# 1. INTRODUCTION

## 1.1 General Introduction

Microorganisms are the largest and the oldest form of all living organisms. They exist as individuals or cell clusters and play an important role as decomposer in our ecosystem. Microorganisms can be divided into five major classes; fungi, bacteria, viruses, protozoa and algae. Not only harmful microorganisms which can be pathogen or parasitic to other living organisms, but there are also useful microbes. Examples of some useful microbes are *Penicillium notatum*, the mold which produces the antibiotic penicillin (discovered by Alexander Fleming in 1928), and the actinomycetes bacteria which produce antibiotics streptomycin and norcardicin. Many important drugs are derived from microbial secondary metabolites and their modified analogs, for example streptomycin, cephalosporins, cyclosporines, kanamycin, erythromycin, chloramphenicol. While the major pharmaceutical companies have been employing combinatorial synthesis in recent years, there are still increased efforts to search for drug leads from natural sources because of the improved techniques in fermentation and sample screening, more importantly, due to the structural diversity and high bioactivity of the secondary metabolites.

Fungi are a large group of microorganisms ranked as a kingdom within the domain Eukaryota. They include conspicuous mushrooms and microscopic forms such as molds and yeasts. Estimate 70,000 species have been described from 1.5 million species of fungi worldwide (Hawksworth, 1995).

In Thailand, many natural product scientists have been working on bioactive compounds from higher plants leading to the discovery of many novel, useful substances, by contrast, there have been few research on bioactive microbial secondary metabolites. The PSU-BIOTEC collaborative research group has been searching for novel bioactive compounds from fungi collected in Thailand and deposited at the BIOTEC Culture Collection (BCC). The biological assays conducted at the BIOTEC Bioassay Research

Facility are antimalarial, anticancer, antitubercular and antiviral. In this doctoral thesis research, an insect pathogenic fungus *Verticillium hemipterigenum* BCC1449 was selected for chemical investigation because its culture broth showed antimalarial activity. In the screening, the broth extract of this fungus exhibited an $IC_{50}$ value of 7.1 μg/mL and the extract from mycelia showed an $IC_{50}$ of 20 μg/mL.

## 1.2 Introduction of *Verticillium*

The genus *Verticillium* is a mold that lacks a known sexual state and thus belongs to the Fungi Imperfecti. *Verticillium* is classified in family Clavicipitaceae, class Hyphomycetes. It is a widely distributed filamentous fungus that inhabits decaying vegetation and soil. Some *Verticillium* species are known to be pathogenic to arthropods, plants and other fungi, and they are used as biocontrol agents, but very rarely cause human disease. It is commonly considered as a contaminant. Colonies of *Verticillium* grow moderately rapidly or rapidly. The colonies are velvety to wooly, at 25 $^{o}$C on potato dextrose agar. From the front, the color is white initially and becomes yellowish, red, pinkish-brown, or green. From the reverse, it is white or brown (rust color) (www.doctorfungus.org).

*Verticillium hemipterigenum* was described (Petch, 1932) as anamorph of *Torrubiella hemipterigena* (Petch, 1923), both encountered on leafhoppers belonging to the family Cicadaellidae. Recently, Hywel-Jones *et al.* (1997) re-described these rarely reported species from collections made on leafhoppers in Thailand. *V. hemipterigenum* BCC 1449 (collected, 2 Feb, 1996; original code, NHJ06076) is one of the isolates used in their taxonomic studies, and later deposited at the BIOTEC Culture Collection (BCC) as BCC 1449.

**stroma on leafhopper** **colony on PDA**

***Verticillium hemipterigenum* BCC1449**

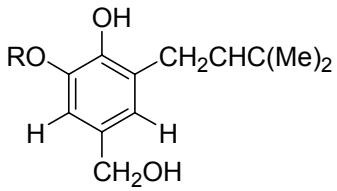
Description of *V. hemipterigenum*

*Colonies* on 10% PDA, slow growing with 2.3-2.5 cm diam. After 14 d at 25°; white, cotton-like aerial mycelium and dense white basal skin or pseudostroma, creamish yellow reverse; sporulation absent to poor; ascomatal initials occasionally formed, in NHJ06076; however, perithecia may develop, often in clumps, which remain significantly smaller than those on the host (maximum size 300 × 120 μm) and effete.

## 1.3 Literature reviews: Bioactive compounds from *Verticillium*

*Verticillium* has been known as a rich source of bioactive secondary metabolites of diverse chemical structures. However, *V. hemipterigenum*, a very rare species, has not been previously chemically explored. In parallel with this research on *V. hemipterigenum* BCC 1449, investigation of another strain, *V. hemipterigenum* BCC 2370, was conducted in the same laboratory, which led to the isolation of eight ascochlorins including two new analogs (Seephonkai *et al.*, 2004).

Bigutol (**1**) and methylbigutol (**2**), isolated from the mycoparasitic biocontrol agent *Verticillium biguttatum*, are antifungal hydroxymethyl-phenols. These compounds inhibited mycelial extension of a range of plant pathogenic fungi at low concentration (Table 1) (Morris *et al*., 1995). Production of these antifungals by *V. biguttatum* suggests that antibiotic may play a role in biocontrol by this mycoparasite, particularly of plant diseases caused by the fungus *Rhizoctonia solani*.

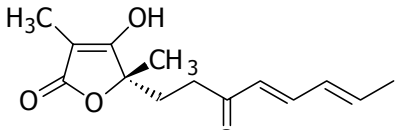



**1** : R = H
**2** : R = Me

**Table 1.** Antifungal activities of bigutol and methylbigutol against five plant pathogens and *V. biguttatum* isolate Vb1 assessed by application of 10-200 µg (28-550 µg metabolite $mL^{-1}$ agar) each metabolite in HPLC grade MeOH to potato dextrose agar blocks of known volume.

| Fungal isolate | Antifungal activity (MIC, µg/mL) | |
|---|---|---|
| | Bigutol | Methylbigutol |
| *Pyrenochaeta lycopersici* | 275 | 550 |
| *Fusarium oxysporum f. sp. narcissi* | >550 | >550 |
| *Botrytis cinerea* | 275 | 550 |
| *Rhizoctonia solani* (AG-3) | 138 | 138 |
| *Fulvia fulva* | >550 | >550 |
| *Verticillium biguttatum* isolate Vb1 | 275 | 550 |

*V. intertextum* was found to produce tetronic acid derivatives, that belong to a class of fungal metabolites known as mycotoxins. Originally, Trifonov isolated four hexaketides, vertinolide (**3**), bisvertinoquinol (**4**), sorbicillin (**5**) and dihydrosorbicillin (**6**) (Trifonov *et al*., 1981, 1982, 1983). Three years later, the same research group isolated four new dimeric vertinolides, bisvertinol (**7**), dihydrobisvertinol (**8**), isohydrobisvertinol (**9**) and bisvertinolone (**10**), from the same fungus (Trifonov *et al*., 1986). Matsuo reported the synthesis of (-)-vertinolide from (*R*)-lactic acid (Matsuo and Sakaguchi, 1996).



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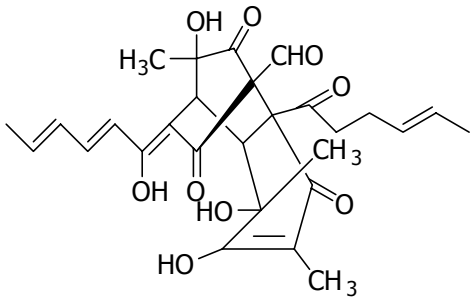

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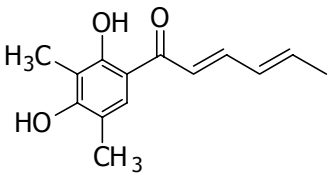

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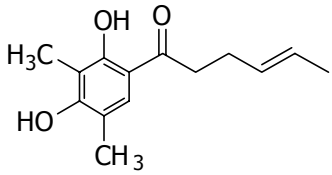

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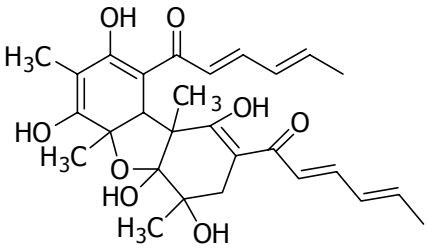

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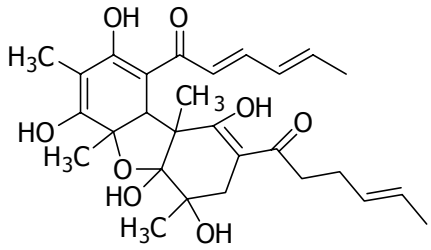

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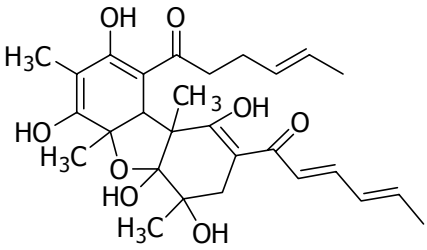

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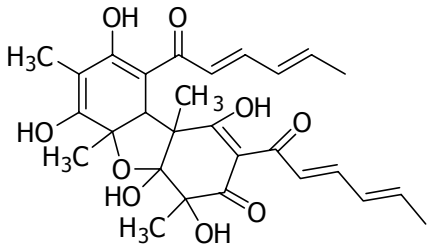
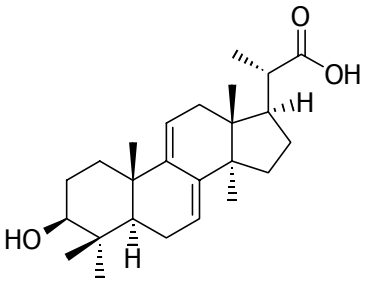


**10**

Two insecticidal $C_{25}$ hydroxy carboxylic acids (compounds **11** and **12**) were isolated from certain entomopathogenic strains of *V. lecanii*. Compound **11** is the less polar analog, and its structure was confirmed by X-ray crystallography (Claydon *et*

*al*., 1984; Grove, 1984a). In the course of an attempt to increase the yields of these acids by incorporating lanosterol in the fermentation medium, a novel pentanorlanost-8-ene (**13**) has been isolated from the mycelium from both lanosterol-enriched and control fermentations (Grove, 1984b).

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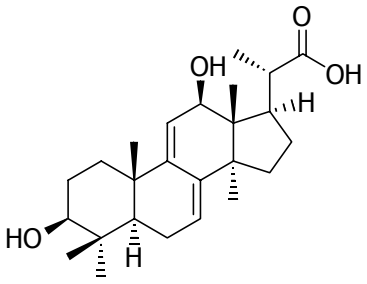


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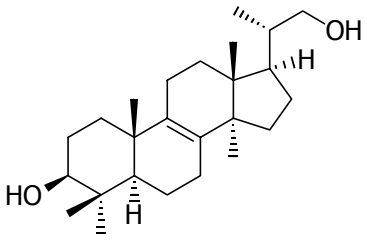
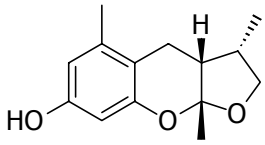


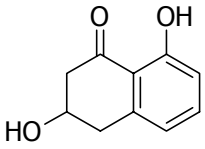

**13**

Compound **14**, named alboatrin, was isolated from the culture filtrate of *V. albo-atrum*. Alboatrin is a phytotoxic metabolite which causes vascular-wilt disease on alfalfa. It inhibited root growth of the host plant (Maris Kabul) 49.0% at 50 ppm (Ichihara *et al*., 1988).



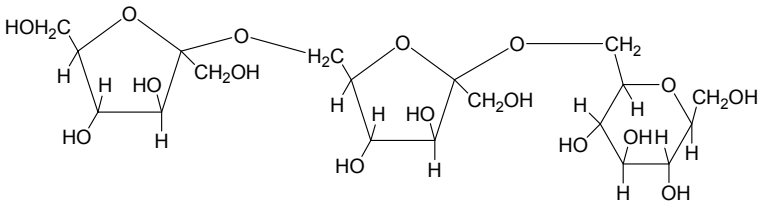

**14**

A pentaketide, (-)-vermelone (**15**), was isolated and identified from culture filtrates of the melanin-deficient brm-1 mutant of *V. dahliae* fed with 1,3,8-trihydroxynaphthalene. (-)-Vermelone is probably the terminal ketone in the biosynthetic pathway from (+)-scytalone to melanin (Stipanovic and Bell, 1976). Several years later, there was a study on melanin biosynthesis. Viviani, *et al*. has taken the 1,3,6,8-tetrahydroxynaphthalene ($T_4HN$) reductase of *V. dahliae* to study in a cell-free system (Viviani *et al*., 1990).



**15**

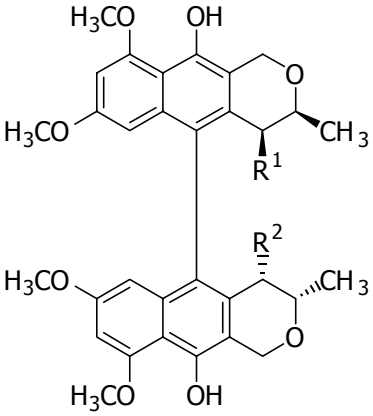
Choy reported the isolation and structure determination of some disaccharides and a trisaccharide (**16**), which were produced under aseptic conditions from sucrose by the plant pathogenic fungus *V. dahliae* (Choy and Unrau, 1971).



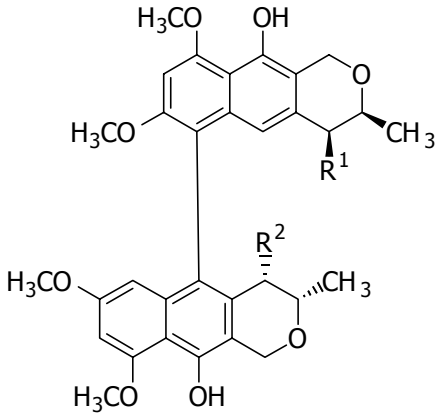
**16**

An orange mutant (M5) obtained from a wild-type isolate of *V. albo-atrum* by UV irradiation was found to contain phytoene, β-carotene, γ-carotene, neo-lycopene A, lycopene, neurosporaxanthin and four unidentified pigments (Valadon and Heale, 1965).

Eight novel bioxanthracenes, ES-242-1 – ES-242-8 (compounds **17**-**24**) were isolated from *Verticillium* sp. SPC 15898 (Toki *et al*., 1992a, 1992b). These compounds are antagonist of *N*-methyl-D-aspartate (NMDA) receptor, inhibiting [$^3$H] thienyl cyclohexylpiperidine ( [$^3$H] TCP) binding to rat crude synaptic membrane in a dose-dependent manner. $IC_{50}$ values for [$^3$H] TCP binding inhibition by ES-242s are listed in Table 2. None of the ES-242s were effective on [$^3$H] kainate binding to its receptor at concentrations up to 10 μM.



(ES-242-1; **17**) : $R^1 = OAc$, $R^2 = H$
(ES-242-2; **18**) : $R^1 = OAc$, $R^2 = OAc$
(ES-242-3; **19**) : $R^1 = OAc$, $R^2 = OH$
(ES-242-4; **20**) : $R^1 = OH$, $R^2 = OH$
(ES-242-5; **21**) : $R^1 = OH$, $R^2 = H$
(ES-242-6; **22**) : $R^1 = H$, $R^2 = H$

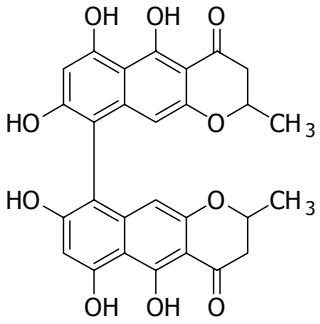


(ES-242-7; **23**) : $R^1 = H$, $R^2 = OH$
(ES-242-8; **24**) : $R^1 = OH$, $R^2 = H$

**Table 2.** Inhibitory effects of ES-242s on the binding of [$^3$H] TCP or [$^3$H] kainate

| compound | $IC_{50}$ (μM) | $IC_{50}$ (μM) |
|---|---|---|
| | [$^3$H] TCP | [$^3$H] kainate |
| ES-242-1 | 0.12 | >15 |
| ES-242-2 | 2.9 | >15 |
| ES-242-3 | 2.9 | not tested |
| ES-242-4 | 25 | >15 |
| ES-242-5 | 1.0 | >15 |
| ES-242-6 | 59 | not tested |
| ES-242-7 | 24 | >15 |
| ES-242-8 | 13 | >15 |

Cephalochromin (**25**), iso-ustilaginoidin A (**26**), and a new antibiotic, dihydroiso-ustilaginoidin A (**27**), were isolated from *Verticillium* sp. (strain K-113). Compounds **25** and **26** have symmetrical dimeric structure, while **27** contains one unit of cephalochromin and iso-ustilaginoidin each (Matsumoto *et al*., 1975). Antimicrobial activity of these compounds was found against Gram-positive bacteria but not against Gram-negative bacteria and fungi (Table 3).



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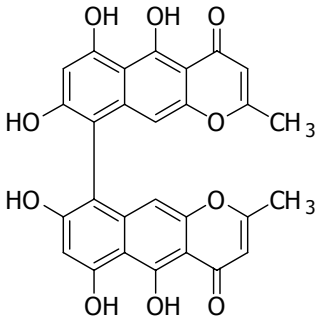

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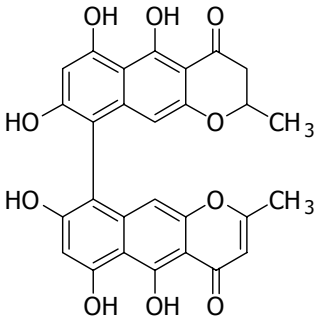
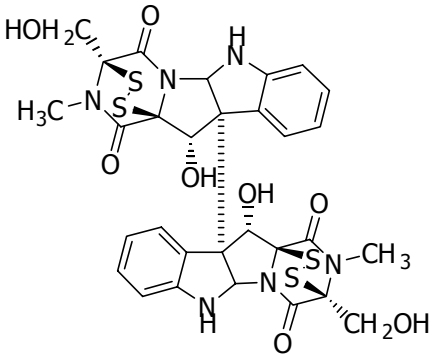


**27**

**Table 3.** *In vitro* antimicrobial activity of compounds **25**, **26** and **27**

| Test organisms | MIC (μg/mL) | | |
|---|---|---|---|
| | **25** | **26** | **27** |
| *Bacillus subtilis* PCI 219 | 10 | 10 | 3 |
| *Staphylococcus aureus* FDA 209 P | 3 | 10 | 3 |
| *Streptococcus pyogenes* C-203 | 3 | 10 | 3 |

A new antibacterial and antimytotic metabolite, 11α,11'α-dihydroxychaetocin (**28**), was isolated from *V. tenerum*. This compound is a symmetrical dimer of epidithiodiketopiperazines (Hauser *et al*., 1972). Verticillin A-C (**29-31**), isolated from *Verticillium* sp. (strain TM-759), are related compounds. Verticillin A is a symmetrical dimer, while verticillin B is an unsymmetrical dimer. Verticillin C (**31**) is thought to be an epitrithio-analogue of verticillin B (**30**). These compounds exhibited antimicrobial activity against Gram-positive bacteria and mycobacteria but not against Gram-negative bacteria and fungi. The cytotoxic effects ($ED_{50}$) of verticillins A and B against HeLa cells were both 0.2 μg/mL (Minato *et al*., 1973 ; Katagiri *et al*., 1970).



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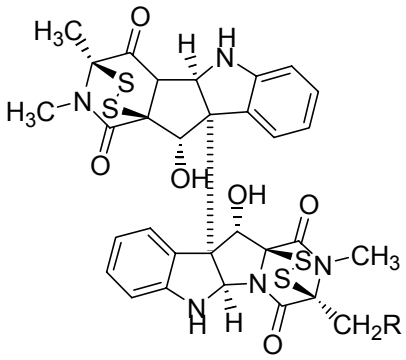
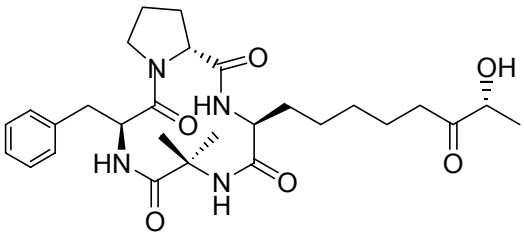


**29** : R = H

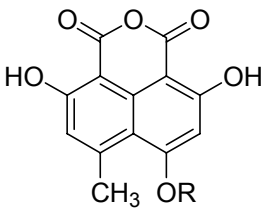
**30** : R = OH

A novel phytotoxic cyclotetrapeptide chlamydocin analogue (**32**) was isolated from *V. coccosporum*. This compound exhibited activity in *L. minor* assay at 0.3 μM and at 1.7 μM in the *Brassica juncea* assay (Gupta *et al*., 1994).



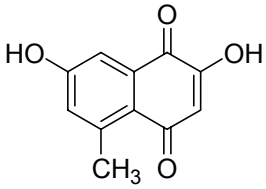
**32**

Lamellicolic anhydride (**33**), its carbonate, 4-*O*-carbomethoxylamellicolic anhydride (**34**), a naphthoquinone, **35**, and a chloro analog, monomethyl 3-chlorolamellicolate (**36**), were isolated from *V. lamellicola* by McCorkindale's group (McCorkindale *et al*., 1973, 1983). Cameron and co-workers synthesized the quinone **35** (Cameron *et al*., 1981).



**33** : R = H

**34** : R = $CO_2Me$



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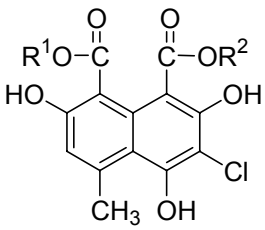
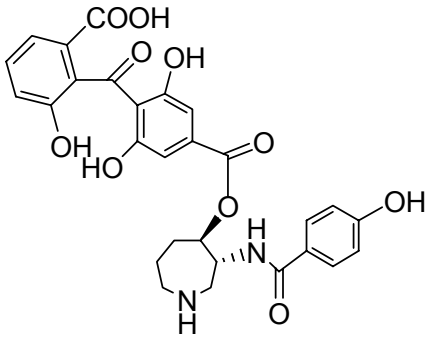


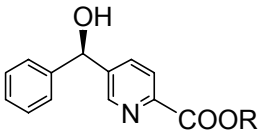
**36** : $R^1$ = H(Me), $R^2$ = Me(H)

Balanol (**37**), isolated from the fungus *V. balanoides* (Kulanthaivel *et al*., 1993), has recently become a popular synthetic target because of its potent inhibitory activity against protein kinase C. The remarkable inhibitory activity of balanol is attributed to its binding to the ATP docking site of protein kinase (Herdeis *et al*., 1999). $IC_{50}$ values of 4-9 nM were examined in assays against human PKC enzymes α, β-I, β-II, γ, δ, ε, and η with the exception of ζ (150 nM) (Kulanthaivel *et al*., 1993). Many research groups have synthesized various balanol analogs in order to find more potent and selective PKC inhibitors (Laursen *et al*., 2002; Narayana *et al*., 1999).



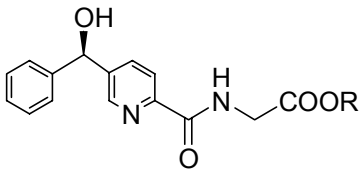

**37**

Vertilecanin A (**38**), vertilecanin A methyl ester (**39**), vertilecanin B (**40**), vertilecanin B methyl ester (**41**), and vertilecanin C (**42**) are five new phenopicolinic acid analogs which were isolated from solid-substrate fermentation cultures of *V. lecanii*. The most abundant component, vertilecanin A, displays antiinsectan activity against *Helicoverpa zea.*, and it also shows some modest antibacterial activity, producing an 8 mm zone of inhibition in a standard disk assay against *Bacillus subtilis* (ATCC 6051) at 100 μg/disk (Soman *et al*., 2001).



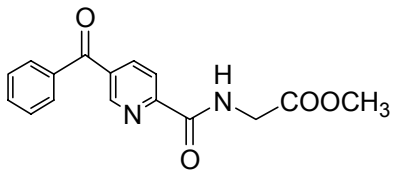

**38** : R = H

**39** : R = $CH_3$



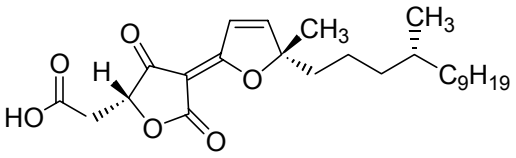

**40** : R = H

**41** : R = $CH_3$

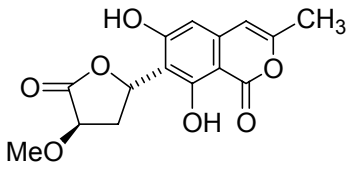



**42**

Lowdenic acid (**43**), a new antifungal metabolite with an unusual structure and a mixed biogenetic origin, has been obtained from *Verticillium* sp. (MYC-406 = NRRL 29280) together with the known antifungal metabolite canescin A (**44**). It is interesting to note that compound **43** occurred as an equilibrium mixture of *E*- and *Z*-isomers (Angawi *et al*., 2003).

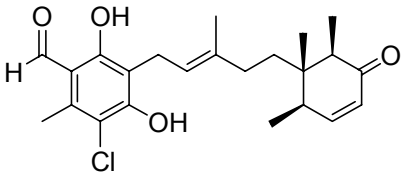


**43**

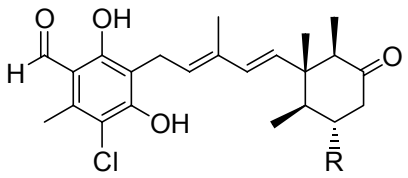


**44**

A new ascochlorin derivative, 8′,9′-dehydroascochlorin (**45**) was isolated from the culture mycelia of *Verticillium* sp. FO-2787 along with five known compounds in this class, LL-Z1272γ (ascochlorin; **46**), LL-Z1272ζ (**47**), LL-Z1272δ (**48**), LL-Z1272ε (**49**) and LL-Z1272β (**50**). These compounds exhibited activity as testosterone 5α-reductase inhibitor ($IC_{50}$ 0.34-1.4 μM) (Takamatsu *et al*., 1994).

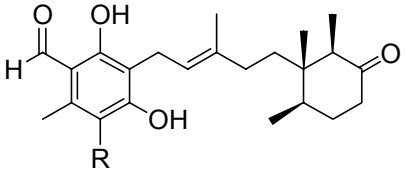


**45**



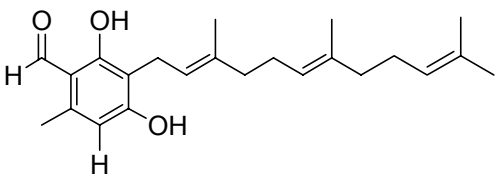
**46** : R = H

**47** : R = OAc



**48** : R = Cl

**49** : R = H



**50**