethylresveratrol and piceid (4- α - β -D-glucosyl resveratrol) showed much less inhibitory effect than oxyresveratrol (**22**) on dopa oxidase activity of mushroom tyrosinase (Shin *et al.*, 1998). Therefore, in the case of stilbenes, the 4-substituted resorcinol skeleton must be the most important feature for revealing potent tyrosinase inhibition.

Among 20 flavonoids, only four flavonoids (**8**, **9**, **18** and **19**), which have 4-substituted resorcinol skeleton at ring B, showed potent tyrosinase inhibitory activity. Glabridin (one of the isoflavans) (Yokota *et al.*, 1998), kurarinone (flavanone), kushenol N (dihydroflavonol), kosamol A (dihydroflavonol) (Lee *et al.*, 1997) and 5-(3-(2,4-dihydroxyphenyl)propyl)-3,4-bis(3-

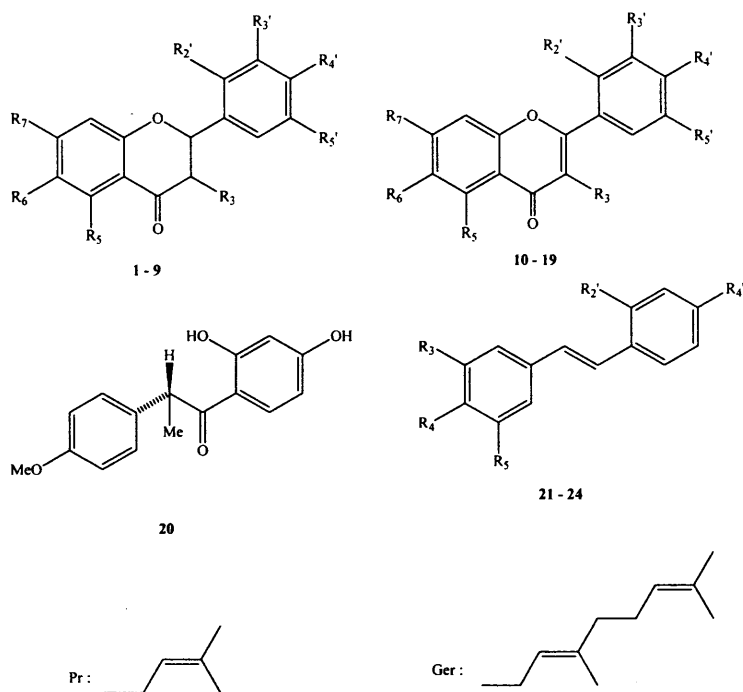


Fig. 8 The chemical structure of 1-24.

methyl-2-butenyl)-1,2-benzenediol (1,3-diphenylpropane derivative) (Jang *et al.*, 1997) were reported as potent tyrosinase inhibitors that have a common 4-substituted resorcinol skeleton. In contrast, **16**, **17** and **20** did not show tyrosinase inhibitory activity, in spite of having 4-substituted resorcinol skeleton at ring B. These results indicate that for flavonoids not only a 4-substituted resorcinol skeleton but also additional structural factors are necessary to reveal tyrosinase inhibitory activity.

In the case of flavonoids having a 4-substituted resorcinol skeleton, except for **20** (which belongs to α -methyldeoxybenzoins), the flavanone type compounds (flavanones and their C-3 substituted derivatives) were more potent inhibitors than were the flavone type compounds (flavones and their C-3 substituted derivatives), e.g. **8** showed a stronger inhibitory effect than did the corresponding flavone **17**. Introduction of a C-3 substituent to the flavanone (**9** $\rightarrow$ **8**) and flavone type (**18** and **19** $\rightarrow$ **16** and **17**) dramatically decreased their activity. Thus, even in flavonoids having 4-substituted resorcinol skeleton, introduction of a C-3 substituent decreased inhibitory activity, probably because of its steric hindrance (Fig. 9).

Compound **20** did not show tyrosinase inhibitory activity, in spite of having a 4-substituted resorcinol skeleton. To clarify which substructure causes inactivity of **20**, the author examined the effects of different C-4 substituents on the tyrosinase inhibitory activity of 4-substituted resorcinols (Table 4). Table 4 demonstrates the powerful influence of the C-4 substituent on the potency of these compounds. Surprisingly, introduction of a carbonyl substituent (**26-30**)

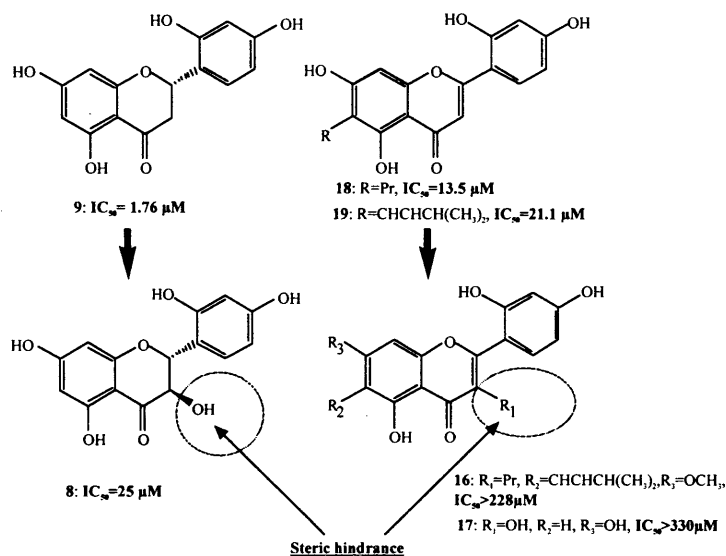
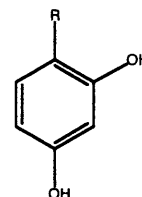


Fig.9 The effect of the introduction of C3 substituent of flavonoids which have 4-substituted resorcinol skeleton on tyrosinase (substrate: L-tyrosine).
Pt: prenyl.

Table 4 Inhibitory activity of 4-substituted resorcinols on tyrosinase (substrate: L-tyrosine)

No	substituent (R)	IC ₅₀ (μM)
26	CHO	>200
27	COCH ₃	>200
28	COOH	>200
29	CONHCH ₂ CH ₂ OH	>200
30	COC ₆ H ₅	>200
31		436
32	H	227
33		185
34		58.0
35	Cl	13.0
36	CH ₃	12.0
37	CH ₂ C ₆ H ₅	2.80
38	CH ₂ (CH ₂) ₄ CH ₃	1.98
39	CH ₂ (CH ₂) ₁₀ CH ₃	1.63
40	CH ₂ CH ₃	1.10
41	CH ₂ CH ₂ CH ₃	0.91



decreased inhibitory activity dramatically. Also, compounds having an azo substituent (**31**, **33**) showed much less inhibitory activity than did **22-25**, in spite of having similar shape to stilbenes concerning the double bond. Both the carbonyl groups at benzyl position and azo substituents are possible to form an intramolecular hydrogen bond with their ortho-hydroxyl group. This intramolecular hydrogen bonding may inhibit the hydroxyl group to bind the enzyme, and therefore, appears to cause inactivation of compounds having 4-substituted resorcinol skeleton. Introductions of chlorine (**35**), alkyl (**36-41**) or phenyl methyl (**34**) substituents at C-4 showed potent inhibitory activities. The non-substituted resorcinol **32** did not show potent inhibitory activity.

Kinetic studies were carried out with the five active compounds (**8**, **9**, **23-25**) from *A. incisus*, as well as the related compounds (**22**, **32** and **40**). The Lineweaver-Burk plot of **23** for DL-DOPA as a substrate is shown in Fig. 10. The mode of inhibition of tyrosinase by **23** was competitive. In addition, similar results were given by **9**, **22**, **24**, **25** and **40** (Table 5). Compounds **8** and **32** did not show typical inhibitory patterns. Interestingly, these compounds (**8** and **32**) exhibited some stimulatory activity to the enzyme at low concentration, similar to a previous report (Kubo *et al.*, 1994). The results obtained so far suggest that (a) **8** and corresponding flavanone **9** not possessing a C-3 hydroxyl group affect mushroom tyrosinase in different ways, and that (b) **32** and corresponding 4-substituted resorcinols (**9**, **22-25** and **40**) affect mushroom tyrosinase in different ways. However, further work is needed to clarify the inhibitory mechanism of **8** and **32**.

Thus, C-4-substituent of resorcinol derivatives and C-3-substituent of flavonoids that have 2',4'-dihydroxyphenyl skeleton seem to significantly affect tyrosinase activity.

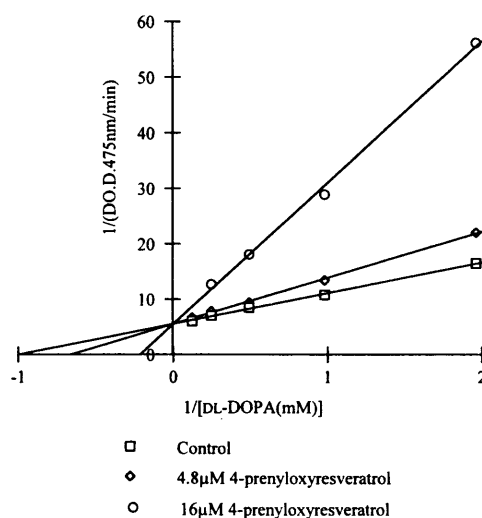


Fig.10 Lineweaver-Burk plots of mushroom tyrosinase and DL-DOPA in the absence or presence of 4-prenyloxyresveratrol (**23**).

Table 5 The tyrosinase inhibitory effects of representative 4-substituted resorcinols tested in reaction using DL-DOPA as a substrate

Compound	IC ₅₀ (μ M)	Ki (μ M)	Type of inhibition
9	90.4	47.8	Competitive
22	20.8	9.24	Competitive
23	17.6	8.70	Competitive
24	19.2	13.4	Competitive
25	6.35	8.49	Competitive
40	3.80	5.39	Competitive
Kojic acid	17.2	11.8	Mix

The tyrosinase inhibitory effects (IC₅₀, Ki and inhibition type) of representative 4-substituted resorcinols using DL-DOPA as a substrate are shown in Table 5. Compound **40** showed stronger inhibitory activity than that of kojic acid, using both L-tyrosine and DL-DOPA as a substrate but the inhibitory effect of **9** and **22-24** were weaker than that of kojic acid using DL-DOPA as a substrate, in spite of showing much stronger inhibitory activity using L-tyrosine as a substrate. Thus, the order of inhibitory effects of these compounds having 4-substituted resorcinol skeleton were different depending on whether L-tyrosine or DL-DOPA was used as a substrate, in comparison with kojic acid. However, it should be noted that these results had been obtained by a simple colorimetric assay method, not by a polarographic assay.

Oxyresveratrol (**22**) showed competitive inhibitory type in this study, although it (**22**) was recently reported as a noncompetitive inhibitor on mushroom tyrosinase with L-DOPA as a substrate (Shin *et al.*, 1998). The difference may be explained as follows. It was reported recently that 4-substituted resorcinols such as 4-ethylresorcinol, 4-hexylresorcinol and 4-dodecylresorcinol could be classified as slow-binding competitive inhibitors of mushroom tyrosinase (Jiménez *et al.*, 1997). Therefore, the difference in the inhibitory type of oxyresveratrol against tyrosinase between us and Shin *et al.*, seems to be due to estimated tyrosinase inhibitory activity by different limited reaction times. To characterize the behavior of these inhibitors completely, a further kinetic study must be needed in order to determine the kinetic parameters (K_1 , K'_1 and k_6) according to Jiménez *et al.* However, in this study, the results of IC₅₀ by using assays with limited reaction time are worthy and valid parameter for understanding the structure-activity relationships.

Some compounds from the moraceous plants have exhibited interesting biological activity (Nomura *et al.*, 1998). It has been reported that some flavonoids isolated from *Artocarpus* species possess inhibitory effects on K⁺-dependent amino acid transport (Parenti *et al.*, 1998), arachidonate 5-lipoxygenase and mouse TNF- α release, cytotoxicity, antiplatelet activity and antibacterial activity against cariogenic bacteria (Nomura *et al.*, 1998). Thus the *Artocarpus* plants are important medicinal resources. In this study, we found a new facet of the biological activity of the *Artocarpus* plant, tyrosinase inhibitory activity.

Some 4-position substituted resorcinols have been reported as inhibitors of enzymatic (polyphenol oxidase) browning in food and beverages (McEvily *et al.*, 1992). However, their structure-activity relationships have been poorly understood. Therefore, our identification of specific compounds having 4-position substituted resorcinol skeleton as potent inhibitors, as outlined above, and the notion that hydrophobic and less bulky substituents were important for controlling the tyrosinase inhibitory effect, may lead to the design and discovery of new tyrosinase inhibitors (Fig. 11). The natural products and synthesized chemicals having 4-substituted resorcinol skeleton should be reinvestigated with regard to their roles as tyrosinase inhibitors. Furthermore, from the chemotaxonomic point of view, specific extracts of plants known as having flavonoids, stilbenes or other types with 4-substituted resorcinol skeleton, for example Moraceae (Jang *et al.*, 1997) or Leguminosae (Yokota *et al.*, 1998; Lee *et al.*, 1997), are candidates for tyrosinase inhibitory materials. Finally it should be noted that these compounds not only inhibit the tyrosinase but also have other properties, such as antioxidant, antimutagen and cancer chemopreventive activities exhibited by resveratrol derivatives (Jang *et al.*, 1997).

2.3.3. Summary

Several flavonoids, stilbenes and related 4-substituted resorcinols, obtained from *A. incisus* and other plants or synthesized, were tested for their inhibitory activity against tyrosinase. The structure-activity relationships suggested that specific natural or synthesized compounds having 4-substituted resorcinol skeleton have potent tyrosinase inhibitory ability. Kinetic studies have

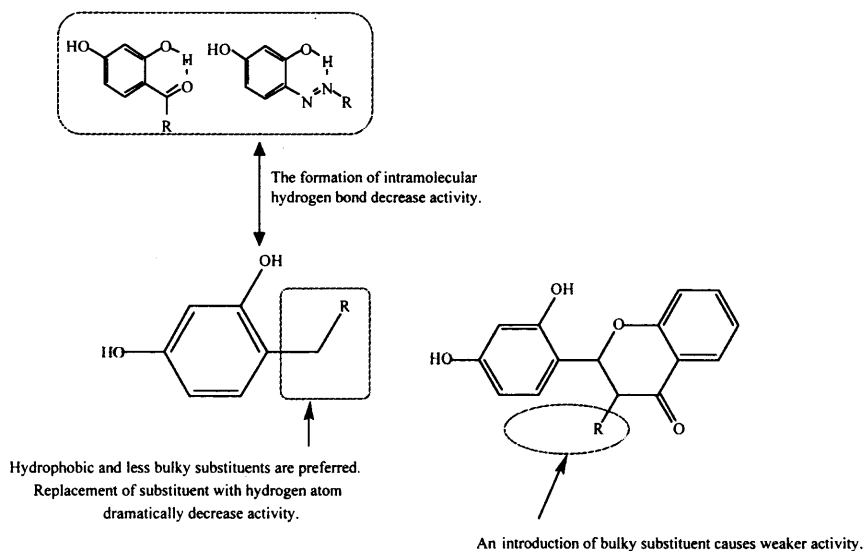


Fig. 11 Summarized structure- activity relationships of compounds having 4-substituted resorcinol skelton.

indicated that such specific compounds exhibit competitive inhibition of the oxidation of DL-DOPA by mushroom tyrosinase. These findings could lead to the design and discovery of new tyrosinase inhibitors.

3. 5 α -Reductase Inhibitors

3.1. Introduction

Δ^4 -3-Oxo-steroid 5 α -oxidoreductase (EC 1.3.99.5; 5 α -reductase) is present in many androgen-sensitive tissues such as the prostate and seminal vesicles; it converts testosterone to a more potent androgen, 5 α -dihydrotestosterone (Anderson *et al.*, 1968; Bruchovsky *et al.*, 1968), which then binds to androgen receptor to exert its biological function (Liao *et al.*, 1989). Inhibition of 5 α -reductase would limit the availability of 5 α -dihydrotestosterone, therefore, 5 α -reductase inhibitors would be useful in selective treatment of androgen-dependent abnormalities, such as benign prostate hyperplasia, prostate cancer, hirsutism, male pattern alopecia and acne, without affecting testosterone-dependant testicular function, sexual behavior, and muscle growth (Russell *et al.*, 1994). Most 5 α -reductase inhibitors are steroid derivatives or compounds with steroid-like structures. Of these, the 4-azasteroids such as 17 β -(N,N-diethyl)carbamoyl-4-methyl-4-aza-5 α -androstane-3-one (4-MA) and finasteride have been the most extensively studied (Brooks *et al.*, 1981, Liang *et al.*, 1985). The steroidal inhibitors have the possibility of an affinity for the androgen receptor and are expected to produce undesirable anti-androgen effects such as impotence, impairment of muscle growth, and gynecomastia. Also, several nonsteroidal inhibitors have been synthesized, such as ONO-3805 (Russell *et al.*, 1994) and LY191704 (Jones *et al.*, 1993). On the other hand, fewer naturally occurring inhibitors have been reported, for example unsaturated fatty acids (Liang *et al.*, 1992) and (-)-epigallocatechin-3-gallate (Liao *et al.*, 1995). Therefore, the author searched for naturally occurring new type 5 α -reductase inhibitors, especially from tropical plants.

We reports here the 5 α -reductase inhibitory components from PNG and Thai plants, respectively, and their structure-activity relationships.

3.2. The 5 α -Reductase Inhibitory Components from Papua New Guinean Woods

3.2.1. Experimental

3.2.1.1. Materials

NADPH was obtained from Sigma (Missouri, USA). The Coomassie brilliant blue dye reagent for protein determination was purchased from Bio-Rad Laboratories (California, USA).

3.2.1.2. Sample woods

The heartwoods of the following 22 PNG wood species were obtained from PNG Forest Research Institute: *Albizia falcataria*, *Alstonia scholaris*, *Amoora* sp., *Anthocephalus chinensis*, *Artocarpus incisus* L. f., *Buchanania* sp., *Cananga adorata*, *Canarium indicum*, *Canarium oleosum*, *Dracontomelon dao*, *Dysoxylum peltigrewianum*, *Eucalyptus deglupta*, *Garcinia latissi-*

ma, *Hibiscus ellipticifolius*, *Intsia bijuga*, *Neonuclea acuminata*, *Octomeles sumatrana*, *Palaquium galactoxylum*, *Pterocarpus indicus*, *Terminalia* sp., *Toona surenii* and *Xanthophyllum papuanum*. All voucher specimens are preserved at the herbarium of the Department of Forest Products, Kyushu University, in Japan. A large amount of wood (45 kg) of *A. incisus* was obtained from Okinawa prefecture, Japan.

3.2.1.3. Isolated compounds from *A. incisus*

Artocarpin (**1**), artocarpesin (**3**), isoartocarpesin (**4**), (+)-norartocarpanone (**5**), (+)-dihydromorin (**6**), chlorophorin (**7**), 4-prenyloxyresveratrol (**8**), and artocarbene (**9**) were isolated from diethyl ether extracts of heartwood of *A. incisus*. Their isolation, purification, and identification were described in chapter 2.

Cycloartocarpin (**2**): Air-dried milled heartwood of *A. incisus* (37 kg) was extracted for 10 days with Et₂O at room temperature, and the extract was concentrated to dryness. The dry Et₂O extract (330 g) was crystallized successively from Et₂O / hexane and MeOH. The yellow solid deposited (240 g) was collected and recrystallized from MeOH / H₂O, yielding compound **1**. The parts of the mother liquor (23.4 g) were separated by open column chromatography (CC) on silica gel (2 kg, 10×150 cm, ethyl acetate/hexane 1/2). A crude fraction containing compound **2** [3.5 g, *t_R* between 2.0 and 4.0 l, TLC (silica gel, ethyl acetate/hexane 1/1, *R_f* 0.6–0.8, UV detection)] was obtained. Compound **2** (20 mg) was isolated from a part of the crude fraction mentioned above (100 mg) by preparative HPLC (Inertsil PREP-ODS: 20 mm i.d. × 250 mm) using H₂O / CH₃CN (20 / 80, 12 ml/min) (detection at 280 nm, *R_t*: 30 min). MS and NMR matched well to published data (Nair *et al.*, 1964).

3.2.1.4. Preparation of liver microsomes

The liver microsomes from female rats were prepared by the method previously reported (Liang *et al.*, 1992) with modification. Two mature Sprague-Dawley female rats (300 g) were killed. The livers were removed and minced in a beaker with a pair of scissors. The minced tissue was then homogenized in 3-tissue volume medium A (0.32 M sucrose, 1 mM dithiothreitol, and 20 mM sodium phosphate, pH 6.5). The homogenate was then centrifuged at 10,000 ×g for 10 min. The resulting pellet was washed with 2-pellet volume medium A. The combined supernatant from the two centrifugations was further centrifuged at 105,000 ×g for 1 h. The washed microsomes were suspended in 4 ml medium A, and the dispersion of microsomes was achieved using a syringe with 18G, 23G, and 25G needles in succession. The microsome suspension was divided into small aliquots and stored at -80°C. The microsomes were diluted with medium A just before use. Protein content in the suspension was determined by the Coomassie brilliant blue dye reagent.

3.2.1.5. 5α-Reductase assay

The standard reaction mixture, in a final volume of 3.0 ml, contained microsomes (1 mg of protein), 150 μM testosterone in 100 μl of ethanol, 167 μM NADPH, and medium A, with or

without the indicated amount of a sample in 100 μ l of DMSO. The reaction was started by the addition of microsomes to the pre-heated reaction solution in a tube. After 10 min the incubation was terminated by adding 100 μ l of 3M NaOH, and then 100 μ l of 1.0 mM cholesterol acetate in *n*-hexane was added as the internal standard for GC-MS. Forty ml of diethyl ether was added to extract metabolites, and the tubes were capped and shaken. The water phase was frozen in a -20°C freezer, and the organic phase was decanted and evaporated under a reduced pressure. Residue was dissolved in 100 μ l ethyl acetate for GC-MS. GC-MS analyses were conducted on a Shimadzu (Kyoto, Japan) GC-17A gas chromatograph equipped with a Neutra Bond-5 (30 m by 0.25 mm; film thickness, 0.4 μ M; GL Sciences Inc., Tokyo, Japan) and coupled to a QP-5000 quadrupole mass spectrometer injector. The mass spectrometer was operated in the electron impact mode at 70 eV. Helium was used as the carrier gas with a flow rate of 0.8 ml/min. The first oven temperature was 240°C, and the temperature was then increased to 300°C at a rate of 10°C/min. The sample (1 μ l) was injected into the GC at an injector temperature of 310°C. The 5 α -reductase activity was measured by analyzing the extent of the conversion of testosterone to 5 α -dihydrotestosterone. The 5 α -reductase inhibitory activity of each sample was calculated as follows: 5 α -reductase inhibitory activity (%) = $(A_0 - A_s) / A_0 \times 100$. A_0 and A_s represent the peak areas of dihydrotestosterone in the absence and presence of the sample, respectively. Here, the peak areas of dihydrotestosterone were normalized to those of the internal standard, cholesterol acetate. The peak areas of other products represented less than 5% of the peak area of dihydrotestosterone formation, within the experimental error. Each experiment was carried out in duplicate or triplicate, and replicate values were usually within 5% of each other. α -Linolenic acid (Sigma, Missouri, USA) was used as a positive standard.

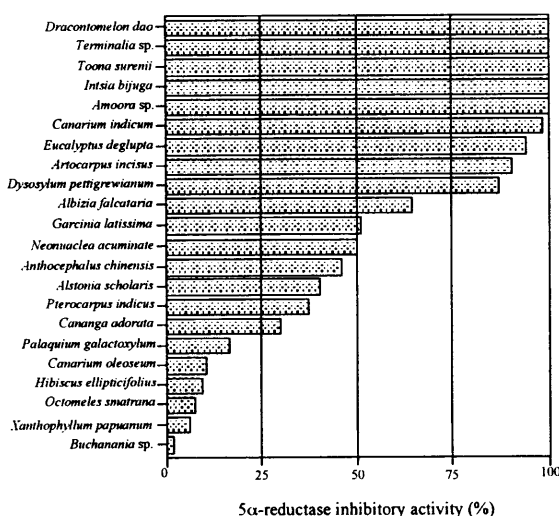


Fig.12 The effect of methanol extracts from heartwoods of PNG woods on 5 α -reductase activity.
(Sample concentration: 100 μ g/ml)

3.2.2. Results and Discussion

In the preliminary screening, the author tested the methanol extracts of the meal of heartwood of 22 PNG wood species for their 5 α -reductase inhibitory activities at the concentration of 100 μ g/ml (Fig. 12). *D. dao*, *Terminalia* sp., *T. surenii*, *I. bijuga*, *Amoora* sp., *C. indicum*, *E. deglupta*, *A. incisus*, and *D. pettigrewianum* showed potent 5 α -reductase inhibitory activity above 85%. These species were further investigated to determine their 5 α -reductase inhibitory activity at the concentration of 50 μ g/ml. As can be seen from Fig. 13, *A. incisus* showed the

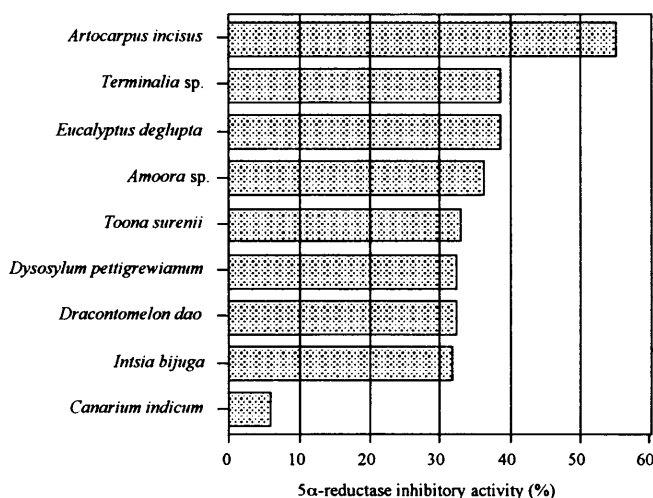


Fig.13 The effect of methanol extracts from heartwoods of PNG woods on 5 α -reductase activity.

(Sample concentration: 50 μ g/ml)

Table 6 Comparison of IC₅₀ of each compound (1-9) from *A. incisus* and α -linolenic acid on 5 α -reductase activity.

Compound	IC ₅₀ (μ M)
Chlorophorin (7)	37
Artocarpin (1)	85
4-prenyloxyresveratrol (8)	128
Artocarpesin (3)	216
Artocarbene (9)	242
Cycloartocarpin (2)	No inhibition at 230 μ M
Isoartocarpesin (4)	No inhibition at 282 μ M
(+)-Norartocarpanone (5)	No inhibition at 347 μ M
(+)-Dihydromorin (6)	No inhibition at 657 μ M
α -Linolenic acid (positive control)	116

highest inhibitory activity, which is why it was selected for further investigation. We had already investigated some constituents in diethyl ether extract of heartwood of *A. incisus* in chapter 2. Also, methanol extracts of the meal that had been extracted by diethyl ether showed much less 5 α -reductase inhibitory activity (data not shown). Therefore, we focused on the diethyl ether extracts of *A. incisus* and investigated the 5 α -reductase inhibitory effects of nine compounds that had been isolated from the diethyl ether extracts (Fig. 14). The results are shown in Table 6. α -Linolenic acid, known as a naturally occurring potent inhibitor, was used as a positive standard. It should be noted that finasteride, which is known as a potent steroidal inhibitor, showed IC_{50} of 0.73 μ M. Artocarpin (1) and chlorophorin (7) showed stronger inhibitory activity than did α -linolenic acid, with chlorophorin (7) showing especially strong inhibitory activity. Artocarpesin (3), 4-prenyloxysveratrol (8), and artocarbene (9) showed moderate inhibitory activity, while cycloartocarpin (2), isoartocarpesin (4), (+)-norartocarpinone (5), and (+)-dihydromorin (6) did not show any inhibitory activity at the indicated concentrations (Table 6).

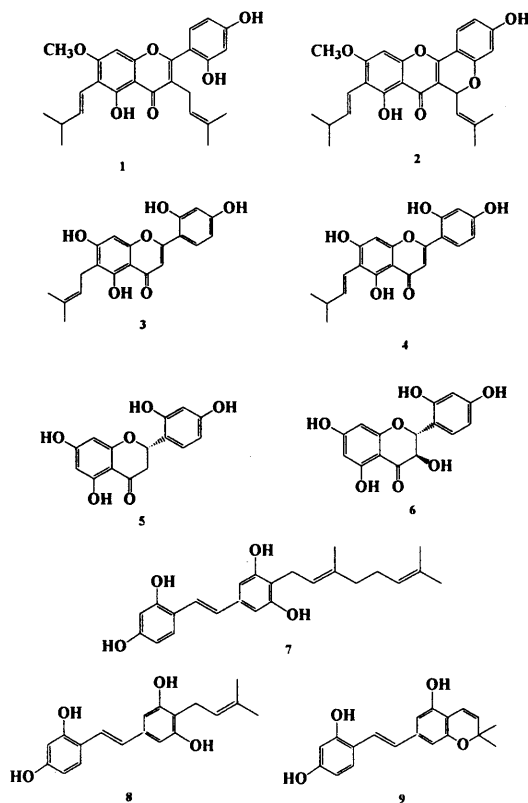


Fig. 14 The chemical structures of compound 1-9.

3.2.3. Summary

The methanol extract of heartwood of *A. incisus* showed potent 5 α -reductase inhibitory activity. The author investigated the 5 α -reductase inhibitory effects of nine compounds isolated from *A. incisus*. Chlorophorin ($IC_{50} = 37 \mu M$) and artocarpin ($IC_{50} = 85 \mu M$) showed more potent inhibitory effects than did α -linolenic acid, which is known as a naturally occurring potent inhibitor.

3.3. The 5 α -Reductase Inhibitory Components from Thai Plants

3.3.1. Experimental

3.3.1.1. Sample plants

The 16 Thai plant species (*Leucaena leucocephala* (lam.) De Wit, *Mitragyna hirsuta* Hav., *Alpinia galanga* Willd., *Ixora maerthyra* Teysm&Binn., *Annona squamosa* L., *Passiflora foetida* L., *Alstonia scholaria* (L.) R. BR., *Piper betle* L., *Tamarindus indica* L., *Eucalyptus* sp., *Artocarpus incisus* (Parkins) Fosberg, *Flagellaria indica* L., *Clitoria ternatea* L., *Albizia myriophylla* Benth., *Citrus hystrix* DC., *Acacia concinna* DC.) were obtained from Kasetsart Agriculture and Agro Industrial Product Improvement Institute, Kasetsart University, Bangkok, Thailand, and all voucher specimens are preserved at the herbarium there.

3.3.1.2. 5 α -Reductase assay

5 α -reductase inhibitory activity was assayed by the method described in chapter 3.2.1.5..

3.3.1.3. Extraction of samples

The leaves (*L. leucocephala*, *M. hirsuta*, *A. galanga*, *I. maerthyra*, *A. squamosa*, *P. foetida*, *A. scholaria*, *P. betle*, *T. indica*, *Eucalyptus* sp., *A. incisus*, *F. indica* and *A. myriophylla*), flowers (*C. ternatea*), vines (*P. foetida*), fruits (*A. concinna*) and skin of fruits (*C. hystrix*) were dried and milled. The 100 g of milled samples were extracted with acetone at room temperature for 24 hrs and then filtrated through the filter paper quality No.4. The extractions were performed for 3 times and acetone extracts were pooled together, then concentrated *in vacuo* at 40 °C.

The juice of *C. hystrix* fruits was obtained by squeezing the peeled fruits. The juice was concentrated *in vacuo* at 50 °C, then freeze-dried.

3.3.1.4. Extraction and separation of extractives of *A. incisus*

The acetone extract (2g) of leaves of *A. incisus* were suspended in water and partitioned with *n*-hexane, diethyl ether and ethyl acetate, successively to give *n*-hexane-soluble (0.438g, 22% of the acetone extract), diethyl ether-soluble (1.452g, 72.6%), ethyl acetate-soluble (0.026g, 1.3%) and aqueous portion (0.086g, 4.3%), respectively. The diethyl ether-soluble portion (1g) was fractionated into 8 fractions (fr. A-H) by column chromatography on silica gel (Wakogel C-200, Wako, Osaka, Japan) (200g) eluting with ethyl acetate-*n*-hexane gradient [ethyl acetate : *n*-hexane = 1 : 3, 0.5 ℓ (fr. A:0.081g, fr. B:0.019g)→1 : 2, 0.5 ℓ (fr. C:0.011g, fr.

D:0.400g)→1 : 1, 0.5 ℓ (fr. E:0.134 g,)→1 : 0.5, 0.5 ℓ (fr. F:0.081g)→ethyl acetate, 0.5 ℓ (fr. G:0.197 g)→MeOH, 0.5 ℓ (fr. H:0.003 g)]. Of these, fr. D (TLC, silica gel, ethyl acetate/*n*-hexane, 1:3, R_f 0.2-0.5) was obtained as the 5 α -reductase inhibitory fraction. Compound **10** (95 mg, 0.53% of dried leaves) and **11** (24 mg, 0.14% of dried leaves) were isolated as the active constituents from a portion of fr. D (320mg) by preparative HPLC (Inertsil PREP-ODS: 20 mm i.d. $\times$ 250 mm) using H₂O/CH₃CN (40/60), 12ml/min.

3.3.1.5. Isolated compounds from *A. incisus*

All nuclear magnetic resonance (NMR) experiments were performed at 400 MHz. Sample was dissolved in acetone-*d*₆ and chemical shifts were refereed to internal TMS for ¹H NMR and to deuterated solvents for ¹³C-NMR.

Compound **10**, 2-geranyl-2',3,4,4'-tetrahydroxy-dihydrochalcone: yellow powder, FAB-MS (glycerol): *m/z* = 409 ([*M*-H]⁻). ¹H-NMR (acetone-*d*₆): δ = 1.54 (3H, *d*, *J* = 0.73 Hz, 9"-H), 1.60 (3H, *d*, *J* = 0.98 Hz, 8"-H), 1.75 (3H, *d*, *J* = 0.98 Hz, 10"-H), 1.97 (2H, *m*, 4"-H), 2.04 (2H, *m*, 5"-H), 2.93 (2H, *m*, β -H), 3.17 (2H, *m*, α -H), 3.45 (2H, *dd*, *J* = 0.73, 6.6 Hz, 1"-H), 5.07 (1H, *m*, 6"-H), 5.19 (1H, *m*, 2"-H), 6.33 (1H, *d*, *J* = 2.4 Hz, 3'-H), 6.41 (1H, *dd*, *J* = 2.4, 8.9 Hz, 5'-H), 6.59 (1H, *d*, *J* = 8.1 Hz, 5-H), 6.65 (1H, *d*, *J* = 8.1 Hz, 6-H), 7.77 (1H, *d*, *J* = 8.9 Hz, 6'-H), 12.82 (1H, *s*, 2'-OH). ¹³C-NMR (acetone-*d*₆): δ = 16.5 (10"-C), 17.7 (9"-C), 25.8 (8"-C), 25.9 (1"-C), 27.5 (5"-C), 28.1 (β -C), 40.2 (α -C), 40.4 (4"-C), 103.1 (3'-C), 108.6 (5'-C), 113.3 (5-C), 113.8 (1'-C), 120.5 (6-C), 124.4 (2"-C), 124.9 (6"-C), 127.3 (2-C), 131.5 (7"-C), 132.1 (1-C), 133.3 (6'-C), 134.9 (3"-C), 143.4 (3-C), 144.1 (4-C), 165.2 (4'-C), 166.1 (2'-C), 204.9 (C=O).

Compound **11**, 3'-Geranyl-2',3,4,4'-tetrahydroxychalcone, Yellow powder. HRFABMS: [*M*+H]⁺ *m/z* = 409.2014 (C₂₅H₂₈O₅ requires *m/z* = 409.2015). ¹H-NMR (acetone-*d*₆): δ = 1.52 (3H, *s*, 9"-H), 1.58 (3H, *d*, *J* = 0.98 Hz, 8"-H), 1.77 (3H, *d*, *J* = 0.98 Hz, 10"-H), 1.94 (2H, *m*, 4"-H), 2.02 (2H, *m*, 5"-H), 3.36 (2H, *d*, *J* = 7.3 Hz, 1"-H), 5.07 (1H, *m*, 6"-H), 5.27 (1H, *m*, 2"-H), 6.51 (1H, *d*, *J* = 8.8 Hz, 5'-H), 6.88 (1H, *d*, *J* = 8.3 Hz, 5-H), 7.20 (1H, *dd*, *J* = 2.0, 8.3 Hz, 6-H), 7.32 (1H, *d*, *J* = 2.0 Hz, 2-H), 7.67 (1H, *d*, *J* = 15.1 Hz, α -H), 7.75 (1H, *d*, *J* = 15.1 Hz β -H), 7.96 (1H, *d*, *J* = 8.8 Hz, 6'-H), 13.9 (1H, *s*, 2'-OH). ¹³C-NMR (acetone-*d*₆): δ = 16.3 (10"-C), 17.7 (9"-C), 22.2 (1"-C), 25.8 (8"-C), 27.4 (5"-C), 40.5 (4"-C), 107.8 (5'-C), 114.2 (1'-C), 115.7 (2-C), 115.9 (3'-C), 116.2 (5-C), 118.3 (α -C), 123.0 (2"-C), 123.1 (6-C), 124.9 (6"-C), 128.0 (1-C), 129.9 (6'-C), 131.3 (7"-C), 134.9 (3"-C), 145.0 (β -C), 146.1 (3-C), 148.9 (4-C), 162.4 (4'-C), 164.9 (2'-C), 192.6 (C=O).

3.3.2. Result and Discussion

The 17 samples prepared from 16 Thai plant species were examined for 5 α -reductase inhibitory activity at the concentration of 200 μ g/ml. The acetone extracts of *A. incisus* and *I. maerothyra* showed potent inhibitory activity at 75% and 79% on 5 α -reductase, respectively (Table 7). In our preliminary activity-guided fractionation of the acetone extract of *I.*

Table 7 The effect of 17 samples prepared from Thai plants on 5 α -reductase activity.
(sample concentration: 200 μ g/ml)

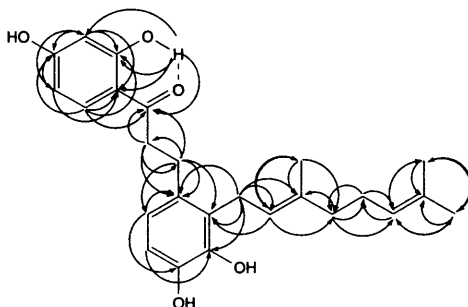
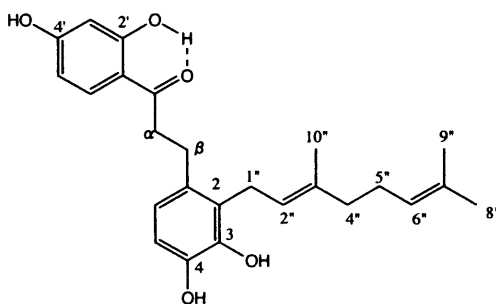
Sample	5 α -reductase inhibitory activity(%)
Acetone extract	
<i>Alstonia scholaria</i> (leaves)	0
<i>Annona squamosa</i> (leaves)	0
<i>Alpinia galanga</i> (leaves)	1
<i>Tamarindus indica</i> (leaves)	1
<i>Albizia myriophylla</i> (leaves)	5
<i>Acacia concinna</i> (fruits)	21
<i>Flagellaria indica</i> (leaves)	23
<i>Leucaena leucocephala</i> (leaves)	25
<i>Citrus hystrix</i> (skin of fruits)	25
<i>Clitoria ternatea</i> (flowers)	29
<i>Eucalyptus sp.</i> (leaves)	47
<i>Passiflora foetida</i> (vines)	47
<i>Piper betle</i> (leaves)	51
<i>Mitragyna hirsuta</i> (leaves)	53
<i>Artocarpus incisus</i> (leaves)	75
<i>Ixora maerothyra</i> (leaves)	79
Juice	
<i>Citrus hystrix</i> (fruits)	14

5 α -reductase inhibitory activity (%)=(A_0 - A_s)/ A_0 ×100

A_0 and A_s represent peak areas of 5 α -dihydrotestosterone in the absence and presence of sample, respectively

maerothyra, α -linolenic acid, which is already known as a potent inhibitor (Liang *et al.*, 1992), was identified as a main active component in it by GC-MS. Therefore, *A. incisus* was selected for further investigation. The acetone extract of leaves of *A. incisus* was partitioned by *n*-hexane, diethyl ether and ethyl acetate. The 5 α -reductase inhibitory activity of *n*-hexane-soluble, diethyl ether-soluble, ethyl acetate-soluble and aqueous portion was 20, 67, 33 and 0% at the concentration of 100 μ g/ml, respectively. The diethyl ether-soluble portion showing the strongest 5 α -reductase inhibitory activity was submitted to further separation by silica gel CC giving 8 fractions [5 α -reductase inhibitory activity (%) at 100 μ g/ml; fr. A (24%), fr. B (21%), fr. C (42%), fr. D (90%), fr. E (46%), fr. F (13%), fr. G (4%) and fr. H (13%)]. Fr. D showed the strongest inhibitory activity. Further separation of fr. D by preparative HPLC gave the active constituents, compound **10** and **11**.

Compound **10** was identified as follows. The ^{13}C -NMR spectrum revealed 25 carbon atoms (DEPT:10×C, 7×CH, 5×CH₂ and 3×CH₃). The FAB mass spectral data ([M-H]⁻= 409) together with the ^{13}C -NMR data suggested the molecular formula to be C₂₅H₃₀O₅. Compound **10** showed characteristic signals for a dihydrochalcone at δ 2.93 (2H, *m*, CH₂- β) and 3.17 (2H, *m*, CH₂- α) in its ^1H NMR spectrum, and δ 28.1 (C- β), 40.2 (C- α) and 204.9 (C=O) in its

Fig.15 HMBC correlations of compound **10**.Fig.16 The chemical structure of compound **10**.

^{13}C NMR spectrum (Garzon *et al.*, 1987, Achenbach *et al.*, 1988). The ^1H -NMR spectrum showed the presence of a geranyl [δ 1.54 (3H, *d*, J = 0.73, CH_3 -9''), δ 1.60 (3H, *d*, J = 0.98, CH_3 -8''), δ 1.75 (3H, *d*, J = 0.98, CH_3 -10''), 1.97 (2H, *m*, CH_2 -4''), 2.04 (2H, *m*, CH_2 -5''), 3.45 (2H, *dd*, J = 0.73, 6.6, CH_2 -1''), 5.07 (1H, *m*, CH-6''), 5.19 (1H, *m*, CH-2'')] and a chelated hydroxyl group [δ 12.82 (1H, *s*, OH)], in addition to a set of ortho-coupled one proton doublets [δ 6.59 and 6.65 (each J = 8.1 Hz, CH-5, 6)] assignable to that of ring A and three protons in an ABX spin system [δ 6.33 (*d*, J = 2.4 Hz, CH-3'), 6.41 (*dd*, J = 2.4, 8.9 Hz, CH-5') and 7.77 (*d*, J = 8.9 Hz, CH-6')] due to ring B. The substituted positions of ring A and B were determined as follow. In the HMBC (Heteronuclear Multiple Bond Connectivity) spectrum (Fig. 15), the methylene protons of the geranyl group at δ 3.45 were coupled with three quaternary carbons at δ 127.3 (C-2), 132.1 (C-1) and 143.4 (C-3). Moreover, the benzyl methylene protons at δ 2.93, assigned to H- β of dihydrochalcone moiety, caused a cross-peak with three quaternary carbons at δ 120.5 (C-6), 127.3 (C-2) and 132.1 (C-1). Consequently, the geranyl group was at C-2. Thus, it was concluded that compound **10** was 2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone (Fig.16). In this connection, ^{13}C NMR data of compound **10** were not completely identical to published data (Fujimoto *et al.*, 1987) of 2-ger-

anyl-2',3,4,4'-tetrahydroxydihydrochalcone. Their ^{13}C NMR data showed δ 58.6 (*t*), but similar value was not in our data. However, it was confirmed that the previous data (Fujimoto *et al.*, 1987) had been erroneously published (Private communication from Fujimoto, Y., Coll. Pharm., Nihon Univ., Japan.). Our MS and ^1H NMR data of compound 10 were almost identical to those of 2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone (Fujimoto *et al.*, 1987). The complete assignment of hydrogen and carbon atoms in the ^1H and ^{13}C -NMR spectra of the compound was accomplished for the first time by HMBC and HSQC (Heteronuclear Single Quantum Coherence).

Compound 10, 2-geranyl-2',3,4,4'-tetrahydroxy-dihydrochalcone, showed more potent 5 α -reductase inhibitory activity ($\text{IC}_{50} = 38 \mu\text{M}$) than α -linolenic acid known as naturally occurring potent inhibitor ($\text{IC}_{50} = 116 \mu\text{M}$). 2-Geranyl-2',3,4,4'-tetrahydroxydihydrochalcone, one of a few naturally occurring C-geranylated dihydrochalcones in nature with highly potent and selective 5-lipoxygenase inhibitory activity, has been isolated from flower part of Indonesian plant, *Artocarpus communis* (= *A. incisus*) (Fujimoto *et al.*, 1987, Koshihara *et al.*, 1987). The total synthesis of this compound has been accomplished (Nakano *et al.*, 1989, Chu-sheng *et al.*, 1998). In this study, its new aspect of biological activity, 5 α -reductase inhibitory activity, was found.

Compound 11 was deduced as having an elemental formula of $\text{C}_{25}\text{H}_{28}\text{O}_5$ by HRFAB mass spectrometry (obsd. $[\text{M}+\text{H}]^+ m/z$ 409.2014). The ^1H NMR spectrum showed characteristic signals of the protons at C- α and C- β positions of the chalcone skeleton [δ 7.67, 7.75 (each 1H, *d*, $J=15.1$ Hz)], and revealed the presence of ABC type aromatic protons [δ 6.88 (1H, *d*, $J=8.3$ Hz), 7.20 (1H, *dd*, $J=2.0, 8.3$ Hz), 7.32 (1H, *d*, $J=2.0$ Hz)], AB type aromatic protons [δ 6.51 (1H, *d*, $J=8.8$ Hz), 7.96 (1H, *d*, $J=8.8$ Hz)], a geranyl group [δ 1.52, 1.58, 1.77 (each 3H, *s*), 1.94, 2.02 (each 2H, *m*), 3.36 (2H, *d*, $J=7.3$ Hz), 5.08, 5.27 (each 1H, *m*)], and a chelated hydroxyl group [δ 13.9 (1H, *s*)]. In the HMBC spectrum, the H-1" proton provided a good starting point for the assignment of the proton and carbon resonances from geranyl function. Proton H-1" exhibited correlations with the C-2', C-3', C-4', C-2" and C-3" carbons. This permitted assignment of the C-3' position of the geranyl group. Accordingly, 11 was identi-

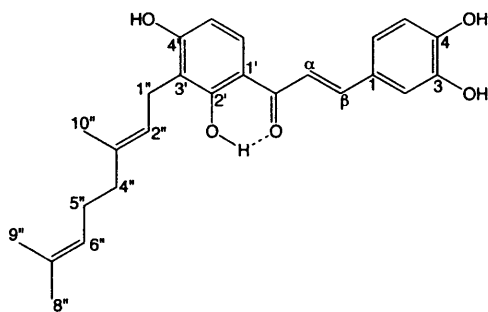


Fig. 17 The chemical structure of compound 11.

fied as a novel 3'-geranyl-2',3,4,4'-tetrahydroxychalcone (Fig. 17). The structure and all assignments were confirmed by ^1H - ^1H COSY, ^{13}C - ^1H COSY and HMBC spectroscopies.

Compound **11** showed more potent inhibitory activity (IC_{50} = 104 μM) than α -linolenic acid (IC_{50} = 116 μM) known as a naturally occurring potent inhibitor (Liang *et al.*, 1992).

3.3.3. Summary

The inhibitory effects of 17 samples prepared from Thai plants on 5 α -reductase activity were examined. The acetone extract of leaves of *A. incisus* showed potent 5 α -reductase inhibitory activity. Fractionation guided by 5 α -reductase inhibitory led to the isolation of 2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone (**10**) and a novel geranylated chalcone, 3'-geranyl-2',3,4,4'-tetrahydroxychalcone (**11**) from the acetone extract of leaves of *A. incisus*. These compounds showed more potent inhibitory effect (2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone (**10**): IC_{50} = 38 μM , 3'-geranyl-2',3,4,4'-tetrahydroxychalcone (**11**): IC_{50} = 104 μM) than α -linolenic acid known as a naturally occurring potent inhibitor.

3.4. Structure-Activity Relationships

3.4.1. Experimental

3.4.1.1. Materials

Oxyresveratrol (**12**) was isolated and identified from *Chlorophora excelsa* by the method described by Christensen *et al.*, 1989. Butein (2',3,4,4'-tetrahydroxychalcone) (**13**) was provided by the Laboratory of Wood Chemistry, Department of Forest Products, Faculty of Agriculture, Kyushu University in Japan, isolated from the heartwood of *Rhus javanica* and its purities and identification had been confirmed by comparison with reference (Imamura *et al.*, 1967). 2',3,4,4'-tetrahydroxydihydrochalcone (**14**) was prepared by reduction of butein (**13**) with 10% Pd/C (Aldrich Chem. Co.) and confirmed by ^1H NMR. Other reagents and methods were described in chapter 3.2. and 3.3..

3.4.1.2. Results and Discussion

To obtain the information on the structure-activity relationships, the author compared the effects of oxyresveratrol (**12**) and its derivatives (**7** and **8**) on 5 α -reductase activity. As shown in Fig. 18, the introduction of prenyl substituent (**8**) and geranyl substituent (**7**) to oxyresveratrol skeleton (**12**) increased 5 α -reductase inhibitory effects dramatically, respectively (**12**: no inhibition $\rightarrow$ **8**: IC_{50} = 128 μM $\rightarrow$ **7**: IC_{50} = 37 μM). The contribution of the inhibitory effect of geranyl substituent was higher than that of prenyl substituent. Also, as shown in Fig. 19, the cleavage of the ring D of cycloartocarpin (**2**), producing prenyl substituent (**1**), caused potent inhibitory activity (**2**: no inhibition $\rightarrow$ **1**: IC_{50} = 85 μM). Furthermore, artocarpesin (**4**), which has 3-methyl-1-butenyl substituent, showed no inhibitory effects, while the same type flavone with prenyl substituent showed potent inhibitory activity (**4**: no inhibition $\rightarrow$ **3**: IC_{50} = 216 μM). The flavonoids with no prenyl or geranyl substituent (**4**, **5** and **6**) did not show potent inhibitory activity. In the case of chalcones and dihydrochalcone, the similar results were obtained. As

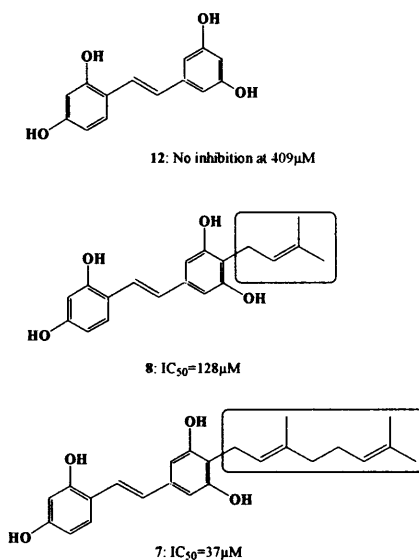


Fig.18 The chemical structures (7,8,12) and 5 α -reductase inhibitory activity.

Square box: prenyl or geranyl substituent.

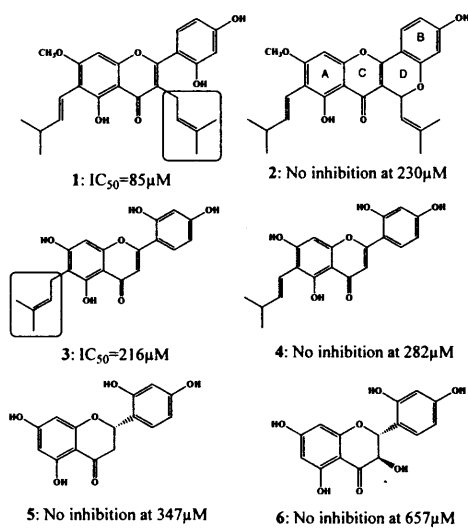


Fig.19 The chemical structures (1-6) and 5 α -reductase inhibitory activity.

Square box: prenyl substituent.

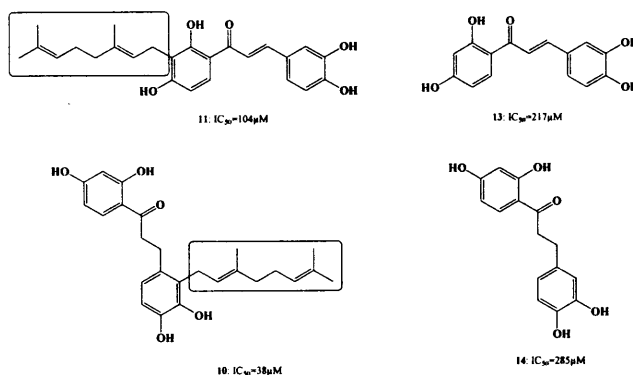


Fig.20 The chemical structures (10,11,13,14) and 5 α -reductase inhibitory activity.

Square box: geranyl substituent.

shown in Fig. 20, the introduction of geranyl substituent to butein (13) and 2',3,4,4'-tetrahydroxydihydrochalcone (14) increased 5 α -reductase inhibitory effects dramatically, respectively (13: IC₅₀ = 217 μ M $\rightarrow$ 11: IC₅₀ = 104 μ M, 14: IC₅₀ = 285 μ M $\rightarrow$ 10: IC₅₀ = 38 μ M). These observations led us to the assumption that, in the case of flavonoids and stilbenes, isoprene derived moieties (prenyl and geranyl) may be the most important skeleton to reveal potent 5 α -reductase inhibitory activity. In particular, geranyl substituent was more desirable than prenyl substituent for revealing potent inhibitory activity.

Some of the isoprenylated compounds from the moraceous plants have exhibited interesting biological activity (Nomura *et al.*, 1998). It has been reported that isoprenoid-substituted flavonoids isolated from *Artocarpus* species possess inhibitory effects on K⁺-dependent amino acid transport (Parenti *et al.*, 1998), arachidonate 5-lipoxygenase and mouse TNF- α release, cytotoxicity, antiplatelet activity, and antibacterial activity against cariogenic bacteria (Nomura *et al.*, 1998). Thus the *Artocarpus* plants are important medicinal resources. In this study, We found a new facet of the biological activity of the *Artocarpus* plant, 5 α -reductase inhibitory activity.

Several naturally occurring, nonsteroidal compounds had been reported as 5 α -reductase inhibitor. The inhibitory effects of the green tea flavonoids, such as epicatechin-3-gallate and epigallocatechin-3-gallate, against 5 α -reductase were examined. Liao *et al* proposed that the gallyl or galloyl group of these tea gallates may interact with a specific site on 5 α -reductase, and other parts of the catechin molecule may also be important for inhibition since simple gallates were not active (Liao *et al.*, 1995). Also, the inhibitory effects of some polyunsaturated fatty acids, such as γ -linolenic acid and α -linolenic acid against 5 α -reductase were examined (Liang *et al.*, 1992). Polyunsaturated fatty acids, but not their mono-unsaturated or saturated counterparts, are also moderately potent inhibitors of 5 α -reductase enzyme activity. 5 α -Reductase is a membrane-bound enzyme that appears to require the unique environment of the lipid bilayer for

activity (Russell *et al.*, 1994). The addition of phospholipids to solubilized enzyme preparations frequently stimulates activity (Cooke *et al.*, 1985), suggesting a role for lipids in modulating enzyme activity. The inhibition of 5 α -reductase enzyme activity by polyunsaturated fatty acids may therefore reflect another property of this modulation, such as perturbation of the lipid matrix of microsomal membranes. It is intriguing that key substructures of active compounds from *A. incisus*, isoprenyl-derived substituents (prenyl and geranyl), have similar shapes to unsaturated alkyl moieties of polyunsaturated fatty acids. Therefore, our present results indicate that isoprenyl derived moieties of active compounds from *A. incisus* may act on 5 α -reductase by a similar mechanism as that of polyunsaturated fatty acids.

It should be noted that the present investigation does not allow any conclusion about the selectivity (type 1 or 2) of 5 α -reductase-inhibition (Russell *et al.*, 1994), as only one enzyme has been investigated.

Our observations of some 5 α -reductase inhibitors having prenyl substituent may lead to the design and discovery of new type of 5 α -reductase inhibitors. Furthermore, from the chemotaxonomic point of view, specific extracts of plants known as having flavonoids, stilbenes, or other types with prenyl substituent, for example Moraceae, Leguminosae, Berberidaceae, Platanaceae, Rubiaceae, and Rutaceae (Wollenweber, 1982), could be candidates for 5 α -reductase inhibitory materials.

3.4.3. Summary

Several flavonoids and stilbenes, obtained from *A. incisus* and other plants or synthesized, were tested for their inhibitory activity against 5 α -reductase. Structure-activity relationship, in the case of flavonoids and stilbenes, suggested that the presence of an isoprene substituent (prenyl and geranyl) would enhance 5 α -reductase inhibitory effects. Our observations of some 5 α -reductase inhibitors having prenyl substituent may lead to the design and discovery of new type 5 α -reductase inhibitors.

4. Conclusion

Tropical forest area occupies 40-50 % of the all forest area in the world. The PNG mainland and surrounding islands, and Thailand possess an extraordinarily rich flora and great diversity of vegetation types that parallel the diverse physiography of lands. A high proportion of the land area has a forest cover, most of it evergreen rain forest. The great diversity of tropical woody plants is reflected in the qualitative and quantitative diversity of extractives of the woods from a chemical point of view of the wood components. Therefore, the tropical woody plants will give us a great chance to find bioactive components.

In this study, we have focused on the inhibitory components on tyrosinase and 5 α -reductase from tropical woody plants for effective and useful use as biomass resources.

In chapter 2, the inhibitory components of *Artocarpus incisus* heartwood which showed the strongest inhibitory activity in 23 PNG wood species, their structure-activity relationships and inhibitory mechanism were evaluated.

First, the inhibitory effects of methanol extracts of heartwood of 23 PNG wood species on tyrosinase activity were examined. The extract of *A. incisus* showed the strongest tyrosinase inhibitory activity which was almost equivalent to kojic acid. The extract apparently inhibited melanin biosynthesis of both cultured B16 melanoma cells without any cytotoxicity and back of brown guinea pig without skin irritation. Thus, the potentiality of the extracts of *A. incisus* is evident both as material of a useful skin whitening agent and as a remedy for the disturbances in pigmentation. Tyrosinase inhibitory activity-guided fractionation led to the isolation of seven active compounds including two new compounds, 3,2',4'-trihydroxy-6'',6''-dimethyl-pyrano(3'',2'':4,5)-*trans*-stilbene, named artocarbene and 6-(3''-methyl-1''-butenyl)-5,7,2',4'-tetrahydroxyflavone, named isoartocarpesin. Other active compounds were (+)-dihydromorin, chlorophorin, (+)-norartocarpanone, 4-prenyloxyresveratrol, artocarbene and artocarpesin. These compounds likely caused melanin biosynthesis inhibitory effects.

Second, several flavonoids, stilbenes and related 4-substituted resorcinols, obtained from *A. incisus* and other plants or synthesized, were tested for their inhibitory activity against tyrosinase. The structure-activity relationships suggested that specific natural or synthesized compounds having 4-substituted resorcinol skeleton have potent tyrosinase inhibitory ability. Kinetic studies have indicated that specific compounds having 4-substituted resorcinol skeleton exhibit competitive inhibition of the oxidation of DL-DOPA by mushroom tyrosinase. These findings could lead to the design and discovery of new tyrosinase inhibitors. From the chemotaxonomic point of view, crude extracts of plants known as containing flavonoids, stilbenes or other types with 4-substituted resorcinol skeleton, for example Moraceae or Leguminosae, may be candidates for tyrosinase inhibitory materials.

In chapter 3, we examined 5 α -reductase inhibitory components from PNG and Thai plants, respectively, and discussed their structure-activity relationship.

First, the methanol extract of heartwood of *A. incisus* showed potent 5 α -reductase inhibitory activity. The author investigated the 5 α -reductase inhibitory effects of nine compounds isolated from *A. incisus* heartwood. Chlorophorin and artocarpin showed more potent inhibitory effects (IC_{50} = 37 μ M and 85 μ M, respectively) than did α -linolenic acid, which is known as a naturally occurring potent inhibitor.

Second, the inhibitory effects of 17 samples prepared from Thai plants on 5 α -reductase activity were examined. The acetone extract of *A. incisus* leaves showed potent 5 α -reductase inhibitory activity. Fractionation guided by 5 α -reductase inhibitory test led to the isolation of 2-geranyl-2',3,4,4'-tetrahydroxy-dihydrochalcone and a novel geranylated chalcone, 3'-geranyl-2',3,4,4'-tetrahydroxychalcone from the acetone extract of *A. incisus* leaves. These compounds showed more potent inhibitory effect (2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone : IC_{50} = 38 μ M, 3'-geranyl-2',3,4,4'-tetrahydroxychalcone : IC_{50} = 104 μ M) than α -linolenic acid known as a naturally occurring potent inhibitor.

Third, several flavonoids and stilbenes, obtained from *A. incisus* and other plants or synthesized, were tested for their inhibitory activity against 5 α -reductase. Structure-activity relationship, in the case of flavonoids and stilbenes, suggested that the presence of an isoprene-derived

substituent (prenyl and geranyl) would enhance 5 α -reductase inhibitory effects. Our observations of some 5 α -reductase inhibitors having prenyl substituent may lead to the design and discovery of new type 5 α -reductase inhibitors.

Early studies on the phenolic constituents of *Artocarpus* plants by Indian researchers showed many flavone derivatives (Venkataraman, 1972). Most of *Artocarpus* flavonoids and stilbenes are characterized by the structure bearing 2', 4'-dioxxygenated pattern in the B ring and the isoprenoid side chain. Also, many *Artocarpus* plants are found in Indonesia and have been used for the Indonesian traditional folk medicine called 'Jamu' (Takahashi, 1988). Therefore, it is very interesting to clarify the relationship between the usage and biological activities of the isoprenoid-substituted phenolic compounds.

In this study, the relationships between the characteristic structures of phenolic compounds in *A. incisus* and tyrosinase and 5 α -reductase inhibitory activities were discussed. As the results of this thesis, it was concluded as follows. 2', 4'-Dioxxygenated pattern in the B ring, which constructs 4-substituted resorcinol, is important to possess the potent tyrosinase inhibitory activity. On the other hand, the isoprenoid side chain is important to reveal the potent 5 α -reductase inhibitory activity. Furthermore, from the chemotaxonomic point of view, crude extracts of plants known as having flavonoids, stilbenes or other types with 4-substituted resorcinol skeleton, for example Moraceae or Leguminosae, may be candidates for tyrosinase inhibitory materials. Also, crude extracts of plants known as having flavonoids, stilbenes, or other types with isoprenoid side chains, for example Moraceae, Leguminosae, Berberidaceae, Platanaceae, Rubiaceae, and Rutaceae, could be candidates for 5 α -reductase inhibitory materials. These findings could lead not only to the design and discovery of superior inhibitors, but also to the effective and useful use of biomass resources, for example woody plants.

References

- ACHENBACH, H., STÖCKER, M. and CONSTENLA, M. A. (1988) : Flavonoid and other constituents of *Bauhinia manca*. *Phytochemistry* **27** : 1835-1841
- ANDERSON, K. M. and LIAO, S. (1968) : Selective retention of 5 α -dihydrotestosterone by prostatic nuclei. *Nature* **219** : 277-279
- BICK, I. R. C., BROWN, R. B. and HILLS, W. E. (1972) : Three flavanones from leaves of *Eucalyptus sieberi*. *Aust. J. Chem.* **25** : 449-451
- BRINKER, A. M. and SEIGLER, D. S. (1991) : Isolation and identification of picea tannol as a phytoalexin from sugarcane. *Phytochemistry* **30** : 3229-3232
- BROOKS, J. R., BAPTISTA, E. M., BERMAN, C., HAM, E. A., HICHENS, M., JOHNSTON, D. B., PRIMKA, R. L., RASMUSSEN, G. H., REYNOLDS, G. F., SCHMITT, S. M. and ARTH, G. E. (1981) : Response of rat ventral prostate to a new and novel 5 α -reductase inhibitor. *Endocrinology* **109** : 830-836
- BROOKS, J. R. (1986) : Treatment of hirsutism with 5 α -reductase inhibitors. *Clin. Endocrin. Metab.* **15** : 391-405
- BRUCHOVSKY, N. and WILSON, J. D. (1968) : The conversion of testosterone to 5 α -androstane-17 β -ol-3-one by rat prostate *in vivo* and *in vitro*. *J. Biol. Chem.* **243** : 2012-2021
- BRUNET, P. C. J. (1980) : The metabolism of the aromatic amino acids concerned in the cross-linking of insect

- cuticle. *Insect Biochem.* **10**: 467-500
- CABABES, J., CHAZARRA, S. and GARCIA-CARMONA, F. (1994): Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. *J. Pharm. Pharmacol.* **46**: 982-985
- CHRISTENSEN, L. P. and LAM, J. (1989): Excelsa octaphenol, a stilbene dimer from *Chlorophora excelsa*. *Phytochemistry* **28**: 917-918
- CHUNG, M. I., LU, C. M., HUANG, P. L. and LIN, C. N. (1995): Prenyl flavonoids of *Artocarpus heterophyllus*. *Phytochemistry* **40**: 1279-1282
- CHU-SHENG, H. and YU-LIN, L. (1998): A facile total synthesis of 2-geranyl-2', 3, 4, 4'-tetrahydroxydihydrochalcone with highly 5-lipoxygenase inhibiting activity. *Chem. Res. Chin. Univ.* **4**: 428-429
- COOKE, G. M. and ROBAIRE, B. (1985): Modulation of epididymal Δ^4 -steroid 5 α -reductase activity *in vitro* by the phospholipid environment. *J. Biol. Chem.* **260**: 7489-795
- DARLEY, C. R. (1984): Recent advances in hormonal aspects of acne vulgaris. *Int. J. Dermatol.*, **23**: 539-541
- DESPHANDE, V. H., SRINIVASAN, R. and RAO, A. V. R. (1975): Wood phenolics of *Morus* species. IV. Phenolics of the heartwood of five *Morus* species. *Ind. J. Chem.* **13**: 453-457
- FRIEDMAN, M. (1996): Food browning and its prevention: An overview. *J. Agric. Food. Chem.* **44**: 631-653
- FUJIMOTO, Y., UZAWA, J., SUHANDA, S., SOEMARTONO, A., SUMATRA, M. and KOSHIHARA, Y. (1987): Isolation and structural elucidation of new lipoxygenase inhibitors from indonesian *Artocarpus communis* (in Japanese). "29th Symposium on the Chemistry of Natural Products", Sapporo, Symposium Papers, 721-728
- GAEFFIELD, W. (1970): Circular dichroism, optical rotatory dispersion and absolute configuration of flavanones, 3-hydroxyflavanones and their glycosides. *Tetrahedron* **26**: 4093-4108
- CANOVAS, F. G., GARCIA-CARMONA, F., SANCHEZ, J. V., PASTOR, J. L. and TERUEL, J. A. (1982): The role of pH in the melanin biosynthesis pathway. *J. Biol. Chem.* **257**: 8738-8744
- GARCIA, M., KNO, M. H. C., VIEIRA, D. M., NASCIMENTO, M. C. D. and MORS, W. B. (1986): Isoflavonoids from *Derris spruceana*. *Phytochemistry* **25**: 2425
- GARZON, N. L., CUCA, S. L. E., MARTINEZ, V. J. C., YOSHIDA, M. and GOTTLIEB, O. R. (1987): Flavonolignoid from the fruit of *Iryanthera* leaves. *Phytochemistry* **26**: 2835-2837
- GRINO, P. B., GRIFFIN, J. E. and WILSON, J. D. (1990): Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. *Endocrinology* **126**: 1165-1172
- HANO, Y., AIDA, M. and NOMURA, T. (1990): Constituents of the Moraceae plants. Part 4. Two new natural Diels-Alder type adducts from the root bark of *Artocarpus heterophyllus*. *J. Nat. Prod.* **53**: 391-395
- HARBORNE, J. B., MABRY, T. J. and MABRY, H. (1975): in *The Flavonoids*, pp. 277-282, Chapman And Hall Ltd, New York.
- HARBORNE, J. B. and MABRY, T. J. (1982): *The Flavonoids: Advances in Research*, p. 57, Chapman and Hall, New York.
- HEARING, V. J. Jr. (1987): Mammalian monophenol monooxygenase (tyrosinase): purification, properties, and reactions catalyzed. *Methods in Enzymology* **142**: 154-165
- IMAMURA, H., KUROSU, H. and TAKAHASHI, T. (1967): The chemistry of wood extractives. XVIII. The heartwood constituents of *Rhus javanica* L.. *Mokuzai Gakkaishi* **13**: 295-299
- IMOKAWA, G., KAWAI, M., MISHIMA, Y. and MOTEGI, I. (1986): Differential analysis of experimental hypermelanosis induced by UVB, PUVA, and allergic contact dermatitis using a brownish guinea pig model. *Arch. Dermatol. Res.* **278**: 352-362
- JANES, N. F., MORGAN and J. W. W. (1960): The occurrence of dihydrokaempferol in *Nothofagus* species.

- J. Chem. Soc. 2560-2565
- JANG, D. I., LEE, B. G., JEON, C. O., JO, N. S., PARK, J. H., CHO, S. Y., LEE, H. and KOH, J. S. (1997) : Melanogenesis inhibitor from paper mulberry. *Cosmetics & Toiletries Magazine* 112 : 59-62
- JANG, M. S., CAI, E. N., UDEANI, G. O., SLOWING, K. V., THOMAS, C. F., BEECHER, C. W. W., FONG, H. H. S., FONG, H. H. S., FARNSWORTH, N. R., KINGHORN, A. D., MEHTA, R. G., MOON R. C. and PEZZUTO, J. M. (1997) : Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 275 : 218-220
- JAYATILAKE, G. S., JAYASURIYA, E. LEE, KOONCHANOK, N. M., GEAHLEN, R. L., ASHENDEL, C. L., MCLAUGHLIN, J. L. and CHANG, C.-J. (1994) : Kinase inhibitors from *Polygonum cuspidatum*. *J. Nat. Prod.* 56 : 1805-1810
- JIMÉNEZ, M. and GARÍ A-CARMONA, F. (1997) : 4-Substituted resorcinols (sulfite alternatives) as slow-binding inhibitors of tyrosinase catecholase activity. *J. Agric. Food Chem.* 45 : 2061-2065
- JONES, C. D., AUDIA, J. E., LAWHORN, D. E., MCQUAID, L. A., NEUBAUER, B. L., PIKE, A. J., PENNINGTON, P. A., STAMM, N. B., TOOMEV, R. E. and HIRSCH, K. S. (1993) : Nonsteroidal inhibitors of human type I steroid 5 α -reductase. *J. Med. Chem.* 36 : 421-423
- KASAI, R., HIRONO, S., CHOU, W. H., TANAKA, O. and CHEN, F. H. (1988) : Sweet dihydroflavonol rhamnoside from leaves of *Engelhardtia chrysolepis*, a Chinese folk medicine, Hung-gi. *Chem. Pharm. Bull.* 36 : 4167-4170
- KIM, M. G., AMOS, L. W. and BARNE, E. E. (1993) : Investigation of a resorcinol-formaldehyde resin by carbon-13 NMR spectroscopy and intrinsic viscosity measurement. *J. Polym. Sci., A. Polym. Chem.* 31 : 1871-1877
- KOISO, I. (1990) : Development of recent melanin control agents and whitening cosmetics (in Japanese). *Fragrance J.* 18 : 39-46
- KONDO, T. (1951) : On the chemical constituents of the heartwood of *Distylium Racemosum* S. et Z. *J. Fac. Agric. Kyushu Univ.* 10 : 79-99
- KOSHIHARA, Y., FUJIMOTO, Y. and INOUE, H. (1987) : A new 5-lipoxygenase selective inhibitor derived from *Artocarpus communis* strongly inhibits arachidonic acid-induced ear edema. *Biochem. Pharmacol.* 37 : 2161-2165
- KROHN, K., MÜLLER, H., ADIWIDJAJA, G., JARCHOW, O. H., SCHMALLE, H. W., HAUSEN, B. M. and SCHULZ, K.-H. (1986) : The structure of the tetraacetate of the allergen chlorophorin. *Z. Kristallogr.* 174 : 283-290
- KUBO, I., KINST-HORI, I. and YOKOKAWA, Y. (1995) : Tyrosinase inhibitors from *Anacardium occidentale* fruits. *J. Nat. Prod.* 57 : 545-551
- LEE, S. H., PARK, J. S., CHUNG, S. R., RO, J. S. and LEE, K. S. (1997) : Inhibitory effect of the natural products isolated from the *Sophora flavescence* on the tyrosinase. in *International Symposium on Natural Medicines*, Kyoto, Japan, p. 182
- LIANG, T., CASCIERI, M. A., CHEUNG, A. H., REYNOLDS, G. F. and RASMUSSEN, G. H. (1985) : Species differences in prostatic steroid 5 α -reductases of rat, dog, and human. *Endocrinology* 117 : 571-579
- LIANG, T. and LIAO, S. (1992) : Inhibition of steroid 5 α -reductase by specific aliphatic unsaturated fatty acids. *Biochem. J.* 285 : 557-562
- LIAO, S., KOKONTIS, J., SAI, T. and HIIPAKKA, R. A. (1989) : Androgen receptors: structures, mutations, antibodies and cellular dynamics. *J. Steroid Biochem.* 34 : 41-51
- LIAO, S. and HIIPAKKA, R. A. (1995) : Selective inhibition of steroid 5 α -reductase isozymes by tea epicatechin-3-gallate and epigallocatechin-3-gallate. *Biochem. Biophys. Res. Commun.* 214 : 833-838

- MABRY, T. J., MARKHAM, K. R. and THOMAS, M. B. (1970a) : in The systematic identification of flavonoids, p. 280, Springer-Verlag New York Inc.
- MABRY, T. J., MARKHAM, K. R. and THOMAS, M. B. (1970b) : in The systematic identification of flavonoids, p. 309, Springer-Verlag New York Inc.
- MAEDA, K. and FUKUDA, M. (1991) : *in vitro* effectiveness of several whitening cosmetic compounds in human melanocytes. J. Soc. Cosmet. Chem. 42 : 361-368
- MAEDA, K. and FUKUDA, M. (1996) : Arbutin: mechanism of its depigmenting action in human melanocyte culture. J. Pharmacol. Exp. Ther. 276 : 765-769
- MALAN, E., SWINNY, E., FERREIRA, D., HALL, A. J. (1988) : Metabolites from *Chlorophora excelsa* : possible intermediates in the biogenesis of a pentasubstituted stilbene. Phytochemistry 27 : 2309-2312
- MCEVILY, A. J., IYENGAR, R., OTWELL, W. S. (1992) : Inhibition of enzymatic browning in foods and beverages. Crit. Rev. Food Sci. Nutr. 32 : 253-273
- MILLER, J. M. and BOHM, B. A. (1979) : Flavonoids of *Leptarrhena pyrolifolia*. Phytochemistry 18 : 1412-1413
- NAIR, P. M., RAO, A. V. R. and VENKATARAMAN, K. (1964) : Cycloartocarpin. Tetrahedron Lett. 2 : 125-128
- NAKANO, J., UCHIDA, K. and FUJIMOTO, Y. (1989) : An efficient total synthesis of AC-5-1 : novel 5-lipoxygenase inhibitor isolated from *Artocarpus communis*. Heterocycles 29 : 427-430
- NOMURA, T., HANO, Y. and AIDA, M. (1998) : Isoprenoid-substituted flavonoids from *Artocarpus* plants (Moraceae). Heterocycles 47 : 1179-1205
- PARENTI, P., PIZZIGONI, A., HANZET, G., HAKIM, E. H., MAKMUR, L., ACHMAD, S. A. and GORDANA, B. (1998) : A new prenylated flavone from *Artocarpus champeden* inhibits the K⁺-dependent amino acid transport in *Bombyx mori* midgut. Biochem. Biophys. Res. Commun. 244 : 445-448
- PAWELEK, J. M. and KORNER, A. M. (1982) : The biosynthesis of mammalian melanin. Am. Sci. 70 : 136-145
- PILOTTI, C. A., KONDO, R., SHIMIZU, K. and SAKAI, K. (1995) : An examination of the anti-fungal components in the heartwood extracts of *Pterocarpus indicus*. Mokuzai Gakkaishi 41 : 593-597
- PRICE, V. H. (1975) : Testosterone metabolism in the skin. A review of its function in androgenetic alopecia, acne vulgaris, and idiopathic hirsutism including recent studies with antiandrogens. Arch. Dermatol. 111 : 1496-1502
- PROTA, G. (1988) : Progress in the chemistry of melanins and related metabolites. Med. Res. Rev. 8 : 525-556
- RUSSELL, D. W. and WILSON, J. D. (1994) : Steroid 5 α -reductase: two genes/two enzymes. Annu Rev Biochem 63 : 25-61
- SÁNCHEZ-FERRER, A., NEPTUNO RODRÍGUEZ-LÓPEZ, N., GARCÍA-CÁNOVAS, F. and GARCÍA-CARMONA, F. (1995) : Tyrosinase: a comprehensive review of its mechanism. Biochim. Biophys. Acta 1247 : 1-11
- SATO, M., FUJIWARA, S., TSUCHIYA, H., FUJI, T., INUMA, M., TOSA, H. and OHKAWA, Y. (1996) : Flavones with antibacterial activity against cariogenic bacteria. J. Ethnopharmacology 54 : 171-176
- SCHULTZ, T. P., BOLDIN, W. B., FISHER, T. H., NICHOLAS, D. D., MCMURTREY, K. D. and POBANZ, K. (1992) : Structure-fungicidal properties of some 3- and 4-hydroxylated stilbenes and bibenzyl analogues. Phytochemistry 31 : 3801-3806.
- SCHULTZ, T. P., HARMS, W. B., FISHER, T. H., MCMURTREY, K. D., MINN, J. and NICHOLAS, D. D. (1995) : Durability of angiosperm heartwood-The importance of extractives. Holzforschung 49 : 29-34
- SCHWEIKERT, H. U. and WILSON, J. D. (1974) : Regulation of human hair growth by steroid hormones. I. Testosterone metabolism in isolated hairs. J. Clin. Endocrinol. Metab. 38 : 811-819
- SHIN, N. H., RYU, S. Y., CHOI, E. J., KANG, S. H., CHANG, I. M., MIN, K. R. and KIM, Y. (1998) :

- Oxyresveratrol as the potent inhibitor on dopa oxidase activity of mushroom tyrosinase. *Biochem. Biophys. Res. Commun.* **243**: 801-803
- TAKAHASHI, S. (1988) : 'Jamu' (in Japanese). Hirakawa Publishing Co., Tokyo
- TAKASUGI, M., MUÑOZ, L., MASAMUNE, T., SHIRATA, A. and TAKAHASHI, K. (1978) : Stilbene phytoalexins from diseased mulberry. *Chem. Lett.* 1241-1242
- TENOVER, J. S. (1991) : Prostates, pates, and pimples. The potential medical uses of steroid 5 α -reductase inhibitors. *Endocrinol. Metab. Clin. N. Amer.* **20** : 893-909
- TOMITA, K., ODA, N., OHBAYASHI, M., KAMEI, H., MIYAKI, T. and OKI, T. (1990) : A new screening method for melanin biosynthesis inhibitors using *Streptomyces bikiniensis*. *J. Antibiotics* **43** : 1601-1605
- VENKATARAMAN, K. (1972) : Wood phenolics in the chemotaxonomy of the Moraceae. *Phytochemistry* **11** : 1571-1586.
- WOLLENWEBER, E. (1982) : Flavonoids with other substituents.: In *The Flavonoids: Advances in Research* (J. B. Harbone and T. J. Mabry, eds.), pp. 219-229, Chapman and Hall, New York.
- YOKOTA, T., NISHIO, H., KUBOTA, Y. and MIZOGUCHI, M. (1998) : The inhibitory effect of glabridin from licorice extracts on melanogenesis and inflammation. *Pigment Cell Res.* **11** : 355-361

6. Acknowledgement

I express my sincerest thanks to Professor Dr. K. Sakai, and Associate Professor Dr. R. Kondo, Department of Forestry and Forest Products Science, Faculty of Agriculture, Kyushu University, for their supervision, encouragement and patience during the whole course of the work. I am extremely grateful to Mr. K. Fujita in the same Department for his guidance in the course of this study and critical advice. I am deeply grateful to Professor Dr. Y. Shoyama, Faculty of Pharmaceutical Sciences, Kyushu University for his critical reading and Mr. H. Sato, Kansai Koso Co. Ltd. for melanin biosynthesis inhibitory test and preparation of rat liver microsome.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education (Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists).

(Received 21 March 2000; Accepted 18 October 2000)

Artocarpus incisus 樹木からのチロシナーゼ及び 5 α -リダクターゼ阻害成分

清水 邦 義

要 旨

世界の森林面積の約40-50%は熱帯林であり、特に熱帯多雨林は蓄積量も多く、かつ構成樹種が極めて多種多様であることを特徴としている。これらの樹種の特徴は、化学成分的に見ると微量成分である抽出成分の量的、質的な大きな変異として現れる。即ち熱帯樹木抽出成分は有用成分の宝庫であり、興味ある成分が見いだされる可能性が高い。本研究では、熱帯産樹木抽出成分から、シミやソバカスなどのメラニン色素沈着症の治療薬になり得るチロシナーゼ阻害成分ならびに前立腺肥大症や男性型脱毛症の治療薬として期待される5 α -リダクターゼ阻害成分の探索を行った。

まず、23種のパプアニューギニア産樹木心材抽出物からのチロシナーゼ阻害成分の探索を行った。その結果、唯一 *Artocarpus incisus* の心材メタノール抽出物のみが、既知の強力な阻害剤であるコウジ酸と同等の阻害活性を示した。さらにB16メラノーマ細胞や茶色モルモットを用いたメラニン生成抑制試験においても色素の著しい淡色効果を示した。チロシナーゼ阻害活性を指標に、活性成分の探索を行い、2つの新規化合物 (artocarbene, isoartocarpesin と命名) を含む7つの強力なチロシナーゼ阻害成分を単離同定した。構造-活性相関を検討し、4位置換レゾルシノール構造が強力な阻害活性発現に重要であることを明らかにした。

次に、パプアニューギニア及びタイ産植物抽出物からの5 α -リダクターゼ阻害成分の探索を行った。その結果、*A. incisus* の心材及び葉部抽出物が高い阻害活性を示した。活性成分の探索を行い、新規化合物 (3'-geranyl-2',3,4,4'-tetrahydroxychalcone) を含む一連のイソプレノイド置換フラボノイド及びスチルベンを単離、同定した。特に, artocarpin, chlorophorin, 2'-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone, 3'-geranyl-2',3,4,4'-tetrahydroxychalcone は既知の天然非ステロイド系阻害剤である α -リノレン酸より高い阻害活性を示した。構造-活性相関検討により、イソプレノイド置換基 (プレニル基またはゲラニル基) の存在が阻害活性発現に重要であることが判明した。

キーワード: *Artocarpus incisus*, クワ科, 心材, artocarbene, チロシナーゼ阻害剤, メラニン生合成阻害剤, isoartocarpesin, 4位置換レゾルシノール, 構造活性相関, 5 α -リダクターゼ阻害剤, 葉, 3'-geranyl-2',3,4,4'-tetrahydroxychalcone