INVESTIGATION OF BIOACTIVE COMPOUNDS FROM ENDOPHYTIC ACTINOMYCETE

By

Asawin Wanbanjob

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

Department of Chemistry

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การแยกองค์ประกอบและศึกษาคุณสมบัติทางเคมีของสารชีวภาพที่สร้างจาก endophytic actinomycete

โดย
นายอัศวิน วันบรรจบ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมีอินทรีย์
ภาควิชาเคมี
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ปีการศึกษา 2551

The Graduate School, Silpakorn University has approved and accredited the Thesis title of "Investigation of bioactive compounds from endophytic actinomycete" submitted by Asawin Wanbanjob as a partial fulfillment of the requirements for the degree of Master of Science, program of organic chemistry.

…….....

(Associate Professor Sirichai Chinatangkul,Ph.D.)

Dean of Graduate School

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(Assoc. Prof. Chutima Limmatvapirat, Ph.D.)

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(Asst. Prof. Thongchai Taechowisan, Ph.D.)

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(Kanok-on Rayanil, Ph.D.)

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(Prof. Pittaya Tuntiwachwuttikul, Ph.D.)

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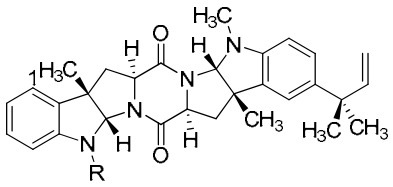
49302206 : MAJOR : ORGANIC CHEMISTRY
KEY WORD : ENDOPHYTIC ACTINOMYCETES; *FICUS BENJAMINA*; MORACEAE;
ALPINIA GALANGA; ZINGIBERACEAE; *STREPTOMYCES* SP. SUC1;
STREPTOMYCES SP. SU238; AERIAL ROOTS
ASAWIN WANBANJOB : INVESTIGATION OF BIOACTIVE COMPOUNDS FROM ENDOPHYTIC ACTINOMYCETE. THESIS ADVISOR : PROF. PITTAYA TUNTIWACHWUTTIKUL, Ph.D. 82 pp.

Four new compounds, Iansai A-D (**1-4**), were isolated from the crude ethyl acetate extract of *Streptomyces* sp. SUC1, which isolated from the aerial roots of *Ficus benjamina*.

A known compound, echinomycin (**5**), was isolated from the crude ethyl acetate extract of *Streptomyces* sp. SU238, which isolated from the roots of *Alpinea ganlanga*.

The structures were determined on the basis of spectroscopic analysis.



Iansai A (**1**) : R = H
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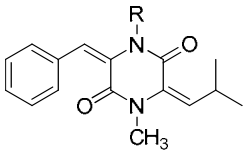

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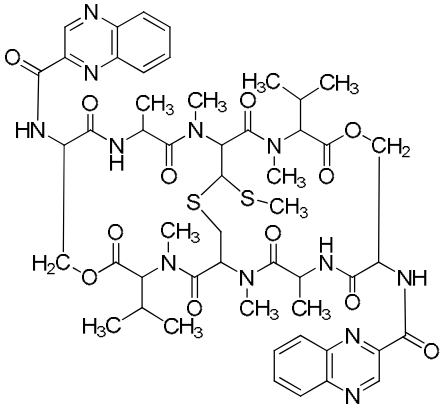


Echinomycin (**5**)

49302206 : สาขาวิชาเคมีอินทรีย์

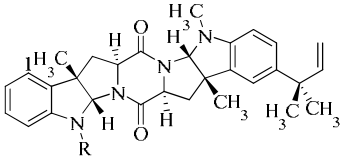
คำสำคัญ : ไทรย้อย; ข่า; รากอากาศ; ENDOPHYTIC ACTINOMYCETES; *FICUS BENJAMINA*; MORACEAE; *ALPINIA GALANGA*; ZINGIBERACEAE; *STREPTOMYCES* SP.SUC1; *STREPTOMYCES* SP. SU238; AERIAL ROOTS

อัศวิน วันบรรจบ : การแยกองค์ประกอบและศึกษาคุณสมบัติทางเคมีของสารชีวภาพที่สร้างจาก endophytic actinomycete. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ศ. ดร. พิทยา ตันติเวชวุฒิกุล. 82 หน้า.

การศึกษาองค์ประกอบทางเคมีของสารชีวภาพที่สร้างจาก *Streptomyces* sp. SUC1 ซึ่งแยกจากเนื้อเยื่อของรากอากาศไทรย้อย (*Ficus benjamina*) พบสารใหม่ 4 ตัวคือ lansai A-D (**1-4**)

การศึกษาองค์ประกอบทางเคมีของสารชีวภาพที่สร้างจาก *Streptomyces* sp. SU238 ซึ่งแยกจากเนื้อเยื่อของรากข่า (*Alpinia galanga*) พบสาร echinomycin (**5**)

พิสูจน์โครงสร้างของสารประกอบดังกล่าวด้วยเทคนิคสเปกโตรสโกปี



lansai A (**1**) : R = H
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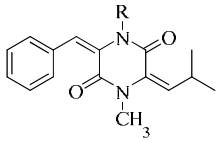

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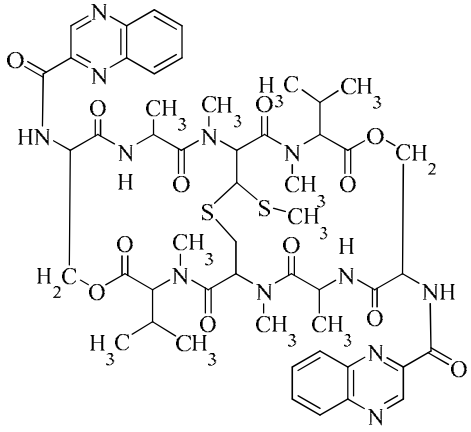


Echinomycin (**5**)

ลายมือชื่อนักศึกษา .....

ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ .....

Acknowledgments

I wish to express my deepest and sincere gratitude to my major-advisor, Prof. Dr. Pittaya Tantiwachwuttikul, and my co-advisor, Asst. Prof. Dr. Thongchai Taechowisan, for their valuable instructions, expert guidance, excellent suggestions and kindness which are more than I can describe here. Everything will always be in my mind. Sincere appreciation goes to Dr. Supachai Supalaknari, Dr. Kanok-on Rayanil and Assoc. Prof. Dr. Chutima Limmatvapirat, the external thesis examiner, for their comments and suggestions.

My sincere gratitude is expressed to all members in Organic Chemistry program for giving me a delightful graduate life and providing a prosperous atmosphere for learning and research. My thanks also go to all staff members of the Department of Chemistry and Department of Microbiology, Faculty of Science, Silpakorn University for their generous help.

Finally, none of this thesis would have been possible without love and encouragement of my family and friends. I thank them all for their kindness and valuable advice. Everything will always kept in my mind.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

The actinomycetes are aerobic, gram-positive bacteria that form branching filaments or hyphae and asexual spores. Although they are a diverse group, the actinomycetes do share many properties. Actinomycetes, when growing on a solid such as agar, the branching network of hyphae developed by actinomycetes grows both on the surface of the substratum and into it to form a substrate mycelium. Septa usually devide the hyphae into long cells (20 μm and longer) containing several nucleoids. Many actinomycetes also have an aerial mycelium that extends above the substratum and forms asexual, thin-walled spores called conidia or conidiospores on the ends of filaments [1], as show in Figure 1.1.

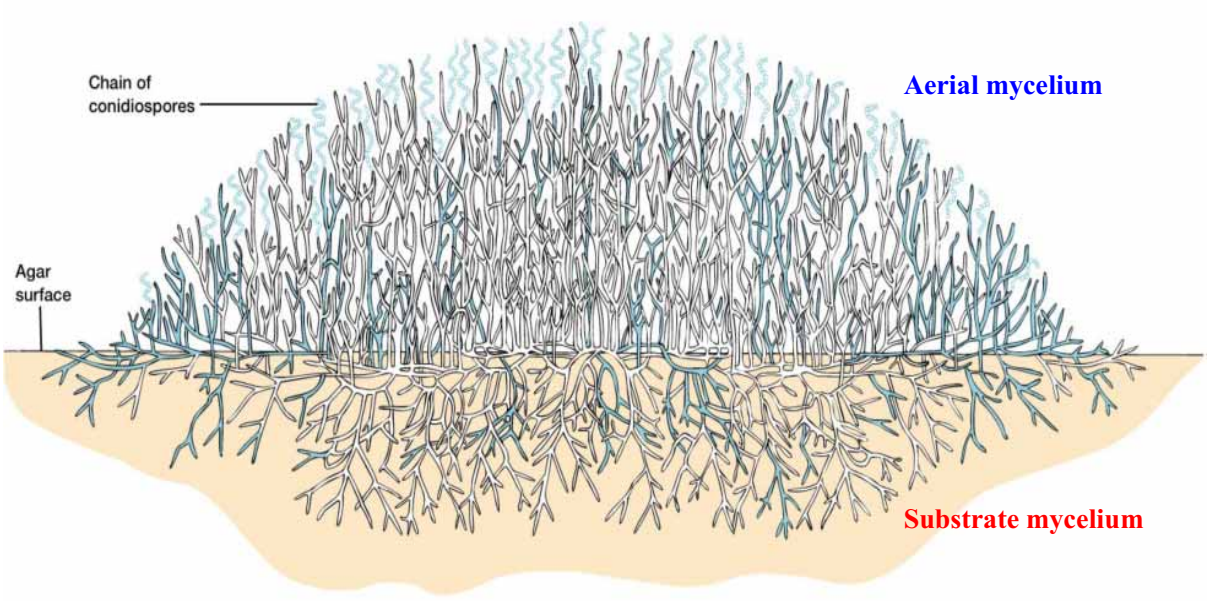


Figure 1.1 An Actinomycete Colony [1].

Actinomycetes are ubiquitous in nature. They are primarily soil inhabitants and are very widely distributed. They can degrade an enormous number and variety of organic compounds and are extremely important in the mineralization of organic matter. Takahashi *et al*.[2] investigated the vertical distribution of actinomycetes in soils taken at 10 cm. intervals between 0 to 1 m. A comparative survey of all isolates demonstrated that the actinomycete population was the largest in soils of the surface layer. And although the number of actinomycetes decreased gradually as depth increased, individual actinomycete strains were present in all soil layers. Actinomycetes produce most of the medically useful natural antibiotics, as show in Table 1.1.

Actinomycetes produce many extracellular enzymes in soil. By decomposing complex mixtures of polymer in dead plant, animal and fungal material. They have the important roles in soil biodegradation and recycling of nutrients associated with recalcitrant polymers [3].

Table 1.1 Important antibiotics from actinomycetes [4]

Antibiotic	Producer	Chemical class	Application
Actinomycin D	*Streptomyces* spp.	Peptide	Antitumor
Antimycin A	*Streptomyces* spp.	Macrolide	Telocidal
Avermectin	*S. avermitilis*	Macrolide	Antiparasitic
Bambermycin	*S. bambergiensis*	Substituted aminoglycoside	Growth promotant
Bialaphos	*S. hygroscopicus*	Peptide	Herbicidal
Bleomycin	*S. verticillus*	Glycopeptide	Antitumor
Candicidin	*S. griseus*	Polyene macrolide	Antifungal
Cephamycin C	*Nocardia lactamdurans*	β-lactam	Antibacterial
Chloramphenical	*S. venezuelae*	*N*-dichloroacyl phenylpropanoid	Antibacterial
Chlortetracycline	*S. aureofaciens*	Tetracycline	Antibacterial
Clavulanic acid	*S. clavuligerus*	β-lactam	Antibacterial
Cycloserine	*S. orchidaceus*	Substituted cyclic peptide	Antibacterial

Table 1.1 Important antibiotics from actinomycetes (cont.)

Antibiotic	Producer	Chemical class	Application
Daptomucin	*S. rodeosporus*	Lipopeptide	Antibacterial
Daunorubicin (daunomycin)	*S. peucetius*	anthracycline	Antitumor
Desferrioxamine	*S. pilosus*	Peptide	Iron purging in iron overload
Doxorubicin	*S. peuctius var. caesius*	Anthracycline	antitumor
Erythromycin	*Sac. Erythraea*	Macrolide	Antibacterial
FK506	*S. hygroscopicus*	Macrolide	Immunosuppressant
Fortimicin	*Micromonospora olivoasterospora*	Aminoglycoside	Antibacterial
Fosfomycin	*Streptomyces* spp.	Phosphoric acid	Antibacterial
Gentamycin	*Micromonospora* spp.	Aminoglycoside	Antibacterial
Hygromycin B	*S. hygroscopicus*	Substituted aminoglycoside	Antihelminthic
Kanamycin	*S. kanamyceticus*	Aminoglycoside	Antibacterial
Lasalocid	*S. lasaliensis*	Polyether	Anticoccidial; growth promotant
Lincomycin	*S. lincolnensis*	Sugar-amide	Antibacterial
Milbemycin	*S. argilaceus*	Macrolide	Antiparasitic
Mithramycin	*S. argillaceus*	Aureolic acid	Antitumor
Mitomycin C	*S caespitosus* *S. verticillatus*	Benzoquinone	Antitumor
Monensin	*S. cinnamonensis*	Polyether	Anticoccidial; growth promotant
Natamycin	*S. nataensis*	Tetraene polyene	Antifungal
Neomycin	*S. fradiae*	Aminoglycoside	Antibacterial

Table 1.1 Important antibiotics from actinomycetes (cont.)

Antibiotic	Producer	Chemical class	Application
Nikkomycin	*S. tendae*	Nucleoside	Antibacterial; insecticidal
Nocardicin	*Nocardia uniformis*	β-lactam	Antibacterial
Nosiheptide	*S. actuosus*	Thiopeptide	Growth promotant
Novobiocin	*S. neveus*	Coumerin glycoside	Antibacterial
Nystatin	*S. noursei*	Polyene macrolide	Antifungal
Oleandomycin	*S. antibioticus*	Macrolide	Antibacterial
Oxytetracycline	*S. rimosus*	Tetracycline	Antibacterial
Paromomycin	*S. rimosus* forma *paromomycinus*	Aminoglycoside	Antibacterial
Phleomycin	*S. verticillus*	Glycopeptide	Antitumor
Polyoxins	*S. cacaoi* var. *asoensis*	Nucleosidepeptide	Antifungal
Pristinamycin	*S. pristimaespiralis*	Peptidic macrolactone + polyunsaturated macrolactone	Antibacterial
Rapamycin	*S. hygroscopicus*	Macrolide	Immunosuppressant
Rifamycin	*Amycolatopsis mediterranei*	Ansamycin	Antibacterial
Ristocetin	*N. lurida*	Glycopeptide	antibacterial
Salimomycin	*S. albus*	Polyether	Anticoccidal; growth promotant
Spectinomycin	*S. spectabilis*	Aminocyclitol	Antibacterial
Spinnosyns	*Sac. Spinosa*	Tetracyclic macrolide	Insecticidal
Spiramycin	*S. ambofaciens*	Macrolide	antibacterial
Streptogramins	*S. graminofaciens*	Macrocyclic lactone	Antibacterial

Table 1.1 Important antibiotics from actinomycetes (cont.)

Antibiotic	Producer	Chemical class	Application
Streptomycin	*S. griseus*	Aminoglycoside	Antibacterial
Teichoplanin	*Actinoplanes teichomyceticus*	Glycopeptide	Antibacterial
Tetracycline	*S. aureofaciens*	Tetracycline	Antibacterial
Thienamycin	*S. cattleya*	β-lactam	Antibacterial
Thiostrepton	*S. azureus*	Thiopeptide	Growth promotant
Tobramycin	*S. tenebarius*	Aminoglycoside	Antibacterial
Tylosin	*S. fradiae*	Macrolide	Growth promotant
Validamycin	*S. hygroscopicus*	Aminoglycoside	Plant protectant
Vancomycin	*A. orientalis*	Glycopeptide	Antibacterial
Virginiamycin	*S. virginiae*	Macrocyclic lactone + macrocyclic peptidolactone	Growth promotant

Endophytes are microorganisms live between the living cells of their respective host and cause no overt tissue damage. Although fungi are the most commonly isolated endophytic microorganisms, but there are also actinomycetes isolated from the tissues of healthy plants [5-7]. In 1980, Baker *et al*. [5] isolated the first endophytic actinomycete from the root of *Elaeagnus umbellate*. Endophytic actinomycetes were increasingly important, because of some actinomycete strains have the ability to produce the bioactive compounds inhibiting some of the pathogenic fungi and bacteria [6]. In addition in 1995, Okazaki *et al.* [8] reported the difference of bioactive compounds and types of actinomycetes between the endophytic actinomycetes and soil actinomycetes.

Okazaki *et al.* [8], Matsukuma *et al.* [9], and Matsumoto *et al*. [10] reported that a variety of actinomycetes inhabit a wide range of plants as symbionts, parasites or saprophytes, most of them belong to the genera, *Streptomyces* and *Microbispora.*

Coombs and Franco [11] reported 38 strains of endophytic actinomycetes, isolated from surface-sterilized root of healthy wheat plant, belonging to *Streptomyces, Microbispora, Micromonospora* and *Nocardia.* Likewise Okazaki [12] also reported a total of 246 strains of actinomycetes isolated from plant origin belonging to *Streptomyces* (97 strains), *Microbispora* (57 strains), *Micromonospora* (18 strains), *Actinomonodura* (4 strains) and *Nocardia* (23 strains).

Rosenblueth *et al.* [13] reported several strains of enophytic actinomycetes can be isolated from a single plant and they are often especially rich in its root. Likewise as Taechowisan *et al.* [14] reported that endophytic actinomycetes, especially *Streptomyces* spp., were the most common isolates recovered, being most prevalent from roots, leaves and less from stems. The evidence shows that plant-associated actinomycetes are quite common in nature. No one knows if microbial communities inside plants interact. It has been speculated that beneficial and/or harmful effects are the combined effect of their activities.

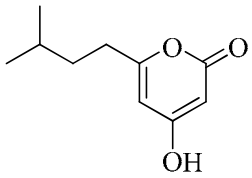
Endophytes colonizing inside plants usually get nutrition and protection from the host plants. In return, endophytes profoundly enhanced the health of the host plants by producing a variety of bioactive metabolites, belonging to antibiotics, plant growth promotor, plant growth inhibitor and enzyme [15-19].

In general, it can be expected that environmental conditions, in which the host plant growth influence the number and variety of endophytic populations. Generally plants growing in unique environmental settings, having special ethnobotanical uses, having extreme age or interesting endemic locations produce novel endophytic microorganisms which can supply new bioactive compounds [20-21].

In 2003, Taechowisan and Lumyong [22] isolated 59 endophytic actinomycetes from the root of *Zingiber officinale* and *Alpinia galanga* and determinded their antifungal activity against *Candida albican* and phytopathogenic fungi. Among them *S. aureofaciens* CMUAc130 showed the most intense antagonism against the tested microorganisms.

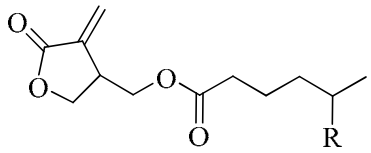
Since 2000, a number of new bioactive compounds have been isolated from endophytic actinomycetes such as

Fistupyrone (**6**), an inhibitor of spore germination of *Alteranria brassicicola*, was isolated from *Streptomyces* sp. TP-A0569. This strain was isolated from a leaf of spring onion, *Allium fistulosum* [23].



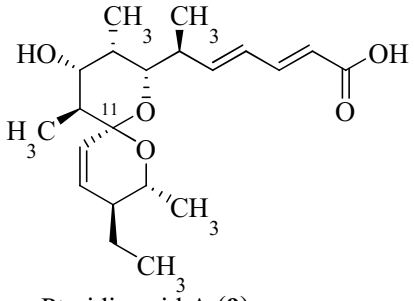
Fistupyrone (**6**)

Cedarmycin A (**7**) and B (**8**), the ester of a butyrolactone, were isolated from *Streptomyces* sp. TP-A0456, which was isolated from a twig of cedar, *Cryptomeria japonica*. Cedarmycin A show antifungal activity against *Candida glabrata* with the MIC of 0.4 μgml^{-1} [24].



Cedarmycin A (**7**) R=Me
Cedarmycin B (**8**) R=H

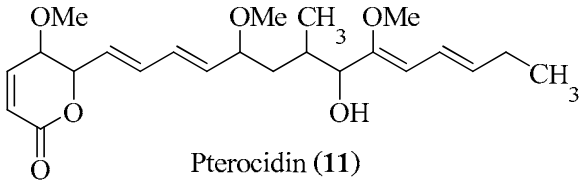
Pteridic acid A (**9**) and B (**10**), the auxin-like plant growth promoter, were isolated from *S. hygroscopicus* TP-A0451 which was isolated from a stem of bracken, *Pteridium aquilinum*. Pteridic acids show the property of inhibiting the rice germination at 100 ppm. Pteridic acid A induces the adventitious root formation of the kidney bean hypocotyl at 1 nM as effectively as indoleacetic acid [25].



Pteridic acid A (**9**)

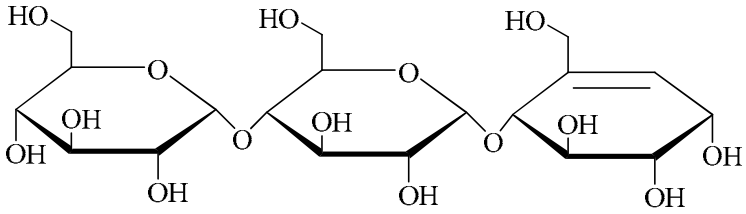
Pteridic acid B (11-epimer) (**10**)

Later in 2006, Igarashi *et al*. [26] reported a new cytotoxic compound, pterocidin (**11**), which isolated from the same strain, *S. hygroscopicus* TP-A0451. This compound showed cytotoxicity against some human cancer cell line with IC_{50} values of 2.9 – 7.1 μM.

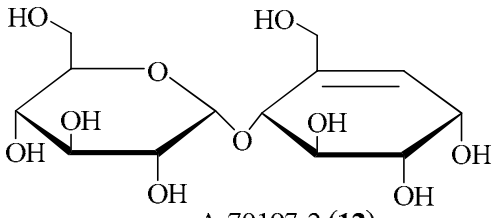


Pterocidin (**11**)

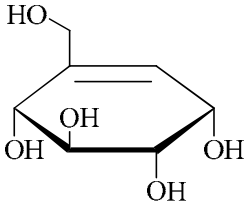
Substance A-79197-2 (**12**) and A-79197-3 (**13**), the plant growth inhibitors, were isolated from *Dacthylosporangium aurantiacum*, which was isolated from a leaf of *Cucubalus* sp. collected at Chiba prefecture. These compounds were determinded to be di- and tri- saccharides consisting of streptol (**14**), they have weak herbicidal activity as streptol (**14**). The minimum concentration of streptol (**14**), A-79197-2 (**12**) and A-79197-3 (**13**) to inhibit the germination of *Brassica rapa* were 8, 18 and 50 ppm, respectively [27].



A-79197-3 (**13**)



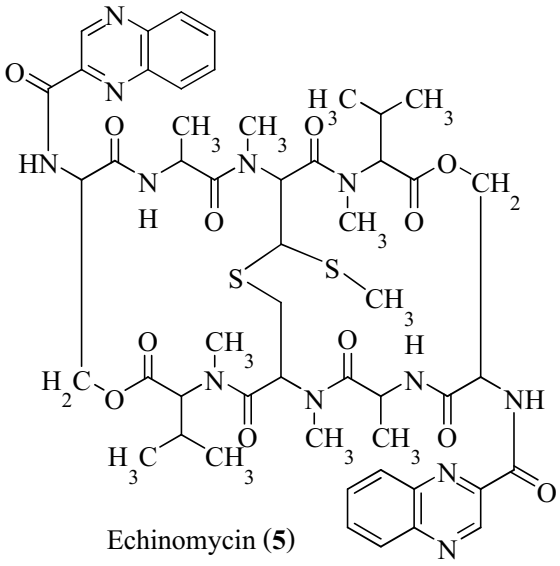
A-79197-2 (**12**)



Streptol (**14**)

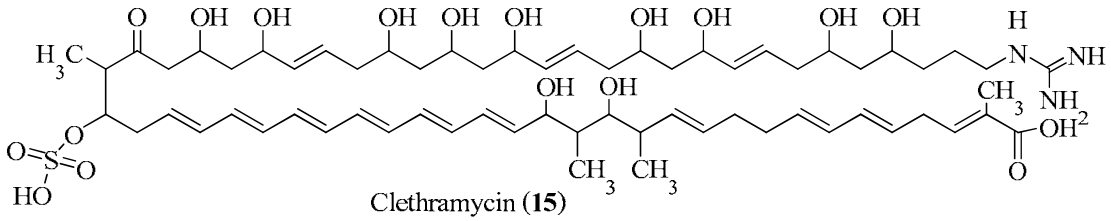
In 2002, Castillo *et al*. [28] isolated four novel peptide antibiotics, called munumbicins A-D, but do not show the structure of these compounds. Munumbicins were isolated from endophytic *Streptomyces* sp. NRRL30562 of a medicinal plant snakevine (*Kennedia nigriscans*). This plant has been used for centuries by Aboriginal people to treat open bleeding wounds to prevent sepsis. These compounds exhibited wide-spectrum activity against many human and phytopathogenic fungi and bacteria.

In 2003, Castillo *et al*. reported the isolation of novel peptide antibiotics named kakadumycins (kakadumycin A and related compounds) which were chemically related to echinomycin (**5**). These compounds were isolated form *Streptomyces* sp. NRRL30566 which was isolated from a fern-leaved greville tree (*Grevillea pteridifolia*) growing in the Northern Territory of Australia. Kakadumycin A has wide spectrum antibiotic activity, especially against Gram-positive bacteria, and it generally displays bioactivity better than echinomycin (**5**). For instance, against *Bacillus anthracis* strains, kakadumycin A has minimum inhibitory concentrations of 0.2-0.3 μgml^{-1} in contrast to echinomycin at 1.0-1.2 μgml^{-1}. Both echinomycin and kakadumycin A have impressive activity against the malarial parasite *Plasmodium falciparum* with LD_{50} in the range of 7-10 $ngml^{-1}$ [29].



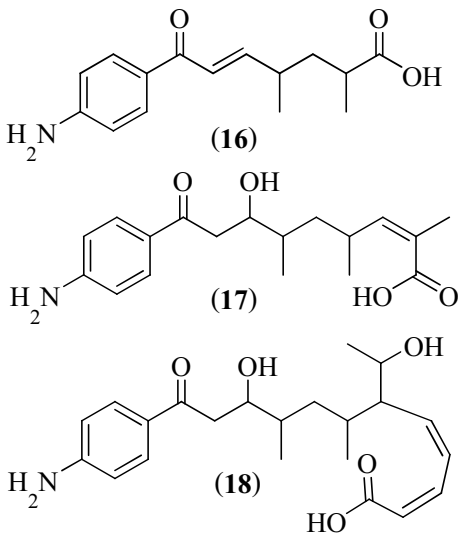
Echinomycin (**5**)

Clethramycin (**15**), an inhibitor of pollen tube growth, was isolated from *S. hygroscopicus* TP-A0623, which was isolated from the root of *Clethra barbinervis* collected in Japan. Clethramycin also shows antifungal activity against *Candida albicans* and *Cryptococcus neoformans* with the MIC of 1 $\mu g ml^{-1}$ [30].

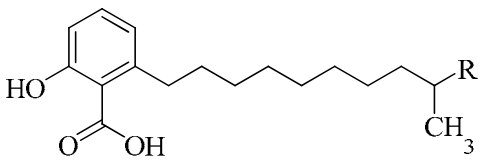


Clethramycin (**15**)

In 2005, Guan *et al*. [31] reported three new *p*-aminoacetophenic acids compost of 7-(4-aminophenyl)-2,4-dimethyl-7-oxo-hept-5-enoic acid (**16**), 9-(4-aminophenyl)-7-hydroxy-2,4,6-trimethyl-9-oxo-non-2-enoic acid (**17**) and 12-(4-aminophenyl)-10-hydroxy-6-(1-hydroxyethyl)-7,9-dimethyl-12-oxo-dodeca-2,4-dieno-ic acid (**18**). These compounds were isolated from *S. griseus* subsp., an endophyte of the mangrove plant *Kandelia candel*.



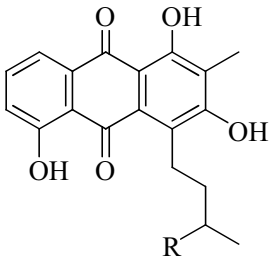
Salaceyins A (**19**) and B (**20**), 6-alkylsalicylic acids, were isolated from *S. laceyi* MS53 which isolated from a surface-sterilized stem of *Ricinus communis* L. Both of salaceyins (**19**) and B (**20**) exhibited modest cytotoxicity against a human breast cancer cell line (SKBR3) with IC_{50} values of 3.0 and 5.5 μgml^{-1}, respectively [32].



Salaceyins A (**19**) : R=CH_3

Salaceyins B (**20**) : R=CH_2CH_3

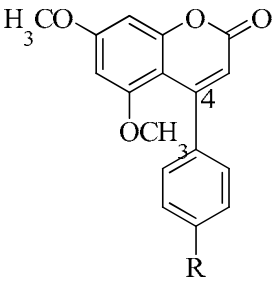
Lupinacidin A (**21**) and B (**22**), the antitumor anthraquinones, were isolated from *M. lupine* sp. *nov*. which was isolated from the root nodules of *Lupinus angustifolius* collected in the mid-west Spain. Both of these compounds show significant inhibitory effects on the invasion of murine colon 26-L5 carcinoma cells without inhibiting cell growth. The IC_{50} values of Lupinacidin A and B are 0.07 and 0.3 μgml^{-1}, respectively [33].



Lupinacidin A (**21**) R=Me
Lupinacidin B (**22**) R=H

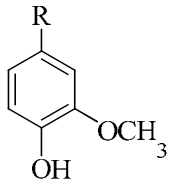
Thailand is in tropical region, plant biodiversity is very large. Endophytic actinomycetes from plants are the good sources for new bioactive compounds. Investigation of endophytic actinomycetes has been increasingly interested. In 2003, Taechowisan *et al.* [14] reported that the isolation of endophytic actinomycetes from the surface-sterilized tissues of 36 plant species, family Acanthaceae (1), Amaranthaceae (1), Cruciferae (2), Cyperaceae (5), Gramineae (6), Iridaceae (1), Labiatae (1), Rubiaceae (1), Rutaceae (1), Taccaceae (1), Umbelliferae (2) and Zingiberaceae (9), and 330 isolates were obtained.

Later Taechowisan *et al.* reported several secondary metabolites and their bioactivity from these isolates such as 5,7-dimethoxy-4-(p-methoxy)-phenylcoumarin (**23**), 5,7-dimethoxy-4-phenylcoumarin (**24**), vanillin (**25**) and 3-methoxy-4-hydroxytoluene (**26**) from *S. aureofaciens* CMUAc130 [34], endophytic actinomycete of *Zingiber officinale*, actinomycin D (**27**) from *Streptomyces* sp. Tc022 [35], endophytic actinomycete of *Alpinia galanga* and kaempferol (**28**), isoscutellarin (**29**), umbelliferone (**30**) and cichriin (**31**) from *Streptomyces* sp. Tc052 [36], endophytic actinomycete of *A. galanga*.



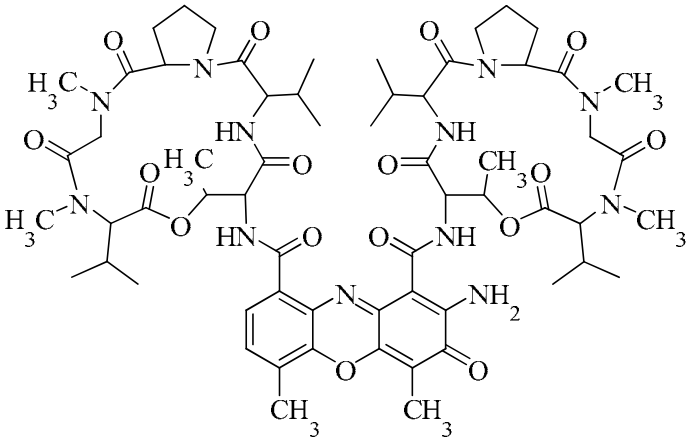
(**23**) R = OCH_3

(**24**) R = H

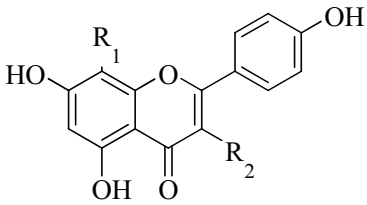


(**25**) R = CHO

(**26**) R = CH_3

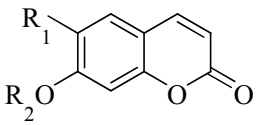


actinomycin D (**27**)



kaempferol (**28**) R_1 = H R_2 = OH

isoscutellarin (**29**) R_1 = OH R_2 = H



umbelliferone (**30**) R_1 = H R_2 = H

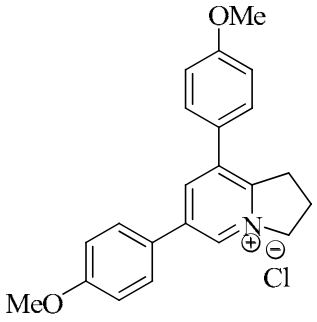
cichoriin (**31**) R_1 = OH R_2 = Glc.

Chapter 2

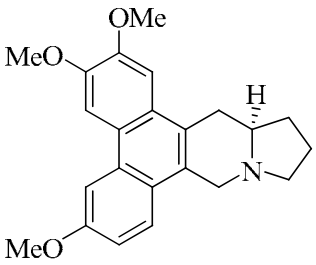
Bioactive compounds from *Streptomyces* sp. SUC1, endophytic on the aerial roots of *Ficus benjamina*

The genus *Ficus* (Moraceae) consists of more than 850 species and is predominantly distributed in the tropical and sub-tropical areas. The chemical constituents of several species of this genus have been studied.

In 1990, Baumgartner *et al*. [37] isolated two indolizidine alkaloids from the methanolic extract of the leaves of *Ficus septica*. A novel alkaloids, ficuseptine (**32**), displayed antibacterial and antifungal activities and another compound is commonly known as antofine (**33**).

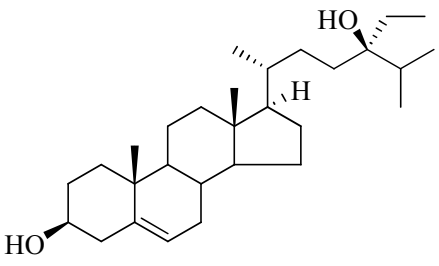


ficuseptine (**32**)

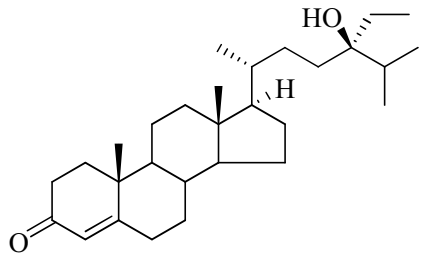


antofine (**33**)

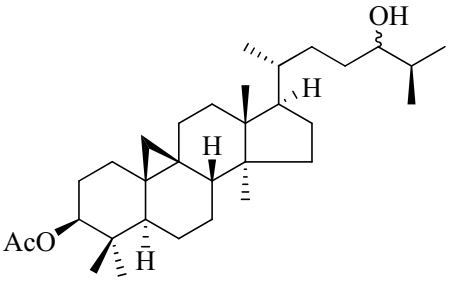
In 1998, Kitajima *et al.* [38] isolated two new sterol, (*24S*)-stigmast-5-ene-3β,24-diol (**34**), (*24S*)-24-hydroxystigmast-4-en-3-one (**35**), two new cycloartane-type triterpinoids, (*24RS*)-3β-acetoxycycloart-25-en-24-ol (**36**), (*23Z*)-3β-acetoxycycloart-23-en-25-ol (**37**) and a new euphane-type triterpenoid, (*23Z*)-3β-acetocyeupha-7,23-dien-25-ol (**38**) from the fruits of *F. pumila*.



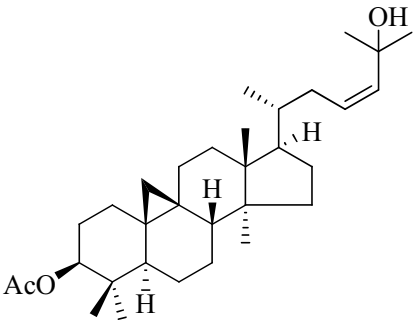
(*24S*)-stigmast-5-ene-3β,24-diol (**34**)



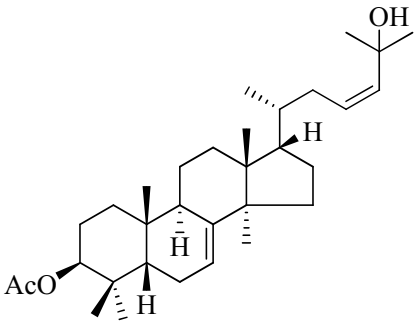
(*24S*)-24-hydroxystigmast-4-en-3-one (**35**)



(*24RS*)-3β-acetoxycycloart-25-en-24-ol (**36**)

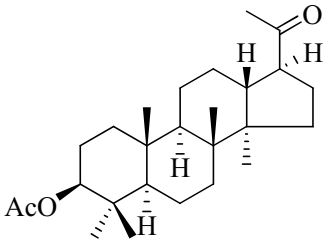


(*23Z*)-3β-acetoxycycloart-23-en-25-ol (**37**)

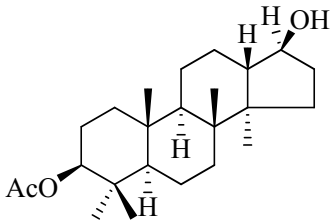


(*23Z*)-3β-acetoxyeupha-7,23-dien-25-ol (**38**)

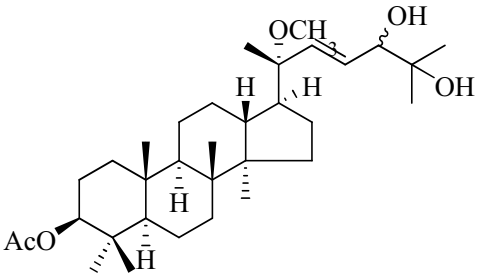
In 1999, Kitajima *et al.* [39] isolated four new compounds, 3β-acetoxy-22,23,24,25,26,27-hexanordammaran-20-one (**39**), 3β-acetoxy-20,21,22,23,24,25,26,27-octa nordammaran-17β-ol (**40**), 3β-acetoxy-(*20R,22E,24RS*)-20,24-dimethoxydammaran-22-en-25-ol (**41**) and 3β-acetoxy-(*20S,22E,24RS*)-20,24-dimethoxydammaran-22-en-25-ol (**42**), from the fruits of *F. pumila*.



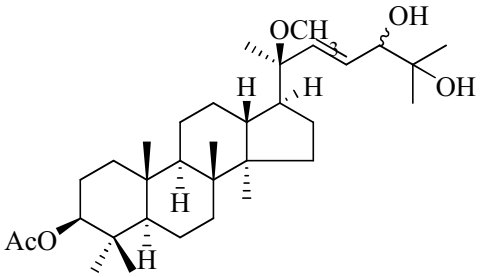
3β-acetoxy-22,23,24,25,26,27-hexanordammaran-20-one (**39**)



3β-acetoxy-20,21,22,23,24,25,26, 27-octanordammaran-17β-ol (**40**)

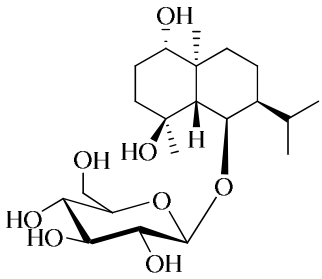


3β-acetoxy-(*20R,22E,24RS*)-20,24-dimethoxydammaran-22-en-25-ol (**41**)

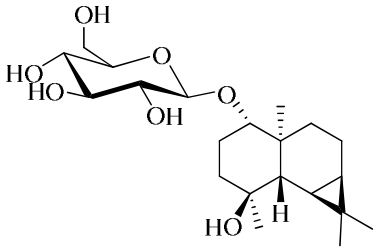


3β-acetoxy-(*20S,22E,24RS*)-20,24-dimethoxydammaran-22-en-25-ol (**42**)

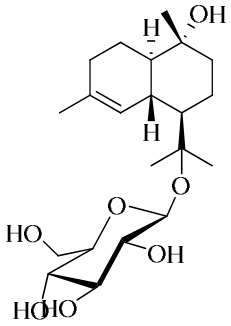
In 2000, Kitajima *et al.* [40] isolated three new sesquiterpenoid glucosides, pumalisides **A-C** (**43-45**) from the fruits of *F. pumila*.



pumilasides A (**43**)



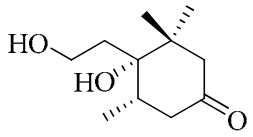
pumilasides B (**44**)



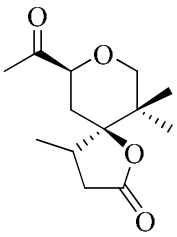
pumilasides C (**45**)

Kuo and Li isolated seven new compounds [41-42], ficusone (**45**), ficuspirolide (**46**), ficusolide (**47**), ficusal (**48**), ficusesqui-lignan A - B (**49-50**) and ficusolide diacetate (**51**), from the heartwood and six new compounds [43], 3β-acetoxy-11α-methoxy-12-ursene (**52**), 3β-acetoxy-11α-ethoxy-12-ursene (**53**), 3β-acetoxy-11α-hydroperoxy-12-ursene (**54**), 3β-

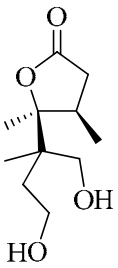
hydroxy-11α-hydroxyperoxy-12-ursene (**55**), 3β-acetoxy-11α-ethoxy-12-oleanene (**56**) and 3β-acetoxy-11α- hydroxyperoxy -12-oleanene (**57**), from the aerial roots of *F. microcarpa*.



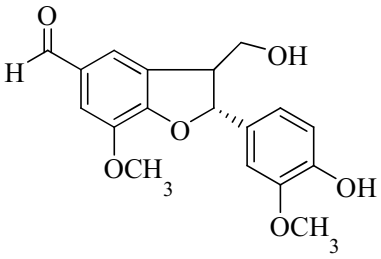
ficusone (**45**)



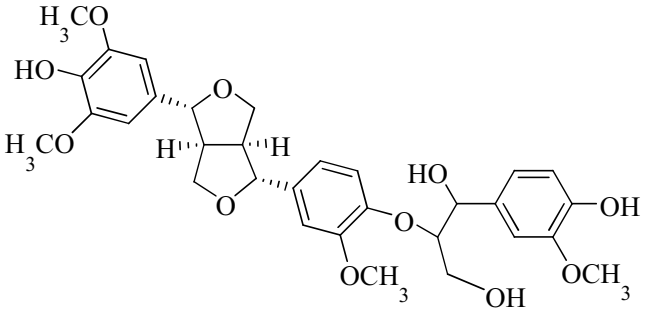
ficuspirolide (**46**)



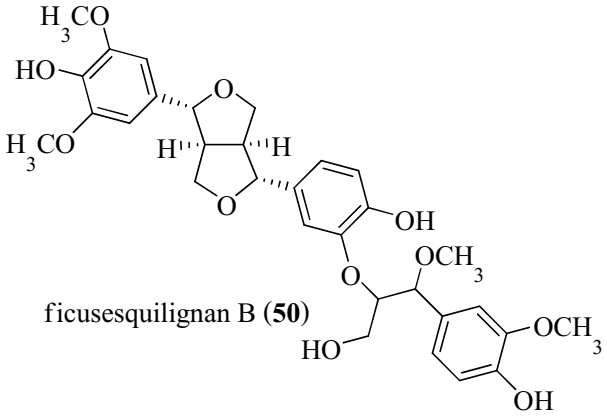
ficusolide (**47**)



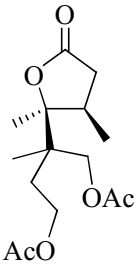
ficusal (**48**)



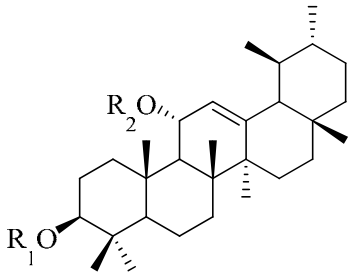
ficusesquilignan A (**49**)



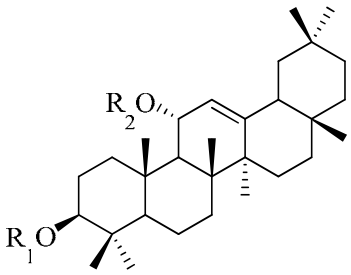
ficusesquilignan B (**50**)



ficusolide diacetate (**51**)

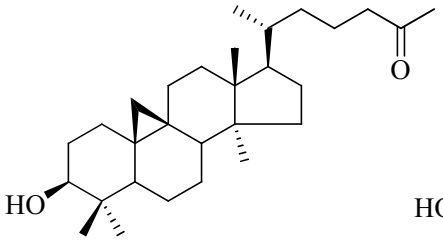


(**52**) R_1 = Ac, R_2 = Me
(**53**) R_1 = Ac, R_2 = Et
(**54**) R_1 = Ac, R_2 = OH
(**55**) R_1 = H, R_2 = OH

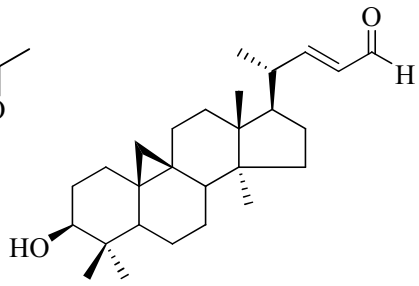


(**56**) R_1 = Ac, R_2 = Et
(**57**) R_1 = Ac, R_2 = OH

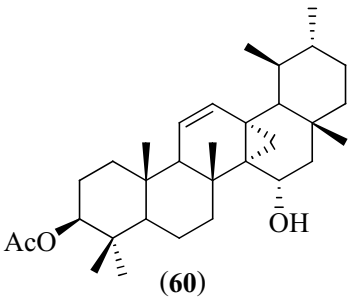
In 2001, Kuo *et al.*[44] isolated four new cyclopropyl-triterpenes, 27-*nor*-3β-hydroxy-25-oxocycloartane (**58**), (*22E*)-25,26,27-*trinor*-3β-hydroxycycloart-22-en-24-al (**59**), 3β-acetoxy-15α-hydroxy-13,27-cyclours-11-ene (**60**) and 3β-acetoxy-12α-formyloxy-13,27-cyclours-11α-ol (**61**), from the aerial roots of *F. microcarpa*.



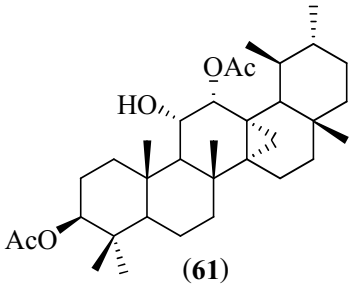
(**58**)



(**59**)



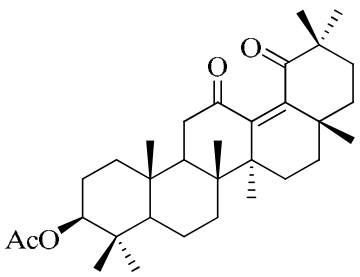
(**60**)



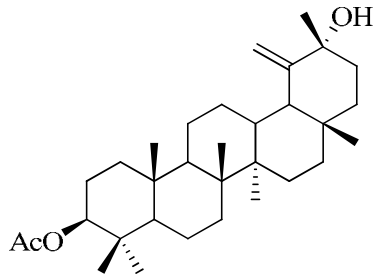
(**61**)

In 2005, Kuo *et al.*[45] isolated six new triterpenes, 3β-acetoxy-12,19-dioxo-13(18)-oleanene (**62**) , 3β-acetoxy-19(29)-taraxasten-20α-ol (**63**), 3,22-dioxo-20-taraxastene (**64**), 3β-acetoxy-21α,22α-epoxyteraxastan-20α-ol (**65**), 3β-acetoxy-11α,12α-epoxy-16-oxo-14-

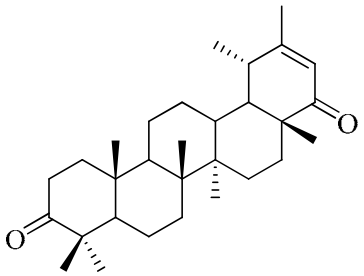
taraxerene (**66**) and 3β-acetoxy-25-methoxylanosta-8,23-diene (**67**), and nine known triterpenes (**68-76**) from the aerial roots of *F. microcarpa*.



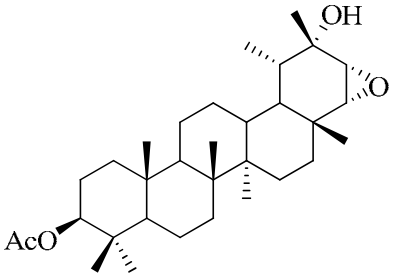
3β-acetoxy-12,19-dioxo-13(18)-oleanene (**62**)



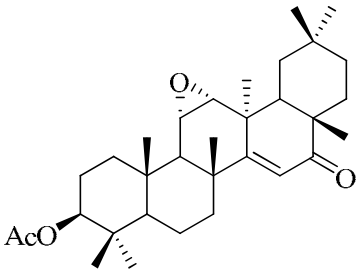
3β-acetoxy-19(29)-taraxasten-20α-ol (**63**)



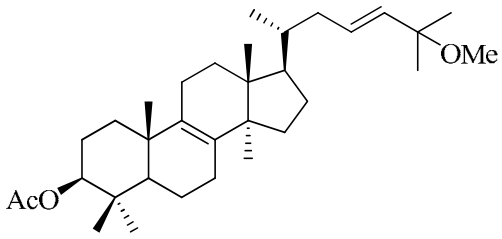
3,22-dioxo-20-taraxastene (**64**)



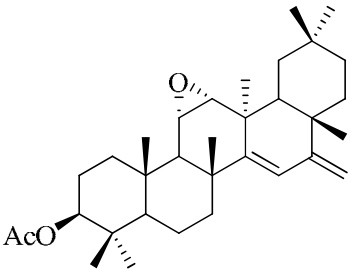
3β-acetoxy-21α,22α-epoxyteraxastan-20a-ol (**65**)



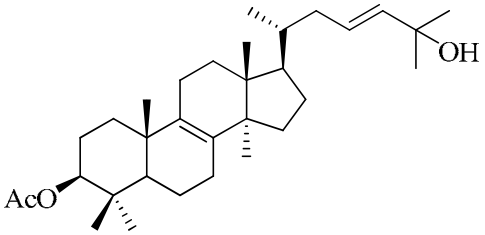
3β-acetoxy-11α,12α-epoxy-16-oxo-14-taraxerene (**66**)



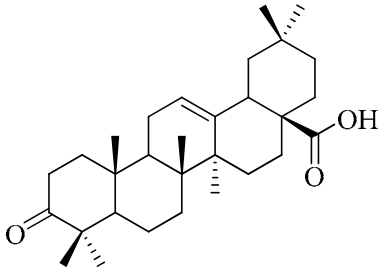
3β-acetoxy-25-methoxylanosta-8,23-diene (**67**)



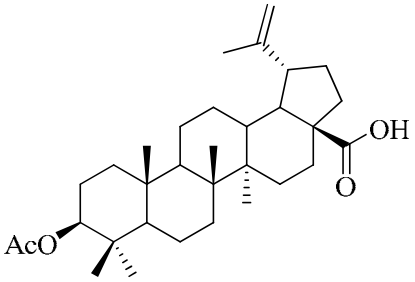
3β-acetoxy-11α,12α-epoxy-14-taraxerene (**68**)



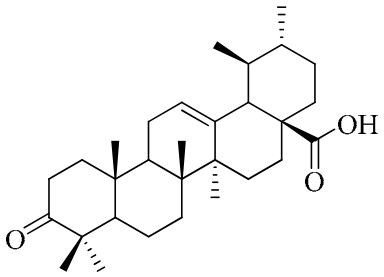
3β-acetoxy-25-hydroxy-lanosta-8,23-diene (**69**)



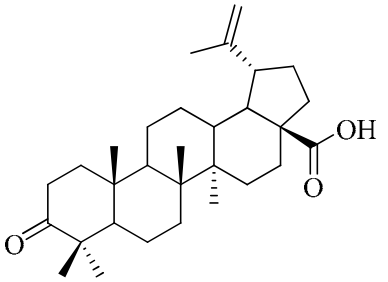
oleanonic acid (**70**)



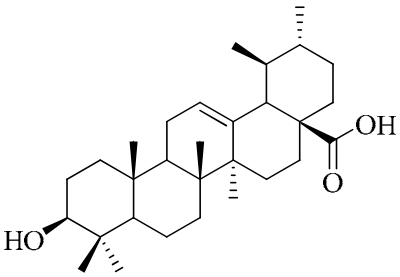
acetylbetulinic acid (**71**)



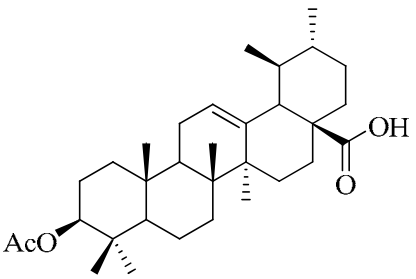
ursonic acid (**72**)



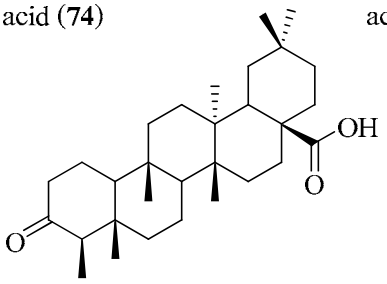
betulonic acid (**73**)



ursolic acid (**74**)

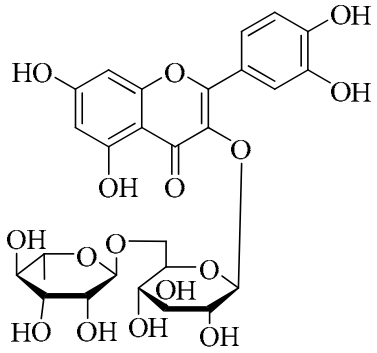


acetylursolic acid (**75**)

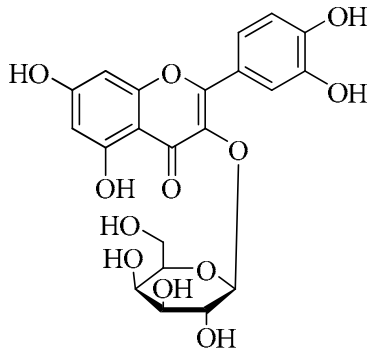


3-oxofriedelan-28-oic acid (**76**)

A number of flavonoids were isolated from *Ficus* species. In 1970, Subramanian and Nair [46] isolated two flavonols, rutin (**77**) and quercetin 3-galactoside (**78**), from the leaves of *F. benjalensis*.

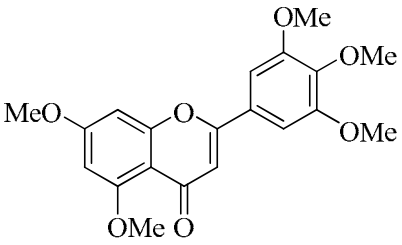


rutin (**77**)

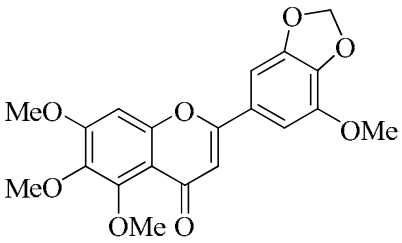


quercetin 3-galactoside (**78**)

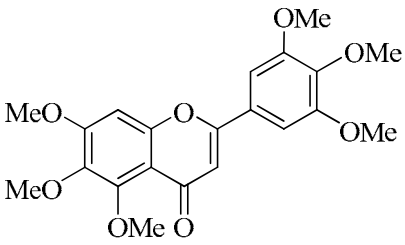
In 1997, Gaspar *et al.* [47] isolated four methoxyflavones, 5,7,3',4',5'-pentamethoxyflavone (**79**), 5,6,7,5'-tetramethoxy-3',4'-methylenedioxyflavone (**80**), 5,6,7,3',4',5'-hexamethoxyflavone (**81**) and 5,6,7,3',5'-pentamethoxy-4'-prenyloxyflavone (**82**) from the leaves of *F. maxima*.



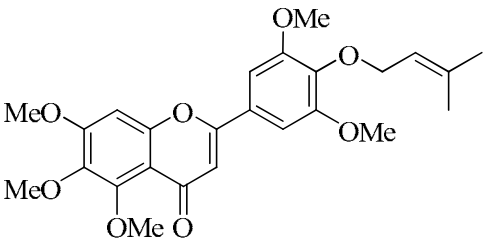
5,7,3',4',5'-pentamethoxyflavone (**79**)



5,6,7,5'-tetramethoxy-3',4'-methylenedioxyflavone (**80**)

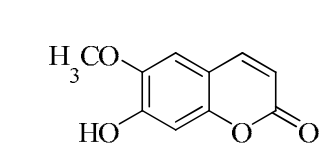


5,6,7,3',4',5'-hexamethoxyflavone (**81**)

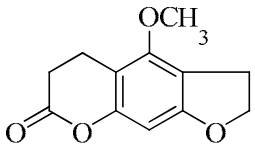


5,6,7,3',5'-pentamethoxy-4'-prenyloxyflavone (**82**)

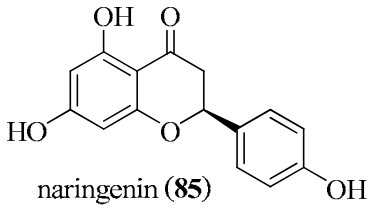
In 2000, Pistelli *et al.* [48] isolated two coumarins, scopoletin (**83**) and bergapten (**84**), ten flavonoids, naringenin (**85**), genistein (**86**), hesperitin (**87**), chrysin (**88**), apigenin (**89**), taxifolin (**90**), tricetin (**91**), luteolin (**92**), 7,4'-dimethoxy-5-hydroxyisoflavone (**93**), 5,7,2',5'-tetrahydroxyflavanone (**94**), and two flavonoid glycosides, rutin (**77**) and isorhamnetin-3-glucoside (**95**), from *F. pumila*.



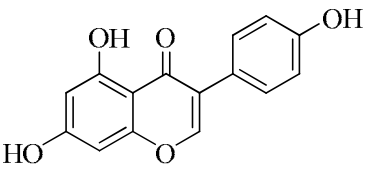
scopoletin (**83**)



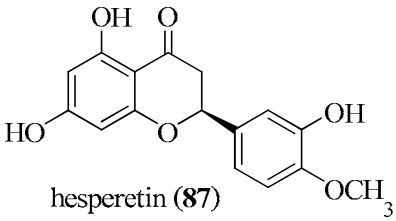
bergapten (**84**)



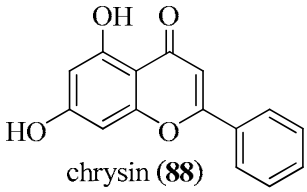
naringenin (**85**)



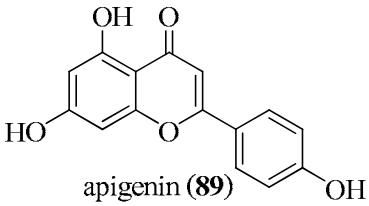
genistein (**86**)



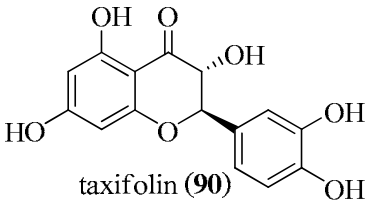
hesperetin (**87**)



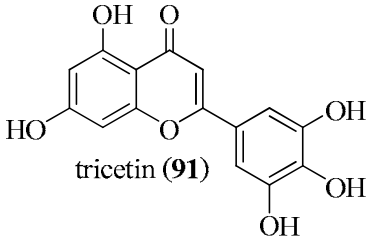
chrysin (**88**)



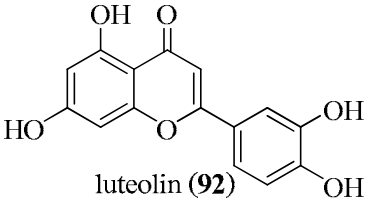
apigenin (**89**)



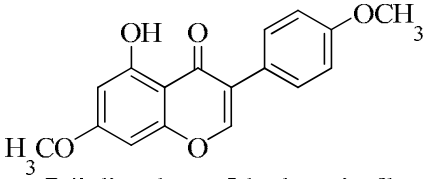
taxifolin (**90**)



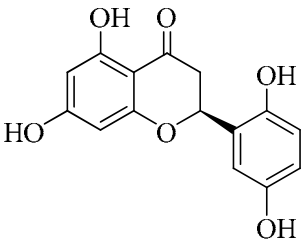
tricetin (**91**)



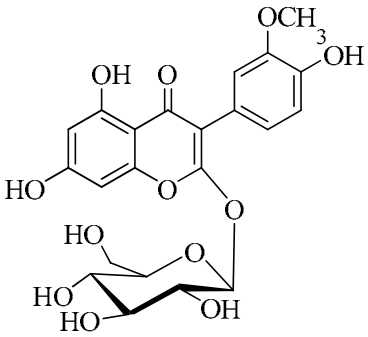
luteolin (**92**)



7,4'-dimethoxy-5-hydroxyisoflavone (**93**)

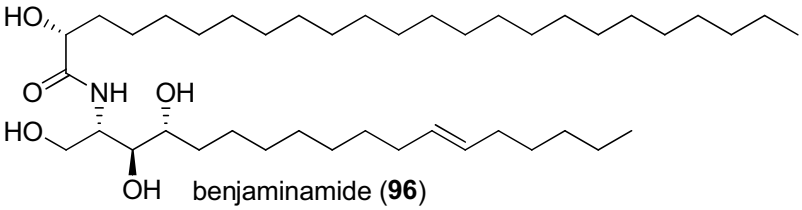


5,7,2',5'-tetrahydroxyflavanone (**94**)

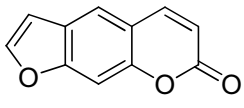


isorhamnetin-3-glucoside (**95**)

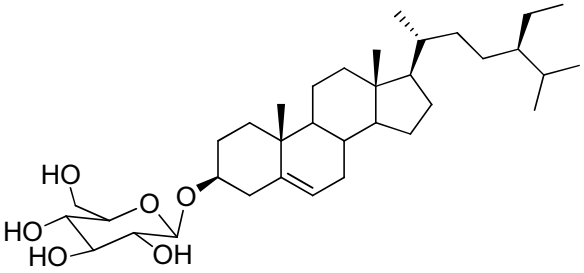
In 2008, Ngadjui *et al*. [49] investigated the constituents of the root barks of *F. benjamina* and isolated a new ceramide, benjaminamide (**96**) together with other compounds, psoralen (**97**), β-sitosterol glucoside (**98**), β-amyrin acetate (**99**), β-amyrin (**100**), lupeol (**101**), betulinic acid (**102**) and platonic acid (**103**).



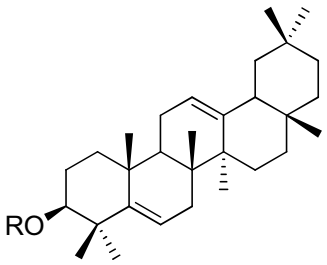
benjaminamide (**96**)



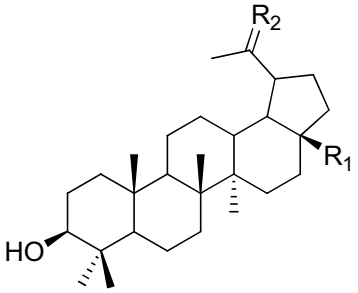
psoralen (**97**)



β-sitosterol glucoside (**98**)



β-amyrin acetate (**99**) R = Ac
β-amyrin (**100**) R = H



	R_1	R_2
lupeol (**101**)	CH_3	CH_2
betulinic acid (**102**)	COOH	CH_2
platanic acid (**103**)	COOH	O

Investigation of endophytic microorganisms of *Ficus* spp. has been reported. In 1995, Bouzar *et al.* [50] isolated new tumoregenic and non-pathogenic agrobacteria, identified as *Agrobacterium tumefaciens*, from crown gall tumors which collected from branches of 1-year-old weeping fig tree (*F. benjamina* L.). In 2001, Suryanarayanan and Vijaykrishna [51] isolated several strains of endophytic fungi from the leaf tissues and aerial roots of *F. benghalensis*.

Bioactive compounds from the endophytic actinomycetes of *F. benjamina* has not yet been investigated. We are now reported the compounds, lansai A-D (**1**-**4**) isolated from *Streptomyces* sp. SUC1, endophyte on the aerial roots of *F. benjamina*.

2.1 EXPERIMENTAL

2.1.1 General procedure

Melting points are uncorrected. Optical rotations were determined with a Jasco digital polarimeter. UV spectra were recorded with a Shimadzu UV-240 spectrophotometer. IR spectra were recorded with a Jasco A-302 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were measured in $CDCl_3$ or $CDCl_3$/DMSO-d_6 on a Bruker AVANCE 300 (300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR) spectrometer. Chemical shifts are given in δ (ppm) with tetramethylsilane (TMS) as an internal standard. Coupling constants (*J*) are given in Hz. The signals in the ^{1}H- and ^{13}C-NMR spectra were assigned unambiguously using 2D NMR techniques: COSY, HMQC and HMBC. MS were recorded on a VG 7070 mass spectrometer operating at 70 eV with VG Quattro triple quadrupole mass spectrometer for the electrospray mass spectra. HR-MS were recorded on a Bruker MicrOTOF mass spectrometer. Column chromatography was carried out using silica gel 60 (Merck, 70-230 or 230-400 mesh). Pre-coated silica gel 60 F_{254} (Merck, layer thickness 0.25 mm) were used for thin-layer chromatography (TLC) and the compounds were visualized under ultraviolet light or sprayed with 1% $CeSO_4$ in 10% aq. H_2SO_4 following by heating. Preparative layer chromatography (PLC) was performed on glass plate using pre-coated silica gel 60 F_{254} (Merck, 20x20 cm, layer thickness 0.25, 0.5 or 1.0 mm). All commercial grade solvents were distilled prior to use and spectral grade solvents were used for spectroscopic measurements.

2.1.2 Isolation of endophytic actinomycetes

Figure 2.1 The aerial roots of *Ficus benjamina* L. (a and b)

Aerial roots (Figure 2.1b, about 0.2-0.4 cm in diameter) of *F. benjamina* growing in the grounds of the Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand, were obtained during the period of September-December, 2004. The samples were washed in running tap water and cut into small pieces of 2 x 2 mm^2. Tissue pieces were rinsed in 0.1% Tween 20 for 30 s, then in 1% sodium hypochlorite for 5 min, and then washed in sterile distilled water for 5 min. Next the tissue pieces were surface sterilized in 70% ethanol for 5 min and air-dried in a laminar flow chamber. Finally the pieces were transferred to dishes of humic acid-vitamin (HV) agar [52] containing 100 μg nystatin and cycloheximide /ml, and incubated at 30^{o}C for about 1 month. The colonies were inoculated onto International Streptomyces Project medium 2 (ISP-2) [53] for purification, and obtained 10 strains of endophytic actinomycetes from the aerial roots of *F. benjamina* L. The small pieces of ISP-2, mostly containing spores, were then stored in l5% (v/v) glycerol at -70^{o}C.

2.1.3 Antifungal activity of the actinomycetes isolates against phytopathogenic fungi and yeast.

All endophytic actinomycetes were screened for antifungal activity using *Colletotrichum musae* , the causative agents of anthracnose of banana and human pathogen yeast *Candida albicans* ATCC90028. *C. musae* was grown on potato dextrose agar (PDA). Mycelial disks of 8 mm diameter were cut from the pathogen colonies and transfer to the ISP-2 plates and positioned 6 cm away from each pre-grown actinomycete colony, as show in Figure 2.2. These plates were incubated at 30 ^{o}C for 3–5 days. The width of inhibition zones between the pathogen and the actinomycete isolates were measured.

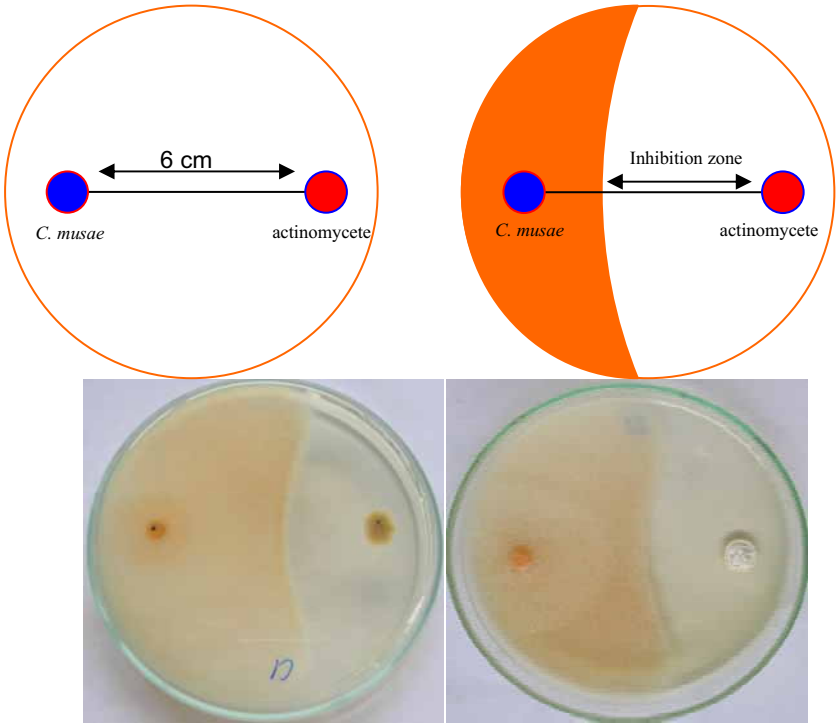


Figure 2.2 Bioactivity test of actinomycete against *C. musae*

For antagonistic activity against *C. albicans* ATCC90028, the yeast was cultured in ISP-2 broth at 30 ^{o}C for 24 h. The cells were diluted to 10^{5} cells ml^{-1} in soft agar, and then were overlayed on pre-grown actinomycete colonies on ISP-2 plates. The plates were incubated at 30 ^{o}C for 24 h, then the width of inhibition zones between the pathogen and the actinomycete isolates were measured, as show in Figure 2.3.

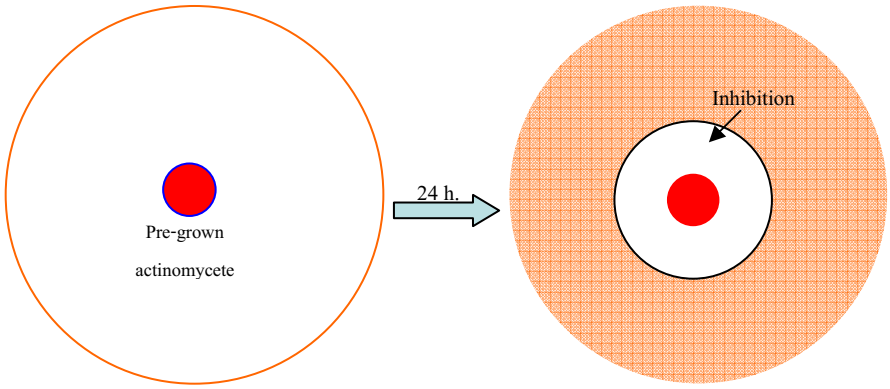


Figure 2.3 Bioactivity test of actinomycete against *C. albicans*

One endophytic actinomycete, *Streptomyces* sp. SUC1, was of primary interest, because of its activity against the plant-pathogenic fungus *Colletotrichum musae in vitro* [22].

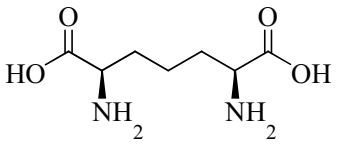
2.1.4 Identification of *Streptomyces* sp. SUC1

2.1.4.1 Morphological observations

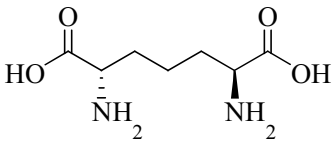
The selected strain of actinomycetes, *Streptomyces* sp. SUC1, was cultured on ISP-2 agar plates at 30^{O}C for 3 days then the cover slides were fixed down the colony and incubated at 30^{O}C for a further 5 days. The selected strain of actinomycetes which had grown on the cover slides were stained with crystal violet for 1 min. The morphology of actinomycete isolates were observed under light microscope. For scanning electron microscopy (SEM) observation, the actinomycete isolates grown on the cover slides was air-dried in a desiccator and mounted on stubs, splutter-coated with gold, and viewed on the SEM (Maxim 20005, CamScan, UK).

2.1.4.2 Amino acid composition of the whole cell extract

Streptomyces sp. SUC1 was grown in ISP-2 medium at 30^{o}C, for 14 days. After the incubation, the cells were collected by filtration with Whatman No.1 filter paper, washed with distilled water and absolute ethanol, and air-dried at room temperature. The dried cells (10 mg) were hydrolysed in 6 N HCl (1ml) in a screw cap tube at 100^{o}C in an oven for 18 h. After cooling, the hydrolysate were filtered, and the solid material was washed with 3 drops of distilled water. The filtrate was dried three consecutive times on a steam bath to remove most of the HCl. The residue was taken up in 0.3 ml of distilled water, and 20 μl of the liquid were spotted on Whatman No. 1 paper. A spot of 10 μl of 0.01 M of *meso*-diaminopimelic acid (**104**) which also contained some of the LL-isomer (**105**) (Wako pure chemical industries Ltd., Japan) was used as the standard. Descending chromatography was carried out overnight by irrigation with methanol/water/10 N HCl/pyridine (80:17.5:2.5:10). Amino acids were detected by dipping the papers in a bath of acetonic ninhydrin (0.1% w/v), followed by heating for 2 min at 100^{o}C. Diaminopimelic acids in the hydrolysate gave purple spots with this reagent. The result was identified by comparing with the R_f values of the standards, as showed in Table 2.1.



meso-diaminopimelic acid (**104**)



LL-diaminopimelic acid (**105**)

Table 2.1 The R_f value of tested amino acid by descending paper chromatography

Amino acid	R_f value
Alanine	0.67
Aspartic acid	0.48
Glutamic acid	0.60
Glycine	0.50
Lysine	0.57
LL- diaminopimelic acid	0.38
meso- diaminopimelic acid	0.31

2.1.4.3 DNA isolation

Genomic DNA was isolated from *Streptomyces* sp. SUC1 using the procedure of Hopwood *et al*. [54] 16S rDNA was amplified by PCR using Taq DNA polymerase (Promega, USA) and primers A 7-26f (5'-CCGTCGACGAGCT- CAGAGTTTGATCCTGGCTCAG-3') and primers B 1523-1504r (5'-CCCGGGTAC-CAAGCTTAAGGAGGTGATCCAGCCGCA-3). The conditions used for thermal cycling were as follows: denaturation at 95^{O}C for 5 min, followed by 35 cycles of denaturation at 95^{O}C for 1 min, annealing at 56^{O}C for 1 min and extension at 72^{O}C for 2 min. At the end of the cycles, the reaction mixture was kept at 72^{O}C for 10 min and then cooled to 4 ^{O}C. The 1.5 Kb amplified 16S rDNA fragment was separated by

agarose gel electrophoresis and purified by using a QiAquick gel extraction kit (QIAGEN, Germany). The purified fragments were cloned into pGEM-T Easy vector (Promega). 16S rDNA nucleotide sequenceswere determined using the dideoxy chain termination method, with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). The primers used for nucleotide sequencing were as follows: T7 promoter, SP6 promoter, C 704-685r (5'-TCTGCGCATTT-CACCGCTAC-3') and D 1115-1100r (5'-AGGGTTGCGCTCGTTG-3). All of the obtained sequences were assembled and then compared with similar sequences from the reference organisms, with the BLAST database (a genome database of the National Center for Biotechnology Information).

2.1.5 Minimum Inhibitory Concentration

The pure compounds (**1-4**) and the crude extract from *Streptomyces* sp. SUC1 were assayed on PDA in Petri dishes to determine the minimum inhibitory concentration against *C. musae*, using the disc diffusion assay. The pure compounds (**1-4**) and the crude extract were dissolved in methanol, serially diluted in the same solvent. The final concentrations of the pure compounds (**1-4**) and the crude extract were 0, 5, 10, 15, 20, 25, 50, 75, 100 μgml^{-1} and 50 μl was applied to sterile 6-mm diameter paper discs (Advantec, Toyo Roshi Kaisha, LTD., Japan) and dried. Each PDA plates were inoculated with a 8-mm diameter plug of fungi, removed from the margin of a 4-day-old colony on PDA, in the center and the treated paper discs were then placed away from the fungi 2.2 cm. Plates were incubated at 30 ^{o}C for 72 h and observed for the presence of an inhibition zone. The experiment was repeated twice.

2.1.6 Anticancer acitivity assay

Cytotoxicity assay against human breast cancer (BC) and human oral cavity cancer (KB) was carried out using the method of Skehan *et al.* [55], the reference substances were ellipticine and doxorubicin.

2.1.7 Extraction and Isolation

Spores of *Streptomyces* sp. SUC1 were used to inoculate on 178 plates of ISP-2 and these were incubated for 14 days at 28^{o}C. The culture medium was then cut into small pieces and extracted with EtOAc (3 x 1 l). The organic solvent was pooled and taken to dryness under rotary evaporation to give a dark brown solid (745 mg).

The solid was separated by flash column chromatography using silica gel 60 [Merck, 0.015-0.040 mm, (diameter x height: 4.4 x 3.5 cm)]. The column was eluted with 30 ml each fraction of hexane, gradients of hexane/EtOAc, EtOAc and CH_2Cl_2/MeOH/H_2O (50:3:1, 30:3:1, 20:3:1, 10:3:1, 7:3:1 and 6:4:1) to give 13 fractions as show in Table 2.2.

Table 2.2 Fractions obtained from the crude extract of SUC1

Fraction No.	Eluent	Weight (mg)	Physical characteristic
C1-1	10% EtOAc / Hexane	4.8	a colorless oil
C1-2	20% EtOAc / Hexane	7.2	a yellow oil
C1-3	40% EtOAc / Hexane	10.6	a yellow oil
C1-4*	60% EtOAc / Hexane	65.2	a yellow oil
C1-5	80% EtOAc / Hexane	34.4	a yellow oil
C1-6	EtOAc	45.7	a yellow wax
C1-7	CH_2Cl_2/MeOH/H_2O (50/3/1)	13.2	a yellow wax
C1-8	CH_2Cl_2/MeOH/H_2O (30/3/1)	18.4	a yellow wax
C1-9	CH_2Cl_2/MeOH/H_2O (20/3/1)	32.6	a brown wax
C1-10	CH_2Cl_2/MeOH/H_2O (10/3/1)	15.4	a brown wax
C1-11	CH_2Cl_2/MeOH/H_2O (7/3/1)	22.3	a brown wax
C1-12	CH_2Cl_2/MeOH/H_2O (6/4/1)	121.5	a brown wax
C1-13	CH_2Cl_2/MeOH/H_2O (6/4/1)	20.5	a brown wax

* Fractions were further investigated.

Each fraction was bioassayed by disk diffusion at 20 mg per disk against *C. musae* and, *C. albicans*. Fraction C1-4 was the only active fraction.

Fraction C1-4, a yellow oil (65.2 mg), was separated on a column to silica gel 60 (Merck, 0.063-0.200 mm). The column was eluted with a gradient of hexane/EtOAc to give 7 fractions, as show in Table 2.3.

Table 2.3 Fractions obtained from C1-4

Fraction No.	Eluent	Weight (mg)	Physical characteristic
C1-4-1*	Hexane	16.9	a colorless oil
C1-4-2	5% EtOAc / Hexane	2.4	a colorless oil
C1-4-3*	10% EtOAc / Hexane	3.6	a white solid
C1-4-4*	15% EtOAc / Hexane	7.4	a white solid
C1-4-5*	20% EtOAc / Hexane	4.1	a white solid
C1-4-6*	25% EtOAc / Hexane	4.4	a white solid
C1-4-7*	30% EtOAc / Hexane	3.1	a white solid

* Fraction was further investigated.

Fraction C1-4-1 (a colorless oil, 16.9 mg) was identified to be lansai C.

Fraction C1-4-3 (a white solid, 3.6 mg) was identified to be lansai D.

Fraction C1-4-7 (a white solid, 3.1 mg) was identified to be lansai A.

Fraction C1-4-4 (a white solid, 7.4 mg) was further separated by preparative TLC (silica gel 60 F_{254}, layer thickness 0.25 mm, 2 plates) using hexane/EtOAc (7:1) as the developing solvent to give lansai D as a white solid (2.4 mg) and lansai B as a white solid (2.8 mg).

Fraction C1-4-5 (a white solid, 4.1 mg) was further purified by preparative TLC (silica gel 60 F_{254}, layer thickness 0.25 mm, 1 plate) using hexane/EtOAc (5:1) as the developing solvent to give lansai B as a white solid (1.2 mg).

Fraction C1-4-6 (a white solid, 4.4 mg) was purified by preparative TLC (silica gel 60 F_{254}, layer thickness 0.25 mm, 1 plate) using hexane/EtOAc (5:1) as the developing solvent to gave lansai A as a white solid (2.5 mg).

Lansai A (**1**). A colorless solid crystallized from MeOH as needles, mp 164–170°C; $[\alpha]_D^{28}$= -497.1° (c 0.105, MeOH); λ_{max} (MeOH) (logε) nm: 249(4.21), 303 (3.81); ν_{max} (KBr) cm^{-1}: 3356, 1666, 1611, 1499, 1418, 1211, 1163, 1002, 911, 745; m/z 482 (100, M^+), 467 (37), 455 (6), 328(6), 270 (6), 239 (6), 210 (43), 198 (18), 171 (6), 144 (12), 97 (6), 69(6), 57 (12); HRESIMS: MH^+, found 483.2769. $C_{30}H_{35}N_4O_2$ requires 483.2760. ^{1}H- and ^{13}C-NMR: see Table 2.4.

Lansai B (**2**). A colorless solid crystallized from MeOH as needles, mp 160–163 °C; $[\alpha]_D^{28}$ = -425.4° (c 0.185, MeOH); λ_{max}(MeOH) (logε) nm: 253(3.99), 306 (3.49); ν_{max} (KBr) cm^{-1}: 1669, 1609, 1496, 1416, 1299, 1207, 1163, 1004, 909, 742; m/z 496 (100, Mþ), 482 (63), 469 (6), 446 (2), 336 (25), 324 (2), 283 (6), 256 (11), 241 (6), 226 (6), 210 (77),198 (35), 183 (14), 158 (32), 145 (37), 129 (12), 97 (8), 73 (18), 57 (28); HRESIMS: MH^+, found 497.2910. $C_{31}H_{37}N_4O_2$ requires 497.2916. ^{1}H- and ^{13}C-NMR: see Table 2.4.

Lansai C (**3**). A colorless solid crystallized from MeOH as plates, mp 146–148°C; $[\alpha]_D^{28}$ =0; λ_{max}(MeOH) (logε) nm: 230 (3.92), 311 (4.04); ν_{max}(KBr) cm^{-1}: 3210, 1682, 1614, 1410, 1377, 1098, 912, 759; m/z 270 (100, M^+-O), 255 (35), 241 (2), 227 (23), 201 (4), 187 (2), 177 (2), 130 (3), 124 (4), 117 (23), 110 (4), 96 (14), 90 (25), 82 (63), 68 (23), 55 (25); HRESIMS: MH^+, found 287.1373. $C_{16}H_{19}N_2O_3$ requires 287.1395. ^{1}H- and ^{13}C-NMR: see Table 2.5.

Lansai D (**4**) A colorless solid crystallized from MeOH as needles, mp 170–176 °C; $[\alpha]_D^{28}$ =0; λ_{max}(MeOH) (logε) nm: 230 (4.07), 319 (4.34); ν_{max} (KBr) cm^{-1}: 3183, 1682, 1630, 1604, 1420, 1395, 1357, 1231, 1106, 860, 765, 723; m/z 270 (100, M^+), 255 (36), 241 (1), 227 (13), 201 (9), 187 (1), 149 (13), 177 (9), 110 (8), 96 (9), 90 (9), 82 (23), 68 (10), 55 (14); HRESIMS: MNa^+, found 293.1272. $C_{16}H_{18}N_2O_2Na$ requires 293.1266. ^{1}H- and ^{13}C-NMR: see Table 2.5.

Table 2.4 ^{1}H- (300 MHz) and ^{13}C-NMR (75 MHz) spectral data of lansai A (**1**) and lansai B (**2**)

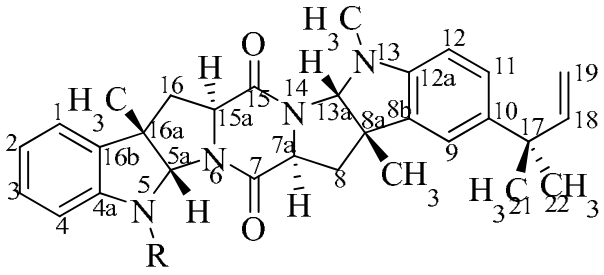
Position	Lansai A		Lansai B	
	(δH)	(δC)	(δH)	(δC)
1	7.10, dd (1.5, 7.8)	122.7	7.06, dd (1.5, 7.8)	122.3
2	6.78, dt (1.5, 7.8)	119.6	6.71, dt (1.5, 7.8)	118.1
3	7.05, dt (1.5, 7.8)	128.7	7.11, dt (1.5, 7.8)	128.8
4	6.56, dd (1.5, 7.8)	109.4	6.36, dd (1.5, 7.8)	105.8
4a	-	148.3	-	150.1
5	5.03, s	-	-	-
5-Me	-	-	2.99, s	32.9
5a	5.25, s	81.2	5.46, s	86.6
7	-	166.6	-	165.6
7a	4.10, dd (6.3, 10.8)	60.2	4.16, m	60.1
8-Ha	2.20, dd (10.8, 12.3)	42.2	2.18, dd (11.3, 12.3)	42.7
8-Hb	2.69, dd (6.3, 12.3)	-	2.72, dd (5.9, 12.3)	-
8a	-	50.5	-	50.5
8a-Me	1.46, s	25.4	1.48, s	25.5
8b	-	132.9	-	132.8
9	7.01, d (1.5)	120.1	7.02, d (1.5)	120.2
10	-	138.6	-	138.7
11	7.08, dd (1.5, 8.1)	126.3	7.08, dd (1.5, 8.1)	126.3
12	6.28, d (8.1)	105.5	6.29, d (8.1)	105.4
12a	-	148.1	-	148.3
13-Me	2.96, s	33.3	2.97, s	33.1
13a	5.39, s	87.1	5.44, s	86.9
15	-	166.0	-	165.8
15a	4.10, dd (6.3, 10.8)	60.5	4.16, m	60.1
16-Ha	2.30, dd (10.8, 12.3)	40.3	2.17, dd (11.3, 12.3)	42.7
16-Hb	2.73, dd (6.3, 12.3)	-	2.72, dd (5.9, 12.3)	-
16a	-	51.8	-	50.3

Table 2.4 ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectral data of lansai A (**1**) and lansai B (**2**) (cont.)

Position	Lansai A		Lansai B	
	(δH)	(δC)	(δH)	(δC)
16a-Me	1.48, s	24.2	1.49, s	25.4
16b	-	132.2	-	132.9
17	-	40.7	-	40.7
18	5.99, dd (10.8, 17.4)	148.6	5.99, dd (10.6, 17.5)	148.6
19	5.00, d (10.8)	110.2	5.01, d (17.5)	110.2
	5.01, d (17.4)	-	5.03, d (10.6)	-
20&21	1.35, s (2x)	25.8 (2x)	1.36, d (2x)	28.5 (2x)

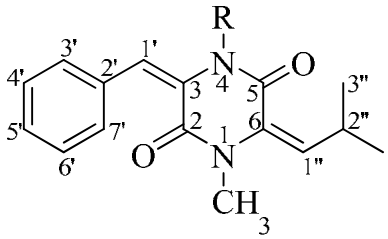
Table 2.5 ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectral data of lansai C (**3**) and lansai D (**4**)

Position	Lansai C		Lansai D	
	(δH)	(δC)	(δH)	(δC)
1-Me	3.31, s	31.1	3.23, s	31.0
2	-	157.8	-	156.8
3	-	125.7	-	125.3
4	8.02, br s	-	8.45, s	-
5	-	157.6	-	159.1
6	-	128.0	-	128.2
1’	7.00, s	115.8	6.40, s	121.9
2’	-	133.3	-	133.8
ArH	7.41, m (5H)	129.4 (2x)	7.33, m (3H)	129.9 (2x)
		128.5 (2x)	7.53, d (7.2, 2H)	127.8 (2x)
		128.6		128.1
1’’	5.51, d (9.6)	134.8	5.50, d (9.6)	134.6
2’’	3.79, d sept (6.6, 9.6)	26.7	3.82, d sept (6.7, 9.6)	26.6
3’’&4’’	1.10, d (6.6)	23.3 (2x)	1.12, d (6.7)	23.3 (2x)



lansai A (**1**) : R = H

lansai B (**2**) : R = CH_3



lansai C (**3**) : R = OH

lansai D (**4**) : R = H

2.2 RESULTS AND DISCUSSION

2.2.1 Isolation and identification of *Streptomyces* sp. SUC1

The aerial roots of *F. benjamina* have been subjected to the procedures for endophytic isolation and yielded 10 strains of actinomycetes. There was only one strain, *Streptomyces* sp. SUC1, that showed inhibitory activity against *Colletotrichum musae*. Close examination of SUC1 by microscopic observation, revealed that this microorganism had sporophores monopodially branched, flexuous, producing open spirals, spores spherical to oval-shaped (1 x 1-1.5 mm) with smooth surfaces, substrate mycelium was extensively branched with non-fragmenting hyphae, aerial mycelium, white, changing to cream without soluble pigment, as show in Figure 2.4.

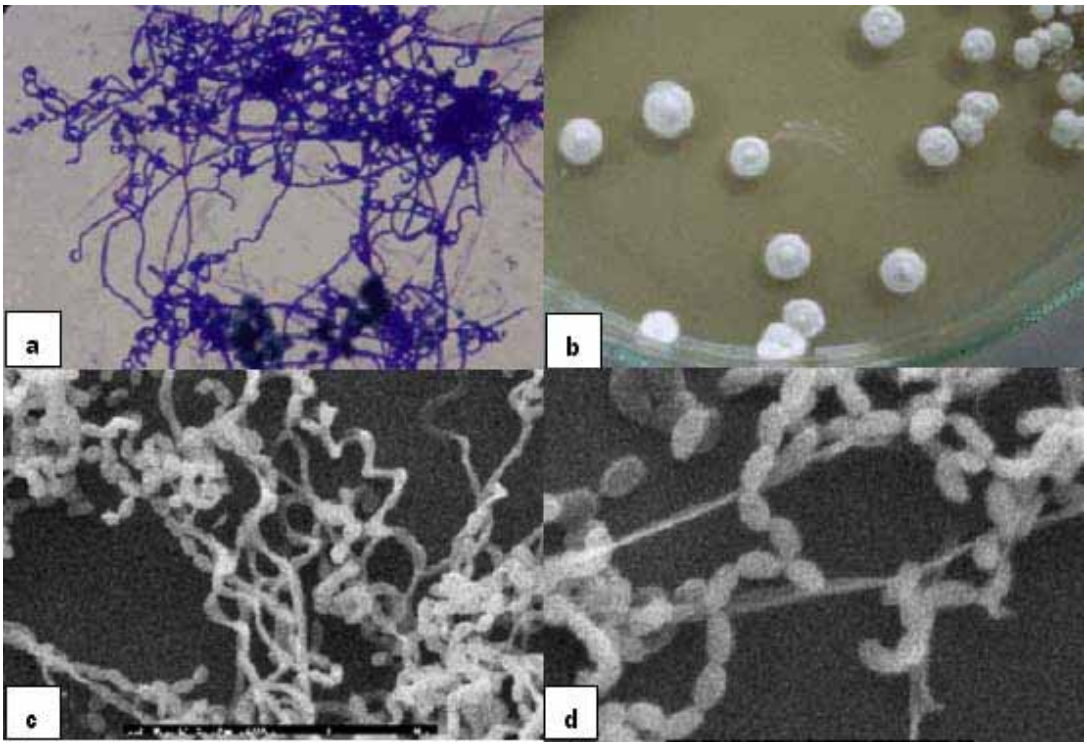


Figure 2.4 Morphology and colony of *Streptomyces* sp. SUC1 (a) morphology of SUC1 observed under light-microscope, (b) a white smooth colony, change to cream without soluble pigment, (c) and (d) SEM observation.

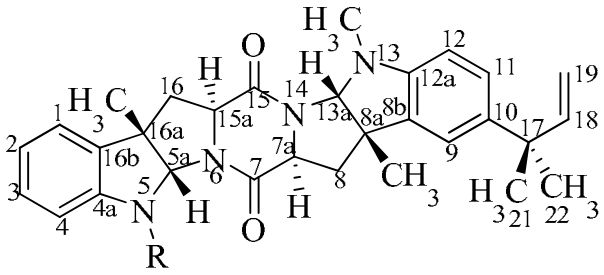
Diaminopimelic acids of the whole-cell extracts was analyzed and identified to be LL-type. Based on results of morphological observation as well as on the presence of LL-diaminopimelic acid (**105**) in the whole-cell extracts, this endophytic actinomycete, SUC1, was identified to be the genus *Streptomyces*.

Almost the complete 16S rDNA sequence was determined for the endophytic *Streptomyces* sp. SUCI (>95% of the *Escherichia coli* sequence) from position 8 to position 1523 (*E. coli* numbering system; Brosius et al. [56]) . BLAST search results for isolate SUC1 came from non-redundant GenBank + EMBL + DDBJ; when reference sequences were chosen, unidentified and unpublished sequences were excluded. The BLAST search results generated from representative strains of the related genera showed that isolate SUC1 had high levels of sequence similarity to the species *S. lateritius* (accession number: AJ781326). 16S rDNA analysis revealed that isolate SUC1 is phylogenetically closely related to *S. lateritius* (the sequence similarity levels were 99.93%). The nucleotide sequence data reported in this paper are deposited in the GenBank, EMBL and DDBJ databases with the accession number AB246922.

2.2.2 Lansai A-D (1-4)

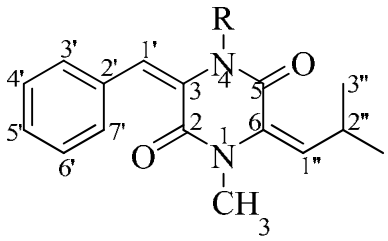
The crude EtOAc extracts of *Streptomyces* sp. SUC1, endophytic on the aerial roots of *Ficus benjamina* L. were separated by chromatographic methods to yield four new compounds, **lansai A–D** (**1-4**).

The structures of **1**-**4** were elucidated by spectroscopic analysis including 1D and 2D NMR techniques.



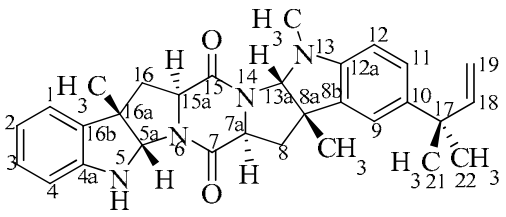
lansai A (**1**) : R = H

lansai B (**2**) : R = CH_3



lansai C (**3**) : R = OH

lansai D (**4**) : R = H



lansai A (**1**)

Lansia A (**1**) was isolated as colorless needles, mp 164–170 ^{o}C, which was shown to be optically active ($[\alpha]_D^{28}$= -497.1^{o}, c = 0.105, MeOH). The compound has molecular formula $C_{30}H_{34}N_4O_2$ by HRESIMS. The UV spectrum showed bands at 249 and 303 nm. The IR spectrum showed absorption bands for NH (3356 cm^{-1}) and amide carbonyl (1666 cm^{-1}). The signals of seven aromatic protons at δ 6.56, 6.78, 7.05, 7.10, 6.28, 7.01, and 7.08 in the ^{1}H-NMR spectrum of 1 (Table 2.4) were ascribed to two indoline units in the molecule. This was consistent with the ^{13}C-NMR spectral data (Table 2.4), which exhibited seven aromatic methine carbons at δ 122.7 (C-1),119.6 (C-2), 128.7 (C-3), 109.4 (C-4), 120.1 (C-9), 126.3 (C-11), and 105.5 (C-12) and five aromatic quaternary carbons at δ 148.3 (C-4a), 132.2 (C-16b), 132.9 (C-8b), 138.6 (C-10), and 148.1 (C-12a). In addition, signals of two ABX systems in the ^{1}H-NMR spectrum of **1** at δ 2.30(1H, dd, *J* = 10.8, 12.3 Hz), 2.73 (1H, dd, *J* = 6.3, 12.3 Hz), and 4.10 (1H, dd, *J* = 6.3, 10.8 Hz) and at δ 2.20 (1H, dd, *J* = 10.8, 12.3 Hz), 2.69 (1H, dd, *J* = 6.3, 12.3 Hz), and 4.10 (1H, dd, *J* = 6.3, 10.8 Hz) were assigned to two β-methylene groups (Hab-16 and Hab-8) and two α-methine protons (H-15a and H-7a) of two α-amino acid functions, respectively. The spectrum also contained two singlets at δ 5.03 (1H) of an NH proton and 2.96 (3H) of an *N*–CH_3 group. Together with the ^{13}C-NMR spectral data, which contained signals for two methylene carbons at δ 40.3 and 42.2, two methine carbons at δ 60.5 and 60.2, and two amide carbonyls at δ 166.0 and 166.6, it appeared that **1** possessed two indoline units, derived from one unit of tryptophan and one unit of *N*-methyltryptophan. The ^{1}H-NMR spectrum of **1** also exhibited two singlets of one hydrogen each at δ 5.25 and 5.39, which were assigned to two methine protons of two –N–CH–N–CO systems. This was consistent with the ^{13}C-NMR spectrum, which showed peaks of two methine carbons at δ 81.2 (C-5a) and 87.1 (C-13a). Two methyl groups on C-16a and C-8a in **1** were appeared as two singlets at δ 1.48 and 1.46,

respectively. The ^{1}H-NMR spectrum also contained a singlet of two methyl groups at δ 1.35 and an ABX system at δ 5.00 (1H, d, *J* = 10.8 Hz), 5.01 (1H, d, *J* = 17.4 Hz), and 5.99 (1H, dd, *J* = 10.8,17.4 Hz), which were due to the presence of a 1,1-dimethyl-2-propenyl group. This was consistent with the ^{13}C-NMR, which exhibited signals for two methyl groups at δ 25.8 (C-20 and C-21), a methylene carbon at δ 110.2 (C-19), an olefinic methine carbon at δ 148.6 (C-18), and a quaternary carbon at δ 40.7 (C-17). The ^{13}C-NMR spectrum of **1** (Table 2.4) was assigned by DEPT, 2D HMQC, and 2D HMBC spectra.

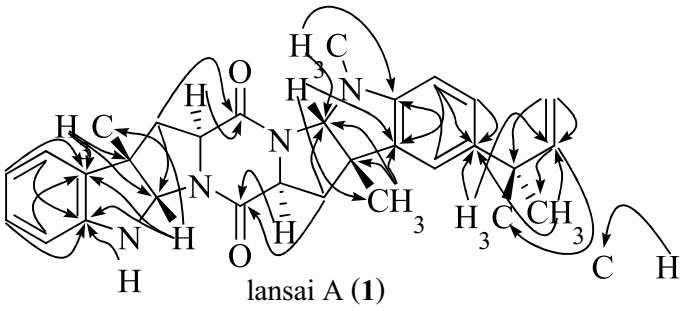


Figure 2.5 HMBC correlations of lansai A (**1**)

The position of 1,1-dimethyl-2-propenyl group at C-10 was established by 2D HMBC correlations (Figure 2.5) between methyl protons (δ 1.35) and C-10 (δ 138.6). That the two methylene groups, Hab-16 at δ 2.30 and 2.73 and Hab-8 at δ 2.20 and 2.69 were attached to C-16a and C-8a of the two indoline nuclei, respectively, was apparent from the correlations between Hab-16 and C-5a (δ 81.2) and Hab-8 and C-13a (δ 87.1). In addition, Hab-16 and H-15a (δ 4.10) showed correlations to the amide carbonyl (C-15) at δ 166.6; similarly, Hab-8 and H-7a (δ 4.10) showed correlations to the amide carbonyl (C-7) at δ 166.6. ^{2}J and ^{3}J correlations were shown between 16a-CH_3 (δ 1.48) and C-16a (δ 51.8) and C-5a (δ 81.2) and also between 8a-CH_3 (δ 1.46) and C-8a (δ 50.5) and C-13a (δ 87.1). Other correlations were observed between H-5a (δ 5.25) and 16a-CH_3 (δ 24.2), C-16b (δ 132.2) and C-4a (δ 148.3) and between H-13a (δ 5.39) and 8a-CH_3 (δ 25.4) and C-8b (δ 132.9) and C-12a (δ 148.1). The NH proton (δ 5.03) showed correlation to C-4a (δ 148.3) while *N*–CH_3 (δ 2.96) showed correlations to C-12a (δ 148.1) and C-13a (δ 87.1).

In the NOEDIFF spectrum of **1** (Figure 2.6), 16a-CH_3 gave enhancements with H-5a and one methylene proton (Ha-16) and H-15a enhanced the signal of the other methylene proton (Hb-16). This indicated that 16a-CH_3 and H-5a were *cis* to each other but *trans* to H-15a. Similarly, irradiation of 8a-CH_3 enhanced the signals of H-13a and Ha-8, while irradiation of H-7a affected Hb-8, indicating that 8a-CH_3 and H-13a were *cis* to each other but *trans* to H-7a in the molecule. On the basis of the above evidence, the tryptophan dimer structure **1** was identified for lansai A with the *N*–CH_3 group attached to N-13.

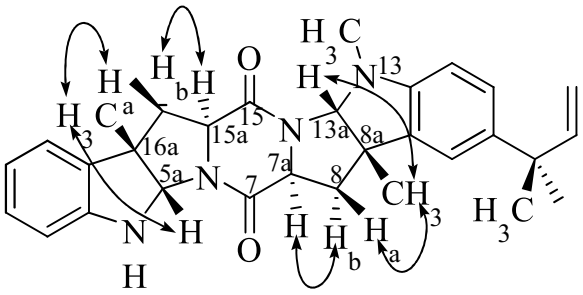
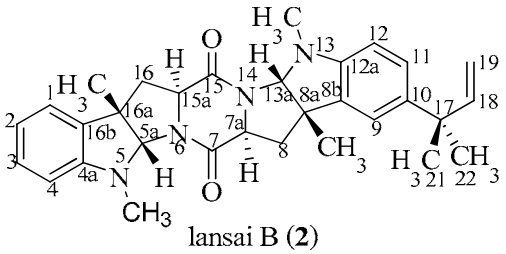


Figure 2.6 NOEDIFF correlations of lansai A (**1**)



lansai B (**2**)

Lansia B (**2**) was isolated as colorless needles, mp 160–163 ^{o}C, which was shown to be optically active ($[\alpha]_D^{28}$= -425.4^{o}, c = 0.185, MeOH). The compound has molecular formula $C_{31}H_{36}N_4O_2$ by HRESIMS. Compound **2** had one carbon and two protons more than compound **1**. This was confirmed by the presence of an extra *N*-methyl signal in the ^{1}H- and ^{13}C-NMR of **2**. Other peaks were almost identical to those of **1** (Table 2.4).

The UV and IR of **2** were similar to those of **1**. The UV spectrum of **2** showed bands at 253 and 306 nm. The IR spectrum of **2** had the absorption band of amide carbonyl at 1666 cm^{-1} and did not show absorption band for NH group.

The signals of seven aromatic protons at δ 6.36, 6.71, 7.11, 7.06, 6.29, 7.02, and 7.08 in the ^{1}H-NMR spectrum of compound **2** (Table 2.4) were ascribed to two indoline units in the molecule. This was consistent with the ^{13}C-NMR spectral data (Table 2.4), which exhibited seven aromatic methine carbons at δ 122.3 (C-1),118.1 (C-2), 128.8 (C-3), 105.8 (C-4), 120.2 (C-9), 126.3 (C-11), and 105.4 (C-12) and five aromatic quaternary carbons at δ 150.1 (C-4a), 132.9 (C-16b), 132.8 (C-8b), 138.7 (C-10), and 148.3 (C-12a). In addition, the signals of two ABX systems in the ^{1}H-NMR spectrum of **2** at δ 2.17 (1H, dd, *J* = 11.3, 12.3 Hz), 2.72 (1H, dd, *J* = 5.9, 12.3 Hz), and 4.16 (m) and at δ 2.18 (1H, dd, *J* = 11.3, 12.3 Hz), 2.72 (1H, dd, *J* = 5.9, 12.3 Hz), and 4.16 (m) were assigned to two β-methylene groups (Hab-16 and Hab-8) and two α-methine protons (H-15a and H-7a) of two α-amino acid functions, respectively. Lansai B (**2**) also contained two singlets at δ 2.99 (3H) and 2.97 (3H) of two *N*-CH_3 groups. Together with the ^{13}C-NMR spectral data, which contained signals for two methylene carbons at δ 42.7 (C-8) and 42.7 (C-16), two methine carbons at δ 60.1 (C-7a) and 60.1 (C-15a), and two amide carbonyls at δ 165.8 and 165.6, it appeared that **2** possessed two indoline units. The ^{1}H-NMR spectrum of **2** also exhibited two singlets of one hydrogen each at δ 5.46 and 5.44, which were assigned to two methine protons of two –N–CH–N–CO systems. This was consistent with the

^{13}C-NMR spectrum, which showed peaks of two methine carbons at δ 86.6 (C-5a) and 86.9 (C-13a). Two methyl groups on C-16a and C-8a in **2** were appeared as two singlets at δ 1.49 and 1.48, respectively. The ^{1}H-NMR spectrum also contained a singlet of two methyl groups at δ 1.36 and an ABX system at δ 5.01 (1H, d, *J* = 17.5 Hz), 5.03 (1H, d, *J* = 10.6 Hz), and 5.99 (1H, dd, *J* = 10.6,17.5 Hz), which were due to the presence of a 1,1-dimethyl-2-propenyl group. This was consistent with the ^{13}C-NMR, which exhibited signals for two methyl groups at δ 28.5 (C-20 and C-21), an oledinic methylene carbon at δ 110.2 (C-19), an olefinic methine carbon at δ 148.6 (C-18), and a quaternary carbon at δ 40.7 (C-17). The ^{13}C-NMR spectrum of **2** (Table 2.4) was assigned by DEPT, 2D HMQC, and 2D HMBC spectra.

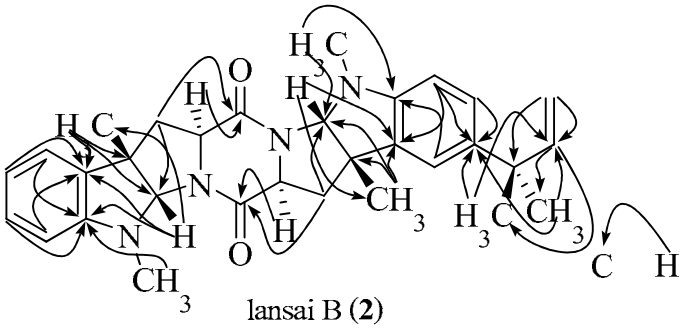


Figure 2.7 HMBC correlations of lansai B (**2**)

The position of 1,1-dimethyl-2-propenyl group at C-10 was established by 2D HMBC correlations (Figure 2.7) between methyl protons (δ 1.36) and C-10 (δ 138.7). That the two methylene groups, Hab-16 at δ 2.16 and 2.72 and Hab-8 at δ 2.20 and 2.72 were attached to C-16a and C-8a of the two indoline nuclei, respectively, was apparent from the correlations between Hab-16 and C-5a (δ 86.6) and Hab-8 and C-13a (δ 86.9). In addition, Hab-16 and H-15a (δ 4.16) showed correlations to the amide carbonyl (C-15) at δ 165.8; similarly, Hab-8 and H-7a (δ 4.16) showed correlations to the amide carbonyl (C-7) at δ 165.6. ^{2}J and ^{3}J correlations were shown between 16a-CH_3 (δ 1.49) and C-16a (δ 50.3) and C-5a (δ 86.6) and also between 8a-CH_3 (δ 1.48) and C-8a (δ 50.5) and C-13a (δ 86.9). Other correlations were observed between H-5a (δ 5.46) and 16a-CH_3 (δ 25.4), C-16b (δ 132.9) and C-4a (δ 150.1) and between H-13a (δ 5.44) and 8a-CH_3 (δ 25.5) and C-8b (δ 132.8) and C-12a (δ 148.3). The *N*-CH_3 proton (δ 2.99)

showed correlation to C-4a (δ 150.1) while *N*–CH_3 (δ 2.97) showed correlations to C-12a (δ 148.3) and C-13a (δ 86.9).

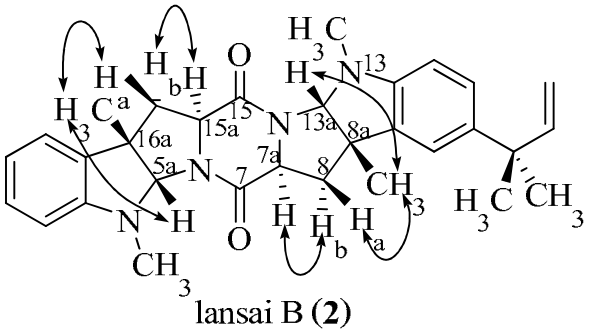
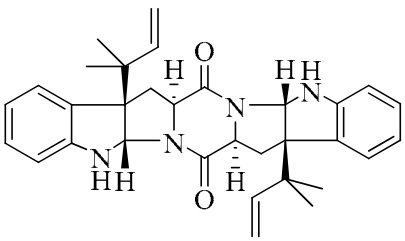


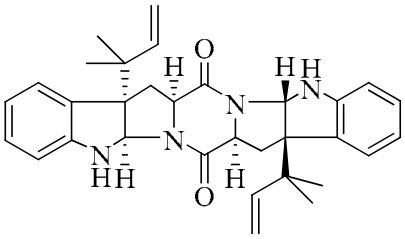
Figure 2.8 NOEDIFF correlations of lansai B (**2**)

In the NOEDIFF spectrum of **2** (Figure 2.8), 16a-CH_3 gave enhancements with H-5a and one methylene proton (Ha-16) and H-15a enhanced the signal of the other methylene proton (Hb-16). This indicated that 16a-CH_3 and H-5a were *cis* to each other but *trans* to H-15a. Similarly, irradiation of 8a-CH_3 enhanced the signals of H-13a and Ha-8, while irradiation of H-7a effected Hb-8, indicate that 8a-CH_3 and H-13a were *cis* to each other but *trans* to H-7a in the molecule. On the basis of the above evidence, lansai B (**2**) had the *N*-methyltryptophan dimer structure **2**.

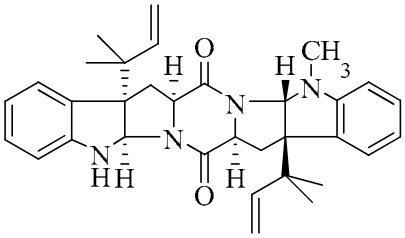
Several piperazinediones derived from tryptophan dimers are known from fungi, e.g., amauromine (**106**) [57], epiamauromine (**107**), and *N*-methylepi-amauromine (**108**) [58]. These compounds have 1,1-dimethylprop-2-ene substituents at C-8a and C-16a rather than methyl groups as in **1** and **2**. The large negative optical rotations found for Lansai A and Lansai B, similar to the value reported for amauromine (**106**) [57], suggest that the compounds have the same absolute stereochemistry as determined for amauromine (**106**) [57], that is the precursor for Lansai A and Lansai B is L-tryptophan (**109**). Acid hydrolysis (HCl), which was used with amauromine (**106**) [57] and epiamauromine (**107**) [58], was not attempted because of the limited amount of lansai A and lansai B.



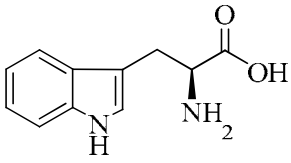
amauromine (**106**)



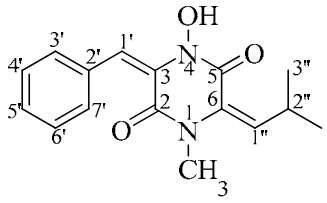
epiamauromine (**107**)



N-methylepiamauromine (**108**)

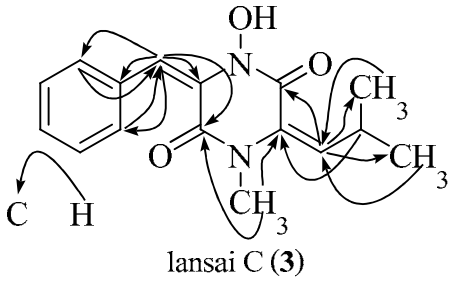


L-tryptophan (**109**)



lansai C (**3**)

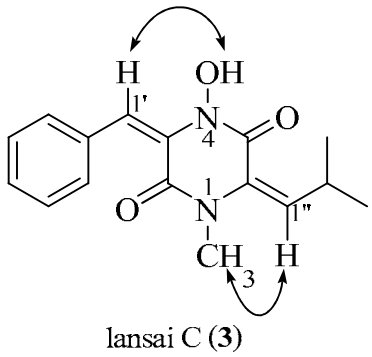
Lansai C (3) was isolated as colorless plates, mp 146–148 °C; it was optically inactive. The molecular formula was $C_{16}H_{18}N_2O_3$. The UV spectrum showed the absorption bands at 230 and 311 nm. Compound **3** had IR bands at 3210 and 1682 cm^{-1} indicating the presence of hydroxyl and amide carbonyl groups, respectively. The ^{1}H-NMR spectrum of **3** (Table 2.5) showed two singlets at δ 8.02 (1H) and 3.31 (3H) from *N*–OH and *N*–CH_3 groups, respectively, and the ^{13}C-NMR (Table 2.5) contained signals for two amide carbonyls at δ 157.8 (C-2) and 157.6 (C-5) and two olefinic quaternary carbons at δ 125.7 (C-3) and 128.0 (C-6), indicating the *N*-hydroxy,*N*-methyl-3,6-dialkylidene-2,5-piperazinedione moiety. The ^{1}H-NMR spectrum of **3** also showed signals from five aromatic protons at δ 7.41 (m) and an olefinic methine proton at δ 7.00 (s). The ^{13}C-NMR showed signals from five aromatic methine carbons at δ 129.4 (2x), 128.5 (2x), 128.6, an aromatic quaternary carbon at δ 133.3, and an olefinic methine carbon at δ 115.8, indicating that compound **3** has a benzylidene unit. The ^{1}H- and ^{13}C-NMR spectra also showed signals due to the presence of an isobutylidene group. The proton signals appeared at δ 1.10 (6H, d, *J* = 6.6 Hz), 3.79 (1H, d sept, *J* = 6.6, 9.6 Hz), and 5.51 (1H, d, *J* = 9.6 Hz) and carbon signals of two methyl carbons at δ 23.3 (C-3'' and C-4''), a methine carbon at δ 26.7 (C-2''), and an olefinic methine carbon at δ 134.8 (C-1'').



lansai C (**3**)

Figure 2.9 HMBC correlation of lansai C (**3**)

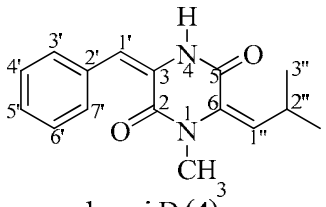
The positions of the benzylidene group at C-3, isobutylidene at C-6, and methyl group on N-1 were established by 2D HMBC correlations (Figure 2.9). The olefinic hydrogen (H-1', δ 7.00) of the benzylidene group showed ^{2}J and ^{3}J correlations to C-3 (δ 125.7) and 2-carbonyl (δ 157.8). The aromatic carbons at δ 133.3 (C-2') and 128.5 (C-3' and C-7') showed correlations to H-1' (δ 7.00). The methyl protons (H-3'' and H-4'') (δ 1.10) and methine proton (H-2'', δ 3.79) of the isobenzylidene group had correlations to olefinic methine carbon (C-1'', δ 134.8) and olefinic quaternary carbon (C-6, δ 128.0), respectively. H-1'' (δ 5.51) had a ^{3}J correlation to the C-5 amide carbonyl (δ 157.6). The *N*-methyl group (δ 3.31) had HMBC correlations to the carbonyl (C-2, δ 157.8) and olefinic quaternary carbon (C-6, δ 128.0).



lansai C (**3**)

Figure 2.10 NOEDIFF correlations of lansai C (**3**)

In the NOEDIFF experiments (Figure 2.10) H-1' and H-1'' gave enhancement with 4-*N*–OH and 1-*N*–CH_3, respectively. Thus, lansai C was characterized as 4-*N*-hydroxy,1-*N*-methyl-3-*E*-benzylidene-6-*E*-isobutylidene-2,5- piperazinedione (**3**).



lansai D (**4**)

Lansai D (**4**) was isolated as colorless plates, mp 170–172°C; it was optically inactive. The molecular formula was $C_{16}H_{18}N_2O_3$, which indicated that **4** had one oxygen less than **3**. The UV spectrum showed the absorption bands at 230 and 319 nm. The IR spectrum displayed the absorption bands for NH and amide carbonyl groups at 3183 and 1682 cm^{-1}, respectively.

The ^{1}H NMR spectrum of **4** (Table 2.5) showed two singlets at δ 8.45 (1H) and 3.23 (3H) for NH and *N*–CH_3 groups, respectively, other peaks in the ^{1}H- and ^{13}C -NMR spectra were almost identical to those **3**. The ^{13}C-NMR (Table 2.5) contained signals for two amide carbonyls at δ 156.8 (C-2) and 159.1 (C-5) and two olefinic quaternary carbons at δ 125.3 (C-3) and 128.2 (C-6), indicating the methyl-3,6-dialkylidene-2,5-piperazinedione moiety. The ^{1}H-NMR spectrum of **4** showed signals from five aromatic protons at δ 7.33 (m) and an olefinic methine proton at δ 6.40 (s). The ^{13}C-NMR showed signals from five aromatic methine carbons at δ 129.9 (2x), 127.8 (2x), 128.4, an aromatic quaternary carbon at δ 133.8, and an olefinic methine carbon at δ 121.9, indicating that compound 4 has a benzylidene unit. The ^{1}H- and ^{13}C-NMR spectra also showed signals due to the presence of an isobutylidene group. The proton signals appeared at δ 1.12 (6H, d, *J* = 6.7 Hz), 3.82 (1H, d sept, *J* = 6.7, 9.6 Hz), and 5.50 (1H, d, *J* = 9.6 Hz) and carbon signals of two methyl carbons at δ 23.3 (C-3'' and C-4''), a methine carbon at δ 26.6 (C-2''), and an olefinic methine carbon at δ 134.6 (C-1'').

The positions of the benzylidene group at C-3, isobutylidene at C-6, and methyl group on N-1 were established by 2D HMBC correlations (Figure 2.11). The olefinic hydrogen (H-1', δ 6.40) of the benzylidene group showed ^2J and ^3J correlations to C-3 (δ 125.3) and 2-carbonyl (δ 156.8). The aromatic carbons at δ 133.8 (C-2') and 127.8 (C-3' and C-7') showed correlations to H-1' (δ 6.40). The methyl protons (H-3'' and H-4'', both at δ 1.12) and methine proton (H-2'', δ 3.82) of the isobenzylidene group had correlations to olefinic methine carbon (C-1'', δ 134.6) and olefinic quaternary carbon (C-6, δ 128.2), respectively. H-1'' (δ 5.50) had a ^3J

correlation to the C-5 amide carbonyl (δ 159.1). The *N*-CH_3 (δ 3.23) had HMBC correlations to the carbonyl (C-2, δ 156.8) and olefinic quaternary carbon (C-6, δ 128.2).

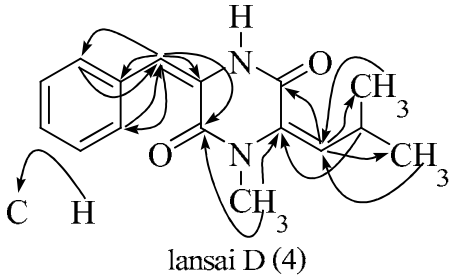


Figure 2.11 HMBC correlations of lansai D (**4**)

In the NOEDIFF spectra (Figure 2.12), H-1' and H-1'' gave enhancement with 4-*N*–H and 1-*N*–CH_3, respectively. Lansai D was thus identified as 1-*N*-methyl-3-*E*-benzylidene-6-*E*-isobutylidene-2,5-piperazinedione (**4**).

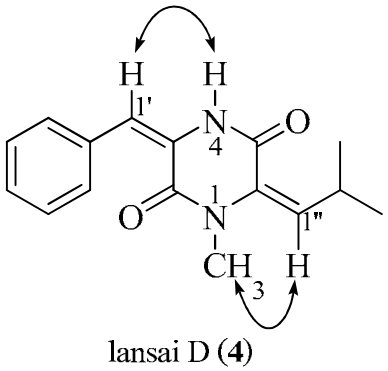


Figure 2.12 NOEDIFF correlations of lansai D (**4**)

A number of 3-ylidene- and 3,6-diylidene-piperazine-2,5-diones have been isolated from various microorganisms, involving amino acids and/or dehydroaminoacids [59].

Table 2.6 Biological activity of lansai A – D (**1**–**4**) and crude EtOAc extract

Compound	IC_{50} (μgml^{-1})		MIC (μgml^{-1})
	BC	**KB**	***C. musae***
crude EtOAc extract	3.41	>20	15
1	>20	>20	inactive
2	15.03	>20	inactive
3	>20	>20	inactive
4	>20	>20	inactive

The crude ethyl acetate extract of the culture of *Streptomyces* sp. SUC1 showed *in vitro* anticancer activity (BC and KB cell lines) with IC_{50} of 3.41 and >20 μgml^{-1}, respectively. The crude extract possessed antifungal activity against *C. musae* with an MIC of 15 μgml^{-1}. Unfortunately, compound **2** was only weakly active against the BC cell line (IC_{50}=15.03 μgml^{-1}); compounds **1**, **3**, and **4** were inactive (IC_{50}>20 μgml^{-1}) (Table 2.6). Compounds **1**–**4** were also inactive against *C. musae* (MIC>100 μgml^{-1}).

Chapter 3

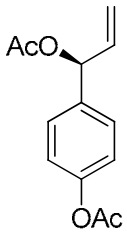
Bioactive compounds from Streptomyces sp. SU238, endophytic on the roots of *Alpinia galanga*

Alpinia galanga is a member of the family Zingiberaceae, which is widely distributed through out the tropics particularly in Southeast Asia. *Alpinia galanga* (Figure 3.1) grows from zhizomes in clumps of stiff stalks up to two meters in height with abundant long leaves. The rhizome has a sharp, sweet taste and smells like a blend of black pepper and pine needles. The red fruit is used in traditional Chinese medicine. In Thailand, *A. galanga* is well known as "Kha". The rhizomes are commonly use in cooking [60].

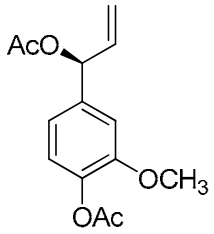
Figure 3.1 Stem and rhizome of *Alpinia galanga*.

The chemical constituents and their biological activity of *A. galanga* have been studied.

In 1976, Mitsui *et al.* [61] isolated two antitumor agents, 1'-acetoxychavicol acetate (**110**) and 1'-acetoxyeugenol acetate (**111**), from the methanolic extract of seeds of *A. galanga*.

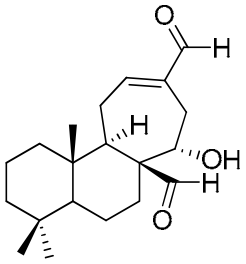


1'-acetoxychavicol acetate (**110**)

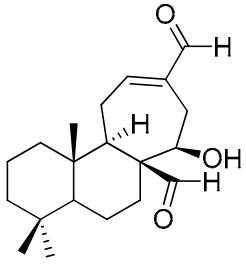


1'-acetoxyeugenol acetate (**111**)

In 1986, Morita and Itokawa [62] isolated two new diterpenes, galanal A (**112**) and B (**113**), from the seeds of *A. galanga*.

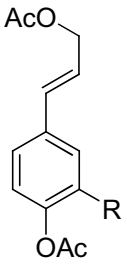


galanal A (**112**)



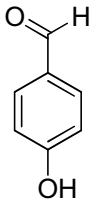
galanal B (**113**)

In 1988, Noro *et al.* [63] isolated xanthine oxidase (XO) inhibitors, *trans*-p-coumaryl diacetate (**114**), *trans*-coniferyl diacetate (**115**), 1'-acetoxychavicol acetate (**110**), 1'-acetoxy-eugenol acetate (**111**) and 4-hydroxybenzaldehyde (**11**6), from the rhizomes of *A. galanga*.



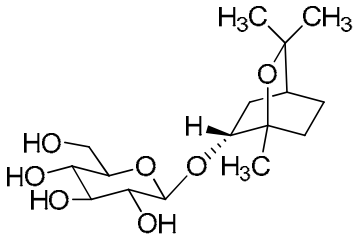
trans-p-coumaryl diacetate (**114**) R = H

trans-coniferyl diacetate (**115**) R = OCH_3

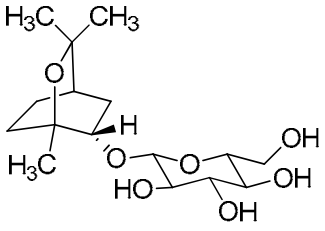


4-hydroxybenzaldehyde (**116**)

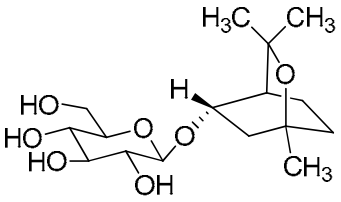
In 2001, Kubota *et al.* [64] isolated three hydroxyl-1,8-cineole glucopyrano-sides, (1*R*,2*R*,4*S*)-*trans*-2-hydroxy-1,8-cineole β-D-glucopyranoside (**117**), (1*S*,2*S*,4*R*) -*trans*-2-hydroxy-1,8-cineole β-D-glucopyranoside (**118**) and (1*R*,3*S*,4*S*)-*trans*-3-hydroxy-1,8-cineole β-D-glucopyranoside (**119**), from the rhizomes of *A. galanga.*



(1*R*,2*R*,4*S*)-*trans*-2-hydroxy-1,8-cineole β-D-glucopyranoside (**117**)

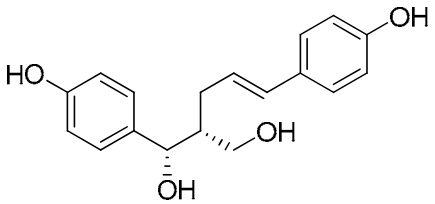


(1*S*,2*S*,4*R*)-*trans*-2-hydroxy-1,8-cineole β-D-glucopyranoside (**118**)

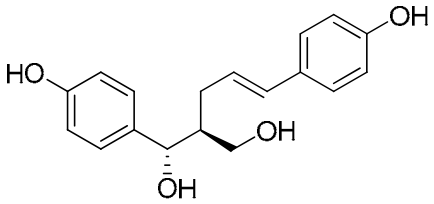


(1*R*,3*S*,4*S*)-*trans*-3-hydroxy-1,8-cineole β-D-glucopyranoside (**119**)

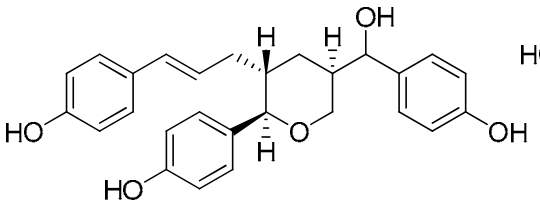
In 2005, Yoshikawa *et al.* [65] isolated four new inhibitors of nitric oxide production, galanganols A-C (**120**-**122**) and galanganal (**123**), and ten known compounds, 1'-acetoxychavicol acetate (**110**), 1'-acetoxyeugenol acetate (**111**), 1'-hydroxychavicol acetate (**124**), chavicol β-D-glucopyranoside (**125**), methyleugenol (**126**), *trans-p*-hydroxycinnamaldehyde (**127**), *trans-p*-coumaryl alcohol (**128**), *trans-p*-hydroxycinnamyl acetate (**129**), *trans-p*-coumaryl diacetate (**114**) and 4-hydroxyben-zaldehyde (**116**), from the rhizomes of *A. galanga*.



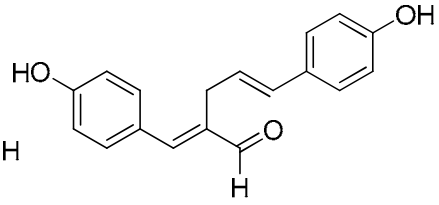
galanganols A (**120**)



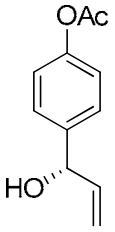
galanganols B (**121**)



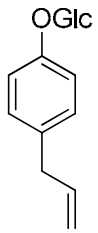
galanganols C (**122**)



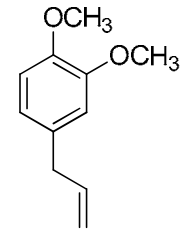
galanganal (**123**)



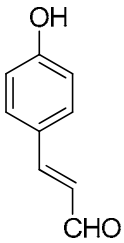
1'-hydroxychavicol acetate (**124**)



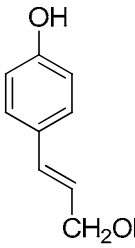
chavicol β-D-glucopyranoside (**125**)



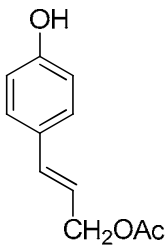
methyleugenol (**126**)



trans-p-hydroxycinnamaldehyde (**127**)



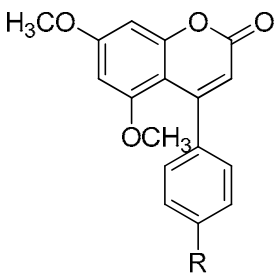
trans-p-coumaryl alcohol (**128**)



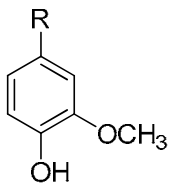
trans-p-hydroxycinnamyl acetate (**129**)

The investigation of bioactive compounds from endophytic actinomycetes of *A. galanga* has been reported.

In 2003, Taechowisan and Lumyong [22] isolated 59 endophytic actinomycetes which isolated from the surface-sterilized roots of *Zingiber officnale* and *A. galanga*. Ten isolates produced bioactive compounds that inhibited both of phytopathogenic fungi, *Colletotrichum musae* and *Fusarium oxysporum*, and nine isolates had activity against yeast, *Candida albicans*. The strain identified as *S. aureofaciens* CMUAc130 was most effective in antifungal activity, inhibited against both of fungi and yeast. Later in 2005, Taechowisan *et al.* [34] reported four bioactive compounds; 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin (**23**), 5,7-dimethoxy-4-phenylcoumarin (**24**), vanillin (**25**) and 3-methoxy-4-hydroxytoluene (**26**), and their antifungal activity from *S. aureofaciens* CMUAc130.

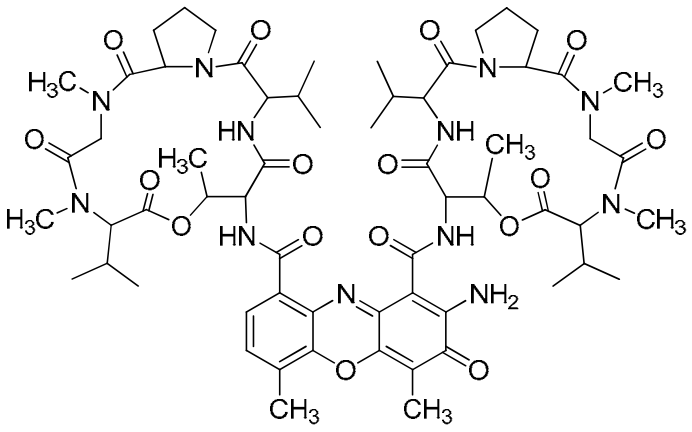


(**23**) R = OCH_3
(**24**) R = H



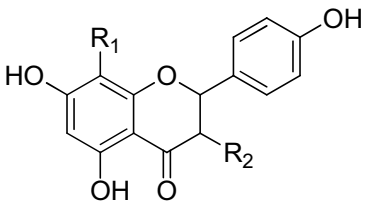
(**25**) R = CHO
(**26**) R = CH_3

In 2006, Taechowisan *et al*. [35] reported the isolation of actinomycin D (**27**) from *Streptomyces* sp. Tc022, endophytic actinomycete of the roots of *A. galanga*.



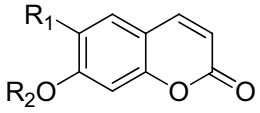
actinomycin D (**27**)

In 2008, Taechowisan *et al*. [36] also isolated four biologically active compounds, kaempferol (**28**), isoscutellarin (**29**), umbelliferone (**30**) and cichriin (**31**), from *Streptomyces* sp. Tc052, an endophyte on the roots of *A. galanga*.



kaempferol (**28**) R_1 = H R_2 = OH

isoscutellarin (**29**) R_1 = OH R_2 = H



umbelliferone (**30**) R_1 = H R_2 = H

cichoriin (**31**) R_1 = OH R_2 = Glc.

3.1 EXPERIMENTAL

3.1.1 General procedure

Melting points are uncorrected. Optical rotations were determined with a Jasco digital polarimeter. UV spectra were recorded with a Shimadzu UV-240 spectro- photometer. IR spectra were recorded with a Jasco A-302 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were measured in $CDCl_3$ or $CDCl_3$/DMSO-d_6 on a Bruker AVANCE 300 (300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR) spectrometer. Chemical shifts are given in δ (ppm) with tetramethylsilane (TMS) as an internal standard. Coupling constants (*J*) are given in Hz. The signals in the ^{1}H- and ^{13}C-NMR spectra were assigned unambiguously using 2D NMR techniques: COSY, HMQC and HMBC. MS were recorded on a VG 7070 mass spectrometer operating at 70 eV with VG Quattro triple quadrupole mass spectrometer for the electrospray mass spectra. HR MS were recorded on a Bruker MicrOTOF mass spectrometer. Column chromatography was carried out using silica gel 60 (Merck, 70-230 or 230-400 mesh). Pre-coated silica gel 60 F_{254} (Merck, layer thickness 0.25 mm) were used for thin-layer chromatography (TLC) and the compounds were visualized under ultraviolet light or sprayed with 1% $CeSO_4$ in 10% aq. H_2SO_4 following by heating. Preparative layer chromatography (PLC) was performed on glass plate using pre-coated silica gel 60 F_{254} (Merck, 20x20 cm, layer thickness 0.25, 0.5 or 1.0 mm). All commercial grade solvents were distilled prior to use and spectral grade solvents were used for spectroscopic measurements.

3.1.2 Isolation of endophytic actionomycetes

The roots of *A. galanga* were collected from the environs of Nakhon Pathom, Thailand during the period of May 2004 – February, 2005. The samples were washed in running tap water and cut into small pieces of 2 x 2 mm^2. Tissue pieces were rinsed in 0.1% Tween 2O for 30 s, then in 1% sodium hypochlorite for 5 min, and then washed in sterile distilled water for 5 min. Next the tissue pieces were surface sterilized in 70% ethanol for 5 min and air-dried in a laminar flow chamber. Finally the pieces were transferred to dishes of humic acid-vitamin (HV) agar, as ascribed by Otoguro *et al*. [52], containing 100 μg nystatin and cycloheximide /ml, and incubated at 30^{O}C for about 1 month. The colonies were inoculated onto International Streptomyces Project medium 2 (ISP-2) [53] for purification, and obtained 42 strains of endophytic actinomycetes from the roots of *Alpinia galanga.* The small pieces of ISP-2, mostly containing spores, were then stored in l5% (v/v) glycerol at -70^{O}C.

3.1.3 Antifungal activity of the actinomycetes isolates against phytopathogenic fungi and yeast.

All endophytic actinomycetes were tested for screening antifungal activity by used the fungal pathogen, *Colletotrichum musae* , the causative agents of anthracnose of banana and human pathogen yeast, *Candida albicans* ATCC90028. *C. musae* was grown on potato dextrose agar (PDA). Mycelial discs of 8 mm diameter were cut from the pathogen colonies and transfer to the ISP-2 plates and positioned 6 cm away from each pre-grown actinomycete colony, as show in Figure 3.2. Then these plates were incubated at 30 ^{o}C for 3–5 days. The width of inhibition zones between the pathogen and the actinomycete isolates were measured.

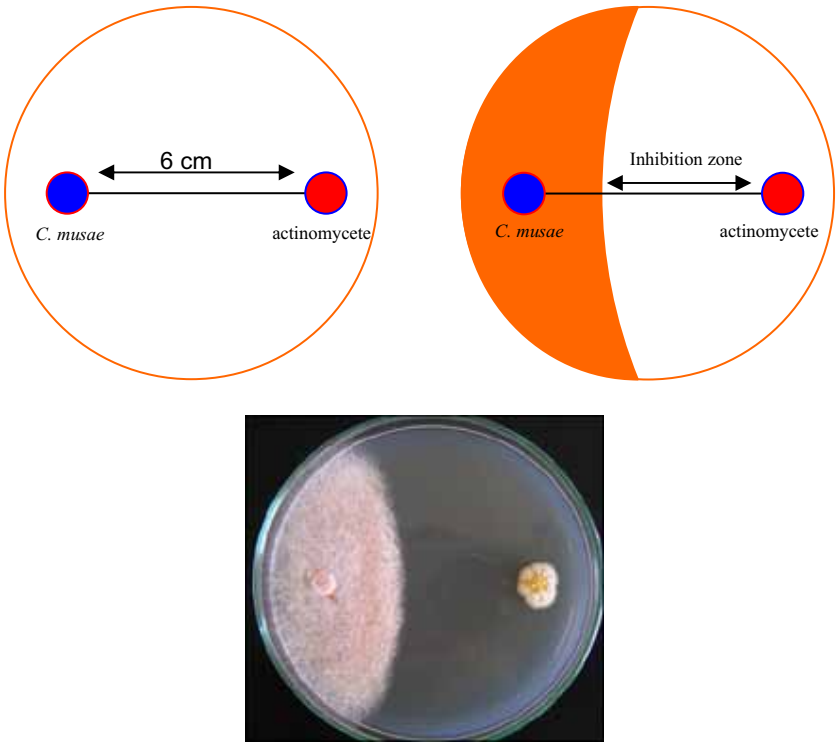


Figure 3.2 Bioactivity test of actinomycete against *C. musae*

For antagonistic activity against *C. albicans* ATCC90028, the yeast was cultured in ISP-2 broth at 30 ^{o}C for 24 h. The cells were diluted to 10^{5} cells ml^{-1} in soft agar, and then were overlayed on pre-grown actinomycete colonies on ISP-2 plates. The plates were incubated at 30 ^{o}C for 24 h, then the width of inhibition zones between the pathogen and the actinomycete isolates were measured, as show in Figure 3.3.

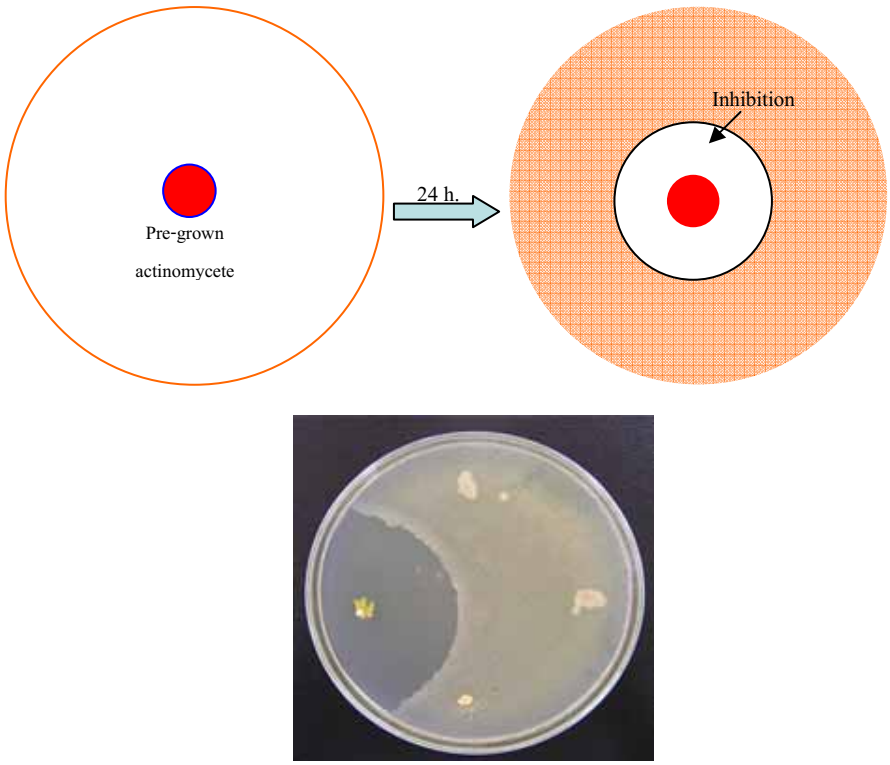


Figure 3.3 Bioactivity test of actinomycete against *C. albicans*

The endophytic actinomycete, identified as *Streptomyces* sp. SU238, was of primary interest, because of its activity against the plant-pathogenic fungus *C. musae* and human pathogen yeast *C. albicans* ATCC90028 *in vitro* [22].

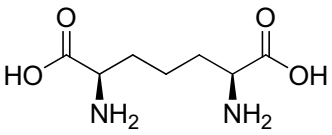
3.1.4 Identification of *Streptomyces* sp. SU238

3.1.4.1 Morphological observations

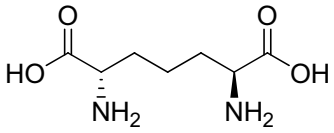
The selected strain of actinomycetes, *Streptomyces* sp. SU238, was cultured on ISP-2 agar plates at 30^{O}C for 3 days then the cover slides were fixed down the colony and incubated at 30^{O}C for a further 5 days. The selected strain of actinomycetes which had grown on the cover slides were stained with crystal violet for 1 min. The morphology of actinomycete isolates were observed under light microscope. For scanning electron microscopy (SEM) observation, the actinomycete isolates grown on the cover slides was air-dried in a desiccator and mounted on stubs, splutter-coated with gold, and viewed on the SEM (Maxim 20005, CamScan, UK).

3.1.4.2 Amino acid composition of the whole cell extract

Streptomyces sp. SU238 was grown in shake culture on ISP-2 medium at 30^{o}C, and the cells were collected at maximal growth after incubation 14 days. After incubation, the cells were collected by filtration with Whatman No. 1filter paper, washed with distilled water and absolute ethanol, and air-dried at room temperature. Dried cells (10 mg) were hydrolysed for 18 h with 1 ml of 6 N HCl in a screw cap tube held at 100^{o}C in an oven. After cooling, the tubes were opened and the contents were filtered through paper. The solid material on the paper was washed with 3 drops of distilled water. The liquid hydrolysate was dried three consecutive times on a steam bath to remove most of the HCl. The residue was taken up in 0.3 ml of distilled water, and 20 μl of the liquid were spotted on Whatman No. 1 paper. A spot of 10 μl of 0.01 M of *meso*-diaminopimelic acid (**104**) which also contained some of the LL-isomer (**105**) (Wako pure chemical industries Ltd., Japan) was used as the standrad. Descending chromatography was carried out overnight by irrigation with methanol/water/10 N HCl/pyridine (80:17.5:2.5:10). Amino acids were detected by dipping the papers in a bath of acetonic ninhydrin (0.1% w/v), followed by heating for 2 min at 100^{o}C. Diaminopimelic acids in the hydrolysate gave purple spots with this reagent. The result was identified by comparing with the R_f values of the standards, as showed in Table 3.1.



meso-diaminopimelic acid (**104**)



LL-diaminopimelic acid (**105**)

Table 3.1 The R_f value of tested amino acid by descending paper chromatography

Amino acid	R_f value
Alanine	0.68
Aspartic acid	0.48
Glutamic acid	0.62
Glycine	0.51
Lysine	0.56
LL- diaminopimelic acid	0.37
meso- diaminopimelic acid	0.32

3.1.4.3 DNA isolation

Genomic DNA was isolated from *Streptomyces* sp. SU238 using the procedure of Hopwood *et al.* [54] 16S rDNA was amplified by PCR using Taq DNA polymerase (Promega, USA) and primers A 7-26f (5'-CCGTCGACGAGCTCAGAGTTTGATCCTGGCTCAG-3') and primers B 1523-1504r (5'-CCCGGGTACCAAGCTTAAGGAGGTGA-TCCAGCCGCA-3'). The conditions used for thermal cycling were as follows: denaturation at 95^{O}C for 5 min, followed by 35 cycles of denaturation at 95^{O}C for 1 min, annealing at 56^{O}C for 1 min and extension at 72^{O}C for 2 min. At the end of the cycles, the reaction mixture was kept at 72^{O}C for 10 min and then cooled to 4 ^{O}C. The 1.5 Kb amplified 16S rDNA fragment was separated by agarose gel electrophoresis and purified by using a QiAquick gel extraction kit (QIAGEN,

Germany). The purified fragments were cloned into pGEM-T Easy vector (Promega). 16S rDNA nucleotide sequenceswere determined using the dideoxy chain termination method, with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). The primers used for nucleotide sequencing were as follows: T7 promoter, SP6 promoter, C 704-685r (5'-TCTGCGCATTT-CACCGCTAC-3') and D 1115-1100r (5'-AGGGTTGCGCTCGTTG-3). All of the obtained sequences were assembled and then compared with similar sequences from the reference organisms, with the BLAST database (a genome database of the National Center for Biotechnology Information).

3.1.5 Minimum Inhibitory Concentration

The pure compound **5** was assayed on PDA in Petri dishes to determine the minimum inhibitory concentration against *C. musae*, using the disc diffusion assay. The pure compound **5** was dissolved in methanol, serially diluted in the same solvent. The final concentrations of the pure compound **5** were 0, 5, 10, 15, 20, 25, 50, 75, 100 μgml^{-1} and 50 μl was applied to sterile 6-mm diameter paper discs (Advantec, Toyo Roshi Kaisha, LTD., Japan) and dried. Each PDA plates were inoculated with a 8-mm diameter plug of fungi, removed from the margin of a 4-day-old colony on PDA, in the center and the treated paper discs were then placed away from the fungi 2.2 cm. Plates were incubated at 30 ^{o}C for 72 h and observed for the presence of an inhibition zone. The experiment was repeated twice.

The determination of minimum inhibitory concentration of this compound against *C. albicans*, Microbroth dilution assay of yeast was performed as described in the NCCLS (1997) protocols [66]. The assay was performed in sterile test tubes, and the total volume per tube was 1 ml. The yeast inoculum was prepared to give approximately $10^4 – 10^5$ CFU per tube and the compound **5** was tested at concentrations from 0.005 to 1.28 $mgml^{-1}$ in twofold step dilution. The actual number of CFU per tube was comfirmed by plating onto Sabouraud agar. Two tubes were incubated for given concentration. The plates were incubated for 16-20 h at 37^{o}C. The MIC was defined as the minimum concentration of compound **5** resulting in no visible growth of the test organism.

3.1.6 Antimycobacterial acitivity assay

The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* $H_{37}Ra$ (ATCC 25177) using the Microplate Alamar Blue Assay (MABA) [67]. Standard drugs, isoniazide (MIC of 0.040-0.090 μgml^{-1}) and kanamycin sulfate (MIC of 2.0-5.0 μgml^{-1}) were used as reference compounds for the assay.

3.1.7 Antiplasmodial activity assay

The *Plasmodium falciparum* (K1, multidrug resistant strain) was cultured continuously according to the method of Trager and Jensen [68]. The quantitative assessment of the antiplasmodial activity *in vitro* was performed by means of the microculture radioisotope technique based upon the method described by Desjardins *et al*.[69]. Standard sample, chloroquine diphosphate was used as reference compound for assay.

3.1.8 Anticancer acitivity assay

Cytotoxicity assay against human breast cancer (BC), human oral cavity cancer (KB), human colon cancer (Coca2) and human liver cancer (HepG2) was carried out using the method of Skehan *et al.* [55], the reference substances were ellipticine and doxorubicin.

3.1.9 Extraction and Isolation

Spores of *Streptomyces* sp. SU238 were used to inoculate 100 plates of ISP-2 and incubated for 14 days at 30°C (Figure 3.4). The culture medium was then cut into small pieces and extracted with EtOAc (3x500 ml). The organic extract was filtered and evaporated to give a brown oil (1.04 g).

Figure 3.4 The maturity of of *Streptomyces* sp. SU238

The oil was separated by flash column chromatography using silica gel 60 (Merck, 0.015-0.040 mm, diameter x height 4.5 x 3.5 cm.) and the column was eluted with 30 ml each fraction with hexane, gradient of hexane/EtOAc and EtOAc to give echinomycin (**5**) (not pure) as a pale yellow solid (143.4 mg). The solid was further purified on a column of siliga gel 60 (Merck, 0.063-0.200 mm, 15 g) using CH_2Cl_2/MeOH/H_2O (150:3:1, 100:3:1, 80:3:1, 50:3:1 and 30:3:1) to give echinomycin (**5**) as a colorless solid (44.0 mg).

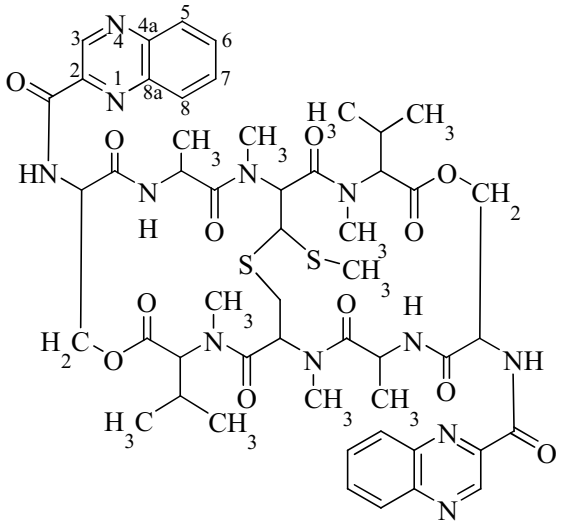
Echinomycin (**5**): m. p. 206 – 214 ^{o}C; $[\alpha]_{D}^{28}$ -537.7^{o} (c 0.0220, MeOH); λ_{max} (MeOH) (log ε) nm : 242 (4.78), 317 (4.04); ν_{max}(KBr) cm^{-1} : 3393, 3062, 2968, 1739, 1683, 1653, 1520, 1492, 1409, 1260, 1136, 1096, 1011, 983, 776; HRTOFMS *m/z* : Calcd for $C_{51}H_{65}N_{12}O_{12}S_2$: 1101.4281. Found : 1101.4287. ^{1}H- and ^{13}C-NMR: see Table 3.2.

Table 3.2 ^{1}H- (300 MHz) and ^{13}C-NMR (75 MHz) spectral data of echinomycin (**5**)

Position	δH	δC	Position	δH	δC
Chain A			**Chain B**		
***N*-Me Valine**			***N*-Me Valine**		
2	5.14, d (12.0)	62.0	2	5.20, d (12.0)	62.7
3	2.34, m	27.8	3	2.34, m	27.7
4	0.88, d (6.5)	18.3	4	0.92, d (6.5)	19.0
3-Me	1.08, d (6.5)	20.4	3-Me	1.10, d (6.5)	20.4
N-Me	3.18, s	31.4	*N*-Me	3.10, s	30.9
C=O	-	171.2	C=O	-	170.9
***N*-Me Cysteine**			***N*-Me Cysteine**		
2	6.13, m	53.5	2	6.48, d (9.0)	60.0
3	2.87, dd(10.9, 15.9)	27.3	3	4.92, d (9.0)	51.8
	3.41, dd(2.1, 15.9)		*N*-Me	2.99, s	32.3
N-Me	3.01, s	29.8	*S*-Me	2.10, s	15.3
C=O	-	170.2	C=O	-	168.8
Alanine			**Alanine**		
2	4.97 (overlapped)	46.2	2	4.83 (overlapped)	46.5
3	1.40, d (7.2)	18.2	3	1.37, d (7.2)	17.2
NH	6.98, d (7.0)	-	NH	6.83, d (7.0)	-
C=O	-	173.6	C=O	-	173.7
Serine			**Serine**		
2	4.92 (overlapped)	53.5	2	4.82 (overlapped)	52.4
3	4.64 (overlapped)	64.8	3	4.69 (overlapped)	65.0
NH	8.66, d (7.6)	-	NH	8.84, d (6.2)	-
C=O	-	167.5	C=O	-	167.3

Table 3.2 ^{1}H- (300 MHz) and ^{13}C-NMR (75 MHz) spectral data of echinomycin (**5**) (cont.)

Position	δ_H	δ_C
Quinoxaline		
2	-	142.3, 142.4
3	9.62, 9.64, both s	143.6, 143.7
4a	-	144.1, 144.2
5	7.93, dd (1.4, 8.4)	129.3
	7.99, dd (1.4, 8.4)	129.5
6	8.18, dt (1.4, 8.4)	129.7 (2X)
7	7.82 (overlapped)	131.1, 131.2
8	7.84 (overlapped)	132.1
	7.89 (overlapped)	132.0
8a	-	140.1, 140.2
C=O	-	164.0, 164.1



Echinomycin (**5**)

3.2 RESULTS AND DISCUSSION

3.2.1 Isolation and Identification of *Streptomyces* sp. SU238

Based on the results of the morphological observation (Figure 3.5) and the presence of LL-diaminopimelic (**105**) acid in the whole-cell extract, endophytic actinomycete SU238 was identified to be the genus *Streptomyces*. Almost of the complete 16S rDNA sequence of the endophytic *Streptomyces* sp. SU238 (>95% of the *Escherichia coli* sequence) was determined. BLAST search results for the strain SU238 came from the non-redundant GenBank + EMBL +DDBJ; when reference sequences were chosen, unidentified and unpublished sequence were excluded. The BLAST results showed that strain SU238 had high levels of sequence similarity (98%) to *S. tendae* (accession number: D638873). The nucleotide sequence data of *Streptomyces* sp. SU238 was deposited in the GenBank, EMBL and DDBJ databases with the accession number AB246727.

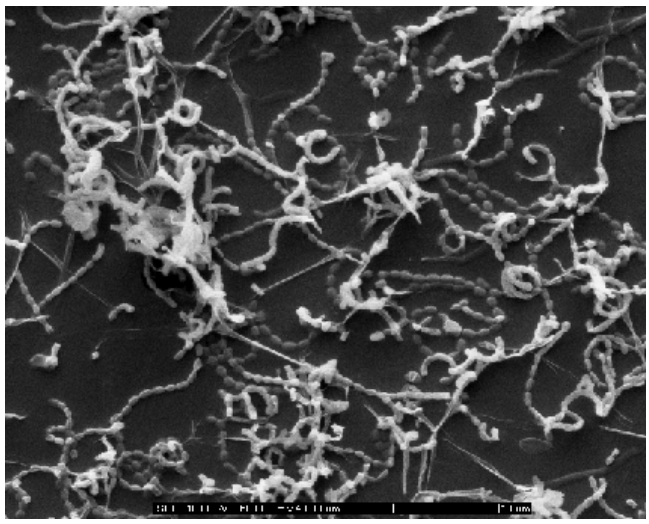
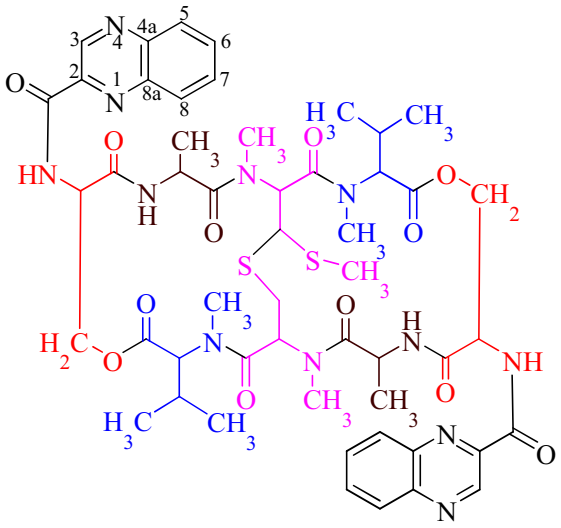


Figure 3.5 Morphology and colony of the *Streptomyces* sp. SU238.
The sporophores have monopodially branched and twisted into open spiral form and its spores are oval shaped under SEM observation, bar = 10 μm.

3.2.2 Echinomycin (5)

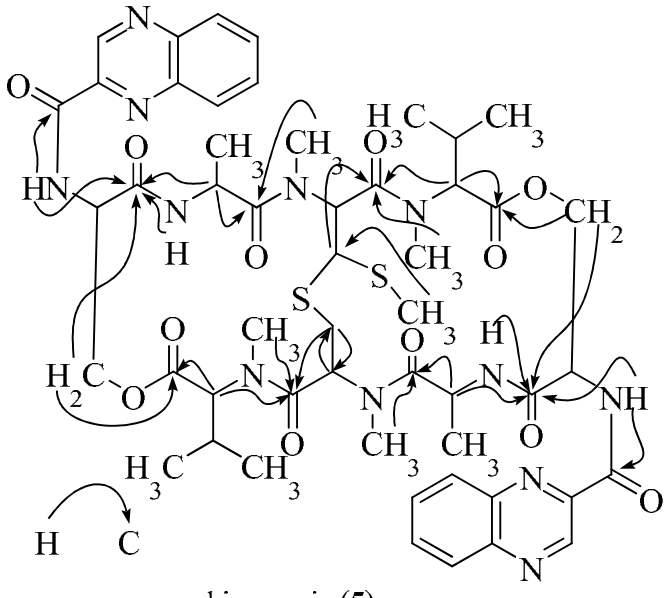
The crude EtOAc extracts from the endophytic actinomycete, Streptomyces sp. SU238, of *A. galanga* were separated by chromatographic methods to yield echinomycin (**5**).



Echinomycin (**5**)

Echinomycin was isolated as a colorless solid, m. p. 206-214°C which was optically active ($[\alpha]_D^{28}$ = -537°, 0.022, MeOH). The compound has the molecular formula $C_{51}H_{64}N_2O_{12}S_2$ by HRTOFMS. The UV spectrum showed bands at 242 and 317 nm. The IR spectrum showed absorption bands for NH (3392 cm^{-1}), ester carbonyl (1740 cm^{-1}) and amide carbonyl (1653 cm^{-1}). The signals of ten aromatic protons at δ 9.62, 9.64, 7.93, 7.99, 8.18 (2H), 7.82 (2H), 7.84 and 7.89 in the ^{1}H-NMR spectrum of echinomycin (Table 3.2) were ascribed to two 2-quinoxalinecarboxyl units in the molecule. This was consistent with the ^{13}C-NMR spectral data (Table 3.2) which exhibited sixteen aromatic carbons at δ 142.3 (C-2), 142.4 (C-2), 143.6 (C-3), 143.7 (C-3), 144.1 (C-4a), 144.2 (C-4a), 129.3 (C-5), 129.5 (C-5),129.7 (C-6 x 2), 131.1 (C-7), 131.2 (C-7), 132.1 (C-8) 132.0 (C-8), 140.1 (C-8a), 140.2 (C-8a) and two carbonyl carbons at δ 164.0 and 164.1. In addition, the ^{1}H-NMR spectrum of echinomycin (**5**) contained four *N*-methyl signals as four singlets at δ 3.18, 3.10, 3.01 and 2.99 and four NH functions as four doublets at δ 8.84 (J = 6.2 Hz), 8.66 (J = 7.6 Hz), 6.98 (J = 7.0 Hz) and 6.83 (J = 7.0 Hz), indicating that echinomycin is an octapeptide containing four *N*-methyl amino acids and four amino acids. This was consistent with the ^{13}C-NMR spectrum of echinomycin (Table 3.2) which showed peaks of

eight amide carbonyls at δ 173.6, 173.4, 171.2, 170.9, 170.2, 168.8, 167.5 and 167.3. In the ^{1}H-NMR spectrum of echinomycin (Table 3.2) showed signals which were indicative for two units each of *N*-methyl valine, alanine and serine and a unit of *S*-methyl-di-*N*-methylcysteinyl-thioacetal. Four doublets (*J* = 6.5 Hz) of three protons each at δ 0.88, 0.92, 1.08 and 1.10, a multiplet of two protons at δ 2.34, two doublets (*J* = 12.0 Hz) of one proton each at δ 5.14 and 5.20 and two singlets of two *N*-methyl groups at δ 3.10 and 3.18 were assigned to two *N*-methyl valine units. The signals of two alanine units appeared as two doublets (*J* = 7.2 Hz), of three protons each at δ 1.37 and 1.40, two overlapped signals of one proton each at δ 4.83 and 4.97 and two doublets (*J* = 7.0 Hz) of two NH groups at δ 6.83 and 6.98. Two methylene groups appeared at δ 4.64 (overlapped) and 4.69 (overlapped), two methine protons at δ 4.82 (overlapped) and 4.92 (overlapped) and two doublets of two NH groups at δ 8.84 (*J* = 6.2 Hz) and 8.66 (*J* = 7 .6 Hz) were assigned to two serine units. The signals of *S*-methyl-di-*N*-methylcysteinylthioacetal unit showed as an ABX system as two doublet of doublets at δ 2.87 (*J* = 10.9, 15.9 Hz) and 3.41 (*J* = 2.1, 15.9 Hz) and a multiplet at δ 6.13, a doublet of an methine proton at δ 6.48 (*J* = 9.0 Hz), a doublet of an acetalic proton at δ 4.92 (*J* = 9.0 Hz) and three singlets of one *S*-mehtyl and two *N*- mehtyl groups at δ 2.10, 2,99 and 3.01, respectively. The ^{13}C-NMR (Table 3.2) was assigned by a combination of DEPT, HMQC and HMBC experiments.



echinomycin (**5**)

Figure 3.6 HMBC correlations of echinomycin (**5**)

The NMR spectrum indicated that echinomycin must have the structure which account for two peptide chains which were bridged by the $CHCH_2SCH(SCH_3)CH$- unit. The methylene group (δH 2.87 and 3.41; δC 27.3) of the unit showed 2D HMBC (Figure 3.6) and correlations with the methine group (δH 6.13; δC 53.5) and the acetalic function (δH 4.92; δC 51.8) and the acetalic carbon had correlation with the *S*-CH_3 protons (δ2.10). The β-protons of the two serine units were shift downfield to δ 4.69 and 4.64, suggesting the ester linkages, which were confirmed by HMBC correlations between β-protons of the two serines and the carbonyl carbons of the two *N*-Me valine at δ 171.2 and 170.9, respectively. The quinoxalinecarbonyls (δ 164.1 and 164.0) showed ^2J correlations with the NH (δ 8.84 and 8.66, respectively) of the two serine units and also ^3J correlations with H-3 (δ 9.62 and 9.64) of the quinoxaline rings, confirming the connectivity between the two serine units and the two 2-quinoxalinecarboxyl units. The HMBC experiments (Figure 3.6) established the connectivity of the amino acids. Each NH proton or *N*-CH_3 protons showed a correlation to the carbonyl of the vicinal amino acid, thereby establishing the sequence of the amino acids.

Echinomycin (**5**) possessed potent *in vitro* antiplasmodial activity with the EC_{50} of 2 $ngml^{-1}$ and *in vitro* antimycobacterial activity with the MIC value of 24 $ngml^{-1}$.The compound also exhibited cytotixicity against KB, BC, Caco2 and HepG2 cell lines with the IC_{50} of 0.006, 0.001, 0.43 and 0.92 $ngml^{-1}$ respectively. Echinomycin (**5**) also showed antifungal activity against *C. musae* and *C. albicans* with the MIC of 10 and 20 μgml^{-1}, respectively.

Bibliography

1. L.M. Prescott, J.P. Harley, D.A. Klein, "Microbiology" 5th ed., McGraw-Hill, Boston, 537-551 (2003).
2. Y. Takahashi, S. Omura, *J. Gen. Appl. Microbiol.*, **49**, 141- 154 (2003).
3. A. J. McCarthy, S. T. Williams, *Gene*, **115**, 189-192 (1992).
4. D. A. Hopwood, T. Kieser, M. Bibb, M. Buttner, K. Chater, "Practical *Streptomyces* Genetics" John Innes Foundation, Norwich, 12-15 (2000).
5. D. Baker, W. Newcomb, J. G. Torrey, *Can J Microbiol*, **26**, 1072-1089 (1980).
6. P. Sardi, M. Saracchi, S. Ouaroni, B. Petrolini, G. E. Borgonovoli, .S. Merli, *Appl. Environ. Microbiol.*, **58**, 2691-2693 (1992).
7. M. Shimizu, Y. Nakagawa, Y. Sato, T. Furumai, Y. Igarashi, H. Onaka, R. Yoshida, H. Kunoh, *J. Gen. Plant. Pathol.*, **66**, 360-366 (2000).
8. T. Okazaki, K. Takahashi, M. Kizuka, R. Enokita, *Ann. Rep. Sankyo Res. Lab.*, **47**, 97-106 (1995).
9. S. Matsukuma, T.Okuda, J.Watanabe, *Actinomycetologica*, **8**, 57-65 (1994).
10. A. Matsumoto, Y. Takahashi, M. Mochizuki, A. Seino, Y. Iwai, S. Omura, *Actinomycetologica*, **12,** 46-48 (1998).
11. J. T. Coombs, C. M. M. Franco, *Appl. Environ. Microbiol.*, **69**, 5603–5608 (2003).
12. T. Okazaki "Studies on actinomycetes isolated from plant leaves. In Selective Isolation of Rare Actinomycetes", National Library of Australia, 102–121 (2003).
13. M. Rosenblueth, E. Martinez-Romero, *Mol. Plant. Microbe Interact.*, **19**, 827-837 (2006).
14. T. Taechowisan, J. F. Peberdy, S. Lumyong, *World J. Microbiol. Biotechnol.*, **19**, 381-385 (2003).
15. Y. Igarashi, T. Iida, Y. Sasaki, N. Saito, R. Yoshida, T. Furumai, *Actinomycetologica* **16**, 9–13, (2002).
16. Y. Igarashi, T. Iwashita, T. Fujita, H. Naoki, T. Yamakawa, R. Yoshida, T. Furumai, *J. Antibiot.*, **56**, 705–706, (2003).
17. T. Furumai, T. Yamakawa, R. Yoshida, Y. Igarashi, *J. Antibiot.*, **56**, 700–704, (2003).

18. K. Gebhardt, J. Schimana, P. Krastel, K. Dettner, J Rheinheimer, A. Zeeck, H.-P. Fiedler, *J. Antibiot.,* **55**, 794–800, (2002).
19. T. Nishimura, S. Hasegawa1, A. Meguro1, M. Shimizu2, H. Kunoh, *Actinomycetologica*, 20, 72–81(2006).
20. G. A. Strobel, *Microbes Infect.*, **5**, 535–544 (2003).
21. G. A. Strobel, B. Daisy, *Microbiol Mol Biol Rev.*, **67**, 491-502 (2003).
22. T. Taechowisan and S. Lumyong, *Ann. Microbiol.*, **53**, 291-298 (2003).
23. Y. Igarashi, M. Ogawa, Y. Sato, N. Saito, R. Yoshida, H. Kunoh, H. Onaka, T. Furumai, *J. Antibiot.*, **53**, 1117-1122 (2000).
24. T. Sasaki, Y. Igarashi, N. Saito, T. Furumai, *J. Antibiot.*, **54**, 567-572 (2001).
25. Y. Igarashi, T. Iida, R. Yoshida, T. Furumai, *J. Antibiot.*, **55**, 764-767 (2001).
26. Y. Igarashi, S. S. Miura, T. Fujita, T. Furumai, *J. Antibiot.*, **5**9, 193-195 (2006).
27. M. Kizuka, R. Enokita, K. Shibata, Y. Okamoto, Y. Inoue, T. Okazaki, *Actinomycetologica*, **16**, 14–16 (2002).
28. U.F.Castillo, G. A. Strobel, E. J. Ford, W. M. Hess, H. Porter, J. B. Jensen, H. Albert, R. Robison, M. A. Condron, D. B. Teplow, D. Stevens, D. Yaver. *Microbiol.*, **148**, 2675-2685 (2002).
29. U.F.Castillo, G. A. Strobel, E. J. Ford, W. M. Hess, H. Porter, J. B. Jensen, H. Albert, R. Robison, M. A. Condron, D. B. Teplow, D. Stevens, D. Yaver, J. K. Harper, J. Sears, K. Alesi, J. Lin, M. Hunter, M. Maranta, H. Ge, D. Millar, *FEMS Microbiol. Lett.*, **224**, 183-190 (2003).
30. T. Furumai, T. Yamakawa, R. Yoshida, Y. Igarashi, *J. Antibiot.*, **56**, 700-704 (2003).
31. S. H. Guan, I. Sattler, W. H. Lin, D. A. Guo, S. Grabley, *J. Nat. Prod.*, **68**, 1198-1200 (2005).
32. D. Lee, N, Kim, J. C. Shin, W. Kim, B. Y. Hwang, B. S. Kim, Y. S. Hong, *J. Antibiot*., **59**, 797-800 (2006).
33. Y. Igarashi, M. E. Trujillo, E. Martínez-Molina, S. Yanase, S. Miyanaga, T. Obata, H. Sakurai, I. Saiki, T. Fujita, T. Furumai, *Bioorg. Med. Chem. Lett.,* **17**, 3702-3705 (2007).
34. T. Taechowisan, C. Lu, Y. Shen, S. Lumyong, *Microbiol.*, **151**, 1691-1695 (2005).

35. T. Taechowisan, A. Wanbanjob, P. Tuntiwachwuttikul, W. C. Talylor, *Ann. Microbiol.*, **56**, 113-117 (2006).

36. T. Taechowisan, A. Wanbanjob, N. Chuaychot, S. Chanaphat, Y. Shen, *Intl. J. Pharmacol.*, **4**, 95-101 (2008).

37. B. Baumgartner, C. A. J Erdelmeier, A. D. Wright, T. Rali, O. Sticher, *Phytochemistry*, **29**, 3327-3330 (1990).

38. J. Kitajima, K. Kimizuka, Y. Tanaka, *Chem. Pharm. Bull.*, **46**, 1408-1411 (1998).

39. J. Kitajima, K. Kimizuka, Y. Tanaka, *Chem. Pharm. Bull.*, **4**7, 1138-1140 (1999).

40. J. Kitajima, K. Kimizuka, Y. Tanaka, *Chem. Pharm. Bull.*, **4**8, 77-80 (2000).

41. Y-H Kuo and Y-C Li, *Chem. Pharm. Bull.*, **4**7, 299-301 (1999).

42. Y-H Kuo and Y-C Li, *Chem. Pharm. Bull.*, **4**8, 1862-1865 (2000).

43. Y-H Kuo and Y-C Li, *Chem. Pharm. Bull.*, **4**8, 593-596 (2000).

44. Y-H Kuo, Y-H Liu, J-K Su, Y-M Chiang, *Chem. Pharm. Bull.*, **49**, 581-583 (2001).

45. Y-H Kuo, Y-M. Chiang, J-Y. Chang, C-C. Kuo, C-Y Chang, *Phytochemistry,* **66**, 495–501 (2005).

46. S. S. Subramanian and A. G. R. Nair, *Phytochemistry*, **9**, 2583-2584 (1970).

47. D.M. Gaspar, C. A. Alberto, S. P. A. Mara, H. M. Adolfo, *Phytochemistry*, **45**, 1697 – 1699 (1997).

48. L. Pistelli, E. E. Chielline, I. Morelli, *Biochem. Syst. Ecol*, **28**, 287-289 (2000).

49. B. T. Ngadjui, C. C. F. Simo, S. F. Kouam, H. M. P. Poumale, I. K. Simo, I. R. Green, K Krohn, *Biochem. Syst. Ecol.*, **36**, 238-243 (2008).

50. H. Bouzar, W. S. Chilton, X. Nesme, Y. Dessaux, V. Vaudequin, A. Petit, J. B. Jones, N. C. Hodge, *Appl. Environ. Microbiol.*, **61**, 65-73 (1995).

51. T. S. Suryanarayanan and D. Vijaykrishna, *Fungal Divers*., **8**, 155–161 (2001).

52. M. Otoguro, M. Hayakawa, T. Yamazaki, Y. Iimura, *J. Appl. Microbiol.*, **91**, 118-130 (2001).

53. E. B. Shirling and D. Gottlieb, *Int. J. Syst. Bacteriol.*, **16**, 313-340 (1966).

54. D. A. Hopwood, M. J. Bibb, K. F. Chater "Genetic Manipulation of Streptomyces: a Laboratory Manual", Crowe and Sons, Norwich, 79-80 (1985).

55. P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, M. R. Boyd, *J. Natl. Cancer Inst.,* **82**, 1107-1112 (1990).

56. J. Brosius, M. L. Palmer, P. J. Kennedy, H. F. Noller, *Proc. Natl. Acad. Sci. USA*, **75**, 4801-4805 (1978).

57. S. Takese, Y. Kawai, I. Uchida, H. Tanaka, H. Aoki, *Tetrahedron*, **41**, 3037–3048 (1985).

58. F. S. Guzman, J. B. Glower, D. T. Wicklow, P. F. Dowd, *J. Nat. Prod.*, **55**, 931–939 (1992).

59. P. G. Sammes, *Progress in the Chemistry of Organic Natural Products*, **32**, 51–118 (1975).

60. http://en.wikipedia.org/wiki/Alpinia_galanga

61. S. Mitsue, S. Kobayashi, H. Nagahori, A. Ogiso, *Chem. Pharm. Bull.*, **24**, 2377-2386 (1976).

62. H. Morita, H. Itokawa, *Chem. Lett.*, **15**, 1205-1208 (1986).

63. T. Noro, T. Sekiya, M. Katoh, Y. Oda, T. Miyase, M. Kuroyanagi. A. Ueno, S. Fukushima, *Chem. Pharm. Bull.*, **36**, 244-248 (1988).

64. K. Kubota, A. Kobayashi, Y. Someya, *Biosci. Biotechnol. Biochem.*, **65**, 950-953 (2001).

65. M. Yoshikawa, T. Morikawa, S. Ando, H. Matsuda, S. Kataoka, O. Muraoka, *Chem. Pharm. Bull.*, **53**, 625-630 (2005).

66. NCCLS – National Committee for Clinical Laboratory Standards, "Reference method for broth dilution antifungal susceptibility testing of yeast. Approved standard M27-A" National Committee for Clinical Laboratory Standards, Wayne, Pa (1997).

67. L. Collins and S. G. Franzblau, *Antimicrob. Agents. Chemother.*, **41**, 1004–1009 (1997).

68. W. Trager and J. B. Jensen, *Science*, **193**, 673 – 675 (1976).

69. R. E. Desjardins, C. J. Canfield, J. D. Haynes, J. D. Chulay, *Antimicrob. Agents. Chemother.*, **16**, 710–718 (1979).

Appendix

δ	=	**chemical shift relative to tetramethylsilane (TMS)**
ε	=	**molar absorptivity coefficient**
λ_{max}	=	**maximum wavelength**
ν_{max}	=	**absorption frequency**
μm	=	**micrometer**
brs	=	**broad siglet**
d	=	**doublet**
dd	=	**doublet of doublets**
dq	=	**doublet of quartets**
$CDCl_3$	=	**deuterochloroform**
$CeSO_4$	=	**cerium sulfate**
CH_2Cl_2	=	**dichloromethane**
CH_3CN	=	**acetonitrile**
COSY	=	**correlation Spectroscopy**
DEPT	=	**Distortionless Enhancement by Polarization Transfer**
EI MS	=	**Electron-Ionization Mass Spectrometry**
EtOAc	=	**ethyl acetate**
eV	=	**Electron Volt**
g	=	**gram**
H_2SO_4	=	**sulfuric acid**
HMBC	=	**Heteronuclear Multiple Bond Correlation**
HMQC	=	**Heteronuclear Multiple Quantum Coherence**
HPLC	=	**High Performance Liquid Chromatography**
Hz	=	**Hertz**
INEPT	=	**Insensitive Nuclei Enhanced by Polarization Transfer**
IR	=	**Infrared**
J	=	**coupling constant**

m	**=**	**multiplet**
MeOH	**=**	**methanol**
MIC	**=**	**Minimum Inhibitory Concentration**
MHz	**=**	**Megahertz**
mg	**=**	**milligram**
ml	**=**	**milliliter**
mm	**=**	**millimeter**
NMR	**=**	**Nuclear Magnetic Resonance**
NOE	**=**	**Nuclear Overhauser Effect**
NOESY	**=**	**Nuclear Overhauser Enhancement Spectroscopy**
PLC	**=**	**Preparative Layer Chromatography**
ppm	**=**	**part per million**
q	**=**	**quartet**
ROESY	**=**	**Rotating frame Overhauser Enhancement Spectroscopy**
RP	**=**	**reverse phase**
s	**=**	**singlet**
TLC	**=**	**Thin Layer Chromatography**
t	**=**	**triplet**
UV	**=**	**Ultraviolet Visible**

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