Phytochemical and molecular studies on *Musa* plants and related species

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Contents

Abbreviations

2PS	2-pyrone synthase
4CL	*p*-coumaryl CoA ligase
Appx	appendix
ACS	acridone synthase
ALS	aloesone synthase
BAS	benzalacetone synthase
β-ME	β-mercaptoethanol
bp	base pair
br. d	broad doublet
br.s	broad singlet
$CDCl_3$	deuterated chloroform
cDNA	complementary deoxyribonucleic acid
$CHCl_3$	chloroform
CHS	chalcone synthase
CoA	coenzyme A
COSY	correlation spectroscopy
Da	dalton
DAD	diode array detector
d	doublet
dd	doublet of doublet
DEPC	diethylpyrocarbonate
DMAP	dimethylaminopyrodine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dt	doublet of triplet
DTT	dithiothreitol
E. coli	*Escherichia coli*
EI-MS	Electron impact mass spectrometry
ESI	Electrospray ionization
EtOH	ethanol
eV	electron volt
Foc	*Fusarium oxysporum* f. sp. *cubense*
Foc4	*Fusarium oxysporum* f. sp. *cubense* race 4
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HSCoA	free coenzyme A
IPTG	Isopropyl β-D-1-thiogalactopyranoside
J	coupling constant
LB	Lucia-Bertani-medium
m-	*meta-*
M^+, M^-	molecular ion (positive or negative)
MeCN	acetronitrile
MeOH-d_4	deuterated methanol
MeOH	methanol
MS	mass spectrometry, Murashige/Skoog (medium)
m/z	mass-to-charge ratio
NAC	N-acetyl-L- cysteine
NH_4Ac	Sodium acetate
NMR	Nuclear magnetic resonance
o-	*ortho-*

$OD_{xxx\ nm}$	Optical density at xxx nm
p-	*para-*
PAL	Phenylalanine-Ammonia-Lyase
PCR	Polymerase chain reaction
pfu	plaque forming unit
PMSF	phenylmethylsulfonylfluoride
ppm	part per milliom
RNA	ribonucleic acid
RNase	ribonulcease
RP	reverse phase
rpm	revolutions per minute
RT	room temperature
R_t	retention time
δ	chemical shift related to TMS
s	singlet
SDS	sodium diodecyl sulfate
STS	stilbene synthase
t	triplet
TAE	tris-acetate EDTA buffer
Taq	*Thermus aquaticus*
TEMED	N,N,N´, N´-tetramethylethylenediamine
TFA	trifluoroacetic acid
TMS	tetramethylsylane
Tris	Tris-(hydroxymethyl)aminoethane
UV	ultraviolet
v/v	volume per volume
MaPKS	polyketide synthase (protein) from *Musa acuminata*
MaPKS	gene polyketide synthase from *Musa acuminata*
w/v	weight per volume

Amino acid

Alanine	Ala	A	Leucine	Leu	L
Asparagine	Asn	N	Lysine	Lys	K
Aspartic acid	Asp	R	Methionine	Met	M
Arginine	Arg	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic acid	Glu	E	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
FHstidine	His	H	Tyrosine	Tyr	Y
Isoluecine	Ile	I	Valine	Val	V

Nucleic acid

Adenine	A
Cytosine	C
Gaunine	G
Thymine	T

Chapter 1 Introduction

1.1 Plant natural products

Plants produce a vast number of chemical compounds that are known as secondary metabolites or natural products. Plant natural products belong to various classes of compounds such as isoprenoids, phenylpropanoids and alkaloids, and are also often found as conjugated products of compounds derived from different biosynthetic pathways. The distribution of secondary metabolites is characteristic of biological groups, such as families or genera, which could be related to evolutionary processes (Dixon, 2001). The compounds are often produced and stored in specific cell compartments. Many of them are biologically active, which is very important to the pharmaceutical industry. Due to their medicinal properties, most studies in natural products chemistry have focused on medical applications, and the role of the compounds in the plant itself has been neglected (Hartmann, 2007).

Secondary metabolites are not essential for plant growth and development. Previously, they were thought to be metabolic waste or detoxification products (Hartmann, 1996). Stahl (1888) was the first to suggest that secondary metabolites play a role in plants' interaction with their environment and other organisms to provide a defence against infection, predation and environmental stress (Rhodes, 1994). In the late 1950s, the role of plant natural products in interactions between plants and insect herbivores was discovered by entomologists. Since then, the functions of plant secondary metabolites have been investigated. The highly diverse structure of secondary metabolites indicates the flexible ways plants have adapted to continuously changing environment (Hartmann, 2007). Many studies have revealed the natural products that have accumulated in plants have clear ecological roles and demonstrate plant adaptation to both biotic and non-biotic environmental stresses (Lewinsohn and Gijzen, 2009).

The ecological functions of secondary metabolites have different aspects (Hartmann, 1996, 2007). For examples, volatile compounds and flower pigments attract insects for pollination (Harborne, 1993). To protect plants against environmental stresses (UV light, cold and draught), flavonoid compounds are efficient scavengers of reactive oxygen species (ROS), which reduce UV radiation (Pourcel, 2006). Natural products function in plant defence mechanism, for example, as phytotoxins to protect plants from feeding insect herbivores (Harborne, 1993, 1999) and as antifungal and antibacterial compounds to protect plants from pathogens. These compounds can be constitutive components or they can be induced after plants are challenged with pathogens. Two groups of these compounds are known as phytoanticipin and phytoalexin, respectively (see **1.2**). Some compounds are stored in pre-active forms for example HCN from cyanogenic glucoside can be released after herbivore attack

(Hartmann, 2007). More than 200,000 secondary metabolites have been isolated and their structures elucidated (Hartmann, 2007). The idea that secondary metabolites have ecological functions is nowadays widely accepted and the ecological functions of plant secondary metabolites are open for investigation.

1.2 Phytoalexins and phytoanticipins

The functions of secondary metabolites have become increasingly interesting in recent decades especially those of metabolites involved in plant defenses such as phytoalexins and/or phytoanticipins. The term 'phytoalexin' was first introduced by Müller and Börger (1940) to refer to compounds produced after parasitic invasion. Phytoalexins usually have a molecular-weight less than 1,000; they are either absent or present in trace amounts in healthy plant but produced and accumulating at higher levels after being challenged by plants' enemies (pathogens and herbivores). The compounds have antifungal or antibacterial activity and play important roles in plants' resistance to disease (Grayer and Kukubon, 2001). Phytoalexins are also inducible by non-biological stress factors such as UV irradiation or chemical treatment (heavy metals, elicitors) (Ingham, 1973). The term 'phytoanticipin' was launched in 1994 (Van Etten *et al.*, 1994) to describe low-molecular-weight compounds that are constitutive defense metabolites. Interestingly, the constitutive compound in one species could be an inducible compound in another species. For example, the flavonoid sakuranetin, a constitutive compound in *Ribes nigra* (Grossulariaceae, black currant) and in *Hebe cupressoides* (Scrophulariaceae), was induced in rice leaves (Grayer and Kukubon, 2001). Phytoalexin production often shows a similar pattern in plants of the same family. For instance, sulphur-containing compounds (e.g. glucosinolates) are main phytoalexins in the Brassicaceae (Morissey and Osbourn, 1999), isoflavonoids are in the Leguminosae, and sesquiterpenes are in the Solanaceae (Dixon, 2001). On the other hand, compounds belonging to one group of secondary metabolites such as phenylpropanoids may act as phytoalexins in many plant families (Dixon, 2001).

1.3 *Musa* sp. and its related species

1.3.1 Taxonomic data

Zingiberales is one of the biggest monocotyledon plant orders, containing around 2,000 species from eight plant families (Sharrock, 1997). Morphological characteristics allow the order to be divided into two groups, the banana families (Musaceae, Heliconiaceae, Strelitziaceae and Lowiaceae) and the ginger families (Zingiberaceae, Costaceae, Cannaceae and

Marantaceae) (Dahlgren and Clifford, 1982). Among these plant families, Zingiberaceae and especially Musaceae are economically important. Fig **1.1** shows a cladogram representing the phylogenetic relationships of the eight plant families of Zingiberales (Manchester and Kress, 1993).

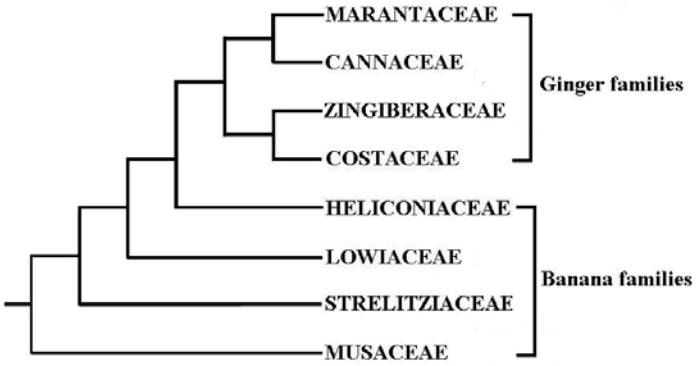


Figure 1.1 Cladogram representing the phylogenetic relationships of the eight plant families of Zingiberales, modified from Manchester and Kress (1993).

The Musaceae are large perennial herbs growing from a sympodial rhizome, with a pseudostem composed of a leaf sheath. The family Musaceae consists of three genera, *Musella*, *Musa* and *Ensete* (Wu and Kress, 2000). The genus *Musa*, covering banana (dessert banana) and plantain (cooking banana), was divided into four sections according to chromosome number (Fig. **1.2**) (Helslop-Harrison and Schwarzacher, 2007). Most of the edible cultivars are in the eumusa group, derived from *Musa acuminata* (genome A) and *Musa balbisiana* (genome B). Intra- and inter-specific hybridization of the two species resulted in various cultivars. The cultivars were classified into genome groups by ploidy and a scoring system based on the contribution of genomes A and B characteristics, for example, AA, AAB, AABB and *etc.* Fig **1.2** shows the genera of the Musaceae and type of *Musa* plants.

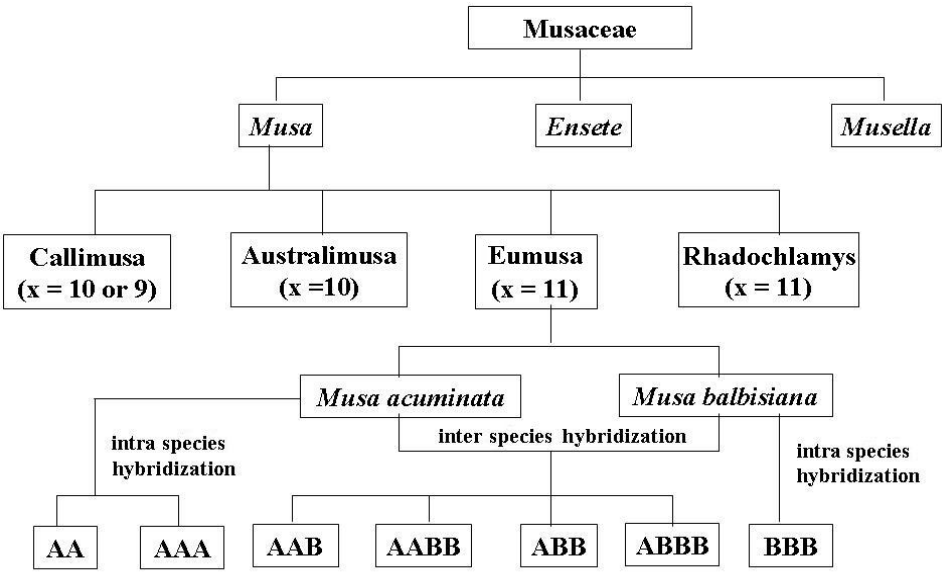


Figure 1.2 Genera of Musaceae and subgroups of *Musa* plants

1.3.2 Economic importance and problems

Banana and plantain originated in Southeast Asia, and later were distributed over the tropics and sub-tropics. They are cultivated for food, fiber (*M. textilis*) and ornamental purposes. The main agriculture sites for bananas are in South America, Africa, Australia and Southeast Asia (Marín *et al.*, 2003). Bananas have become economically important in international trading (http://www.unctad.org). Planting *Musa* can be problematic as they are susceptible to devastating diseases. *Fusarium* wilt or Panama disease caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) was one of the most serious diseases in the 1930s to 1950s. Foc could not be controlled by fungicides, so the replacement with a resistant variety was the only solution. This led to the substitution of the susceptible variety Gros Michel (AA) with the resistant Cavendish (AAA) in the 1950s to 1960s. Nowadays, the Cavendish banana is a major product on the world market. Recently, the pathogen Foc race 4 (Foc4) was also found to attack the Cavendish (Ploetz, 2000). Black Sigatoka, caused by the fungus *Mycosphaerella fijiensis*, is considered the most destructive disease in recent years. The infection can cause up to 50% of crop losses (Helslop-Harrison and Schwarzacher, 2007). Bacterial infection, such as bacterial wilt caused by *Xanthomonas* sp. (Reeder *et al.*, 2007), viral diseases such as banana bunchy top virus (Thomas *et al.*, 1994), and burrowing nematode *Radopholus similis* (Price, 2006) also cause banana production to decline.

1.3.3 Chemical constituents

Phytochemical studies reported that natural products found in *Musa* are derived from various biosynthetic pathways including terpenoids (Knapp *et al.*, 1969) and phenylpropanoids such as flavonoids (Pascual-Villalobos and Rodriguez, 2007; Lewis *et al.*, 1999). Phenylphenalenones (see **1.4)** derived from the phenylpropanoid biosynthetic pathway were also reported as secondary plant metabolites produced in response to pathogen attacks (Luis *et al.*, 1996; Kamo *et al.*, 1998b, 2001).

1.4 Diarylheptanoids and phenylphenalenones

1.4.1. Chemical structures of diarylheptanoids and phenylphenalenones

Diarylheptanoids and phenylphenalenones are derived from the phenylpropanoid biosynthetic pathway. Diarylheptanoids (Fig **1.3a**) possess two aryl rings linked with a chain of seven carbon atoms. Curcumin, the most studied diarylheptanoid compound, is used as a health-promoting substance (Chattopadhyay *et al*., 2004). Phenylphenalenones are polycyclic diarylheptanoids. Their chemical structure consists of a tricyclic phenalene nucleus, ketone groups (on ring A or ring B) and a lateral phenyl substituent. Phenylphenalenones can be categorized into two groups according to oxidation patterns on the tricyclic phenalene nucleus. Compounds of each of the two groups may possess additional hydroxyl or methoxyl substituents in the lateral phenyl ring.

- *Phenylphenalenones bearing oxygenated functional groups (ketone/hydroxyl group) only on ring A (Fig **1.3b**):*

This group can be divided into sub-groups according to the position of the lateral phenyl ring, for examples 4-phenylphenalenones, 9-phenylphenalenones and 8-phenylphenalenones. This group also includes phenylphenalenone derivatives that have lost a carbon atom on ring A, such as phenyl-naphthalic anhydrides and phenyl-naphthalic dicarboxylic acids.

- *Phenylphenalenones bearing oxygenated functional groups (ketone/hydroxyl group) on both ring A and ring B (Fig **1.3c**):*

This group is also called the 7-phenylphenalenones due to the position of lateral phenyl rings according to their numbering system (starting from the ketone group on ring B). They are tri- and tetrahydroxygenated phenylphenalenones. Oxabenzochrysenones, which are tetra-

hydroxygenated phenylphenalenone-derived compounds and possess an ether bridge between the phenalenone tricycle and the lateral phenyl ring, also belong to this group; the group covers phenylphenalenones that have lost one of the carbon atoms from ring B, such as phenylbenzoisochromenones and phenyl-napthalic anhydrides. Phenylbenzoisoquinolinediones, in which C-2 has been replaced by nitrogen atom, are also in this group.

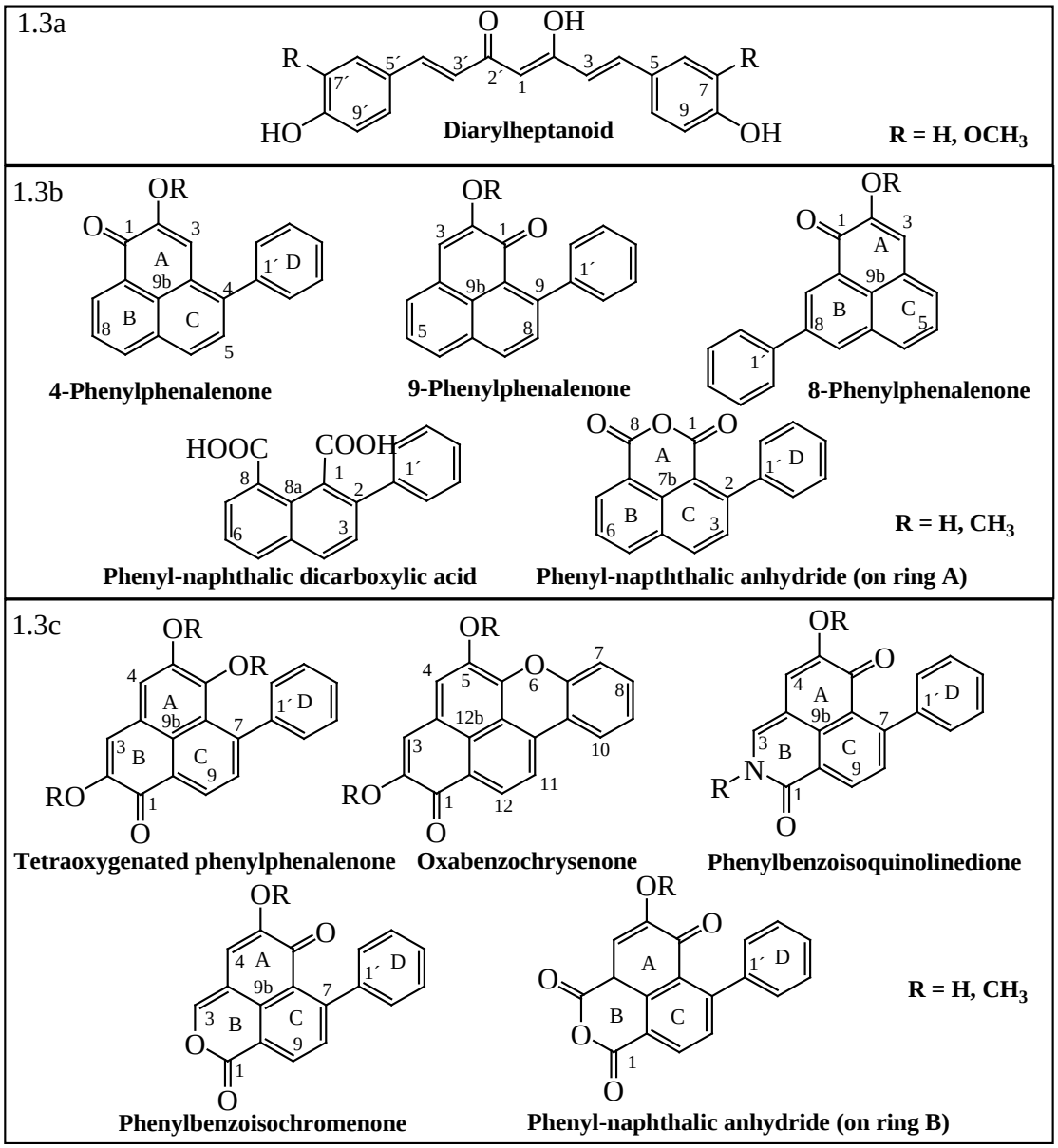


Figure 1.3 Chemical structures of linear diarylheptanoids (**1.3a**) and phenylphenalenones bearing oxygenated functional groups only on ring A (**1.3b**) and phenylphenalenones bearing oxygenated functional groups on both ring A and ring B (so-called 7-phenylphenalenones) (**1.3c**).

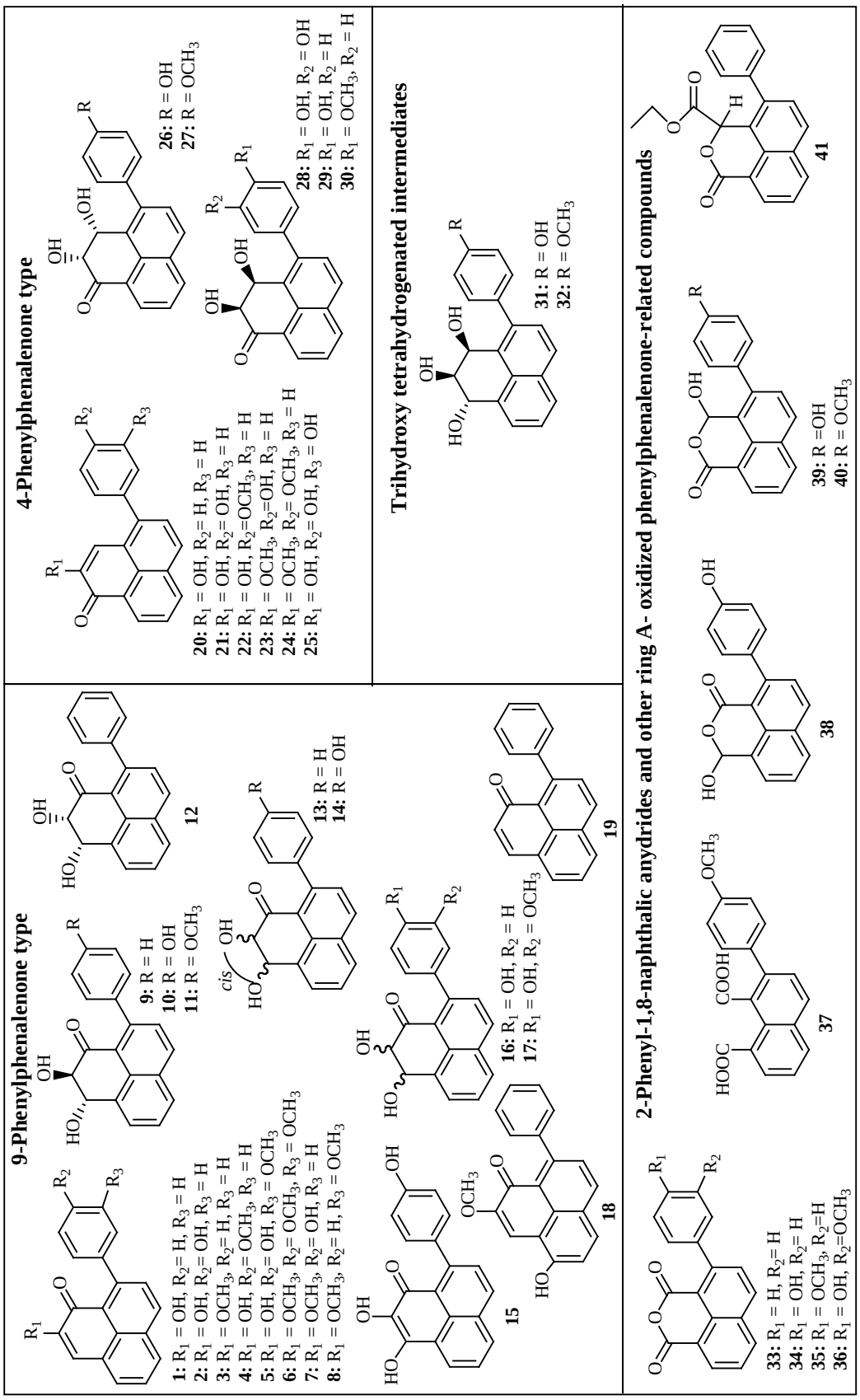


Figure 1.4a Structures of phenylphenalenones and related compounds isolated from *Musa sp.*

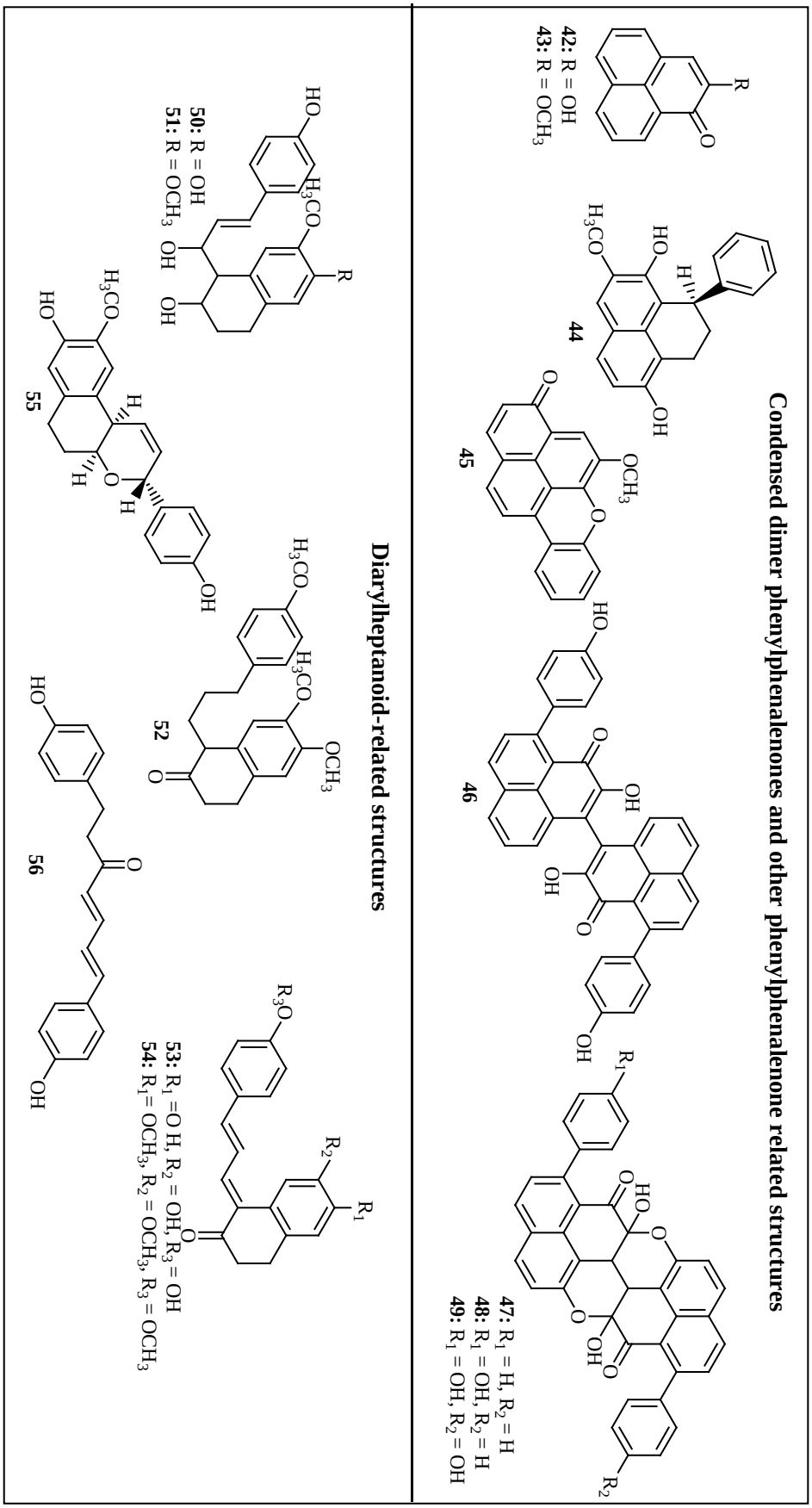


Figure 1.4b Structures of phenzlphenalenones, diarylheptanoids and related compounds isolated from *Musa* sp.

1.4.2 Natural occurrence

Diarylheptanoids were found in both dicotyledonous and monocotyledonous families. The Zingiberaceae family is the main source of linear diarylheptanoids (curcuminoids) in monocotyledon plants (Giang *et al.*, 2005; Tezuka *et al.*, 2001). In addition, some dicotyledonous families such as, Aceraceae, Juglandaceae and Betulaceae also contain linear and alicyclic diarylheptanoids (Zhu *et al.*, 2000). Phenylphenalenones seem to be limited in monocotyledon plant families. In 1955, Cooke and Segal reported the first phenylphenalenones isolated in a higher plant, the glycoside haemocorin from *Haemodorum corymbosum* (Haemodoraceae). After that, many phenylphenalenone-type compounds were isolated from Haemodoraceae (Cooke and Edward, 1981), Strelitziaceae (Hölscher and Schneider, 2000), Musaceae (Luis *et al.*, 1996; Kamo *et al.*, 2001; Otálvaro, 2004) and Pontederiaceae (Della-Greca *et al.*, 1992a, b, 2008; Hölscher and Schneider, 2005).

1.4.3. Phenylphenalenones in Musaceae in comparison to phenylphenalenones in other monocotyledon families

Two main phenylphenalenone-rich monocotyledon families are Haemodoraceae and Musaceae. In Haemodoraceae, covering the genera *Anigozanthos*, *Dilatris*, *Haemodorum*, *Lachnanthes*, *Xiphidium* and *Wachendorfia* (Cooke and Edward, 1981; Dora *et al.*, 1993; Hölscher and Schneider, 1997; Opitz and Schneider, 2002), phenylphenalenones including their dimers and glycosides were found. Most phenylphenalenones of the Haemodoraceae bear oxygenated functional groups on ring A and are mainly of the 9-phenylphenalenone type (see **1.4.1** and Fig **1.3b**). Phenylphenalenones that have oxygenated functional groups at both ring A and ring B (see **1.4.1** and Fig **1.3c**), such as tetraoxygenated phenylphenalenones and their glycosides, have also been reported (Opitz, 2002).

In Musaceae, mainly phenylphenalenones bearing oxygenated functional groups on ring A (see **1.4.1** and Fig **1.3b**) were found. 9-Phenylphenalenones were found in all three genera, *Musella* (Qin *et al.*, 2006), *Ensete* (Hölscher and Schneider, 1998) and *Musa* (Luis *et al.*, 1996; Kamo *et al.*, 1998a, b, 2001; Otálvaro *et al.*, 2002a, 2007; Del Rio *et al.*, 2006, Jang *et al.*, 2002). Additionally, 4-phenylphenalenones, which are unknown from the Haemodoraceae, were mainly found in the genus *Musa* together with 2-phenyl-1,8-naphthalic anhydrides (Hirai *et al.*, 1994; Kamo *et al.*, 1998b, 2001). A compound of the oxabenzochrysenone type (see **1.4.1** and **Fig 1.3c)**, which was previously reported only from the Haemodoraceae (Edward and Weiss, 1974; Cooke and Dagley, 1979), was recently isolated from *Musa acuminata* (Opitz *et al.*, 2002). Fig **1.4a**, **b** show structures of phenyphenalenones and diarylheptanoid-related compounds found in banana plants.

1.4.4. Phenylphenalenones as inducible compounds in *Musa*

Many phenylphenalenones are pigments, occurring constitutively in the Haemodoraceae where they accumulate in roots. Phenylphenalenones are produced in *M. acuminata*, where they accumulated after the plants were challenged with pathogens, such as *Colletrotrichum musae* (Kamo *et al.*, 1998b, 2001), *Fusarium oxysporum* (Luis *et al.*, 1994, 1996) and *Mycosphaerella fijiensis* (Luis *et al.*, 1993). Infestation with the nematode *Radopholus similis* also stimulated phenylphenalenone production (Binks *et al*., 1997). Treatment with the antibiotic kanamycin also enhanced the production of phenylphenalenones (Luis *et al.*, 1993). These compounds were isolated from roots, banana fruit peel and infection sites on leaf tissues, indicating that phenylphenalenones may act as phytoalexins and play an important role in the defense of banana plants.

1.5 Biological activity of inducible phenylphenalenones against plant pathogens

As phenylphenalenone were produced in *Musa* after infection, the activity of induced compounds in response to challenges from pathogens was tested. *cis*-2,3-Dihydro-2,3-dihydroxy-4-(4´-methoxyphenyl)phenalen-1-one (**27**), 4´-*O*-methylirenolone (**22**), musanolones C (**16**), D (**17**), E (**15**), and F (**5**), induced by treatment with Foc4 (*Fusarium* wilt), strongly inhibited growth of fungal germinal tube (Luis *et al*., 1994, 1996). 2-(4´-Hydroxyphenyl)-1,8-naphthalic anhydride (**34**) and its methylated products, (+)-*cis*-2,3-dihydro-2,3-dihydroxy-4-(4´-hydroxyphenyl)phenalen-1-one (**26**), irenolone (**21**) and hydroxyanigorufone (**2**) showed antifungal activity against *Colletotrichum musae* (anthracnose disease) by the TLC autobiography method. In this method, phenyl-naphthalic anhydride and phenyl-naphthalene dicarboxylic acid derivatives were more effective than 4-phenylphenalenones and 9-phenylphenalenones, respectively (Hirai *et al.*, 1994; Kamo *et al.*, 1998b, 2001). Anigorufone (**1**), isoanigorufone (**20**), hydroxyanigorufone (**2**) and irenolone (**21**) inhibited the mycelial growth and spore germination of *Mycosphaerella fijiensis* (Black Sigatoka disease) but less than perinaphthenone, a synthetic substance, did (Quiñones *et al*., 2000). Recently, 2-hydroxy-1*H*-phenalen-1-one (**42**) and 2-methoxy-1*H*-phenalen-1-one (**43**), perinaphthenone compounds, were isolated from *M. acuminata* var. “Yagambi km 5” (AAA) (Otálvaro *et al.*, 2007). These compounds also exhibited stronger activity against *M. fijiensis* than phenylphenalenones. Phenylphenalenones lacking substitution on a phenyl ring, such as anigorufone (**1**) and methoxyanigorufone (**3**), were reported to be only moderately active in an antifungal assay (Otálvaro *et al.*, 2007).

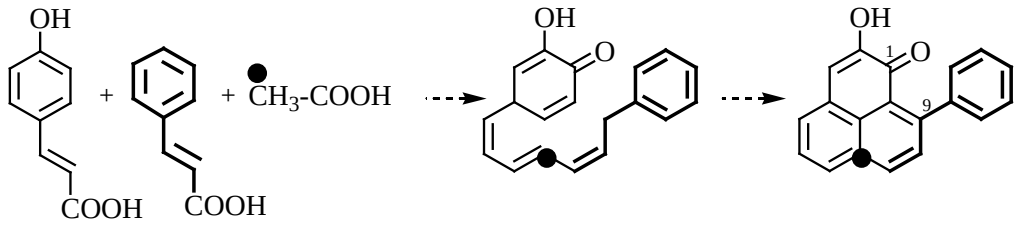
1.6 Biosynthesis

1.6.1 Early biosynthetic hypotheses and studies

Phenylphenalenones are natural products with relatively simple core structures. Originally, there were two hypotheses for the biosynthesis of phenylphenalenones (Thomas, 1961):

- a polyketide pathway using phenylpropanoyl-CoA (or a benzoyl-CoA) as a starter and 5 (or 6 malonyl-CoA units, respectively) as extender units;
- condensation of two phenylpropanoyl-CoA and a malonyl- or acetyl-CoA.

Based on radiolabelling (Thomas, 1971) and stable isotope feeding experiments (Harmon *et al.*, 1977) in *Lachnanthes caroliana* and *Haemodorum corymbosum*, a biosynthetic pathway of phenylphenalenones, made up of one molecule each of phenylalanine, tyrosine and acetic acid, was proposed (Thomas, 1973). The incorporation of phenylalanine, tyrosine, and acetate into haemocorin and lachanthoside, respectively, was observed; this disproved the polyketide hypothesis. Phenylalanine and tyrosine were converted to cinnamic acid and *p*-coumaric acid by phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase, respectively. Condensation of the two phenylpropanoid molecules with one molecule of malonyl- or acetyl-CoA, which provide the central carbon atom from C-2 unit, resulted in the formation of the diarylheptanoid intermediate. The intermediate went through structural modification and then cyclized to 9-phenylphenalenone structure (Scheme **1.1**) (Thomas, 1971, 1973). Later biosynthetic studies of phenylphenalenone were mostly done in *Anigozanthos preissii* (see **1.6.2**) and *Musa* (see **1.6.3**).



Scheme 1.1 Biosynthetic pathway of 9-phenylphenalenones as hypothesized by Thomas (1973).

1.6.2. Phenylphenalenone biosynthesis in *Anigozanthos preissii* root culture

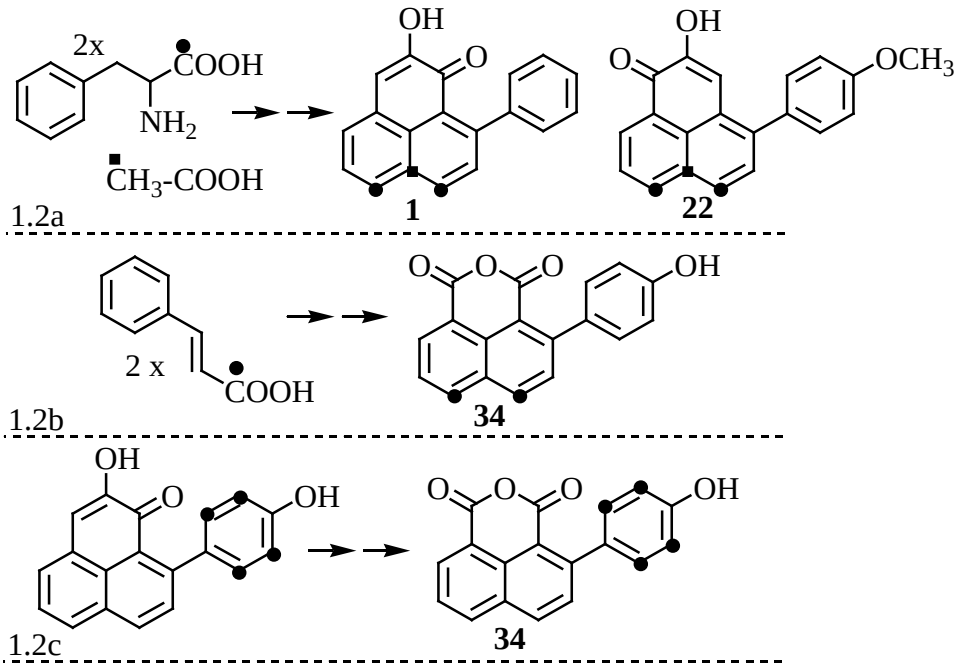
In *A. preissii*, Hölscher and Schneider (1995b) demonstrated the incorporation of [2-^{13}C]cinnamic and [2-^{13}C]*p*-coumaric acids into hydroxyanigorufone (**2**) as shown in the enhancement of ^{13}C NMR signals of C-5 and C-8. The C-2 of [2-^{13}C]acetate was incorporated into C-6a, the central carbon atom located between the two phenylpropanoid. This confirmed

the hypothesis of Thomas (1973), namely that phenylphenalenones are formed from two phenylpropanoid units and a one-carbon unit derived from acetate or malonate. The incorporation of [2-^{13}C]1-phenyl-7-(3,4-dihydroxyphenyl)hepta-1,3-dien-5-one into anigorufone (**1**) proved that phenylphenalenones are formed via a diarylheptanoid intermediate by an intramolecular [4+2]cycloaddition, probably of the Diels-Alder type (Hölscher and Schneider, 1995a).

1.6.3. Phenylphenalenone biosynthesis in *Musa* sp.

In addition to 9-phenylphenalenones, 4-phenylphenalenones and phenyl-naphthalic anhydrides were also found in *Musa*. The biosynthesis of phenylphenalenones in *Musa* was studied and discussed, especially the relationship between 4- and 9-phenylphenalenones.

The labelling experiments showed the incorporation of ^{13}C-labelled phenylalanine, phenylpropionic acids and [2-^{13}C]acetate into anigorufone (**1**), hydroxyanigorufone (**2**), 4´-*O*-methylirenolone (**22**) and 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) (Scheme **1.2a** and **b**) (Kamo *et al.*, 2000; Otálvaro, 2004). This finding revealed the closely related biosynthetic pathway of phenylphenalenones in *Musa* and *Anigozanthos*. The C_6C_3 units and acetate or malonate were precursors of both 4- and 9-phenylphenalenones including phenyl-naphthalic anhydrides (Kamo *et al.*, 2000; Otálvaro, 2004).



Scheme 1.2 Study of the biosynthesis of phenylphenalenone using feeding experiments with labelled precursors in *Musa* (Kamo *et al.*, 2000; Otálvaro, 2004).

Based on further labelling experiments 9-phenylphenalenones were suggested to be converted to 4-phenylphenalenones via dihydroxy-phenylphenalenones or 1,2,3-trihydroxy phenylphenalene intermediate through slightly different mechanisms (Kamo *et al.*, 2001; Otálvaro, 2004). This hypothesis needs to be confirmed by further studies. Phenyl-naphthalic anhydrides were derived from 9-phenylphenalenones by oxidative decarboxylation (Scheme **1.2c**) (Kamo *et al*., 2000).

1.7 Plant type III polyketide synthases

1.7.1 General

Polyketide synthases (PKS) are a family of enzymes that synthesize structurally diverse natural products. Polyketide-derived compounds are found in plants, bacteria, fungi and animals. The compounds play important roles in the life cycle of the organisms that produce them, including chemical defenses (Rimando and Baerson, 2006). Many polyketide compounds have therapeutic properties and are valuable in the pharmaceutical industry. Despite their diverse chemical structures, polyketides are biosynthesized from common reactions. The biosynthesis begins with the decarboxylative condensation of the starter-CoA ester and extender units (malonyl-CoA). The condensation is repeated, extending to the polyketide chain. Structural modification appears in different steps depending on the type of PKSs. There are three types of polyketide synthases in various organisms. Type I and type II PKS are more likely evolutionarily related to their type I and II fatty acid synthase (FAS) counterparts (Rimando and Baerson, 2006).

Type I PKS are multifunctional enzymes organized in modules. Each module or subunit contains multiple active sites (catalytic domains) which are usually responsible for the catalysis of one polyketide elongation cycle (Stuanton and Weissman, 2001).

Type II PKS, also known as bacterial aromatic polyketide synthases, are multienzyme complexes of mono-functional enzymes. The proteins carry subunits that participate in the iterative series of condensation reactions to form a specified polyketide chain length; after this reaction the polyketide chains are folded and cyclized to yield the final products (Shen, 2000; Rimando and Baerson, 2006).

1.7.2 Type III PKS or chalcone synthase/stilbene synthase type III PKS super family

Type III PKSs are involved in the biosynthesis of a large number of secondary metabolites in higher plants. They are smaller and less complex than type I and II PKS. Plant type III PKS show a high similarity in their amino acid sequences. They are homodimeric proteins

of 40-45 kDa with the catalytic triad Cys-His-Asn. Type III PKSs accept a broad spectrum of physiological starters including aromatic, aliphatic and fatty acid CoA-esters. The different number of condensations, substrate selectivity, and the following cyclization and aromatization reactions lead to the structural diversity of polyketides. Although the type III PKS is a super family of enzymes specific to higher plants, recently they have been reported in bacteria and fungi; these, however, share only 25% similarity of their amino acid sequences with the sequences of plant type III PKS (Austin and Noel, 2003).

1.7.3 Examples of plant type III PKS

- *Chalcone synthases (CHS) and stilbene synthases (STS)*

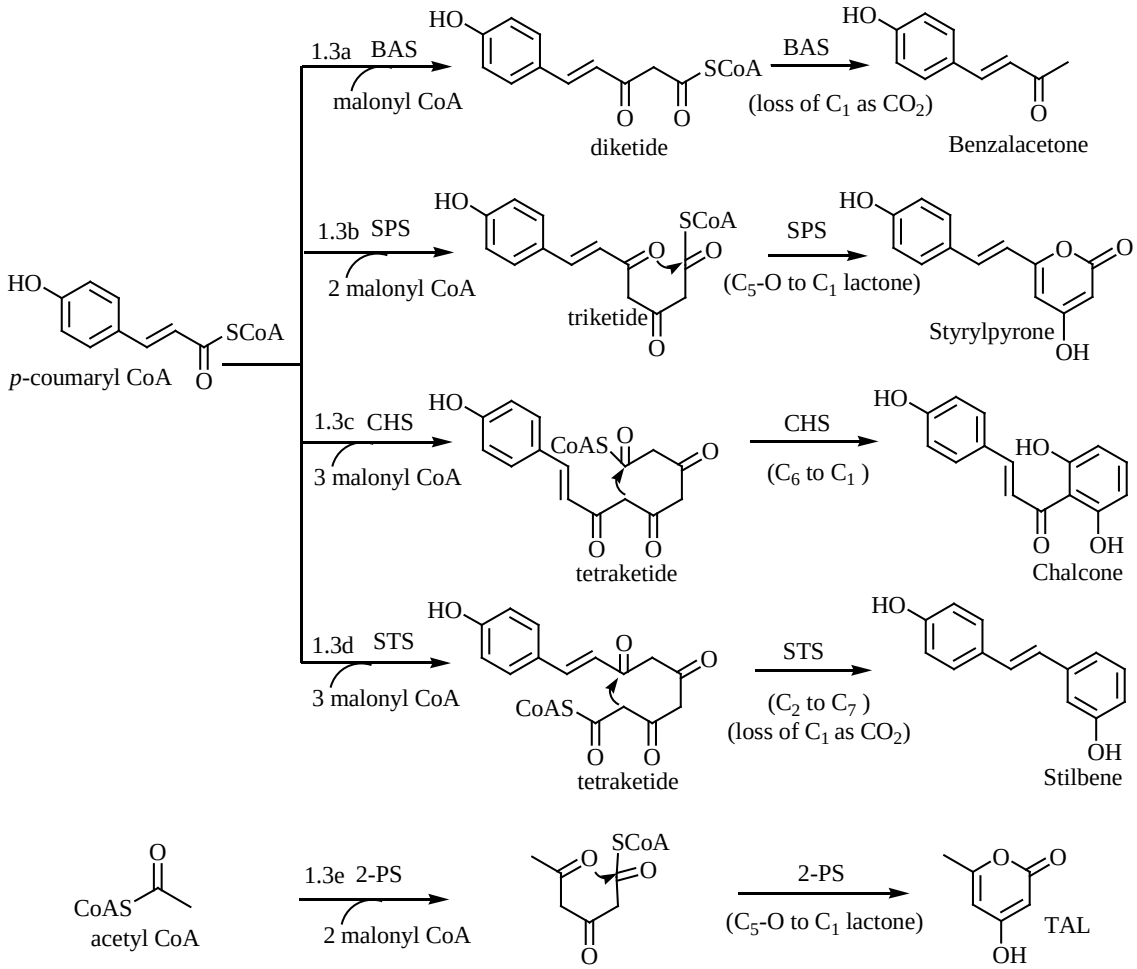
The chalcone synthase (CHS) is the prototype of plant type III PKS. CHS are found in all gymnosperms and angiosperms. The enzyme catalyses the first committed step of the flavonoid biosynthetic pathway. CHS uses *p*-coumaryl-CoA as a starter to condense with three molecules of malonyl-CoA and form a phenylpropanoid tetraketide intermediate, which later undergoes the C6→C1 Claisen cyclization to form 4,2´,4´,6´-tetrahydroxychalcone (chalcone) (Scheme **1.3c**) (Austin and Noel, 2003).

Stilbene synthase (STS) appears to be evolved from CHS, sharing more than 65% identity of its amino acid sequences. Like CHS, STS catalyses three condensation steps of *p*-coumaryl-CoA with malonyl-CoA, but the intermediate undergoes C2→C7 aldol cylization with the decarboxylative loss of C_1 to form stilbene (Scheme **1.3d**). Unlike the occurrence of CHS, the occurrence of STS is limited in some plant species. Resveratrol, a typical stilbene, functions as a phytoalexin in host plants (Austin and Noel, 2003).

- *Other plant type III PKS*

Benzalacetone synthase (BAS) uses *p*-coumaryl-CoA as a starter for single-step condensation with malonyl-CoA to form diketide intermediate, which is then followed by decarboxylation to form benzalacetone (Scheme **1.3a**) (Borejsza-Wysocki and Hrazdina, 1996). 2-Pyronesynthase (2PS) uses acetyl-CoA as a starter for double condensations with malonyl-CoA, resulting in triketide intermediate. This process is followed by lactonization (Scheme **1.3e**) (Eckermann *et al.*, 1998). Styrylpyrone synthase (SPS) uses *p*-coumaryl-CoA as a starter for double condensations. As with 2PS, this process is followed by lactonization (Scheme **1.3b**) (Beckert *et al.*, 1997; Herderich *et al.*, 1997). Type III PKS that condense more than three steps usually use acetyl- or malonyl-CoA as starter substrates. They include, for example, pentaketide synthase (PCS), hexaketide synthase (HKS), aleosone synthase (ALS), or

heptaketide synthase and octaketide synthase (OKS) (Austin and Noel, 2003; Rimando and Baerson, 2006; http://www.biologie.uni-freiburg.de/data/bio2/schroeder).



Scheme 1.3 Example of plant type III PKSs with their reactions and products; BAS = benzalacetone synthase; SPS = styrylpyrone synthase; CHS = chalcone synthase; STS = stilbene synthase; 2-PS = 2-pyrone synthase; TAL = triacetic acid lactone (scheme modified from Austin and Noel, 2003).

1.7.4 Type III PKS involved in diarylheptanoid and phenylphenalenone biosynthesis

Schröder (1997) proposed that type III PKS is likely to be involved in diarylheptanoid/phenylphenalenone biosynthesis. The enzyme was suggested to catalyse the first committed step by condensing a phenylpropanoid-CoA starter with one malonyl-CoA to produce a phenylpropanoid diketide intermediate analogue to BAS (see **1.7.3**). Unlike BAS, the diketide intermediate was thought to condense with a second phenylpropanoid molecule to form a diarylheptanoid, which later undergoes the structural modification and intra-molecular cyclization that results in phenylphenalenones. Brand *et al.* (2006) reported a type III PKS involved in phenylphenalenone biosynthesis. WtPKS1 (*Wachendorfia thyrsiflora* polyketide

synthase 1) was isolated from *W. thyrsiflora* (Haemodoraceae). The recombinant WtPKS1 was able to condense phenylpropanoid-CoA starters with one molecule of malonyl-CoA, resulting in benzalacetones, the diketide intermediate-derived products, suggesting that WtPKS1 catalyzed the first step of phenylphenalenone biosynthesis. Pyrones from double condensation were also found as enzymatic products, but neither diarylheptanoids nor phenylphenalenones were detected. The authors argued that the formation of phenylphenalenones could be the function of downstream enzymes (Brand *et al*., 2006).

The enzymatic study in *W. thyrsiflora* revealed that type III PKS are involved in diarylheptanoid/phenylphenalenone biosynthesis. In *Musa,* which produces phenylphenalenones as inducible compounds, such an enzyme has so far not been isolated. Hence, the function of type III PKS in the biosynthesis of phenylphenalenones in *Musa* is still opened for investigation. Recent developments in diarylheptanoid biosynthetic enzyme identification will be discussed in Chapter **7**.

1.8 Aim of this work

Plants in the Zingiberaceae and Musaceae families are economically important, especially *Musa* (banana), one of the world's main fruit crops. The cultivation of *Musa* faces serious problems from infectious diseases that can dramatically decrease yield. Solutions to this problem include efficient pesticides and the development of resistant cultivars, among others. Secondary metabolites in plants play an important role in plant survival and resistance to pathogens. The study of natural constituents in *Musa* could provide useful information in plant development to help scientists better understand the function of secondary metabolites. Interestingly, plants of Musaceae and Zingiberaceae are characterized by the occurrence of phenylphenalenones and diarylheptanoids, respectively, phenylpropanoid-derived natural products. This work studies compounds in plants from these two families in detail, mostly focusing on species of *Musa*. The scope of this work is as follows.

The idea that plants from Zingiberaceae and Musaceae develop from common ancestor is still being discussed. *Alpinia oxymitra,* a Zingiberaceae member distributed in Thailand and Southeast Asia, possesses banana-like fruitlets. It has closely related morphological characteristics to the extinct species *Spirematospermum,* which is believed to be the ancestor of the two families. Thus, this plant is a good candidate with which to study the chemotaxonomic relationship between Zingiberaceae and Musaceae. As a part of the present work, a phytochemical study of *A. oxymitra* was conducted in order to search for diarylheptanoids and/or phenylphenalenones. Phytochemical studies of *A. oxymitra* and *Alpinia zerumbet* (Pers.) B.L. & R.M. Sm. (Zingiberaceae) are described in Chapter **3**.

In many banana-producing countries, vast *Musa* plantations are cultivated in monocultures to supply the worlds' fruit markets. Banana cultivation in Thailand, however, is mostly for local consumers and therefore, more variable cultivars are grown there. The large diversity of *Musa* cultivars in Thailand makes information about the diversity natural products easy to come by. The distribution of phenylphenalenones in *Musa* and related species from Thailand was investigated to understand the occurrence of these compounds in different *Musa* cultivars and in order to correlate the chemical composition of different *Musa* cultivars with their phytopathological susceptibility. The methods used in this phytochemical screening are isolation and separation by TLC and HPLC, and structural elucidation of chemical constituents by spectroscopic techniques. Moreover, the isolation of new phenylphenalenone derivatives is also one of the purposes of the study. The phytochemical screening of *Musa* and related species (Musaceae) from Thailand is described in Chapter **4**.

Phenylphenalenone-type compounds are natural products found in many species of *Musa*. The compounds are produced highly in infected plants, suggesting the ecological function of the phenylphenalenones in *Musa* as phytoalexins. To understand the function of phenylphenalenones, induced phytoalexins in banana plants, sterile *in vitro* plants of *Musa acuminata* was treated with a chemical elicitor (jasmonic acid, JA) and micro-organisms. Responses to biotic and non-biotic elicitors of natural products' profiles, especially phenylphenalenones in *M. acuminata*, were investigated using phytochemical analyses. The response of *M. acuminata* to elicitation is described in Chapter **5**.

Understanding some biosynthetic aspects of phenylphenalenones is another aim of this project, such as the structural variation in phenylphenalenones induced by *O*-methylation. *O*-Methylation can change physical properties and alter the bioactivity of the compounds. Methylated products may function in plants differently from their hydroxyl analogues. The origin of the methyl group in *O*-methyl phenylphenalenones is also an interesting point in phenylphenalenone biosynthesis. In this study, feeding experiments with ^{13}C-labelled methionine together with elicitation with JA and with $CuCl_2$ were done to find out the origin of the *O*-methyl group in *O*-methyl phenylphenalenones and check whether or not *O*-methylation is affected by elicitors and precursor supply. The biosynthetic aspect of methyl groups in phenylphenalenones is described in Chapter **6**.

In addition to feeding experiments, the biosynthetic enzymes involved in the formation of phenylphenalenones were studied, especially the type III PKS that probably catalyse the biosynthesis of diarylheptanoids and phenylphenalenones in *M. acuminata*. RT-PCR homology-based methods were used to isolate type III PKS from *Musa*. Chapter **7** reports on these experiments and describes some trials that prove the function of the isolated type III PKS.

Chapter 2 General materials and methods

2.1 Plant materials

In vitro-plants of *Musa acuminata* "Cavendish" were obtained from Mr. F. Novak, IAEA (International Atomic Energy Agency) Laboratories, Austria; Universidad Católica de Oriente, Cra 46 No 40B 50 Barrio Santa Ana, Rionegro, Antioquia, Colombia and from Prof. Wanchai De-Eknamkul, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The plants were maintained on Murashige & Skoog (MS) agar (100 ml) (see Appx **6.1**) under a 28 ^{o}C and 12 h dark-light cycle. Plants were transferred to new culture media every 8 weeks.

2.2 Chromatography

2.2.1. Analytical HPLC

Identification and quantitative analysis of phenylphenalenones from *Musa* extracts were carried out using analytical HPLC (Agilent series HP1100; binary pump G1312A; autosampler G1313A; diode array detector (DAD) G1315B), *Anal. HPLC system_1-5* (Appx **1.1**).

Enzymatic products were analyzed by on a radio-HPLC (Agilent series HP1100; binary pump G1312A; autosampler G1313A; VWD G1314A; Flow scintillation analyzer, PACKARD) UV 280 nm/ flow scintillation analyser (CanberraPackard, Dreieich, Germany) coupled to HPLC and scintillation liquid Ultima-Flo AP (Perkin Elmer, Wellesley, MA, USA) (1 ml/min). *Anal. HPLC system_6 -7*. HPLC conditions are shown in Appx **1.1**.

2.2.2 Semi-preparative HPLC

Purification of natural products was done by semi-preparative HPLC (Agilent series HP1100; binary pump G1312A; auto sampler G1313A; DAD G1315B). HPLC conditions are shown in Appx **1.2**.

2.2.3 Preparative HPLC

Isolation of natural products was done on a Merck Hitachi LiChrograph chromatography system (L-6200A gradient pump, L-4250 UV-Vis detector). Isolation and purification of natural products were done on reversed phase RP18 column (on a column LiChrospher® RP18, 10 μm, 250 x10 mm); UV detector 254 nm. HPLC conditions are given in Appx **1.3**.

2.2.4 HPLC-SPE-NMR

Identification of natural products in part was done by HPLC-SPE-NMR (Agilent series HP1100, QautPump G1311A, auto sampler G1313A, DAD: J&M TIDAS). HPLC-SPE was accomplished by Agilent series HP1100 using DAD: J&M TIDAS. Samples were collected by post column SPE using a PROSPEKT2 (Spark Holland). The NMR measurement was recorded on a Bruker AV 500 using a CryoFit^{TM} insert (60 ml). For HPLC conditions, see Appx **1.4**.

2.3 Spectroscopic methods

2.3.1 UV-Visible absorption

UV-Vis spectra were recorded from an Agilent G1315B diode array detector during analytical HPLC in MeCN-0.1% TFA-H_2O solvent mixture. All spectra were recorded from 200-700 nm.

2.3.2 Optical rotation

The isolated compounds were dissolved in MeOH or $CHCl_3$ (1 ml) and measured for their optical rotation on a Polarimeter Jasco P-1030 (polarimeter cell Ø 0.35 cm x 10 cm).

2.3.3 Nuclear Magnetic Resonance (NMR)

^{1}H NMR spectra were recorded on a Bruker Avance DRX 500 NMR, operating at 500.13 MHz using 5 mm probe. ^{1}H NMR, ^{13}C NMR, HMQC, ^{1}H-^{1}H COSY and HMBC spectra were recorded on a Bruker AV 500 using a Cryo-TCI 5 mm probe head. The spectra were recorded in DMSO, $CDCl_3$ or acetone-d_6. Chemical shifts (δ) are given in ppm values relative to TMS internal standard. Acetonitrile-d_3 was uses as solvent for HPLC-SPE-NMR.

2.3.4 Mass spectrometry (MS)

Electron impact mass spectra (EIMS) and high resolution spectra (HREIMS) were recorded on MasSpec sector field mass spectrometer (Waters, Manchester, UK) with direct insertion probe (70 eV). The electrospray ionization mass spectra and high resolution spectra (HRESIMS) were recorded on Micromass Quattro II-Tandem Quadrupol mass spectrometer (Waters, Manchester, UK).

2.4 Other methods and analytical data

More details on additional methods and analytical data can be found in the various chapters of the Material and methods as follows.

Extraction, isolation and identification of natural products from *Alpinia oxymitra* and *A. zerumbet* are described in Chapter **3**. Extraction, isolation and identification of phenylphenalenones from *Musa* and its related species from Thailand are described in Chapter **4**. Elicitation and treatment of *in-vitro* plants of *Musa acuminata*, including extraction, isolation and identification of phenylphenalenones from the treated plants are described in Chapter **5**. Feeding experiments with labeled precursors and treatment with elicitors in order to study biosynthetic *O*-methylation in phenylphenalenones are described in Chapter **6**. Molecular biology and biochemistry methods to study type III PKS, involved in phenylphenalenone biosynthesis are described in Chapter **7**.

Chapter 3 Phytochemical studies of *Alpinia oxymitra* K. Schum. and *Alpinia zerumbet* (Pers.) B.L. & R.M. Sm. (Zingiberaceae)

3.1 Introduction

Zingiberaceae (ginger family) is one of the most frequently studied monocotyledon plant families due to its members' medicinal properties. Phytochemical studies of Zingiberaceae plants detected various groups of natural products, such as terpenoids, flavonoids, and diarylheptanoids (Prasain *et al*., 1997, 1998; Dong *et al*., 1998; Jasril *et al.*, 2003; Joshi *et al*., 2008; Tao *et al*., 2008). The Zingiberaceae and Musaceae families are members of the order Zingiberales. Both Musaceae and Zingiberaceae are characterized by the occurrence of diarylheptanoids: Numerous linear and alicyclic diarylheptanoids have been found in Zingiberaceae species, and phenylphenalenones, which are a biosynthetically related polycyclic group of diarylheptanoids, were found in *Musa* plants (Luis *et al*., 1994; Kamo *et al.*, 1998a, b, 2000). The taxonomical relationship between Musaceae and Zingiberaceae has been recognized (Manchester and Kress 1993), but the evolutionary origin of the two plant families from a common ancestor is still a matter of debate. A candidate ancestor is the extinct plant *Spirematospermum drexlerii*. Because of its banana-like seeds and fruitlets, which were frequently found as fossils in Denmark (Friedrich and Koch, 1970) and various other parts of the world, it can be taxonomically grouped close to the Musaceae. *Alpinia oxymitra* K. Schum. (synonym *Cenolophon oxymitrum* (Schum.) Holttum.), a perennial herb distributed in Thailand and Malaysia (http://www.ars-grin.gov/cgi-bin/npgs/html), is considered a living relative of *Spirematospermum.* It is distinguished from other *Alpinia sp*. by its bracteole shape and longitudinal groove ovary (Saensouk, 2006); moreover, its fruits are very similar to those of *Spirematospermum*. Furthermore, the seeds of *A. oxymitra* closely resemble those of *Spirematospermum* (Boyd, 1992). Due to these morphological relationships, *A. oxymitra* is of special interest for chemotaxonomic studies and could be a key to the chemical relationship between Zingiberaceae and Musaceae. In Thailand, the plant is used as a vegetable and traditional medicine as a carminative or against diarrhea. Phytochemical studies of *A. oxymitra* are reported here for the first time.

Alpinia zerumbet (Pers.) B.L. & R.M. Sm. or *A. speciosa* K. Schuman -- common name, shell flower -- is a perennial plant with strong odour. It originated in Asia and is distributed in many tropic and subtropic areas (www.efloras.org, flora of China). *A. zerumbet* has been traditionally used as an aromatic stomachic and a treatment for pain in China and Japan. In Brazil, the plant was infused and decocted for use as a diuretic and antihypertensive medicine. The isolation of natural products such as sesquiterpenes, labdane diterpenes, kawa pyrones and some other phenolic acids have been reported (Xu *et al.*, 1995; Kuster *et al.*,

1999; Lahlou *et al*., 2003; Elzaawely *et al*., 2007). Here we report on the re-investigation of the CH_2Cl_2 extract from rhizomes of *A. zerumbet* using HPLC-SPE-NMR in order to detect phenylphenalenones and diarylheptanoids, and to study biosynthetic relationships. Although biosynthetically related to diarylheptanoids, phenylphenalenones never have been found in any *Alpinia* species. The reason may be that one or more of the biosynthetic enzymes catalyzing the formation of the cyclic phenylphenalenones from their linear precursors are not expressed in *Alpinia*. Since these enzymes are not yet characterized, an expression analysis did not seem to be useful. Instead, a phytochemical approach seemed to be reasonable.

3.2 Materials and methods

3.2.1 Plant material

A. oxymitra K. Schum was kindly provided by Prof. Wanchai De-Eknamkul, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The plants were identified by Dr. Surapon Saensouk and were kept in the greenhouse of the Max Planck Institute for Chemical Ecology, Jena, Germany. *A. zerumbet* was collected from Khon Kaen province, northeastern Thailand, in 2004. The plant was identified by Dr. Surapon Saensouk, and Prof. Dr. Pranom Chantaranothai, Faculty of Sciences, Khon Kaen University, Khon Kaen province, Thailand (Fig **3.1**).

Figure 3.1 Pictures of *Alpinia oxymitra* (A and B) and *Alpinia zerumbet* (C and D)

3.2.2 Extraction and isolation

3.2.2.1 *Alpinia oxymitra*

Rhizomes (78.5 g, fresh weight) were cut into small pieces, macerated with MeOH separately and exhaustively extracted. The MeOH extract was evaporated to dryness with a rotary evaporator (≤50 ^{o}C). The residues were suspended in 50% MeOH in H_2O and parti-

tioned with *n*-hexane, $CHCl_3$ and EtOAc, respectively. The fractions were analysed by TLC in comparison to authentic samples of diarylheptanoids (curcumin), gingerols, and phenylphenalenones (anigorufone (**1**), hydroxyanigorufone (**2**) and methoxyanigorufone (**3**)). The EtOAc fraction (1.063 g) and $CHCl_3$ fraction (239 mg) of rhizomes were further separated and purified.

The EtOAc fraction was fractionated by gel filtration chromatography (Sephadex LH20) using MeOH as an eluent. Fractions were collected (10 ml each). Fractions 15 to 20 were pooled, evaporated to dryness and the residue again subjected to Sephadex LH20 column using 50% CH_2Cl_2 in MeOH as an eluent. Fractions were collected (5 ml each). Fractions 22-28 were pooled and further separated by preparative HPLC using *prep. HPLC system 1* (see Appx **1.3**). Compound **57** (0.9 mg) was eluted at R_t 19.5 min. Fractions 29-37 were pooled and purified by preparative HPLC using *prep. HPLC system_2* (see Appx **1.3**). Compound **58** (1 mg) was eluted at R_t 19.1 min.

The $CHCl_3$ extract was chromatographed on Sephadex LH20. The fractions were eluted with mixtures (20 ml each) of CH_2Cl_2 in 50%, 60%, 70%, and 80% MeOH, and pure MeOH. Fractions were collected (5 ml each). Fractions 21-28 were pooled, again applied to Sephadex LH20, and eluted with 100% MeOH. Compound **59** (12.6 mg) was obtained in a pure form.

3.2.2.2 *Alpinia zerumbet*

Fresh rhizome material of *A. zerumbe*t (160 g) was cut into small pieces and then macerated with 95% EtOH (3 x 200 ml). The macerate was evaporated to the final volume of 50 ml. Then the extract was partitioned with *n*-hexane and CH_2Cl_2, respectively. The CH_2Cl_2 faction was analysed by analytical HPLC for diarylheptanoids and phenylphenalenones using *anal. HPLC system_1* (see Appx **1.1**). The CH_2Cl_2 fraction (about 0.8 mg) was then further analyzed by HPLC-SPE-NMR using *HPLC-SPE-NMR-system_1* (see Appx **1.4**).

3.3 Results

3.3.1 Phytochemical study of *A. oxymitra*

From the EtOAc extract of the rhizome, two pure compounds were isolated. Structure elucidation of the isolated compounds was established by means of 1D- and 2D-NMR and MS techniques. According to spectroscopic data, compounds **57** and **58** were identified as epicatechin (**57**) and galloepicatechin (**58**) respectively (Fig **3.2**).

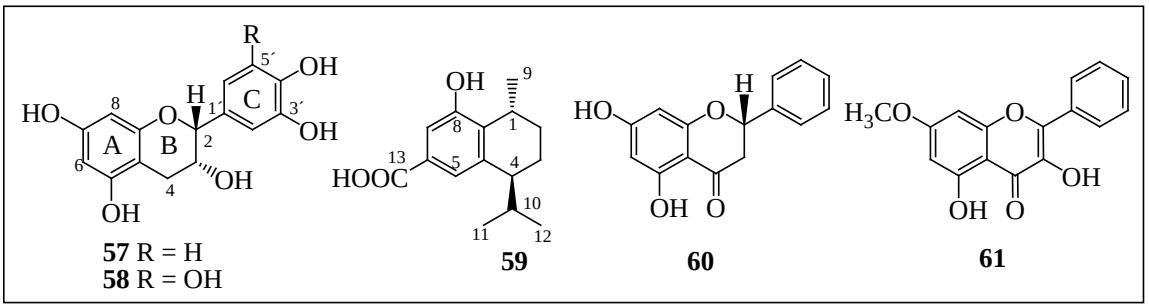


Figure 3.2 Chemical structures of the compounds isolated from *A. oxymitra* and *A. zerumbet*

The HR-ESIMS spectrum of compound **59** showed an $[M]^+$ ion at *m/z* 248.1415, indicating a molecular formula of $C_{15}H_{20}O_3$ (calc. 248.1412) of a sesquiterpene. The ^{1}H NMR spectrum of compound **59** ($CDCl_3$, unless otherwise noted) showed three doublet methyl signals at δ 0.82, 0.98 and 1.21 assigned to H-12, H-11 and H-9, respectively. ^{1}H NMR signals of two methylene groups appeared at δ 1.82, 1.55 and 1.97 assigned to H-3, H-2b and H-2a, respectively. Three signals of aliphatic methine protons at δ 2.02, 2.57 and 3.19 were assigned to H-10, H-4 and H-1, respectively. Two signals of aromatic methines at δ 7.53 and 7.28, assigned to H-5 and H-7, completed the ^{1}H NMR spectrum. ^{1}H,^{1}H COSY and TOCSY spectra indicated that all aliphatic signals belonged to one spin system, and the signals at δ 7.53 and 7.28 were due to an aromatic AX spin system. HSQC and HMBC correlations of the two AX protons established the aromatic ring, the position of the carboxyl and hydroxyl group. The HMBC spectrum displayed cross peaks of H-5 with C-7 (δ 113.2), C-8a (δ 136.1), the carboxyl group (δ 170.8), and a methine carbon at δ 43.1 assignable to C-4 of the aliphatic part of the molecule.

Long-range correlations of H-7 through three bonds were observed with C-5 (δ 124.4), C-8a and the carboxyl group. A correlation of H-7 through two bonds with δ 153.1 assigned this low-field carbon to the hydroxylated C-8. HMBC correlations of the hydroxyl proton (measured in DMSO-d_6) with C-7, C-8, and C-8a confirmed the position of the hydroxyl group at C-8. The eight carbon atoms of the aliphatic part of the molecule were also assigned from their HMBC, HSQC and ^{1}H,^{1}H COSY correlations. The methyl proton signal at δ 1.21 (H-9) coupled with C-8a and showed a strong unresolved cross signal with C-1 (δ 27.1) and C-2 (δ 26.5). The two other methyl signals (H-12, δ 0.82; H-11, δ 0.98), mutually correlated by HMBC, showed further cross peaks with C-10 (δ 33.2) and C-4 (δ 43.1) and therefore were attributed to the isopropyl side chain attached to C-4. The methine proton H-4 (δ 2.57) coupled not only with the isopropyl carbons C-10 (δ 33.2), C-11 (δ 21.9) and C-12 (δ 19.6) but also with C-4a (δ 141.6), C-5, and C-8a of the aromatic ring and methylene carbons C-2 (δ 26.5) and C-3 (δ 18.8). From these data (Table **3.1**), the constitution of compound **59**

was determined as 1,2,3,4-tetrahydro-8-hydroxy-4-isopropyl-1-methylnaphthalene-6-carboxylic acid (8-hydroxy-13-calamenenoic acid), a new natural product of the calamenene type. Since, due to missing ROESY correlation between the geminal methyl groups of the isopropyl moiety and the secondary methyl group (C-9, δ 20.7), *cis*-orientation is unlikely, (1*R*,4*R*)- and (1*S*,4*S*)-configuration can be excluded.

Table 3.1 ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), ^{1}H,^{1}H COSY, and HMBC data of (–)-(1*R*,4*S*)-8-hydroxy-13-calamenenoic acid (**59**) ($CDCl_3$, TMS).

Position	**^{13}C (δ)**	**^{1}H** (δ, integral, mult., coupling constant)	**^{1}H,^{1}H-COSY**	**HMBC**
1	27.1	3.19 (1H, m)	H-2a, H-2b, H-9	C-2, C-3, C-4a, C-8, C-8a, C-9
2	26.5	a: 1.97 (1H, m) b: 1.55(1H, br. d, *J* =12.6 Hz)	a: H-1, H-2b, H-3 b: H-1, H-2a, H-3	a: C-1, C-3, C-4 b: C-8a, C-3, C-4, C-9
3	18.3	1.82 (2H, m)	H-2a, H-2b, H-4	C-1, C-2, C-4, C-10
4	43.1	2.57 (1H, br.s)	H-3, H-10	C-2, C-4a, C-5, C-10, C-11, C-12
4a	141.6	-	-	-
5	124.4	7.53 (1H, br.s)	H-7	C-4, C-7, C-8a, C-13
6	126.3			
7	113.2	7.28 (1H, br.s)	H-5	C-5, C-6, C-8, C-8a, C-13
8	153.1	-	-	-
8a	136.1	-	-	-
9	20.7	1.21 (3H, d, *J* = 6.9 Hz)	H-1	C-1, C-2, C-8a
10	33.2	2.02 (1H, m)	H-4, H-11, H-12	C-4, C-4a, C-11, C-12
11	21.9	0.98 (3H, d, *J* = 6.6 Hz)	H-10	
12	19.6	0.82 (3H, d, *J* = 6.9 Hz)	H-10	C-4, C-10, C-12
13	170.8	0.98 (3H, d, *J* = 6.6 Hz)	-	C-4, C-10, C-11

The absolute stereochemistry of **59** was assessed based on data reported from closely related calamenenes. The optical rotation $[\alpha]_D^{22}$ = -10.95 (c 0.00065, $CHCl_3$) of compound **59** was in good agreement with that of synthetic (1*R*,4*S*)-8-hydroxycalamenene $[\alpha]_D$ = -24° (c 1.06, $CHCl_3$) but clearly different from the positive optical rotation values $[\alpha]_D$= +38° of (1*S*,4*R*)-hydroxycalamenene and $[\alpha]_D$ = +40° of (1*R*,4*R*)-8-hydroxycalamenene (Nishizawa *et al*., 1983). Moreover, 6-substituted (1*S*,4*R*)-8-hydroxy calamenenes from *Tarenna madagascariensis* displayed positive optical rotation values (Salmoun *et al*., 2007). Hence, compound **59** is (–)-(1*R*,4*S*)-8-hydroxy-13-calamenenoic acid (**59**) (Fig **3.2**) (Jitsaeng *et al*., 2009).

3.3.2 Phytochemical study of *Alpinia zerumbet*

Using HPLC-SPE-NMR technique (*HPLC-SPE-NMR_system 1*, Appx **1.4**), two compounds were identified from the rhizomes of *A. zerumbet*. By comparison with reference data (in house data base), compounds **60** and **61** were identified as pinocembrin (**60**) (R_t 28.0 min) and izalpinin (**61**) (R_t 30.5 min), respectively (Fig **3.2**).

3.3.3 Spectroscopic data of the isolated compounds

Spectroscopic data of the known compounds **57**, **58**, **60**, and **61**, see Appx **3.**

(–)-(1*R*,4*S*)-8-Hydroxy-13-calamenenoic acid (59)

^{1}H NMR (500 MHz, $CDCl_3$, TMS as an internal standard): see Table **3.1**.; $[\alpha]_D^{22}$ = -10.95° (*c* 0.00065, $CHCl_3$); ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.45 (1H, br. s, 13-COOH), 9.51 (1H, s, 8-OH), 7.21 (1H, d, *J* = 1.6 Hz, H-5), 7.18 (1H, d, *J* = 1.6 Hz, H-7), 3.09 (1H, m, H-1), 2.47 (1H, br. s, H-4), 1.91 (1H, m, H-10), 1.85 (1H, m, H-2a), 1.76 (1H, m, H-3a), 1.72 (1H, m, H-3b), 1.44 (1H, br. d, *J* = 13.2 Hz, H-2b), 1.10 (3H, d, *J* = 7.0 Hz, H-9), 0.92 (3H, d, *J* = 6.6 Hz, H-11), 0.76 (3H, d, *J* = 6.9 Hz, H-12); HR-ESIMS: *m/z* (rel. int. %): 248.1415 $[M]^+$ (100) (calc. for $C_{15}H_{20}O_3$ 248.1412), corresponding to the molecular formula $C_{15}H_{20}O_3$.

3.4 Discussion

In order to acquire more information about the chemotaxonomic relationship between the plant families Zingiberaceae and Musaceae and their evolution, the chemical constituents of two zingiberaceous plant species were studied. *A. oxymitra* is a species that is considered to be closely related to banana (Musaceae) due to its banana-like fruitlets (Boyd, 1992). No phytochemical study of this plant has been reported before. By combining conventional chromatographic techniques and preparative HPLC, three pure compounds were isolated. There were two flavonoids, epicatechin (**57**) and galloepicatechin (**58**). These two compounds are well-known flavonoids, widely distributed in higher plants not only in Zingiberaceae. The antioxidant activity of many flavonoids has been proven and the compounds are widely used as a health supplement (Lila, 2004; Khan and Mukhtar, 2007). Based on this phytochemical result, *A. oxymitra* could be developed as a source of natural flavonoids for health supplements. Together with the two flavonoids, one sesquiterpene derivative, (–)-(1*R*,4*S*)-8-hydroxy-13-calamenenoic acid (**59**), was isolated. This compound is a new natural product. Terpenoids were found as main components in the volatile oils of zingiberaceous plants (Habsah *et al.*,

2000; Behura and Srivastava, 2004). Their antimicrobial activities have been reported (Medeiros *et al.*, 2003). It would be interesting to study the bioactivity of compound **59**.

From the CH_2Cl_2 rhizome extract of *A. zerumbet*, two known flavonoids, pinocembrin (**60**) and izalpinin (**61**), were isolated. Unlike in the phytochemical study of *A. oxymitra*, the HPLC-SPE-NMR technique was used. Microgram amounts of extract were sufficient to identify these two main metabolites.

As stated above, phytochemical studies of these two zingiberaceous plants were conducted in order to investigate the hypothetical chemical relationship between Zingiberaceae and Musaceae. If diarylheptanoids occurred in these plants, they would have been a valuable indicator of a chemical relationship between Zingiberaceae and Musaceae. However, in *A. oxymitra* and *A. zerumbet*, diarylheptanoids were neither reported in the literature nor found in the present study. In addition, the treatment of *A. oxymitra* with JA to induce the formation of natural products and enhance the concentration of minor components was performed, but no significant changes in the profile of natural products were observed (data not shown). However, the occurrence of diarylheptanoids in other plants of the Zingiberaceae family, especially in the genera *Alpinia*, *Zingiber* and *Curcuma* (Prasain *et al*., 1997; Ma *et al*., 2004; Ma and Gang, 2006, Jiang *et al*., 2006; Sun *et al*., 2008; Wang *et al*., 2008) as well as the isolation of phenylphenalenones from *Musa sp*. (Musaceae) (Luis *et al*., 1994, 1996; Kamo *et al.*, 1998b, 2001; Otálvaro, 2004) continues to be reported. Therefore the study of the chemical relationship between these two families, focused on the distribution of diarylheptanoid derivative, is still challenging.

Chapter 4 Phytochemical screening of *Musa* sp. and related species from Thailand

4.1 Introduction

In Thailand, the third largest banana producer in Asia, various banana and plantain cultivars are widely cultivated throughout the country. The products are mostly consumed locally. Silayoi and Babpraserth (1983) reported 59 Thai banana cultivars of six different genome groups, AA, AAA, AAB, AB, ABB, ABBB and BBB, classified by the method of Simmonds and Sheperd (1955). Later, new cultivars were bred and imported inpart because banana plantations in Thailand faced problems from devastating plant disease (see also **1.2**) (Silayoi, 2002). The INIBAP (International Network for the Improvement of Bananas and Plantains) started the International *Musa* Testing Programme (IMTP) to improve bananas and plantains (Orjeda, 1998). The variability of *Musa* cultivars has increased our interest in screening natural products in different *Musa* cultivars, as some could play important roles in plant resistance to diseases. Here we mainly focus on the occurrence of phenylphenalenones, diarylheptanoids and related compounds in *Musa* cultivars and related species. The occurrence of phenylphenalenones may be linked to plant resistance to disease. In order to study the occurrence of phenylphenalenones in different banana cultivars and related species, plants of the Musaceae family from two genera, *Musa* and *Ensete,* were collected. *Musa* plants included edible bananas and plantains of different genome groups, including the ornamental species *Musa coccinea* and *M. ornata. Ensete glauca,* a non-edible wild species related to banana, was also analyzed in this study. The differences among phenylphenalenone profiles of *Musa* and related species were investigated.

4.2 Materials and methods

4.2.1 Plant material

Plant material from fifty-four Musaceae plants was collected from Queen Sirikit Botanic garden, Bangkok, Thailand, and Pakchong Research Institute, Nakorn RatchaSima, Thailand. Plant material was collected separately from above-ground and underground parts. Plants belonged to six different genome groups, AA, AAA, AAB, AB, ABB, ABBB and BBB, as categorized by method of Simmonds and Shepherd (1955). Names of the cultivars are given by genus, genome group and common Thai names. Bracketed ciphers denote consecutive numbers as used later on in the text. The list of cultivars is shown in Appx **2**.

4.2.2 Extraction

Plant material (ca. 100 g fresh weight) of above-ground and underground parts of banana plants was collected. Plant material was then exhaustively extracted by 95% EtOH. The extracts were evaporated to a final volume of 50 ml. The extracts from underground parts were partitioned with CH_2Cl_2 (2 × 50 ml), while the above-ground extracts were partitioned with *n*-hexane 50 ml (2 × 50 ml) and later with CH_2Cl_2 (2 × 50 ml). The fractions were evaporated to dryness using a rotary evaporator (≤50 ^oC). CH_2Cl_2 extracts were analysed with TLC in the primary screening and later by HPLC for further screening.

4.2.3 TLC analysis

TLC developing system: 15 % MeOH in CH_2Cl_2; Running distance: 8 cm; Sample loading: 15 µl (10 mg/ml); Standard compounds: anigorufone (**1**) 5 µl (1 mg/100 µl), methoxyanigorufone (**3**) 5 µl (1 mg/100 µl), hydroxyanigorufone (**2**) 5 µl (1 mg/100 µl), anigopreissin (**AP**) 5 µl (1 mg/100 µl).

4.2.4 HPLC analysis for phenylphenalenones in Thai *Musa* cultivars

The CH_2Cl_2 extracts from rhizomes and leaves of the cultivars selected by TLC (see **4.3.1**) and the extracts from some economically important cultivars such as Nam Wa, Hom Thong, Khiew, Leb Mue Nang or those with interesting morphological characters such as Roi Wi and Nag Daeng banana plants were dissolved in MeOH (5 mg/ml; 50 µl injection volume) and then preliminarily analysed for natural product pattern by means of HPLC using *anal. HPLC system_1* (see Appx **1.1**). The presence of selected phenylphenalenones in the extracts was verified by comparing their retention times and UV spectra with those of authentic references using *anal. HPLC system_2* (see Appx **1.1**). The rhizome extracts were dissolved in MeOH (10 mg/ml; 25 µl injection volume) and analysed for phenylphenalenones. Authentic phenylphenalenones were used as standard compounds (Table **4.2a, b**).

4.2.5 Isolation and purification of phenylphenalenones and related compounds

Six *Musa* cultivars and *Ensete glauca* were selected and their phenylphenalenones were isolated and purified. The CH_2Cl_2 extracts of underground part were first fractionated by RP18 short column (2×4 cm). Mixtures of MeOH and H_2O were used as an eluent. The fractions were further isolated and purified using semi-preparative HPLC and preparative HPLC (Table **4.1**) to obtain phenylphenalenone compounds in pure forms.

Table 4.1 List of plants and isolation methods to purify phenylphenalenones (methods' details are shown in Appx **1**)

Plants	Compounds: Isolation methods
Ensete glauca "Nual"	**7:** *prep. HPLC system_3* then *prep. HPLC system_4* **3:** *prep. HPLC system_5*
Musa (AA) "Thong Ngoei"	**47:** *prep. HPLC system_6;* **20, 48:** *semiprep HPLC system_3*; **39**, **21**, **62:** *prep. HPLC system_7* then *semiprep. HPLC system_1*; **33**, **49:** *semiprep HPLC system_1*; **63:** *semiprep HPLC system_1* then *semiprep HPLC system_5*; **3**, **1:** *semiprep. HPLC system_2*
Musa (ABB) "Hakmuk"	**49, 48:** *prep. HPLC system_8*
Musa (BBB) "Thep Panom"	**62**, **3**, **20:** *prep. HPLC system_9* then *semiprep. HPLC system_4*
Musa (ABBB) "Theparos"	**63:** *prep. HPLC system_10*; **47:***prep. HPLC system_11*
Musa (ABB) " Leb Chang Kud"	**48:** *prep. HPLC system_13*; **33:** *prep. HPLC system_7*
Musa "Rattagatli"	**2:** *prep. HPLC system_9*; **49:** *prep. HPLC system_14*

4.3 Results

4.3.1 Plant collection and TLC analysis

In this study, 54 plants from the Musaceae family were collected from Thailand; they included original Thai cultivars, imported edible bananas and ornamental plants. Primary phytochemical screening was done using TLC. The same amounts of CH_2Cl_2 extracts (10 mg/ml, 15 µl) were loaded on TLC plates for analysis. Authentic phenylphenalenones (1 mg/ml, 5 µl), anigorufone (**1**), hydroxyanigorufone (**2**), methoxyanigorufone (**3**), and anigopreissin (**AP**) were used as standards. TLC patterns (Fig **4.1**) of *Musa* plant extracts were compared. Some extracts were selected for further analysis by HPLC. Selected samples were grouped according to the complexity of the profiles of their natural products.

- **Group I**: **complex profiles with high levels of natural products:**

Under UV detection (254 nm), the TLC patterns of the extracts in this group showed many spots and high intensity, especially in the R_f of authentic phenylphenalenones. The samples belonged to this group were; *Musa* (AA)"Hom Phol San" (8) and "Thong Ngoei" (9); *Musa* (AAA) Hochuchu" (12); *Musa* (AAB) "Khom" (24); *Musa* (ABB) "Hakmuk" (31); *Musa* (ABBB)"Theparos" (38); *Musa* (BBB) "Thep Panom" (41); *Ensete glauca* (54)

- **Group II: simple profiles with low levels of natural products:**

Under UV detection (254 nm), the TLC patterns of the extracts in this group showed few spots and low intensity, comparing to the R_f of authentic phenylphenalenones, the extracts

showed very weak signals The samples belonged to this group were; *Musa* (AA) "Hom Chan" (3); *Musa* (AAB) "Nam" (23); *Musa* (ABB) "Nam Wa Dam"(36); *Musa* (BBB) "Leb Chang Kud" (39); *Musa* "Bua Si Muang" (20); *M. itinerans* "Hok" (45); *M. coccinea* "Ratta-gatli" (17).

The numbers shown in the brackets correspond to the numbers of lanes in the TLC analysis. Phenylphenalenone-type compounds in *Musa* cultivars analyzed by TLC and other methods are shown in Table **4.2a** and **b**.

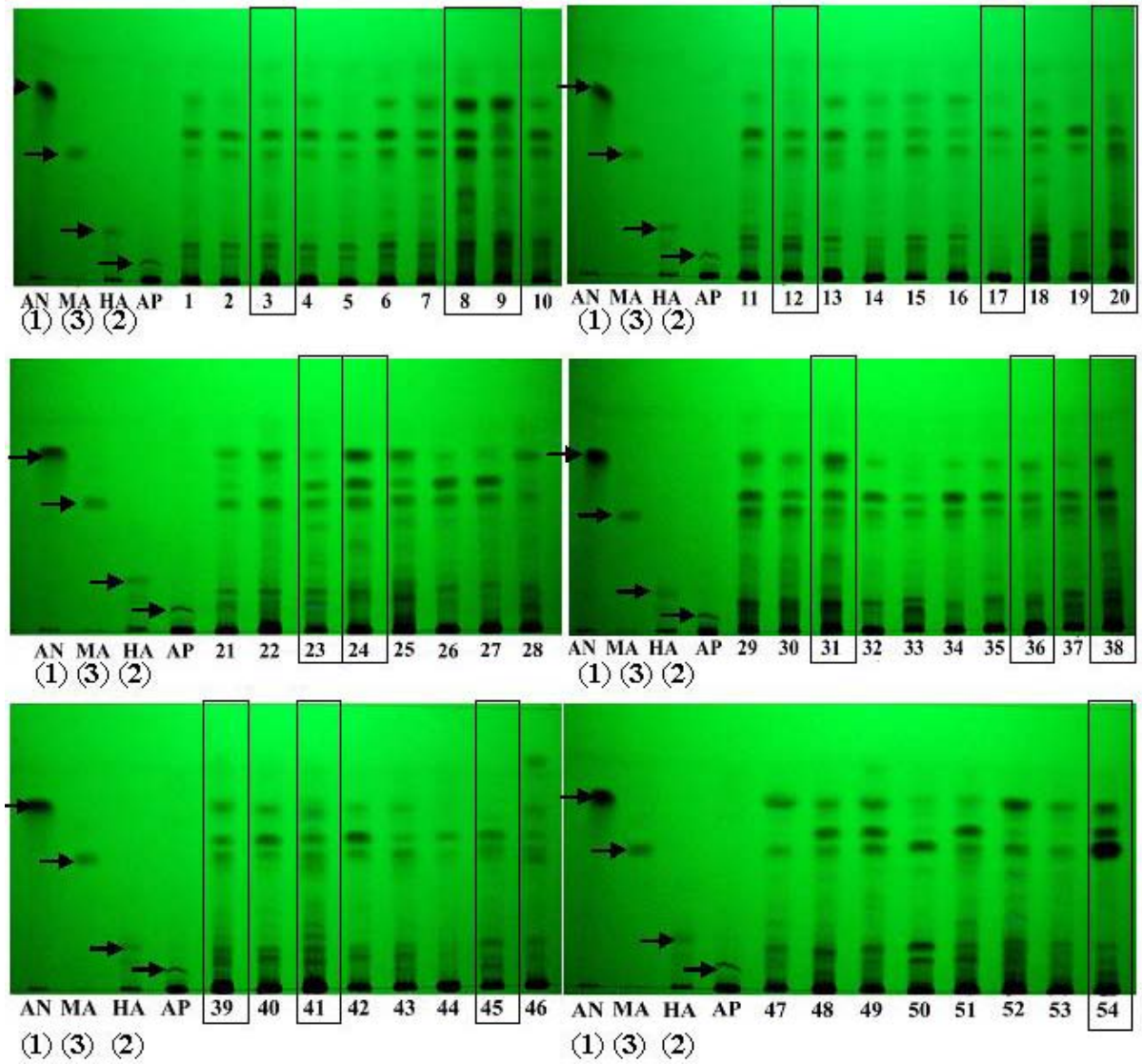


Figure 4.1 TLC analysis of CH_2Cl_2 extracts from *Musa* sp. and related species collected in Thailand. The cultivars selected for further analysis are marked with rectangular frames. 15% MeOH in CH_2Cl_2 was used as a developing solvent mixture; detection: UV 254 nm. The numbers of TLC lanes are correlated to number of the collected species. Standard compounds used in this TLC analysis are AN (**1**) = anigorufone, MA (**3**) = methoxyanigorufone; HA (**2**) = hydroxyanigorufone and AP = anigopreissin (*Anigozanthos preisseii*, Haemodoraceae).

4.3.2 Screening for phenylphenalenones in Thai *Musa* using HPLC

The CH_2Cl_2 extracts of leaves and rhizomes of the selected *Musa* cultivars (see **4.3.1**) were analyzed by HPLC for the occurrence of phenylphenalenones. Rhizome extracts were good sources of phenylphenalenones. Phenylphenalenones were identified by comparing R_t and UV spectra of extract components with authentic phenylphenalenones. Results showed that the most frequently found phenylphenalenones in almost all cultivars except *Ensete glauca* and *M. coccinea* belonged to the condensed dimer phenylphenalenones. In *Ensete glauca,* two main components, methoxyanigorufone (**3**) and 9-(4´-hydroxyphenyl)-2-methoxyphenalen-1-one (**7**), were found; these belong to 9-phenylphenalenones. In *M. coccinea* "Rattagatli", which is an ornamental plant, the main components detected were hydroxyanigorufone (**2**) and 4′,4″-dihydroxyanigorootin (**49**) (Table **4.2a** and **b**).

In cultivars that produce edible fruits, the dimeric phenylphenalenones, anigorootin (**47**), 4′-hydroxyanigorootin (**48**), and 4′,4″-dihydroxyanigorootin (**49**) were detected. In order to visualize the pattern of dimeric phenylphenalenones **47 - 49** in *Musa* cultivars, their ratios were calculated from HPLC peak integrals (Fig. **4.2**). The ratios and levels of dimeric phenylphenalenones in selected *Musa* cultivars was variable and independent from the genome groups. Economic important cultivars such as "Hom Thong" (AAA), "Nam Wa" (ABB) and "Leb Mue Nang" (AA) contained low levels of the compounds. According to the ratios and levels of the three dimeric phenylphenalenones, *Musa* cultivars can be categorized into three groups:

- *Anigorootin dominant, present at high levels:* the ratio of the signal belonging to the dimeric phenylphenalenones was about 1:2:3 (**49**:**48**:**47**). The cultivars that had this characteristic were *Musa* (AA) "Thong Ngoei", "Hom Phol San"; *Musa* (AAB) "Khom"; *Musa* (ABB) "Thong Ma Eng".

- *All three condensed dimer phenylphenalenones present at approximately equal levels*: The ratio of the signals of the condensed dimer phenylphenalenones was close to 1:1:1. The cultivars that had this characteristic mostly belonged to *Musa* (ABB) "Hakmuk", "Nam Wa Dam", "Nom Mi"; *Musa* (BB) "Tani", "Tani Dam"; *Musa* (BBB) "Leb Chang Kud" and *Musa* (ABBB) "Theparos"

- *4′,4″-Dihydroxyanigorootin dominant: Musa coccinea* "Rattagatli" and *Musa* "Bua Si Muang". Both cultivars are ornamental plants.

Table 4.2a Phenylphenalenones identified from the selected Thai *Musa* cultivars and *Ensete glauca* using HPLC, TLC and spectroscopic techniques. The R_t of the compounds shown in this table were detected by HPLC analysis (*anal. HPLC_system_2*, see Appx **1.1**).

	R_t (min)	Hom Chan, 3	Sa, 5	Leb Mue Naang, 6	Hom Phol Sam, 8	Thomg Ngoei, 9	Khai Thong Raung, 10	Hom Thong, 11	Hochuchu, 12	Khiew, 13	Nak Daeng, 14	Roi Wi, 22	Nam, 23	Khom, 24	Nom Mi, 29	Nang Paya, 30
Anigorufone (**1**)	33.7	x	?	x	x	x	x	x	?	x	?	x	x	x	x	x
Hydroxyanigorufone (**2**)	21.0	x	?	x	x	x	x	x	x	x	x	x	?	x	?	x
Methoxyanigorufone (**3**)	32.0	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
9-(4'-Hydroxyphenyl)-2-methoxyphenalen-1-one (**7**)	19.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Isoanigorufone (**20**)	34.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Irenolone (**21**)	19.5	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-
2-Phenyl-1,8-naphthalic anhydride (**33**)	30.2	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-
3-Hydroxy-4-(4'-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**39**)	22.3	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-
3-Hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (**62**)	22.2	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-
Anigorootin (**47**)	53.8	x	x	x	x	x	x	x	?	x	x	x	x	x	x	x
4´-Hydroxyanigorootin (**48**)	43.8	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
4´,4´´-Dihydroxyanigorootin (**49**)	33.0	x	?	?	x	x	x	x	x	x	x	x	x	x	x	x
2-Phenylnaphthalene-1,8-dicarboxylic acid (**63**)	11.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: Authentic phenylphenalenone compounds used as standard compounds in HPLC analysis under the limit of detection in this experiment are: Musanolone F (**5**), R_t 21.4 min, 4´-*O*-methylirenolone (**22**), R_t 36.1 min, 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**), R_t 17.5 min; 7-(4´-methoxyphenyl)-naphthal-8-formyl-1-carboxylic anhydride (**40**), R_t 23.5 min. These compounds were not detected in the selected plant materials. Numbers after plants' names correspond to TLC lanes. X = detected, ? = not clearly defined, - = not found.

Table 4.2b Phenylphenalenones identified from selected Thai *Musa* cultivars and *Ensete glauca* using HPLC, TLC and spectroscopic techniques. The R_t of the compounds shown in this table were detected by HPLC analysis (*anal. HPLC_system_2*, see Appx **1.1**).

	R_t (min)	Hakmuk, 31	Nam Wa, 34	Nam Wa Khom, 35	Nam Wa Dam, 36	Nam Wa Khom, 35	Thong Ma Eng, 37	Theparos, 38	Leb Chang Kud, 39	Thepanom, 41	Tani, 43	Tani Dam, 44	Ratagatli, 17	Bua Si Muang, 20	Hok, 45	*Ensete glauca*, 54
Anigorufone (**1**)	33.7	x	x	x	x	x	x	x	x	x	x	?	?	?	-	x
Hydroxyanigorufone (**2**)	21.0	x	?	-	?	-	x	x	x	x	x	x	x	x	x	x
Methoxyanigorufone (**3**)	32.0	x	x	x	x	x	x	x	x	x	x	x	?	x	x	x
9-(4'-Hydroxyphenyl)-2-methoxyphenalen-1-one (**7**)	19.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	x
Isoanigorufone (**20**)	34.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Irenolone (**21**)	19.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Phenyl-1,8-naphthalic anhydride(**33**)	30.2	-	-	-	-	-	-	-	x	-	-	-	-	-	-	-
3-Hydroxy-4-(4'-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**39**)	22.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-Hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (**62**)	22.2	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-
Anigorootin (**47**)	53.8	x	x	x	x	x	x	x	x	x	x	x	?	x	x	-
4′-Hydroxyanigorootin (**48**)	43.8	x	x	x	x	x	x	x	x	x	x	x	x	x	?	-
4′,4′′-Dihydroxyanigorootin (**49**)	33.0	x	x	x	x	x	x	x	x	x	x	x	x	x	?	-
2-Phenylnaphthalene-1,8-dicarboxylic acid (**63**)	11.8	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-

Note: Authentic phenylphenalenone compounds used as standard compounds in HPLC analysis under the limit of detection in this experiment are: Musanolone F (**5**), R_t 21.4 min, 4′-*O*-methylirenolone (**22**), R_t 36.1 min, 2-(4′-hydroxyphenyl)-1,8-naphthalic anhydride (**34**), R_t 17.5 min; 7-(4′-methoxyphenyl)-naphthal-8-formyl-1-carboxylic anhydride (**40**), R_t 23.5 min. These compounds were not detected in the selected plant materials. Numbers after plants' names correspond to TLC lanes. X = detected, ? = not clearly defined, - = not found.

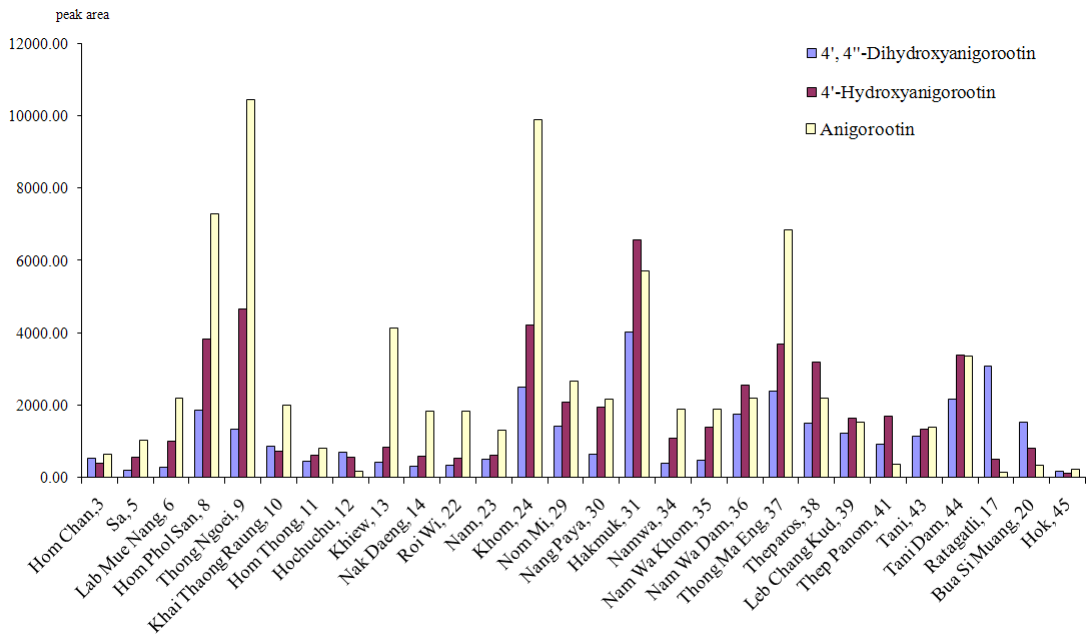


Figure 4.2 Ratios of HPLC signals (*anal. HPLC system_2*, see Appx **1.1**) of the three condensed dimer phenylphenalenones, 4´,4´´-dihydroxyanigorootin (**49**), 4´-hydroxyanigorootin (**48**) and anigorootin (**47**), in rhizome extracts of *Musa* cultivars.

4.3.3 Isolation and structure elucidation of phenylphenalenones isolated from *Musa* and related species from Thailand

Six *Musa* cultivars were selected and their natural products were isolated and purified: Musa (AA) "Thong Ngoei"; *Musa* (ABB) "Hakmuk" and "Leb Chang Kud", *Musa* (ABBB) "Theparos", *Musa* (BBB) "Thep Panom" , *M. coccinea* "Rattagatli", and *Ensete glauca* "Nual" in order to identify phenylphenalenones. Thirteen phenylphenalenone-type compounds were isolated. The chemical structures (Fig **4.3)** of the compounds were elucidated using spectroscopic techniques. The detection of phenylphenalenone-type compounds in *Musa* cultivars by HPLC is shown in Table **4.2a** and **b**.

4.3.3.1 Structure elucidation of 9-phenylphenalenones

Structure elucidation using NMR and MS techniques takes advantage of common characteristics of phenylphenalenones. The ^{1}H NMR patterns of 9-phenylphenalenones exhibited the AB (H-7, H-8) and AMX spin systems (H-4 – H-6). The proton signal located most downfield is assignable to H-7 of the AB spin system (Fig. **4.5** A). In 4-phenylphenalenones, H-9, the proton in *peri* position to carbonyl appeared most downfield (Fig. **4.5** B).

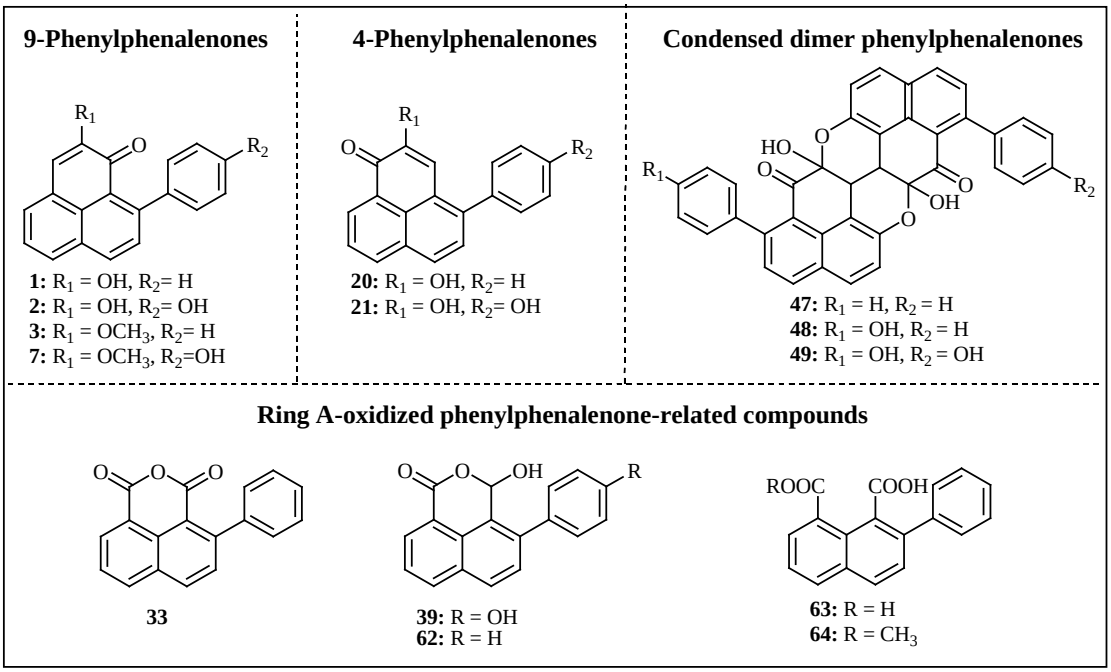


Figure 4.3 Chemical structures of isolated phenylphenalenones from Thai *Musa sp.* and related species. Compound **64** is a methylated product of compound **63** by diazomethane.

The EIMS spectrum of 9-phenylphenalenones showed the higher intensity of the $[M-H]^+$ ion than the $[M]^+$ ion (Thomas 1971; Bick and Blackman, 1973; Kamo *et al.*, 1998b) as formation of $[M-H]^+$ stabilized the molecule by forming an ether bridge (Fig. **4.4**). The downfield chemical shift of proton signals of H-7 in 9-phenylphenalenones and of H-9 in 4-phenylphenalenones and the intense $[M-H]^+$ ion were used to discriminate 9-phenylphenalenones from isomeric 4-phenylphenalenones.

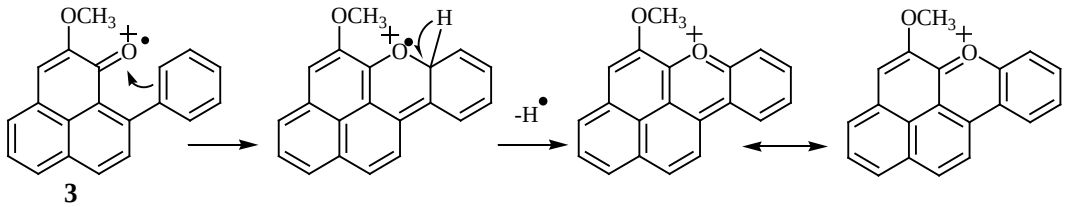


Figure 4.4 Formation of $[M-H]^+$ ion of methoxyanigorufone (**3**) under positive ionization conditions in EIMS.

Compounds **1**, **2** and **3**, the ^{1}H NMR spectra of these compounds showed proton signals of 9-phenylphenalenone characteristics. By comparing the ^{1}H NMR spectra to those of authentic phenylphenalenones, compounds **1**, **2** and **3** were identified as anigorufone (**1**), hydroxyanigorufone (**2**) and methoxyanigorufone (**3**), respectively.

The EIMS spectrum of compound **7** showed an $[M]^+$ ion at *m/z* 302 corresponding to the molecular formula of $C_{20}H_{14}O_3$. The NMR data of compound **7** match those of 9-(4´-hydroxyphenyl)-2-methoxyphenalen-1-one, which was reported by Kamo *et al.*, (1998b), but not the isomeric 4-(4´-hydroxyphenyl)-2-methoxyphenalen-1-one reported in the same publi-

cation (Kamo *et al.*, 1998b) and also not the corresponding (4´-methoxyphenyl)-phenalenones [9-(4´-methoxyphenyl)-2-hydroxyphenalen-1-one (**3**) and 4-(4´-methoxyphenyl)-2-hydroxyphenalen-1-one] (**22**), for which reference spectra were available inhouse.

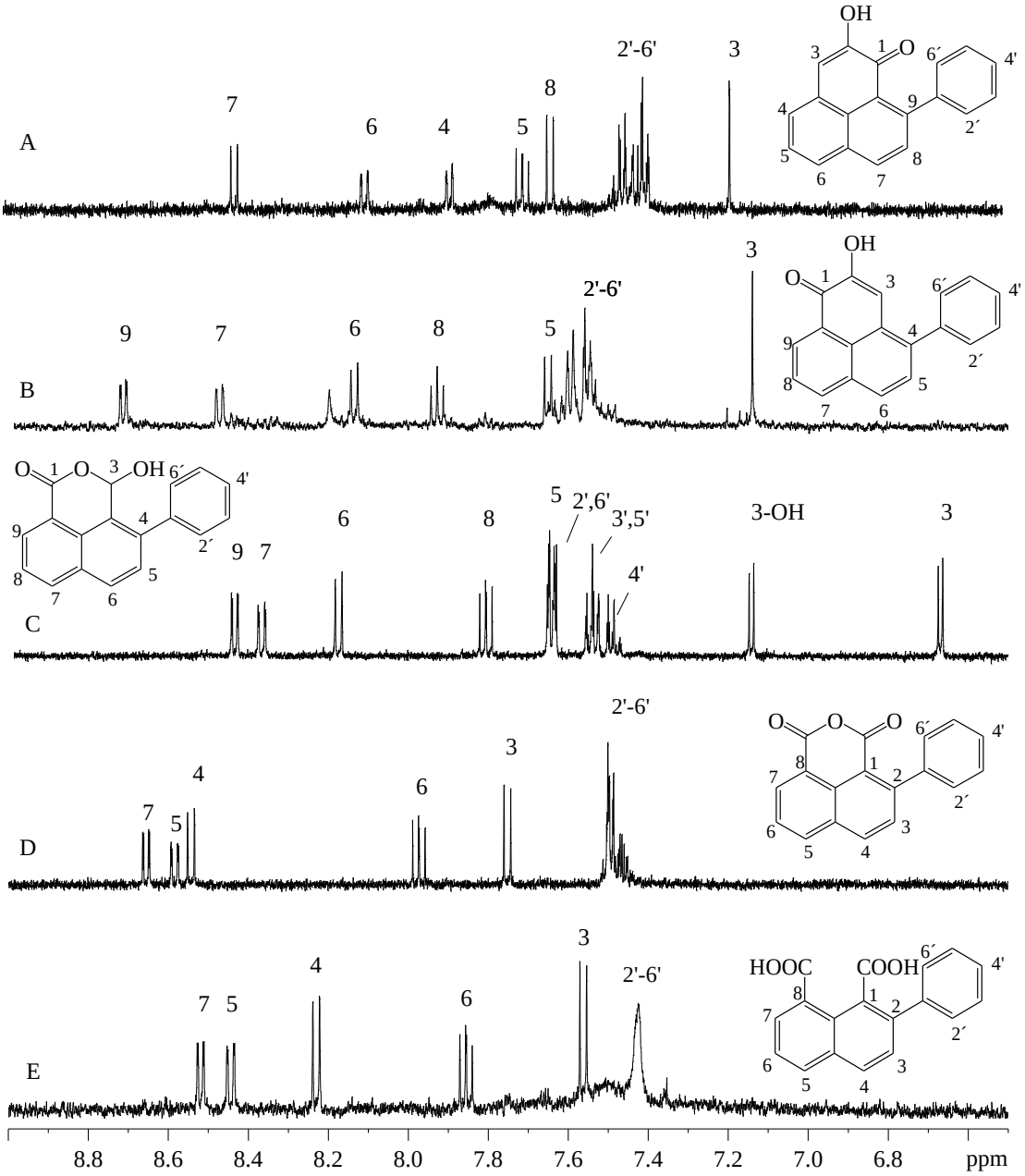


Figure 4.5 A comparison of ^{1}H NMR spectra shows characteristics of phenylphenalenones from different groups. **A**) Anigorufone (**1**), a 9-phenylphenalenone; **B**) isoanigorufone (**20**), a 4-phenylphenalenone; **C**) 3-hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (**62**); **D**) 2-phenyl-1,8-naphthalic anhydride (**33**), **E**) 2-phenylnaphthalene-1,8-dicarboxylic acid (**63**). Compounds **62** (spectrum **C**), **33** (spectrum **D**) and **63** (spectrum **E**) are phenylphenalenone derivatives with different degrees of oxidation.

4.3.3.2 Structure elucidation of 4-phenylphenalenones

Structure elucidation by means of NMR and MS techniques revealed the common characteristics of 4-phenylphenalenone. Like the ^{1}H NMR spectra of 9-phenylphenalenones, the ^{1}H NMR signal pattern of 4-phenylphenalenones exhibited AB and AMX spin systems. However, in contrast to 9-phenylphenalenones, the most downfield signal of 4-phenylphenalenone is not H-7 (part of AB system) but the doublet of doublet signal of H-9 (J_1 ~7.3 Hz, J_2 ~ 1.3 Hz), which is part of the AMX spin system. The downfield shift of H-9 is due to the strong deshielding effect of carbonyl group in the *peri* position (Fig **4.5** B). The EIMS showed the higher intensity of the $[M]^+$ ion than the $[M-H]^+$ ion (Thomas 1971; Bick and Blackman, 1973; Kamo *et al.*, 1998b).

Compound **20**, the MS spectrum showed a $[M]^+$ ion at *m/z* 272 corresponding to the molecular formula of $C_{19}H_{12}O_2$. Its ^{1}H NMR spectrum matched that of authentic isoanigorufone (**20**). Compound **21**, the MS spectrum showed a $[M]^+$ ion at *m/z* 288 corresponding to the molecular formula of $C_{19}H_{12}O_3$. By comparing the NMR data with reference data (Luis *et al.*, 1996), this compound was identified as irenolone (**21**).

4.3.3.3 Structure elucidation of the ring A- oxidized phenylphenalenone-related compounds

This group includes 2-phenyl-1,8-naphthalic anhydrides, 2-phenyl-naphthalene dicarboxylic acids and 2-phenylbenzo[*de*]isochromen-1-ones. The ^{1}H NMR spectra of 2-phenyl-1,8-naphthalic anhydride derivatives showed specific characteristics. The two doublets of doublet proton signals of the AMX spin system (J_1~ 7.3 Hz, J_2~1.3 Hz) appear in the downfield region. The most downfield signals are those of the protons at the *peri* position to carbonyl groups (Fig. **4.5** C-E). These signals are attributed to H-7 of dicarboxylic acids (for example, **63** in Fig. **4.5** E) and 1,8-naphthalic anhydrides (for example **33** in Fig. **4.5** D) and H-9 of phenyl–benzo[*de*]isochromen-1-one (for example, **62** in Fig. **4.5** C). The effect of the other carbonyl group (C1-C=O) in the 2-phenyl-1,8-naphthalic anhydrides led to the downfield shift of the doublet proton signal of the AB spin system (H-4) when compared to the other ring A-oxidized phenylphenalenone derivatives.

Compound **33**, the EIMS spectrum showed a $[M]^+$ ion at *m/z* 274, indicating the molecular formula of $C_{18}H_{10}O_3$. The ^{1}H NMR spectra matched those of 2-phenyl-1,8-naphthalic anhydride (**33**).

Compound **62** ($[\alpha]_D^{22}$ = +17.18^o) is a new natural product. The chemical structure was identified using 1D and 2D NMR and MS. The ^{1}H NMR spectrum (acetone-d_6) of compound **62** exhibited signals of ten non-exchangeable protons. The signals of the AB spin system ap-

peared at δ 7.64 and 8.18 and were assigned to H-5 and H-6. The signals of the AMX spin system, appearing at δ 8.38, 7.81 and 8.44, were attributed to H-7, H-8 and H-9, respectively. The multiplet signals at δ 7.65 (2H), 7.55 (2H) and 7.49 (1H) were assigned to H-2´/6´, H-3´/5´and H-4´of the phenyl ring, respectively. The spectrum displayed two additional doublets at δ 6.67 and 7.15 that were coupled with each other. The latter disappeared and the doublet at δ 6.67, a methine signal (H-3), collapsed to a singlet signal when the ^{1}H NMR of compound **62** was measured in MeOH-d_4. This indicated that the doublet signal at δ 7.15 was from an exchangeable proton and therefore was assigned to 3-OH. The proton signal (H-9) of the AMX spin appeared at the most downfield area, suggesting the *peri* position of the carbonyl group. But the deshielding effect of the carbonyl in **62** is less pronounced than in other 4-phenylphenalenones, whose H-9 signal appeared more downfield than 8.5 ppm. Moreover, the singlet signal of H-3 in the spectrum of compound **62** was also largely shifted upfield compared to the singlet signal of H-3 of isoanigorufone (**20**). A related structure with a similar ^{1}H NMR spectrum, namely 3-hydroxy-4-(4'-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**39**), was reported as a racemic mixture (Kamo *et al.*, 2001).

Table 4.3 ^{1}H NMR (500 MHz), ^{13}C NMR data (125 MHz) and HMBC correlations of 3-hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (**62**) (acetone-d_6, TMS).

Position	**^{13}C (δ)**	**^{1}H** (δ, integral, mult., coupling constant)	**HMBC**
1	164.4	-	-
3	95.2	6.67 (1H, d, J = 5.8 Hz)	C-1, C9b
3a	127.2	-	-
4	139.8	-	-
5	130.4	7.64 (1H, d, J = 8.5 Hz)	C-3a, C-4, C-6a, C-1´
6	129.5	8.18 (1H, d, J = 8.5 Hz)	C-4, C-6a, C-7, C-9b
6a	132.4	-	-
7	134.7	8.38, (1H, dd, J_1 = 8.3 Hz, J_2 = 1.1 Hz)	C-6, C-9, C-9b
8	127.3	7.81 (1H, dd, J_1 = 8.3 Hz, J_2 =7.2 Hz,)	C-6a, C-9, C-9a
9	130.7	8.44 (1H, dd, J_1 = 7.2 Hz, J_2 = 1.1 Hz)	C-1, C-6a, C-7, C-9b
9a	121.7	-	-
9b	128.5	-	-
1´	132.5	-	-
2´, 6´	130.4	7.65 (2H, m)	C-4, C-1´, C-4´
3´, 5´	129.4	7.55 (2H, m)	C-1´, C-4´
4´	129.0	7.49 (1H, m)	C-2´, C-6´
3-OH	-	7.15 (1H, d, J = 5.8 Hz)	

The chemical structure of **62** was further elucidated using HMQC and HMBC experiments. The HMBC signals of H-7 to C-6, C-9 and C-9b, H-6 to C-4, C-7 and C-9b, H-9 to C-9b and C-7, H-5 to C-3a and C-6a, H-8 to C-6a and C-9a confirmed the connection of the AB and AMX spin systems and thereby the naphthalene moiety (rings B and C) . The long-range

correlation signals of H-3 to the carbonyl carbon C-1 and C-9b, 3-OH to C-1, and H-9 to C-1 established the structure of the 3-hydroxy-benzo[*de*]isochromen-1-one. The HMBC signals of H-2´/6´ to C-4 and H-5 to C-1′ indicated the phenyl substitution at C-4 position (Table **4.3**). HR-ESIMS of compound **62** showed a $[M+Na]^+$ ion at *m/z* 299.0676 (calc. for $C_{18}H_{12}O_3Na$ 299.0684) corresponding to the molecular formula of $C_{18}H_{12}O_3$. From these data, compound **62** was identified as 3-hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (**62**). In order to compare the chemical shifts and coupling pattern of the proton signals of **62** to 4-phenylphenalenone, the numbering system of compound **62** used in this work does not follow the IUPAC system.

Compound **39**, the ^{1}H NMR spectrum displayed an AA´BB´ spin system at δ 7.50 and 7.01, indicating a *para*-substituted phenyl ring instead of a mono-substituted phenyl in **62**. The HR-ESIMS spectrum of **39** showed a $[M-H]^-$ ion at *m/z* 291.0653 (calc. for $C_{18}H_{11}O_4$ 291.0657), indicating the molecular formula of $C_{18}H_{12}O_4$. From these data, compound **39** was identified as the above-mentioned 3-hydroxy-4-(4'-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**39**). Comparing the spectrum to reference ^{1}H NMR data (Kamo *et al*., 2001) confirmed this result.

Compound **63**, the HR-ESIMS spectrum showed a $[M-H]^-$ ion at *m/z* 291.0653 (calc. for $C_{18}H_{11}O_4$ 291.0657), indicating the molecular formula of $C_{18}H_{12}O_4$. The ^{1}H NMR spectrum of **63** closely resembled that of 2-(4-methoxyphenyl)-naphthalene-1,8-dicarboxylic acid (**37**) (Otálvaro, 2004). The difference in the ^{1}H NMR signals of the lateral phenyl ring of compounds **37** and **63** indicated that the last compound was a de-*O*-methyl analogue of (**37**). Hence, the structure of compound **63** was established as 2-phenylnaphthalene-1,8-dicarboxylic acid. Interestingly, a broad ^{1}H NMR signal of H-2'/6' of compound **37** confirmed the close spatial proximity between 1-COOH and the phenyl substituent attached to C-2 in 2-phenylnaphthalene-1,8-dicarboxylic acids. To confirm the structure of compound **63**, a methyl derivative was prepared by applying an ethereal solution of diazomethane for 20 min. The *O*-methylated product was isolated and analysed by ^{1}H NMR. The signals of the AB system were observed at δ 8.26 (H-4) and 7.65 (H-3). The signals of the AMX system appeared at δ 8.28, 7.71 and 8.04, and were assigned to H-5, H-6 and H-7. The signal of the phenyl ring changed from a broad singlet to two multiplets. An additional methyl ester signal appeared at δ 3.72, indicating methylation of only one of the two carboxyl groups. Unfortunately, too little methyl ester was available to unambiguously assign the methylation position by HMBC. However, the resonance signal at δ 3.72 was closer to the values of δ 3.79 – 3.85 found for 8-OCH_3 than to the values of δ 3.48 – 3.51 found for 1-OCH_3 in methyl esters of 4-hydroxyphenylnapththalene-dicarboxylic acids (Kamo *et al.*, 1998b). Thus, the methyl ester **64** is likely 8-(methoxycarbonyl)-2-phenylnaphthalene-1-carboxylic acid.

4.3.3.4 Structure elucidation of the condensed dimer phenylphenalenones

By comparing ^{1}H NMR to reference data, compound **47** was identified as anigorootin (**47**). Compound **48** showed the ^{1}H NMR and ^{1}H,^{1}H COSY spectra that matched those of 4´-hydroxyanigorootin (**48**) (Otálvaro *et al.*, 2002b), a non-symmetrical phenylphenalenone of the condensed dimer type. Compound **49** was the most polar compound, comparing to **47** and **48**. Comparisons to reference NMR data (Otálvaro *et al*., 2002b) helped identify compound **49** as 4′,4″-dihydroxy anigorootin (**49**).

4.3.4 Spectroscopic data of the isolated compounds

Spectroscopic data of anigorufone (**1**), hydroxyanigorufone (**2**), methoxyanigorufone (**3**), 9-(4´-hydroxyphenyl)-2-methoxyphenalen-1-one (**7**), isoanigorufone (**20**), irenolone (**21**), 2-phenyl-1,8-naphthalic anhydride (**33**), 3-hydroxy-4-(4´-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**39**), anigorootin (**47**), 4´-hydroxyanigorootin (**48**), 4,4´-dihydroxyanigorootin (**49**), and 2-phenylnaphthalene-1,8-dicarboxylic acid (**63**), see Appx **3**. The data matched those of authentic reference compound available in our inhouse database.

3-Hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (62)

UV (MeCN-H_2O): λ_{max} 225, 250, 321 nm; $[\alpha]_D^{22}$ = +17.18° (*c* 0.00022, MeOH); ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): See Table **4.3**.; ^{13}C-NMR (125 MHz, acetone-d_6, TMS as internal standard): See Table **4.3**.; HR-ESIMS: *m/z* (rel. int. %) $[M+Na]^+$ 299.0677 (100), (calc. for $C_{18}H_{12}O_3Na$ 299.0684), corresponding to the molecular formula $C_{18}H_{12}O_3$.

8-(Methoxycarbonyl)-2-phenylnaphthalene-1-carboxylic acid (64)

UV (MeCN-H_2O): λ_{max} 209, 241, 288 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.28 (1H, d, *J* = 8.2, 1.3 Hz, H-5), 8.26 (1H, d, *J* = 8.5 Hz, H-4), 8.04 (1H, dd, *J* = 7.2, 1.1 Hz, H-7), 7.71 (1H, dd, *J* = 8.2, 7.2 Hz, H-6), 7.65 (1H, d, *J* = 8.5 Hz, H-3), 7.46 (3H, m, H-3´,4´,5´), 7.29 (2H, m, H-2´,6´), 3.72 (3H, s, 8-$COOCH_3$).

4.4 Discussion

4.4.1 Phenylphenalenones were identified as natural products in *Musa* sp. and *Ensete glauca* in Thailand

Thai *Musa* sp. and related species have been phytochemically screened in order to obtain information about the profiles of natural product, especially phenylphenalenone-type compounds. Banana and plantains are economically important plants in Thailand. *Musa* culti-

vars in Thailand are more diverse than in other main banana-producing countries, where only a few cultivars for example, *Musa* AAA "Cavendish" -- supply the world's fruit markets. Even though banana cultivation in Thailand is mostly for local consumption, there are 59 different cultivars from different genome groups, according to Silayoi and Babpraserth (1983). Studies of Thai bananas have so far focused mostly on classification and the relationship among cultivars (Ruangsuttapha *et al*., 2007; Kidamrongsont *et al.*, 2008). The diversity of banana cultivars in Thailand and the diverse profiles of natural products could be interesting in terms of plant resistance to diseases. Here, we report the occurrence of phenylphenalenones and related natural products as chemical constituents in Thai *Musa* and their related species, *Ensete glauca,* collected in Thailand for the first time.

Musa sp. and *Ensete glauca* were collected from agricultural fields and botanical gardens. The green parts and the rhizomes of plants were separated and extracted. Primary screening by TLC and further screening by HPLC using leaf extracts and rhizome extracts were conducted. The rhizome extracts were more dominant in natural products according to the TLC patterns and HPLC chromatograms, especially in the range (R_f for TLC and R_t for HPLC) that is closely related to that of authentic phenylphenalenone compounds. Therefore, only rhizome extracts were further investigated. Identification of phenylphenalenones in Thai *Musa* sp. and *Ensete glauca* was done by chromatographic and spectroscopic methods including 1D, 2D NMR and MS (Luis *et al*., 1996; Kamo *et al*., 2001; Otalvaro *et al*., 2002b).

Phenylphenalenone-type compounds were identified as main natural products in Thai *Musa* and *Ensete glauca*. This study revealed that 9-phenylphenalenones and dimeric phenylphenalenones were more dominant than 4-phenylphenalenones and the ring A-oxidized phenylphenalenone-related compounds. The structures of the isolated compounds are shown in Fig. **4.3**). For the occurrence of phenylphenalenones analysed by HPLC methods, two groups of plants showed different patterns.

- *Dimeric phenylphenalenone-dominant group*

The edible banana cultivars selected in this study belonged to the condensed dimer phenylphenalenone dominant group, in which three dimeric phenylphenalenones **47-49** were dominantly detected. Bananas of different genome groups showed slightly different phenylphenalenone patterns. Condensed dimeric phenylphenalenones **47-49** were previously isolated from *Anigozanthos sp* (Hölscher and Schneider, 1999) as well as from *M. acuminata* (Otálvaro *et al*., 2002b). 9-Phenylphenalenone-type compounds with hydroxyl substitution at C-4 position such as 4-hydroxyanigorufone, which was found in *A. flavidus*, might be the monomeric precursor (Otálvaro *et al.*, 2002b). 4-Hydroxy-2-methoxy-9-phenyl-1*H*-phenalen-1-one (**18**), a compound closely related to 4-hydroxyanigorufone, was also found in *Musa* (Otálvaro, 2004).

The correlation between the content of phenylphenalenone (4- and 9- phenylphenalenones) and phytopathological properties in *Musa* has been studied by Otálvaro *et al.* (2002a). Four *Musa* varieties from different genome groups were analysed by these authors for phenylphenalenone occurrence. *Musa acuminata* (ABB) "Pelipita", a non-commercial variety but highly resistant to *Microsphaerella fijiensis* and *Fusarium oxysporum* contained a high amount of phenylphenalenones.

In Thailand, the susceptibility of the commercially important banana cultivars to infectious diseases has been reported (Silayoi, 2002). The susceptibility of *Musa* cultivars varies widely. For example, *Musa* (AAA) "Hom Thong" is susceptible to both *Fusarium* wilt and Black Sigatoka; *Musa* (ABB) "Nam Wa" is susceptible to *Fusarium* wilt but more resistant to Black Sigatoka. Phytochemical screening in this study revealed that the Thai commercial cultivars, *Musa* (AA) "Leb Mue Nang", Musa (AAA) "Hochuchu" and *Musa* (ABB) "Nam Wa", contained very low levels of condensed dimer phenylphenalenone derivatives. Even though there is nothing reported about bioactivity of condensed dimer phenylphenalenones, their presence in *Musa* could contribute to banana's resistance to pathogens. Note that apart from the formation of phenylphenalenones as phytoalexins, other groups of natural products for example, flavonoids may also play important role in plant resistance to pathogens; other defense mechanism such as cell wall reinforcement may also be involved (De Ascensao and Dubery, 2000).

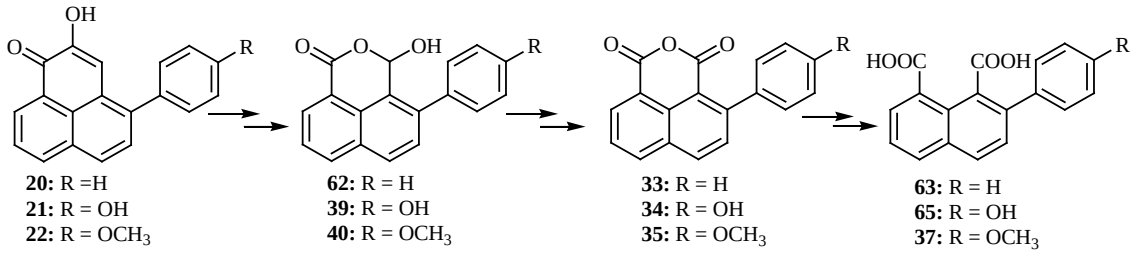
- *Monomeric phenylphenalenone-dominant group*

The dominant monomeric phenylphenalenone group in this study contained only two species. They are the ornamental bananas *M. coccinea* "Rattagatli" and *Ensete glauca*. In these two species, 9-phenylphenalenones were dominant. In *M. coccinea* (Rattagatli), hydroxyanigorufone (**2**) was detected as a main component together with 4′,4′-dihydroxyanigorootin (**49**). In *Ensete glauca*, very high levels of two *O*-methylated 9-phenylphenalenones, methoxyanigorufone (**3**) and 9-(4´-hydroxyphenyl)-2-methoxyphenalen-1-one (**7**), were found. Hölscher and Schneider (1998) reported the first phytochemical study of the genus *Ensete* in *E. ventricosum* from Ethiopia. In this plant, non-methylated 9-phenylphenalenones such as anigorufone (**1**) and hydroxyanigorufone (**2**) were found to be main constituents along with phenylphenalenones bearing a phenyl ring at the C-6 position (Hölscher and Schneider, 1998). The present investigation of *Ensete glauca* from Thailand is the second report of phenylphenalenones from this genus and the first from the Asian subgenus. Unlike *E. ventricosum* in Ethiopia, *E. glauca* is a wild plant whose natural habitat is the northern part of Thailand (Silayoi, 2002). Nowadays, this plant is used ornamentally. *Ensete* was reported to be susceptible to bacterial wilt caused by *Xanthomonas campestris* pv. *musacearum* (Xcm) (Ndungo *et al*., 2006). The high content of methoxyanigorufone (**3**) and

9-(4´-hydroxyphenyl)-2-methoxyphenalen-1-one (**7**) in *E. glauca* suggests that 9-phenylphenalenones are major compounds playing a role in response to pathogenic attacks.

4.4.2 New phenylphenalenone derivatives and biosynthetic implications of isolated phenylphenalenone derivatives

3-Hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (**62**) is a new 4-phenylphenalenone derivative isolated from the rhizomes of *Musa* (AA) "Thong Ngoei". It was found together with its known 4´-hydroxyl derivative 3-hydroxy-4-(4'-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**39**). The biosynthetic pathway of phenylphenalenones in Musaceae, especially conversion between 9-phenylphenalenones and 4-phenylphenalenones as well as the biosynthesis of 2-phenyl-1,8-naphthalic anhydrides, has been discussed and proposed (Luis *et al.*, 1995; Kamo *et al.*, 2000; Kamo *et al.*, 2001). Biosynthetic studies by feeding labelled precursors to *Musa* plant revealed that 4-phenylphenalenones and 9-phenylphenalenones are precursors of 2-phenyl-1,8-naphthalic anhydrides (Kamo *et al.*, 2000). The hypothetical biosynthesis of 4-phenylphenalenones from 9-phenylphenalenones via dihydroxy intermediates such as 2,3-dihydro-2,3-dihydroxyphenylphenalenones (for example, **12** and **29**, Fig. **1.4a**) and trihydroxylated intermediates such as **31** and **32** were also proposed by Kamo *et al.* (2000). Feeding [4´-$O^{13}CH_3$]2-hydroxy-9-(4´-methoxyphenyl)-phenalen-1-one (**4**) to *in-vitro* plants of *M. acuminata* revealed the conversion of the precursor to oxidized compounds **11**, **35** and **37** (Fig. **1.4a**) (Otálvaro, 2004) but not to 4-phenylphenalenones. The author suggested that the dihydroxy and trihydroxy phenylphenalenones were intermediates of the oxidative pathway of 9-phenylphenalenone to form 2-phenyl-1,8-naphthalic anhydrides (Otálvaro, 2004). Considering its oxidative state, compound **62** could be formed from **20** and then oxidized to give compound **33**. The anhydride **33** could then be converted to dicarboxylic acid **63** (Scheme **4.1**). The discovery of compounds **62**, **63**, and **33** in *Musa* (AA) "Thong Ngoei" and *Musa* (BBB) "Thep Panom" supported this hypothesis. The formation of compounds **39**, **34**, and a putative compound **65** from **21** and **40**, **35**, **37** from **22**, are probably possible in a parallel oxidative pathway. The first step in this pathway may be an auto-oxidative process rather than an enzyme-catalyzed process (see Chapter **6**).



Scheme 4.1 Hypothetical oxidative biosynthetic pathways in *Musa.*

Chapter 5 Responses of *Musa acuminata* to microorganisms and chemical elicitation

5.1 Introduction

Plants protect themselves from environmental stresses and pathogen attack by various defense mechanisms including the formation of phytoalexins (Dixon, 2001) (see **1.2**). *Musa acuminta* (banana) is an important crop plants facing serious problems from infectious diseases. The enhanced levels of phenylphenalenones in banana *(Musa sp*., Musaceae) after plants were infected by pathogenic fungi (Luis *et al*., 1994, 1996; Kamo *et al*., 1998b, 2000) suggested the role of these compounds as phytoalexins (see also Chapter **4**). To study the response of *Musa* plants to microbial challenge and chemical elicitation, sterile *in-vitro* plants of *M. acuminata* were used. Here, the modification of natural products' profiles, especially phenylphenalenones, was investigated.

5.2 Materials and methods

5.2.1 Plant materials

In-vitro Musa acuminata were grown on MS agar and transferred to 100 ml liquid MS medium 7-14 days before being treated and kept under the conditions described in **2.1**.

5.2.2 Microbial treatment and chemical elicitation

Sporobolomyces salmonicolor and *Pseudomonas fluorescens* were kindly provided from the Hans Knöll Institute, Jena, Germany. The microorganisms were maintained on potato-dextrose-agar (*S. salmonicolor*) or nutrient agar (*P. fluorescens*) at 28 ºC. Suspensions of conidia of *S. salmonicolor* were prepared from 5 day-old liquid culture in potato-dextrose broth. The cultures were filtered through sterile cotton wool. Cell concentrations were determined using a cell-counting apparatus. The cell suspension was centrifuged and re-suspended in 0.9% NaCl solution before inoculation. The cell concentration of an overnight culture of *P. fluorescens* was measured at OD_{600} compared to standard Mc Farland. The cell suspensions were centrifuged and re-suspended in sterile 0.9% NaCl before inoculation. Suspensions of microorganisms were inoculated under sterile conditions into the culture and adjusted to a total concentration of 10^6 cells/ml medium. The same volume of sterile 0.9% NaCl solution was used for control plants. Plants were kept under the conditions described in **5.2.1.** Plants treated with *S. salmonicolor* were harvested after 5-7 days, while plants treated with *P. fluorescens* were harvested after three weeks.

Jasmonic acid (JA) was prepared as an aqueous solution (10 mM) and sterilized by filtering (0.22 µm filter). Sterile JA solution (10 mM) was added to the plant cultures and adjusted to a total concentration of 100 µM. Sterile water instead of JA solution was used in control plants. Plants were kept under the conditions described in **5.2.1**. After 7 days of treatment, plants were harvested.

5.2.3 Extraction

Plants were separated to root and leaf parts and then exhaustively extracted with 95% EtOH. The extracts were evaporated (≤ 50 ^{o}C) to dryness. The residues were re-suspended in a mixture of MeOH and H_2O (1:1) (30 ml) and partitioned with *n*-hexane (3 x 30 ml), CH_2Cl_2 (3 x 30 ml) and EtOAc (3 x 50 ml). The fractions were evaporated (≤ 50 ^{o}C) to dryness. The MS media (100 ml) from both control and treated plants were filtered and then partitioned with EtOAc (3 x 100 ml). The EtOAc extracts were evaporated to dryness (≤ 50 ^{o}C).

5.2.4 HPLC analysis of natural products in *Musa acuminata*

5.2.4.1 HPLC screening of phenylphenalenones

The CH_2Cl_2 extracts obtained from **5.2.3** were dissolved in MeOH (5 mg/ml). The methanol solutions (aliquots of 50 µl) were analyzed for a change in the profile of natural products by HPLC using *anal. HPLC system_1* (see Appx **1.1**). Phenylphenalenones in the extract were identified by comparing the R_t with those of authentic compounds.

5.2.4.2 Quantitative analysis of phenylphenalenones

***Calibration curves*:** Calibration curves for quantifying hydroxyanigorufone (**2**) and 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) were created as follows. Methanolic solutions of the compounds were prepared in five different concentrations. Hydroxyanigorufone (**2**): conc. 0.005, 0.01, 0.02, 0.05, 0.08 mg/ml; 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**): 0.05, 0.10, 0.20, 0.40, 0.80 mg/ml. Pyrene (0.05 mg/ml) was added as an internal standard. The solutions were subjected to HPLC using *anal. HPLC system_3* (see Appx **1.1**). Calibration curves were created by plotting the concentrations of the phenylphenalenones versus the ratio of the HPLC peak areas (UV 254 nm) normalized to pyrene.

***Modification of the natural products' profiles in response to S. salmonicolor*:** Plants were divided into two groups: *S. salmonicolor*-treated group (8 plants) and control group (8

plants). Treatment was done as described in **5.2.2**. At day 1, 3, 5 and 7 after treatment, plants both from control group (2 plants) and treated group (2 plants) were extracted as described in **5.2.3**. In order to quantify total phenylphenalenones, the extracts were partitioned with EtOAc. The residues of EtOAc fractions were dissolved in MeOH (2.5 mg/ml). Aliquots (15 µl) of the MeOH solutions were analyzed by HPLC using *anal. HPLC system_3* (see Appx **1.1**). Pyrene (0.05 mg/ml) was added as an internal standard.

Modification of the natural products' profiles in response to JA: Plants were divided into two groups: JA-treated group (3 plants) and control group (3 plants). Treatment was done as described in **5.2.2**. Lyophilized plant material (100 mg) was extracted with EtOAc (3 x 15 ml). The residues were dissolved in MeOH (400 µl) and aliquots (15 µl) were analysed by HPLC using *anal. HPLC system_3* (see Appx **1.1**). Pyrene (0.05 mg/ml) was added as an internal standard.

5.2.5 Isolation and purification of phenylphenalenones from *M. acuminata*

Four *in-vitro* plants of *M. acuminata* were treated with *S. salmonicolor*. Four control plants were kept under identical conditions. Seven days after treatment, plants were harvested; roots and leaves of control (roots 47.0 g, leaves 29.4 g) and treated plants (roots 55.5 g, leaves 35.0 g) were separately cut in pieces and then extracted as described in **5.2.3**. The extracts were analyzed by HPLC as described in **5.2.4.1**.

Isolation and purification of natural products were carried out as follows: The EtOAc extract of media was subjected to prep. HPLC using *prep. HPLC system_15* (see Appx **1.3**). Compounds **34** (430 µg) and **33** (100 µg) were obtained. The CH_2Cl_2 extract from roots (30 mg) was fractionated by RP18 short column (∅2 x 4 cm) using mixtures of MeOH 20% (50 ml), 40% (120 ml), 60% (200 ml), 80% (200 ml) in H_2O and pure MeOH (100 ml). The fraction eluted with 50% MeOH in H_2O showed the signals of the induced compounds corresponded to the preliminary HPLC screening (*anal. HPLC system_1* (see Appx **1.1**). The faction was further purified using *prep. HPLC system_18* (see Appx **1.3**). Compounds **34** (540 µg) and **66** (100 µg) were obtained. The fraction eluted with 60% MeOH in H_2O (2.5 mg) was further separated by *prep. HPLC system_16* (see Appx. **1.3**). Compound **2** (2 mg) was obtained. The peak collected at R_t 34.9 min was purified by *semiprep. HPLC system_6* (see Appx **1.2**). Compounds **7**, **21** and **39** were obtained. Compound **1** was obtained by *semiprep. HPLC system_7* (see Appx **1.2**). The EtOAc fraction (29.1 mg) was separated *prep. HPLC system_17* (see Appx **1.3**). Compound **67** (1 mg) was obtained.

5.3 Results

5.3.1 HPLC analysis of natural products in *M. acuminata* after challenging plants with biotic and non-biotic elicitors

HPLC (*anal. HPLC system_1*, see Appx **1.1**) and 1D and 2D NMR techniques were used to detect change in the profile of natural products in *M. acuminata*. The methanol-soluble fractions of the extracts from treated and non-treated plants were prepared in the same concentration. The same volumes of the solutions were subjected to HPLC analysis. The HPLC chromatograms showed the increase of peak numbers and peak intensities, indicated that natural products were induced in treated plants (Fig. **5.1**).

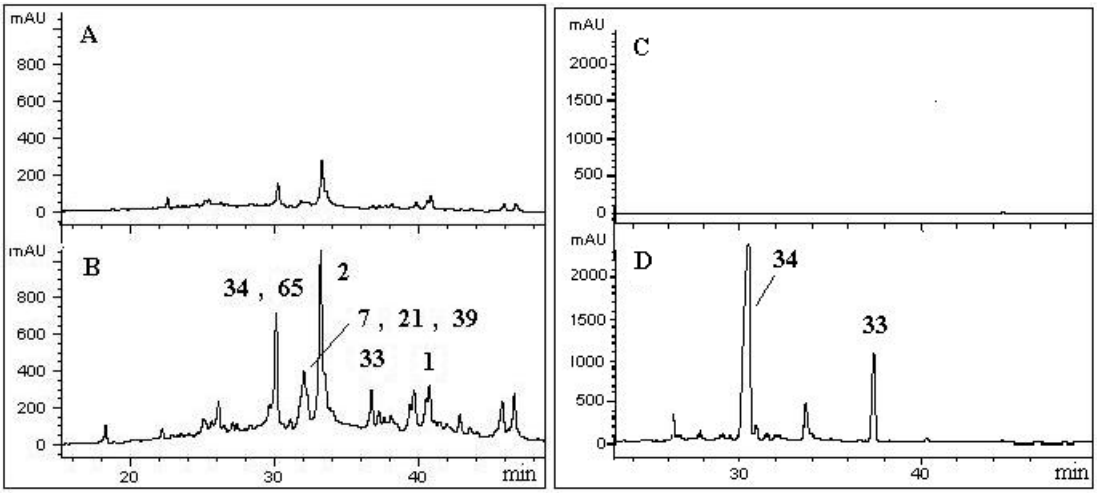


Figure 5.1 Selected HPLC chromatograms (UV 254 nm, *anal. HPLC system_1*, see Appx **1.1**) of *M.acuminata* plants treated with *Sporobolomyces salmonicolor*: CH_2Cl_2 fraction from control rhizome (**A**) and treated rhizome (**B**); EtOAc fraction from culture media of control (**C**) and treated *M. acuminata* (**D**).

Table 5.1 Phenylphenalenone-related compounds detected as induced natural products in *M. acuminata* after challenging with elicitors

Treatments	Extracts	Compounds							
		1	2	7	21	33	34	39	66
Sporobolomyces salmonicolor	leaf	-	-	-	-	-	?	-	-
	root	x	x	x	x	x	x	x	x
	media	?	?	?	?	x	x	?	?
Pseudomonas fluorescens	leaf	-	?	-	-	-	?	-	-
	root	-	x	x	x	x	x	x	?
	media	-	-	?	?	x	x	?	-
Jasmonic acid (JA)	leaf	-	-	-	-	-	-	-	-
	root	-	?	-	-	x	x	-	-
	media	?	?	?	?	?	?	?	?

Note: x = detected; ? = not clearly defined or presented in low level; - = not detected.

M. acuminata treated with biotic elicitors, the non-pathogenic strains, *Sporobolomyces salmonicolor* and *Pseudomonas fluorescens* as, as well as with a non-biotic elicitor, JA, showed a similar pattern of induced natural products. The compounds were identified based on R_t from HPLC analysis (*anal. HPLC system_1*, see Appx **1.1**) and by comparing UV absorption of the extract components to authentic phenylphenalenones. The results showed that all the induced natural products are phenylphenalenone-related compounds (Table **5.1)**.

5.3.2 Phenylphenalenone levels in response to elicitation

Quantification of phenylphenalenones in response to elicitation was done by HPLC method. Levels of two main phenylphenalenones, hydroxyanigorufone (**2**) and 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**), in rhizome extracts were calculated from calibration curves of authentic compounds (see Appx **5.1.**).

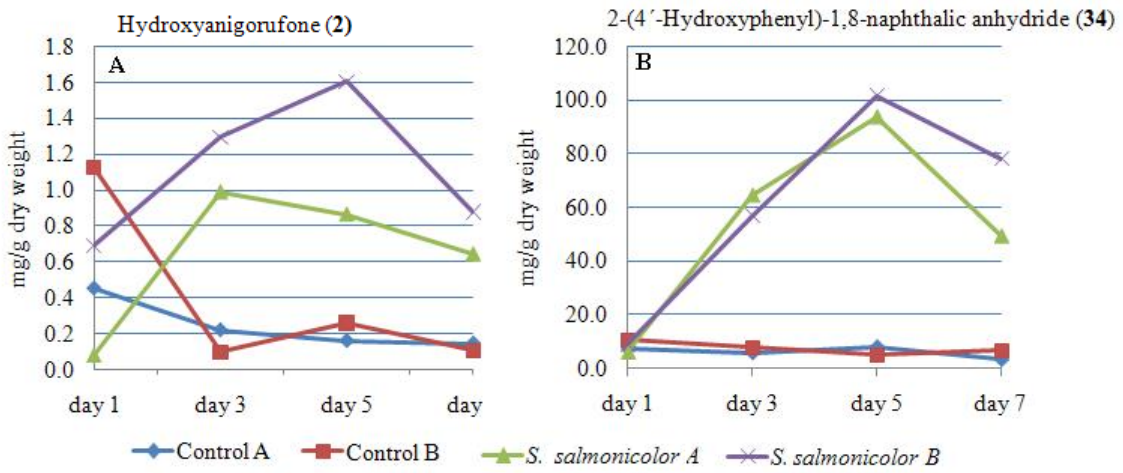


Figure 5.2 Change of levels of 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) and hydroxyanigorufone (**2**) after treatment with *S. salmonicolor*, analyzed by HPLC (*anal. HPLC system_3*, see Appx **1.1**) using pyrene (0.05 mg/ml) as an internal standard.

***Elicitation with Sporobolomyces salmonicolor*:** At day 1, 3, 5 and 7 after elicitation, the increase of the concentration of **2** and **34** were observed. The level of **2** reached maximum at day 3 and 5 while the level of **34** were at the maximum at day 5. The levels of both compounds dropped by day 7 after elicitation (Fig **5.2**, for more detail see Appx **5.2**).

Elicitation with JA: Quantitative analysis of *M. acuminata* after JA treatment for seven days showed that the concentration of 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) increased to 360 µg/g dry weight (control 148 µg/g), while the concentration of hydroxyanigorufone (**2**) was increased to 122 µg/g (control 14 µg/g).

5.3.3 Isolation, purification and structure elucidations of the isolated natural products

Nine natural products were isolated and purified from root extracts of the *S. salmonicolor*-treated plants. Using spectroscopic techniques, all the isolated compounds were identified as phenylphenalenone-related compounds. From the CH_2Cl_2 fraction compounds **1**, **2**, **7**, **21**, **33**, **34**, **39** and **66** were detected by HPLC as induced natural products as described in **5.3.1**. Compound **67** was isolated from EtOAc extract; however the HPLC chromatogram did not clearly indicate whether or not **67** was induced. The chemical structures of the isolated natural products are shown in Fig **5.3**.

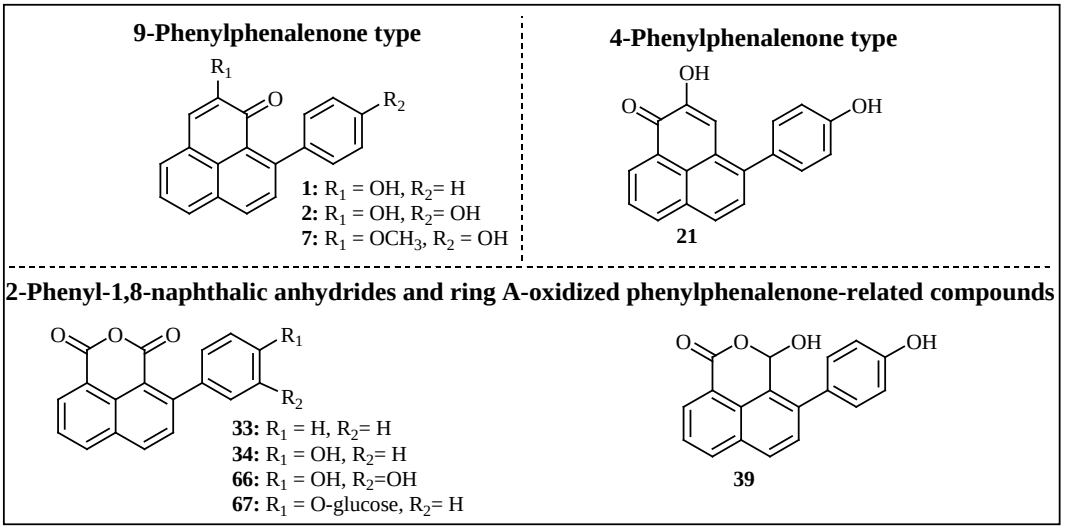


Figure 5.3 Induced phenylphenalenones in *Musa acuminata* after elicitation with JA or biotic elicitors (*S. salmonicolor* and *P. fluorescens*). The compounds were identified by HPLC (*anal. HPLC system_1, 3* see Appx **1.1**), 1D and 2D NMR and MS. Compound **67** was not clearly defined as an induced compound.

The isolated compounds were identified as anigorufone (**1**), hydroxyanigorufone (**2**), 9-(4'-hydroxyphenyl)-2-methoxyphenalen-1-one (**7**), irenolone (**21**) and 3-hydroxy-4-(4'-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**39**). Structure elucidation of these compounds was already described in Chapter **4.3**. The EIMS spectrum of compound **34** displayed an $[M]^+$ ion at *m/z* 290 corresponding to the molecular formula of $C_{18}H_{10}O_4$. From these data, compound **34** was identified as 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**). ^{1}H NMR,^{1}H,^{1}H- COSY, HSQC and HMBC date were in agreement with those published by Hirai *et al*. (1994).

Compound **66** showed a ^{1}H NMR pattern that was similar to compound **34**, suggesting that they were related compounds. ^{1}H NMR and ^{1}H,^{1}H COSY spectra revealed the typical AMX and AB spin systems of 2-phenyl-1,8-naphthalic anhydrides. The signals of the AMX spin system appeared at δ 8.57, 7.96 and 8.64 and belonged to H-5, H-6 and H-7. The AB spin

system at δ 7.78 and 8.53 was assigned to H-3 and H-4. Instead of the AA´BB´ spin system of the *para*-hydroxylated phenyl ring of compound **34**, the spectrum of compound **66** displayed an ABX spin system at δ 7.34, 7.14 and 7.54 assignable to H-6´, H-5´and H-2´, respectively. The three bond-HMBC correlations of H-2´ and H-6´ to the oxygenated C-4´ (δ 154.4) and H-5´ to the oxygenated C-3´ (δ 154.0) suggested the presence of a 3´,4´-dihydroxyphenyl ring. The attachment of the 3´,4´-dihydroxyphenyl ring to C-2 position of 1,8-naphthalic anhydride skeleton was assigned by the long-range correlation of H-2´and H-6´ to C-2. The EIMS of compound **66** had an $[M+H_2O]^+$ ion of *m/z* 324, showing that compound **66** had one additional oxygen atom comparing to compound **34**. On the basis of MS and NMR data (Table **5.2**), compound **66** was identified as 2-(3´,4´-dihydroxyphenyl)-1,8-naphthalic anhydride (**66**).

Table 5.2 ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), ^{1}H,^{1}H COSY and HMBC correlations of 2-(3´,4´-dihydroxyphenyl)-1,8-naphthalic anhydride (**66**) (acetone-d_6, TMS), n.d. = non detected.

Position	**^{13}C (δ)**	**66**	**^{1}H,^{1}H COSY**	**HMBC**
		^{1}H (δ, integral, mult., coupling constant)		
1	n.d.	-	-	
1a	116.7	-	-	
2	149.0	-	-	
3	132.6	7.78 (1H, d, *J* = 8.5 Hz)	H-4	C-1a, C-4a, C-1´
4	135.4	8.53 (1H, d, *J* = 8.5 Hz)	H-3	C-2, C-5, C-7b
4a	132.4	-		
5	136.4	8.57 (1H, dd, J_1 = 8.2, 1.2 Hz)	H-6	C-4, C-7b, C-7
6	128.1	7.96 (1H, dd, J_1 = 8.2 , 7.3 Hz)	H-5, H-7	C-7a, C-4a
7	134.0	8.64 (1H, dd, J_1 = 7.3, 1.2 Hz)	H-6	C-5, C-7b, C-8
7a	120.5	-	-	
7b	132.1	-	-	
8	162.0	-	-	
1´	134.2	-	-	
2´	131.3	7.54 (1H, d, *J* = 2.2 Hz)	H-6´	C-4´, C-6´, C-2
3´	154.0	-	-	
4´	154.4	-	-	
5´	117.3	7.14 (1H, d, *J* = 8.3 Hz)	H-6´	C-1´, C-3´
6´	129.6	7.34 (1H, dd, J_1= 8.3, 2.2 Hz)	H-2´, H-5´	C-2´, C-4´, C-2

Compound **67** was isolated from the EtOAc fraction. It was the most polar compound among the isolated phenylphenalenones. The ^{1}H NMR spectrum of compound **67** showed 14 proton signals integrating to 16 protons in total. The signals in the non-aromatic region clearly showed the characteristics of a ß-glucose (Table **5.3**). The aromatic region of the ^{1}H NMR spectrum again showed the characteristics of 2-phenyl-1,8-naphthalic anhydrides similar to the aglycone part of compound **67**. The ^{1}H chemical shifts were closely related to those of 2-

(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**). The signals of the AB spin system at δ 7.77 and 8.52 were assigned to H-3 and H-4, respectively; those of the AMX spin system at δ 8.56, 7.95 and 8.64 are assigned to H-5, H-6 and H-7, respectively; and the signals of the AA´,BB´ spin system at δ 7.46 and 7.17 were assigned to H-2´/6´and H-3´/5´, respectively, of the *para*-substituted phenyl ring. The ^{1}H,^{1}H COSY, HMBC and HMQC data shown in Table **5.3** are consistent with the 2-phenyl-1,8-naphthalic anhydride skeleton.

Table 5.3 ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), ^{1}H,^{1}H COSY and HMBC correlations of 2-(4´-*ß*-glucosyloxyphenyl)-1,8-napthalic anhydride (**67**) (acetone-d_6, TMS), n.d. = non detected.

Position	**67**			
	^{13}C (δ)	**^{1}H** (δ, integral, mult., coupling constant)	**^{1}H,^{1}H COSY**	**HMBC**
1	n.d.	-	-	-
1a	116.6	-	-	-
2	148.0	-	-	-
3	132.8	7.77 (1H, d, *J* = 8.5 Hz)	H-4	C-1a, C-4a, C-4, C-1´
4	135.3	8.52 (1H, d, *J* = 8.5 Hz)	H-3	C-5, C-2, C-7b
4a	132.0	-	-	-
5	136.4	8.56 (1H, dd, *J* = 7.3, 1.0 Hz)	H-6	C-4, C-7, C-7b
6	128.0	7.95 (1H, dd, *J* = 8.2, 7.3 Hz)	H-5, H-7	C-7a, C-4a
7	133.8	8.64 (1H, dd, *J* = 8.2, 1.0 Hz)	H-6	C-5, C-8, C-7b
7a	120.5	-	-	-
7b	132.1	-	-	-
8	160.9	-	-	-
1´	135.1	-	-	-
2´/6´	130.9	7.46 (2H, d, *J* = 8.8 Hz)	H-3´, 5´	C-2, C-4´, C-6´
3´/5´	116.9	7.17 (2H, d, *J* = 8.8 Hz)	H-2´, 6´	C-1´, C-4´, C-5´
4´	158.8	-	-	-
1″	101.9	5.09 (1H, d, *J* = 7.5 Hz)	H-2″	C-4´
2″	74.7	3.52 (1H, m)	H-1″, H-3″	
3″	70.7	3.65 (1H, m)	H-2″, H-4″	
4″	71.4	3.49 (1H, m)	H-3″, H-5″	C-5″
5″	78.0	3.56 (1H, m)	H-4″, H-6″	C-4″
6″a	62.6	3.91 (1H, dd, *J* = 11.5, 2.4 Hz)	H-6″b, H-5″	-
6″b		3.74 (1H, dd, *J* = 11.5,5.5 Hz)	H-6″a, H-5″	C-5″

The aglycone part was confirmed by HR-EIMS showing an $[M]^{+}$ ion at *m/z* 290.0597 (calc. for $C_{18}H_{10}O_4$ 290.0579), corresponding to the molecular formula of $C_{18}H_{10}O_4$. From these NMR and MS data, the aglycone part was identified as 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**). The downfield shift (0.15-0.20 ppm) of the protons belonging to the AA´,BB´spin system suggested that the sugar moiety is attached to the 4´-*O*-position. The

glycoside structure was further confirmed by the HMBC correlation of H-1″ to C-4´ (δ 158.8), which was shifted downfield due to the de-shielding effect of the glucoside substituent. The coupling constant (J = 7.5 Hz) of the signal of the anomeric proton at δ 5.09 indicated *β*-configuration of the anomeric proton. On the basis of the data presented here, compound **67** was identified as 2-(4´–*ß*-glucosyloxyphenyl)-1,8-naphthalic anhydride (**67**). The molecular ion of the whole glucoside was not detected due to the cleavage of the sugar moiety in the HR-EIMS condition. Compound **67**, which is a new natural product, is the first 2-phenyl-1,8-naphthalic anhydride glycoside isolated from plants.

5.3.4 Spectroscopic data of the isolated compounds

Spectroscopic data of anigorufone (**1**), hydroxyanigorufone (**2**), 9-(4´-hydroxyphenyl)-2-methoxyphenalen-1-one (**7**), irenolone (**21**), 2-phenyl-1,8-naphthalic anhydride (**33**), 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**), and 3-hydroxy-4-(4´-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**39**) matched those of compounds previously isolated from Thai *Musa* sp. (Chapter **4**, see also Appx **3**).

2-(3´,4´-Dihydroxyphenyl)-1,8-naphthalic anhydride (66)

^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): see Table **5.2**; ^{13}C-NMR (125 MHz, acetone-d_6, TMS as an internal standard): see Table **5.2**; EIMS *m/z* (rel. int. %): 323.9 $[M+H_2O]^+$ (100), corresponding to the molecular formula $C_{18}H_{10}O_5$.

2-(4´-*ß*-Glucosyloxyphenyl)-1,8-naphthalic anhydride (67)

^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): see Table **5.3**; ^{13}C-NMR (125 MHz, acetone-d_6, TMS as an internal standard): see Table **5.3**; HR-EIMS *m/z* (rel. int. %): 290.0597 $[M-C_6H_{10}O_5]^+$ (20); (calc. for $C_{18}H_{10}O_4$ 290.0579), corresponding to the molecular formula $C_{24}H_{20}O_9$.

5.4 Discussion

5.4.1 Responses of *Musa acuminata* to elicitation

To study the response of *M. acuminata* to elicitation as well as the function of phenylphenalenones-related natural products in *Musa* plants, sterile *in-vitro* plants were treated with a non-pathogenic yeast strain, *Sporobolomyces salmonicolor*, a non-pathogenic bacteria, *Pseudomonas fluorescens*, and a chemical elicitor, JA. After plants were treated, plants were extracted and analyzed for the presence of natural products. HPLC analysis

showed an increase of natural products in root and media extracts from all treated plants. The HPLC chromatograms of treated plants showed more UV signals with higher intensities than did the HPLC chromatograms of control plants.

From the CH_2Cl_2 root extracts and EtOAc media extracts, induced compounds were isolated and identified. They belonged to phenylphenalenone-related compounds, including 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**), anigorufone (**1**), hydroxyanigorufone (**2**), 2-phenyl-1,8-naphthalic anhydride (**33**), 9-(4´-hydroxyphenyl)-2-methoxyphenalen-1-one (**7**), irenolone (**21**) and 3-hydroxy-4-(4´-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**39**). The site of phenylphenalenone biosynthesis in *M. acuminata* is not yet known. From literature reports, it is known that phenylphenalenones accumulate in the rhizomes, fruits and leaves of infected plants (Simmonds, 1982; Luis *et al*., 1995, 1996; Hirai *et al.*, 1994; Kamo *et al*., 1998b, 2001). In our experiment, we observed a dramatic increase of phenylphenalenone accumulation in the roots, which microorganisms directly contact.

Luis *et al*. (1994, 1996) reported hydroxyanigorufone (**2**) was a main phenylphenalenone together with other 9-phenylphenalenones and 4-phenylphenalenones in rhizomes of *Musa* after infection with *Fusarium oxysporum* F. sp. *cubense* race 4 (Foc4), the Panama disease causing fungus. The phenylnaphthalic anhydrides- were not detected in these studies (Luis *et al.*, 1994, 1996). 2-(4´-Hydroxyphenyl)-1,8-naphthalic anhydride (**34**) was the first phenylnaphthalic anhydride-related compound isolated from unripe banana fruit peel treated with *Colletotricum musae,* the anthracnose causing fungus (Hirai *et al.*, 1994). Phenylnaphthalic anhydrides and 4- and 9-phenylphenalenones were induced in unripe banana fruits after wounding and wounding followed by inoculation with the same pathogen (Kamo *et al.*, 1998b). Kamo *et al.* (1998b) suggested that the enzyme involved in the biosynthesis of phenylnaphthalic anhydride by C-2 elimination would be expressed only in fruits. This suggestion was disproved as phenyl-naphthalic anhydride derivatives were detected in banana roots (Otálvaro, 2004) and also found as induced metabolites together with hydroxyanigorufone (**2**) in response to elicitation in this study. A biosynthetic study of 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) in banana fruits (Kamo *et al.*, 2000) and roots (Otálvaro, 2004) showed that hydroxyanigorufone (**2**) was a precursor of 2-(4-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) (Kamo *et al.*, 2000; Otálvaro, 2004).

- *Response to biotic elicitors*

Used as biotic elicitors, non-pathogenic microorganisms such as *S. salmonicolor* and *P. fluorescens* were able to induce phenylphenalenones in *in-vitro M. acuminata*. *P. fluorescens* has been reported to be a bio-control agent (Rajappan *et al*., 2002; Kavino *et al*., *2008)*. Recently, Saravanan *et al.* (2004b) reported that *P. fluorescens* isolated from the rhizosphere of banana was able to reduce *Fusarium* wilt symptom when plants were pre-inoculated with *P.*

fluorescens before being exposed to the pathogen *F. oxysporum.* The extract of soil inoculated with rifampicin-resistant *P. fluorescens* also inhibited the spore germination of *F. oxysporum* (Saravanan *et al.*, 2004b).

The activity of peroxidase, phenylalanine ammonia lyase (PAL) and polyphenol oxidase increased in banana plants treated with *P. fluorescens* alone and in plants treated with *P. fluorescens* and challenged with *Fusarium oxysporum* (Saravanan *et al.*, 2004a). PAL is involved in phenylpropanoid biosynthesis and the enhancement of its activity suggested an increase in the formation of phenylpropanoid-derived natural products including phenylphenalenones. The accumulation of compounds **2** and **34** that we detected in our study after plants were treated with *P. fluorescens* could be involved in plant resistance to *F. oxysporum.* However, some strains of *P. fluorescens* were reported to produce antibiotics such as phenazine and phoroglucinol, which are known to be important in plants' ability to resist pathogenic fungi (Broadbent *et al.*, 1976; Thomashow *et al.*, 1988; Saravanan *et al.*, 2004b). The yeast strain *Sporobolomyces salmonicolor* is commonly isolated from environmental sources. The strain so far has not been used as a bio-control agent.

The induction of phenylphenalenone compounds in *M. acuminata* in response to non-pathogenic strains was observed in this study for the first time. However, *in-vitro* plants may respond to the non-pathogenic strains differently than do those in natural habitats.

- *Response to chemical elicitor, JA*

The treatment of *M. acuminata* with JA also increased the levels of phenylphenalenones. The main compounds were 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) and hydroxyanigorufone (**2**). JA is known as a plant signal compound which induces phytoalexin production in many plant species (Farmer and Ryan, 1990; Gundlach *et al.*, 1992; Choeng and Choi, 2003). Under the conditions used in our experiments, the effect of JA seemed to be less pronounced than that of microbial treatment. An aminoglycoside antibiotic, kanamycin, was able to induce phenylphenalenone production in banana leaves and green fruits (*M. paradisiaca* var. Grand dwarf and Velry). The major compounds detected in banana treated with kanamycin were irenolone (**21**) and hydroxyanigorufone (**2**) (Luis *et al.*, 1993; 1995). Recently, $CuCl_2$ was also used to elicit the formation of phenylphenalenones in *Musa* (Otálvaro, 2004 and Chapter **6**).

- *Excretion of induced phenylphenalenone compounds into the culture medium of in vitro plants of Musa acuminata*

To gain more information about how *Musa* reacts to stress-inducing factors or pathogens, the media in which treated plant were grown and the media in which control plants were grown were analyzed. The two phenyl naphthalic anhydride derivatives **33** and **34** were found

in media extracts of plants after challenging with *S. salmonicolor* and *P. fluorescens* but not in media of control plant. Root exudates are important for plant-microbe interaction in underground zone (Bais *et al.*, 2002; Walker *et al.*, 2003) and play an important role in plant defense. Cell damage caused by infection could be the reason that *Musa* plants secreted antifungal compounds, in this case phenylphenalenones, into the medium. Study of chemical constituent of root exudates from *M. acuminata* after elicitation by using a non-cell damage system would be one possibility to confirm the ecological role of this group of natural products important in underground plant defense.

- *Induction of phenylphenalenones as plant's response to elicitation*

Results from both biotic and non-biotic elicitation of *in vitro* plants of *M. acuminata* in this chapter, all the induced compounds were phenylphenalenones. Hence, they can be considered as the general inducible defense compounds of *M. acuminata.* The phenyl naphthalic anhydrides **34**, and **33** and hydroxyanigorufone (**2**) were the main induced phenylphenalenones formed in this study.

The bioactivity of phenylphenalenones was previously tested against *Musa* pathogens, *Fusarium oxysporum* f. sp. *cubense* (Foc) (Luis *et al.*, 1994), *Colletotrichum musae* (Kamo *et al.*, 1998b, 2000) and *Microsphaerella fijiensis* (Otálvaro *et al.*, 2007). Phenylphenalenones that have antifungal activity in this experiment were anigorufone (**1**), hydroxyanigorufone (**2**), 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**), irenolone (**21**) (Luis *et al.*, 1994; Kamo *et al.*, 1998b, 2000; Otálvaro *et al.*, 2007). These results confirmed that phenylphenalenones play an essential role as defense compounds in *Musa*.

5.4.2 New phenylphenalenone glucoside from *M. acuminata*

From the EtOAc root extract a new phenylphenalenone glucoside, 2-(4´–ß-glucosyloxyphenyl)-1,8-naphthalic anhydride (**67**) was isolated. However, the HPLC analysis comparing the extracts from treated plants to the extracts from control plants did not characterize compound **67** as induced. Phenylphenalenone glycosides are known as constitutive metabolites of other phenylphenalenone-rich species such as *Wachendorfia thyrsiflora* (Opitz, 2002) and *Xiphidum caeruleum* (Haemodoraceae) (Opitz *et al.*, 2003). However, no phenyl-1,8-naphthalic anhydride glucoside has so far been known from plants. Hence, compound **67** is the first glucoside of that particular type and the first phenylphenalenone-related glucoside from *Musa*. The formation of a phenylphenalenone glucoside in *Musa* and its role in plant defence is still an open question worth to be studied in more detail.

Chapter 6 Biosynthetic aspects of *O*-methylation in phenylphenalenones

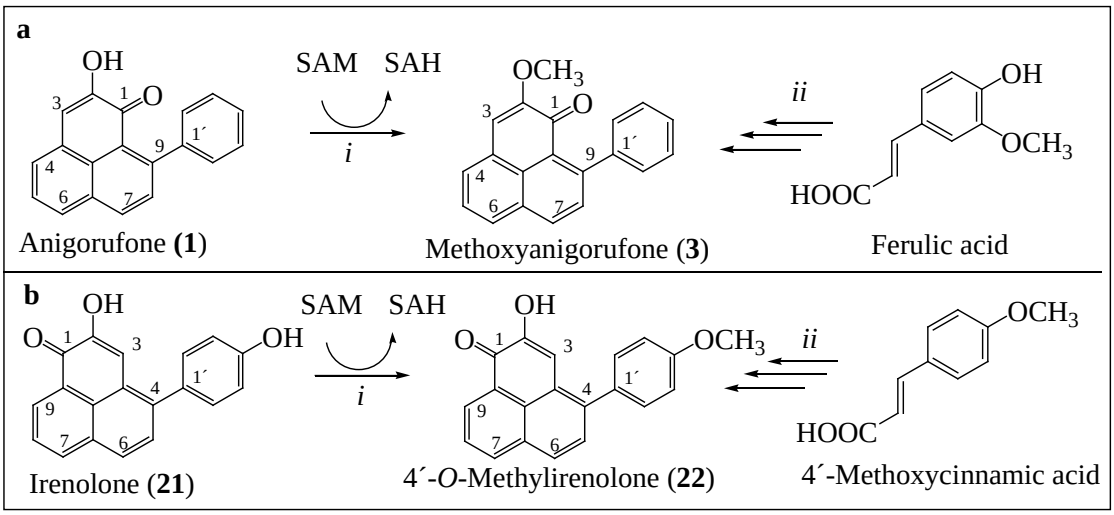
6.1 Introduction

Structural variation within phenylphenalenone subtypes such as 9-phenylphenalenones, 4-phenylphenalenones and phenyl-napththalic anhydrides (see more details in **1.4.1**) is generated, for example, by hydroxylation and *O*-methylation both on ring A and on the lateral phenyl ring (for ring designations, see Fig. **1.3**). *O*-Methylation is an important reaction in the biosynthesis of natural products, as it can change the physical and chemical properties as well as the biological activity of the compounds (Roje, 2006; Ferrer *et al.*, 2008). *O*-Methylation in general is catalyzed by *S*-adenosyl-L-methionine (SAM)-dependent *O*-methyltransferases, which have been classified according to their substrate preference and molecular architecture (Ibrahim *et al.*, 1998; Noel *et al.*, 2003).

Pairs of hydroxyphenylphenalenones and their *O*-methyl derivatives were found in *Musa* sp., for example, anigorufone (**1**)/methoxyanigorufone (**3**) and irenolone (**21**)/4´-*O*-methylirenolone (**22**). The incorporation of [*methyl*-^{13}C]methionine into 4´-*O*-methylirenolone (**22**), musanolone F (**5**), (2*S*,3*S*)-(+)-2,3-dihydro-2,3-dihydroxy-4-(4´-methoxyphenyl)phenalene-1-one (**30**), 4-hydroxy-2-methoxy-9-phenyl-phenalen-1-one (**18**) and 2-(4´-hydroxy-3´-methoxyphenyl)naphthalene-1,8-dicarboxylic anhydride (**36**) (for chemical structures, see Fig. **1.4a**) confirmed that L-methionine is the origin of the methyl group in *O*-methyl phenylphenalenones (Otálvaro, 2004). However, there are two possible ways to form *O*-methyl phenylphenalenones, **a)** by incorporating intact *O*-methyl-phenylpropanoid precursors such as ferulic acid or 4´-methoxycinnamic acid during early biosynthetic step (Scheme **6.1a-*ii***, **6.1b-*ii***), or **b)** by incorporating L-methionine from *S*-adenosyl-L-methionine (SAM) during *O*-methylation of the hydroxyl phenylphenalenone in a downstream biosynthetic step (Scheme **6.1a-*i***, **6.1b-*i***).

A biosynthetic study in a related species, *Anigozanthos preissii*, revealed the incorporation of ferulic acid into musanolone F (**5**) but not methoxyanigorufone (**3**) (Schmitt *et al.*, 2000); hence the formation of methoxyanigorufone (**3**) likely occurred at the late state by the *O*-methylation of anigorufone (**1**) (Scheme **6.1a-*i*)** (Otálvaro, 2004). If the latter is the case, the concentration of the L-methionine / SAM pools may limit the extent of *O*-methylation. To prove this hypothesis, the elicitation of *in vitro* plants of *M. acuminata* with and without [*methyl*-^{13}C]methionine feeding was conducted. Elicitors were applied to *M. acuminata* in order to probe plants for their ability to induce phenylphenalenone biosynthesis and to investigate whether *O*-methylation is more stimulated than usual. Elicitation experiments with and without administering [*methyl*-^{13}C]methionine were conducted, and the levels of hydroxy-phenylphenalenones and their *O*-methyl derivatives were analyzed. JA was used because it is

involved in the oxylipin biosynthesis cascade and its external application generally tends to stimulate secondary metabolite biosynthesis (Farmer and Ryan, 1990; Gundlach *et al.*, 1992). $CuCl_2$ was previously demonstrated to be an effective elicitor of phenylphenalenone formation in *Musa* (Otálvaro, 2004).



Scheme 6.1 Hypothetical formation of *O*-methyl phenylphenalenone by *O*-methylation of the hydroxyl group of phenylphenalenones (*i*) or formation from *O*-methyl phenylpropanoids (*ii*). SAM: *S*-adenosyl-L-methionine, SAH: *S*-adenosylhomocysteine

6.2 Materials and methods

6.2.1 Feeding experiments

In vitro plants of *M. acuminata* (from Thailand; see **2.1**) were transferred to 30 ml MS media in 250 ml Erlenmeyer flasks. Plants were kept under the same conditions as described in **2.1** for one week before the experiment started. Aqueous solutions of [*methyl*-^{13}C]methionine (1 mg/ml), $CuCl_2$ (1 mg/ml) and JA (10 mM) were filtered (0.2 µm) to sterility and added to the cultures. Two plants were used for each treatment as follows.

Control: non-treated plants

Treatment 1: [*methyl*-^{13}C]methionine (0.3 mg/plant)

Treatment 2: [*methyl*-^{13}C]methionine (0.3 mg/plant) with $CuCl_2$ (0.1 mg/plant)

Treatment 3: [*methyl*-^{13}C]methionine (0.3 mg/plant) with JA (100µM)

Treatment 4: $CuCl_2$ (0.1 mg/plant)

Treatment 5: JA (100 µM)

After six days of treatment, plants were harvested. Leaves and roots were separated and lyophilized. Since phenylphenalenones mostly accumulate in roots, only roots were analyzed.

6.2.2 Extraction and isolation

Dry plant rhizomes were extracted three times with 96% EtOH (1 ml solvent/10 mg dry plant material) by shaking at 150-175 rpm for 30 min. The extracts were evaporated to dryness (≤50 ^{o}C), re-dissolved in 20 ml of a MeOH-H_2O mixture (1:1) and then partitioned with *n*-hexane (3 x 20 ml), CH_2Cl_2 (3 x 20 ml) and EtOAc (3 x 20 ml), respectively. The fractions were analyzed for the occurrence of phenylphenalenones using HPLC. The R_t and UV absorption of the extracts' component were compared to those R_t and UV absorption of authentic compounds. *Anal.HPLC system_1* (Appx **1.1**) was used in this preliminary analysis.

The *n*-hexane and CH_2Cl_2 fractions, which contained phenylphenalenones, were fractionated again with *prep. HPLC system_10* (see Appx **1.3**). Fractions were collected over time as follows: F01 (0-18 min, 30-46% MeCN in H_2O), F02 (18-24 min, 46-52% MeCN in H_2O), F03 (24-33 min, 52-60% MeCN in H_2O), F04 (33-42 min 60-68% MeCN in H_2O) and F05 (42-51 min, 68-77% MeCN in H_2O). Phenylphenalenones were isolated from F03 and F04 of plants treated with [*methyl*-^{13}C]methionine and elicitators. The F03 were purified by HPLC using *semiprep. HPLC system_8* (see Appx **1.2**). Compounds **21** and **2** were eluted at R_t 31.0 and 33.0 min, respectively. The F04 were purified by HPLC using *semiprep. HPLC system_9* (see Appx **1.2**). Compounds **1** and **22** were eluted at R_t 40.1 and 42.0 min, respectively. The mixture collected at R_t 42.0 min (containing compounds **22** and **20**) was rechromatographed using *semiprep. HPLC system_8* to separate compounds **40** (R_t 28.5 min) and **62** (R_t 27.0 min).

6.2.3 HPLC analysis and quantification of phenylphenalenones

Phenylphenalenones were quantified by analytical HPLC using *anal. HPLC system_4* (see Appx **1.1**) for hydroxyanigorufone (**2**) and irenolone (**21**) in F03 and *anal HPLC system_5* (see Appx **1.1**) for anigorufone (**1**) and 4´-*O*-methylirenolone (**22**) in F04. Calibration curves were created from integral ratios of HPLC peaks (UV 254 nm) of authentic compounds normalized to internal standard versus concentration as follows:

- *Hydroxyanigorufone (**2**)*: conc. 0.005, 0.0075, 0.01, 0.02, 0.04 mg/ml; *irenolone (**21**):* conc. 0.01, 0.02, 0.04, 0.06, 0.1 mg/ml; 3-phenylpropionic acid (1 mg/ml) was used as an internal standard and *anal. HPLC system_4* (Appx **1.1**) was used for analysis.
- *Anigorufone (**1**)*: conc. 0.01, 0.03, 0.06, 0.09, 0.12 mg/ml; *4´-O-methylirenolone (**22**)*: conc. 0.01, 0.02, 0.04, 0.08 mg/ml; pyrene (0.1 mg/ml) was added as an internal standard and *anal. HPLC system_5* (Appx **1.1**) was used for analysis.

The analytical data and the created calibration curves are shown in Appx **5.3**.

6.3 Results

6.3.1 Biosynthetic origin of *O*-methyl phenylphenalenones

O-methyl groups in *O*-methyl phenylphenalenones were derived from L-methionine as demonstrated by Otálvaro (2004). However, the *O*-methyl groups may be introduced at different steps in *O*-methyl phenylphenalenone biosynthesis, either from the intact *O*-methylphenylpropanoid precursors or by the methylation of the hydroxyphenylphenalenones. To investigate the *O*-methylation step of phenylphenalenone biosynthesis, *in vitro* plants of *M. acuminata* were used. Plants were treated with elicitors (JA, $CuCl_2$), an elicitor in combination with [*methyl*-^{13}C]methionine, or [*methyl*-^{13}C]methionine only (see **6.2.1**). Non-treated plants were used as negative controls. After treatments for six days, plants were harvested, dried and extracted. The extracts were fractionated by preparative HPLC. The fractions were analyzed for the occurrence of phenylphenalenones, especially *O*-methyl phenylphenalenones, using HPLC and ^{1}H NMR.

6.3.2 Purification and identification of phenylphenalenones

HPLC and ^{1}H NMR analysis of fractions F03 and F04 from different treatments showed quantitative and qualitative differences in the profiles of natural products. Phenylphenalenones were detected in plants treated with [*methyl*-^{13}C]methionine alone, [*methyl*-^{13}C]methionine with $CuCl_2$, [*methyl*-^{13}C]methionine with JA and with $CuCl_2$ alone, while in control plants and JA treatment no significant levels of phenylphenalenones were found. Fig **6.1** shows the selected HPLC chromatograms of phenylphenalenone analysis in F03 and F04 from *Musa* plants.

To clearly identify phenylphenalenone structures, the compounds were purified from fractions F03 and F04 of plants treated with $CuCl_2$ by semiprep. HPLC (see **6.2.2**) (Fig. **6.1)**. Hydroxyanigorufone (**2**, R_t 31.0 min) and irenolone (**21,** R_t 33.0 min) were obtained from F03. From F04 anigorufone (**1**, R_t 40.1 min) and 4′-*O*-methylirenolone (**22**, 42.0 min) were obtained. Signals of a co-eluting minor compound, assignable to isoanigorufone (**20**), were detected in the NMR spectra of fractions containing compound **22**. Compound **22** was the only *O*-methyl phenylphenalenone detected in these experiments. The chemical structures of phenylphenalenones identified in this chapter are shown in Fig **6.2**.

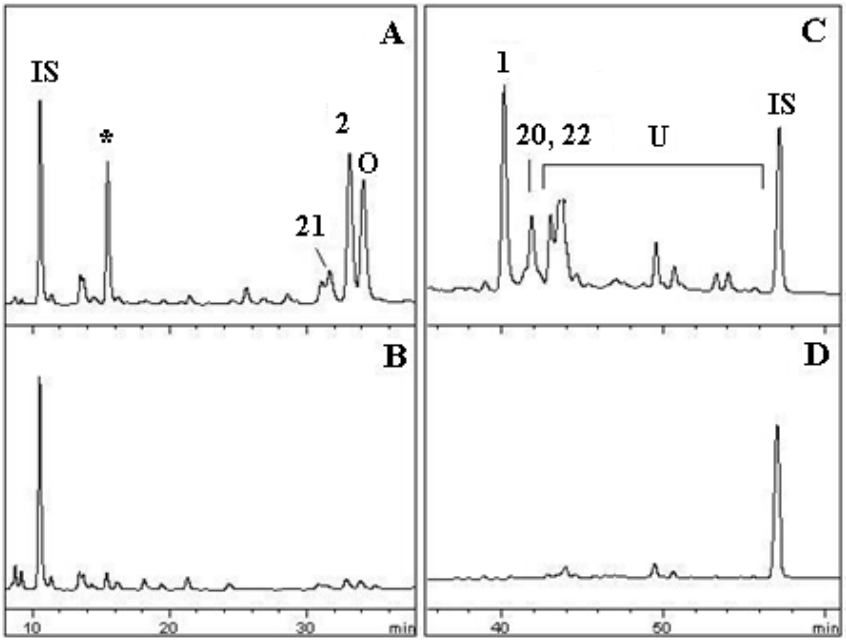


Figure 6.1 HPLC chromatograms of fractions F03 (*anal. HPLC system_4*, see Appx **1.1**) and F04 (*anal. HPLC system_5*, see Appx **1.1**) for phenylphenalenone analysis comparing non-treated and treated plants; A) F03 of [*methyl*-^{13}C]methionine/$CuCl_2$-treated plants, B) F03 of non-treated plants, C) F04 of [*methyl*-^{13}C]methionine/$CuCl_2$-treated plants, D). F04 of non-treated plants.

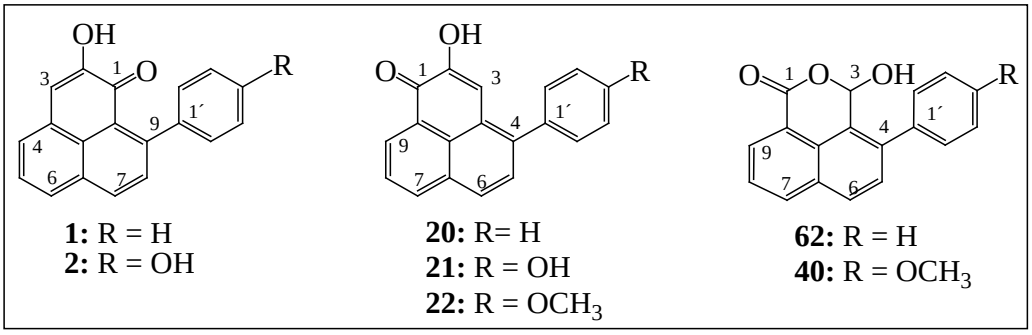


Figure 6.2 Phenylphenalenones isolated from [*methyl*-^{13}C]methionine feeding experiments: anigorufone (**1**), hydroxyanigorufone (**2**), irenolone (**21**), 4´-*O*-methylirenolone (**22**) co-eluted with isoanigorufone (**20**), and the degradation derivatives of **22** and **20**, 3-hydroxy-4-(4´-methoxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**40**) and, 3-hydroxy-4-(phenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**62**), respectively.

6.3.3 Quantification of phenylphenalenones

Quantification of phenylphenalenones in *M. acuminata* roots was done in order to determine the efficiency of elicitors in phenylphenalenone induction in general and to identify the increase of *O*-methyl phenylphenalenone levels in plants co-treated with [*methyl*-^{13}C]methionine/elicitors in comparison to plants treated with elicitors alone. Where *O*-methyl phenylphenalenones are formed in the final step by the *O*-methylation of the hydroxyl

phenylphenalenone analogues, the administration of L-methionine may increase the formation of *O*-methyl phenylphenalenones.

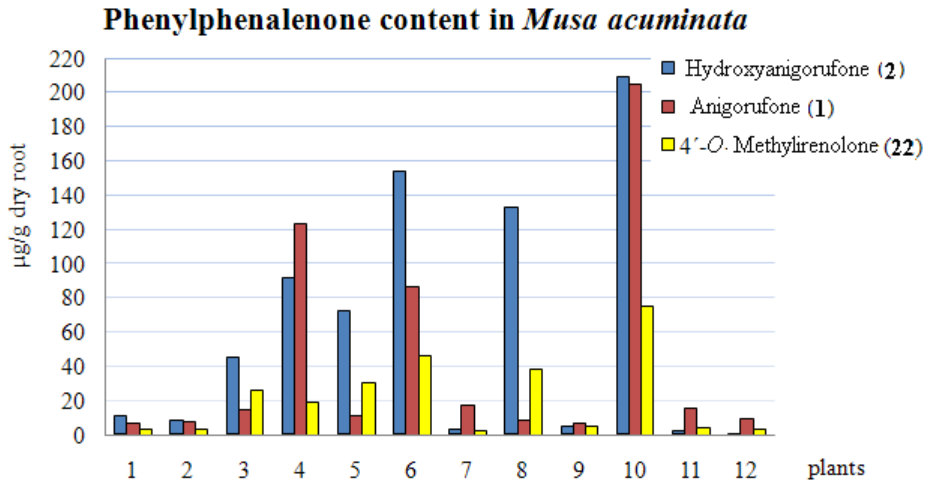


Figure 6.3 Hydroxyanigorufone (**2**), anigorufone (**1**) and 4'-*O*-methylirenolone (**22**) content from *M. acuminata;* plants 1 and 2: control; plants 3 and 4: [*methyl*-^{13}C]methionine; plants 5 and 6: [*methyl*-^{13}C]methionine+$CuCl_2$; plants 7 and 8: [*methyl*-^{13}C]methionine+JA; plants 9 and 10: $CuCl_2$; plants 11 and 12: JA; concentrations in µg/g dry root.

Phenylphenalenone-type compounds were quantified by HPLC analysis and by comparing the peaks areas to calibration curves (see **6.2.3**). The average values of duplicate experiments (less than 15% variation) were taken to create calibration curves (see Appx **5.3**). Concentrations of phenylphenalenones in the extracts (Fig. **6.3**, see alsoAppx **5.4**) obtained from different treatments were calculated. The observed responses within the same treatment groups varied greatly, but in general, treated *in vitro*-plants of *M. acuminata* seemed to produce higher levels of phenylphenalenones than did non-treated plants. $CuCl_2$ induced mainly anigorufone (**1**) and hydroxyanigorufone (**2**). 4´-*O*-Methylirenolone (**22**), the *O*-methyl phenylphenalenone, increased less than **1** and **2** did. Treatment with [*methyl*-^{13}C]methionine, [*methyl*-^{13}C]methionine/JA and [*methyl*-^{13}C]methionine/$CuCl_2$ increased levels of **2** and **22** but to a lesser extent than treatment with $CuCl_2$ alone did. Anigorufone (**1**) was induced only in treatment with [*methyl*-^{13}C]-methionine and [*methyl*-^{13}C]methionine/JA. Plants treated with JA alone showed no reproducible increase in phenylphenalenone production.

Irenolone (**21**) was detected as a constituent in *M. acuminata* by ^{1}H NMR analysis, but the level of **21** was roughly estimated due to the poor and non-resolved signals in UV absorption. The amount of **21** was more than 25 µg/g dry root in all treated plants except plants treated with JA when compared to control plant.

6.3.4 Oxidative modification of phenylphenalenone-related compounds

During the isolation of 4´-*O*-methylirenolone (**22**) and isoanigorufone (**20**), structural modification to produce **40** and **62**, respectively, was observed. When an inseparable co-eluting mixture of 4´-*O*-methylirenolone (**22**) and isoanigorufone (**20**) was subjected to HPLC in *anal. HPLC system_5*, two additional peaks were observed at R_t 27.0 and 28.5 min (for HPLC chromatogram see Appx **4**). Compounds **40** and **62** were isolated by using *semi-prep. HPLC system_8* (Appx **1.2**) and identified as 3-hydroxy-4-(4´-methoxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**40**) (Otálvaro, 2004) and 3-hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (**62**) (see **4.3.2.3**), respectively. Thus compounds **40** and **62** seemed to be oxidative products of 4´-*O*-methylirenolone (**22**) and isoanigorufone (**20**) as observed from HPLC analysis under acidic conditions (0.1% TFA-H_2O).

6.4 Discussion

6.4.1 Phenylphenalenones are induced by treatment with chemical substances

Phenylphenalenones were induced in *Musa sp*. after either 1) fungal infection (Luis *et al*., 1994, 1996; Kamo *et al*., 1998b, 2001), 2) challenging with non-pathogenic strains of *Sporobolomyces salmonicolor* and *Pseudomnas fluorescen*s (see Chapter **5**) or 3) elicitation with chemical elicitors such as kanamycin (Luis *et al.*, 1993) and JA (see Chapter **5**). In this study, *Musa* plants responded to different chemical treatments by increasing their phenylphenalenone formation. 9-Phenylphenalenones, anigorufone (**1**) and hydroxyanigorufone (**2**) were identified as main compounds together with three minor compounds of the 4-phenylphenalenone type, irenolone (**21**) and 4´-*O*-methylirenolone (**22**), co-eluted with isoanigorufone (**20**) in a ratio of 2:1 obtained from integrating signals in ^{1}H NMR spectrum.

In order to determine the efficiency of elicitors in phenylphenalenone induction, phenylphenalenones were quantified. $CuCl_2$ seemed to be the most effective elicitor in this study as phenylphenalenone levels were highly induced both in treatment with $CuCl_2$ alone and in treatment with $CuCl_2$/[*methyl*-^{13}C]methionine. Treatment with [*methyl*-^{13}C]methionine was able to induce phenylphenalenone but was less effective than treatment with $CuCl_2$. JA treatment showed no reproducible change in phenylphenalenone levels but the treatment of JA and [*methyl*-^{13}C]methionine showed higher levels of hydroxyanigorufone (**2**) than did plants treated with *methyl*-^{13}C]methionine alone.

Heavy metals, e.g. copper ions, have been shown to induce natural product in many plant species (Kodama *et al.*, 1988; Rakwal *et al.*, 1996; Kim *et al.*, 1999; Tebayashi *et al.*, 2001). Although JA is known as a plant signalling molecule in plant defense (Farmer and

Ryan, 1990; Gundlach *et al.*, 1992; Choeng and Choi, 2003; Zhoa *et al.*, 2004) it was not reproducibly able to induce phenylphenalenone formation. However, the effect of JA in phenylphenalenone induction could not be ruled out as higher levels of hydroxyanigorufone (**2**) were induced in plants treated with JA and [*methyl*-^{13}C]methionine than in plants treated with [*methyl*-^{13}C]methionine alone. The responses of plants were diverse even in parallel experiments. Moreover, the induction of phenylphenalenones by JA has been clearly demonstrated in *M. acuminata* (Columbia) as described in Chapter **5**. In this chapter, plants' responses to elicitation were individual. Plants also responded variably in $CuCl_2$ treatments, but in light of the induction effect, $CuCl_2$ must be considered a potent elicitor of phenylphenalenone biosynthesis, including 4´-*O*-methylphenylphenalenone (**22**).

6.4.2 Inducibility of *O*-methyl phenylphenalenone biosynthesis

In the biosynthesis of *O*-methyl phenylphenalenones, L-methionine has been proven to be the origin of the 3´- or 4´-*O*-methoxy groups of phenylphenalenones (Otálvaro, 2004). However, it may be that the *O*-methyl group of phenylphenalenones is introduced at two different biosynthetic steps, as hypothesized in Scheme **6.1**.

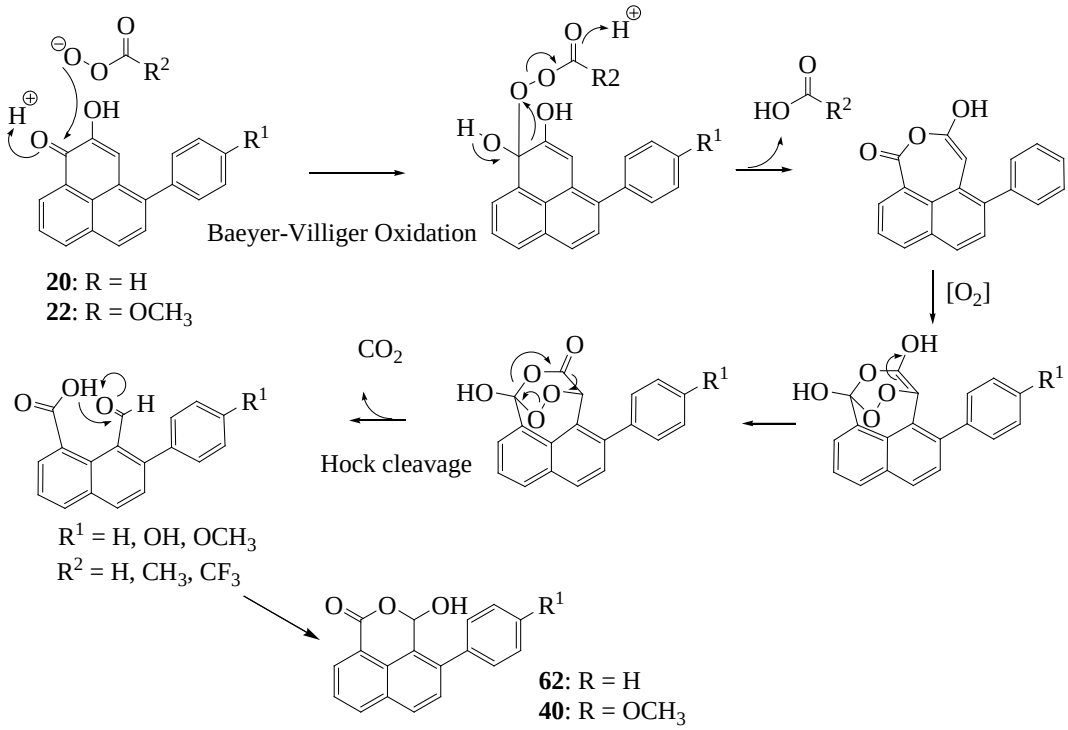
The incorporation of ferulic acid to rings B and D of musanolone F (**5**) but not into rings A and B of methoxyanigorufone (**3**) (Fig. **1.4a**; for ring designation, see Fig. **1.3**) has been previously demonstrated in *Anigozanthos preissii* (Schmitt *et al.*, 2000), suggesting the origin of the 3´-*O*-methyl group from the intact methyl phenylpropanoid precursors. Because ferulic acid was not incorporated into rings A and B of **3**, we hypothesized that 2-OCH_3 of **3** was likely derived from L-methionine by SAM-dependent *O*-methylation onto 2-OH of anigorufone (**1**). Thus, the availability of L-methionine should increase **3** in plants treated with [*methyl*-^{13}C]methionine/elicitor when compared to plants treated with elicitor alone.

However, methoxyanigorufone (**3**) was not detected in this study. The only *O*-methyl phenylphenalenone detected was 4´-*O*-methylirenolone (**22**). Although the origin of 4´-OCH_3 in **22** from L-methionine has been proven by Otálvaro (2004) it was unclear whether **22** was formed by direct methylation onto 4´-OH of irenolone (**21**) (Scheme **6.1-b*i***) or by intact 4´-*O*-methoxycinnamic acid (Scheme **6.1-b*ii***). Otálvaro (2004) reported that 4´-*O*-methoxy cinnamic acid was not incorporated into **22**. Instead it was converted to 3´,4´-methylenendioxycinnamic acid. This result did not clearly prove the first hypothesis. In order to prove the second hypothesis (Scheme **6.1-b*ii***), quantitative analysis of **22** was done in plants from different treatments as described above. The levels of **22** in plants treated with [*methyl*-^{13}C]methionine/elicitor were not higher than in plants treated with elicitor alone. Moreover, the responses of plants within the same treatment group varied considerably and

the experiments were not always reproducible. As methionine can also be used in other metabolic pathways, the expected evidence may not be easily observed.

6.4.3 Autoxidation of 4-phenylphenalenones

Oxidative conversion of 4´-*O*-methylirenolone (**22**) to **40** and isoanigorufone (**20**) to **62** was observed during HPLC analysis in MeCN-0.1% TFA/H_2O. The autoxidation of **20** and **22** spontaneously occurs in the presence of acid by Baeyer-Villiger oxidation followed by Hock cleavage, yielding the 3-hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-ones, **40** and **62** (Scheme **6.2**). Related reaction mechanisms have been reported for 3-keto steroids (Alvarez *et al.*, 1986) and triterpenes such as lupane derivatives (Urban *et al.*, 2005) and betulinic acid (Urban *et al.*, 2004). The Hock cleavage is also involved in the autoxidative conversion of dihydroartemisinic acid (Sy and Brown, 2002). However, compounds such as **40** and **62** could probably also be formed enzymatically by *M. acuminata*. Thus, more detailed studies of the biosynthesis of phenylphenalenones should be undertaken.



Scheme 6.2 Hypothetical mechanism of autoxidative conversion of 4-phenylphenalenones to 3-hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-ones via the Bayer-Villiger reaction and Hock cleavage.

Chapter 7 Type III Polyketide synthases from *Musa acuminata*

7.1 Introduction

Phenylphenalenones are formed from two molecules of phenylpropanoyl-CoA and one malonyl-CoA via a diarylheptanoid intermediate (see **1.6**). However, many biosynthetic details are not yet understood. Schröder (1997) proposed that type III PKSs are involved in diarylheptanoid and phenylphenalenone biosynthesis. These enzymes condense a phenylpropanoyl-CoA with malonyl-CoA, yielding a phenylpropanoid diketidyl-CoA intermediate. The diketide intermediate is condensed with a second phenylpropanoyl-CoA molecule, resulting in a diarylheptanoid, followed eventually by cyclization to form phenylphenalenones (http://www.biologie.uni-freiburg.de/data/bio2/schroeder). That type III PKS are involved in phenylphenalenone biosynthesis in *Wachendorfia thysiflora* (Haemodoraceae) has been demonstrated by Brand *et al.* (2006). The recombinant WtPKS1 is able to form a diketide intermediate from phenylpropanoid-CoA and malonyl-CoA, but no diarylheptanoids or phenylphenalenones were detected (Brand *et al.*, 2006). Katsuyama *et al.* (2007) reported a type III PKS, CUS (curcuminoid synthase) from rice (*Oryza sativa*, Poaceae) that was able to synthesize curcuminoids from *p*-coumaryl-CoA and malonyl-CoA, but so far neither diarylheptanoids nor phenylphenalenones have been found in rice. Recently, plant type III PKS involved in curcuminoid biosynthesis in *Curcuma longa*, a diarylheptanoid-rich species, have been reported (Katsuyama *et al.*, 2009).

In this study, a RT-PCR homology-based approach was used to isolate candidate type III PKS which may be involved in phenylphenalenone biosynthesis in *M. acuminata.*

7.2 Materials and methods

7.2.1 Materials

***Media and buffers*:** Media used in these experiments: LB, NZY, NZCYM, 2xYT, TB, NZY-top agar. Buffers used: SM, 0.5xTAE, 20xSCC and etc. The components and preparations are shown in Appx **6.1-2**.

***List of material and equipments*:**

Company	**Material**
Invitrogen GmbH, Karlsruhe, Germany	Reverse transcriptase-SuperScript III, RNAse inhibitor-RNAse OUT, 100bp DNA ladder, 1kb DNA ladder, Low mass DNA ladder, TOPO-TA-cloning-Kit, pCR4-TOPO, *E. Coli* TOP10, Acrylamide solution, IPTG, primers
Amersham Biosci., Buckinghamshire, UK	APS, TEMED, *E. coli* NM522, *E. coli* NP66, λExCell Not I/ Eco RI/ CIP, Ready-to-go DNA labelling beads, ProbeQuant G-50 micron column, HiTrap Chelating Columns, Sephadex^{TM}G-35M columns, HybondN+
Novex Electrophoresis GmbH, Frankfurt, Germany	Colloidal blue stain kit
Stratagene, La Jolla, CA, USA	*E. coli* BL21-CodonPlus (DE3)-RIL
New England Biolabs, Beverly, MA, USA	[^{32}P]dCPT
Qiagen GmbH, Hilden, Germany	Plant RNeasy kit, QIAquick gel purification kit, PCR purification kit
Macherey-Nagel GmbG&Co.KG. Düren, Germany	NucleospinPlasmid-Kit
Applied Biosystems, Foster city, CA, USA	Big Dye
Bio-Rad Laboratories GmbH, Munich, Germany	Bio-Rad Protein Assay
Hartmann Analytics, Braunschweig, Germany	[2-^{14}C]Malonyl-CoA
Agilent Technologies, Waldbronn, Geramny	RNA 6000 Nano lapchipkit

Equipment, model	**Company**
Agilent Bioanalyzer,2100	Agilent Technologies , Palo Alto, CA, USA
HPLC-system, HP 1100 with VWD or DAD	Agilent Technologies, Palo Alto, CA, USA
Radioactive detector, flow scintillation analyzer	Canberra-Packard, Dreieich, Germany
Thermomixer, Thermomixer comfort	Eppendorf AG, Hamburg, Germany
Thermocyler, BiometraTgradient PCR	Biometra GmbH, Göttingen, Germany
Electrophoresis unit, *i*-mupid mini gel (for DNA)	Eurogentec GmbH, Colonge, Germany
Electrophoresis apparatus, Mini Protean III	Biorad Laboratories, Hercules, CA, USA
French press, Amnico	SLM Instr. Inc., Rochester, NY, USA
UV linker, Stratalinker	Stratagene, La Jolla, CA, USA
Gel documentation system, Gene genius	Syngene, Cambridge, UK
Sequenzer, ABI Prism-3100, 16-Capillary	Applied Biosystems, Foster City, CA, USA
Film developer, SRX101	Konica Europe GmbH, Hoenbrumm, Germany

7.2.2 RNA extraction

The plant material was collected separately from leaves, petioles and roots of *in vitro*-plant of *Musa acuminata* (AAA) “Cavendish” Columbia (see Chapter **2.1**). The materials were immediately frozen in liquid N_2 and ground to fine powder. RNA was extracted from 100 mg of the frozen plant material using Plant RNeasy kit. RNA was eluted with 40 µl of TE buffer. The aliquots of total RNA samples (1 µl) were loaded onto RNA nano Lapchip. Quantification of the extracted total RNAs was verified by capillary gel electrophoresis on Agilent Bioanalyzer 2100 according to the manufacturer’s protocol. The quality of total RNA was determined by the ratio of 28S-rRNA to 18S-rRNA.

7.2.3 cDNA synthesis

First-strand cDNA was synthesized from total RNA (1 µg) by reverse transcriptase, SuperScript III. Anchored-poly dT primer [dT(18)v] (5′-GCG AAT TCT AGA TCT CGA GGT ACC TTT TTT TTT TTT TTT TTT V-3´) was used. Recombinant RNase inhibitor, RNAseOUT, was added to the reaction mixture in order to prevent degradation of the target RNA due to the ribonuclease contamination from RNA preparation. cDNA was synthesized following the SuperScript III manufacturer's protocol.

7.2.4 Polymerase chain reaction (PCR)

Amplification of cDNA fragments was done by PCR. For a list of the primers used to amplify DNA fragments, see Appx **7.1**.

The components of PCR reaction (20 µl):

1	µl	cDNA-fragment (template)
1	µl	forward primer (10 pmol/µl)
1	µl	reverse primer (10 pmol/µl)
2	µl	10×PCR buffer
0.5	µl	dNTPs (10 nmol/µl)
0.25	µl	*taq*-polymerase (1U/µl)
13.75	µl	sterile H_2O

Thermo cycle of the PCR amplification condition:

Step	1.	denaturation	5 min	96 °C	
	2.	denaturation	30 sec	96 °C	←
	3.	annealing	1 min	50 °C	
	4.	amplification	1 min	72 °C	×35 from step 2.
	5.	amplification	10 min	72 °C	

7.2.5 Gel electrophoresis

Agarose gel electrophoresis for RNA analysis: RNA capillary gel electrophoresis was done on an Agilent Bioanalyzer 2100. The agarose gel was prepared using RNA 6000 Nano lapchip kit following the manufacturer's protocol.

Agarose gel electrophoresis for DNA analysis: Agarose gel (1% w/v) was suspended in 0.5xTAE buffer (see Appx **6.2**) and melted to solution by microwave. Ethidium bromide (0.5 mg/ml) was added after the gel solution was cooled to ~50 °C. The gel was left to settle for 30-45 min before being used. The sample was mixed with loading buffer (1 µl/5 µl sample) before being transferred into the gel-well. DNA was analyzed by comparing it to standard DNA, 100bp DNA ladder. Electrophoresis was run at 135 V for 15 min. Gel photographs were taken by gel documentation Gene Genius.

50x Tris acetate-EDTA buffer (TAE):

2.0 M	Tris
1.0 M	acetic acid
0.1 M	EDTA

adjusted to pH 8.3

5x DNA loading buffer:

30% (v/v)	glycerine
0.25% (w/v)	bromophenol blue
0.25%	xylene cyanol

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) for proteins: SDS-PAGE was used in protein analysis with Mini-PROTEAN-III Electrophoresis Cell. Ingredients for SDS-PAGE preparation are as follows:

	Separating gel	*Collecting gel*
sterile H_2O	4.85 ml	6.4 ml
Tris/HCl	2.5 ml 1.5 M Tris/HCl pH 8.8	2.5 ml 0.5 M Tris/HCl pH 6.8
10% SDS	100 µl	100 µl
10% APS	50 µl	50 µl
TEMED	5 µl	10 µl
acrylamide gel solution	2.5 ml	1 ml

The separation gel was prepared by mixing sterile water, 1.5 M Tris/HCl and 10% SDS. Then acrylamide gel solution was added, followed by APS and TEMED. The resulting solution was mixed gently before being transferred to the gel form (~ 3.5 ml/gel). Then the gel was overlaid with isopropanol (1 ml) and left to polymerize for 1 h. Collecting gel was prepared in the same manner, then applied (~1 ml/gel) on top of separating gel and left for polymerization before being used. The bacterial cell pellet of the protein expression strain (*E. coli* BL21 containing pHis8_MaPKS1, see **7.2.13**) was denaturated with protein-loading buffer (5 µl) at 95 °C and left on ice to cool. Protein standard marker (5 µl) (Roti-Mark Standard) was used to compare protein size. Electrophoresis was run in 1x electrode buffer at 200 V. After electrophoresis analysis, the gel was placed in staining solution (Colloidal blue staining kit based on Coomassie blue, Neuhoff *et al.*, 1988) and shaken for 1 h, and then washed with water. Gel photo was taken by gel documentation Gene Genius.

5x Electrode buffer (1 1):

15.0 g	Tris
72.2 g	glycine
5.0 g	SDS

Staining solution: (Colloidal blue stain kit):

55 ml	H_2O
20 ml	MeOH
20 ml	solution A
5 ml	solution B

Protein loading buffer:

4.8 ml	H_2O
1.2 ml	collecting gel buffer
2.0 ml	SDS (10%)
1.0 ml	glycerine
0.5 ml	bromophenol blue (0.5%)

β-ME (50 µl/ml) was added prior to use

7.2.6 Purification of DNA

After PCR amplification, the DNA to be purified was separated from excess primers. The PCR product (for cloning purpose) was analyzed by gel electrophoresis (see **7.2.5**), 100 V, 20 min. The DNA fragment was excised from the gel and purified with a QIAquick gel extraction kit, according to the manufacturer's protocol for gel extraction. Purification of the PCR products for sequencing reaction and the digested plasmid DNAs was done with a QIAquick Gel Extraction Kit, according to the manufacturer's protocol for PCR purification.

7.2.7 Cloning and transformation

7.2.7.1 Plasmids

pCR4-TOPO: The plasmid encodes ampicillin and kanamycin resistance. It contains sequences of pUC origin as well as *lac*Z-gene with *lac*-promotor (TOPO TA Cloning kit, user manual, version O, April 2006).

pHis8: (constructed by J. Noel and kindly provided by J. Schröder). The plasmid was used to create a histidine tag to the 5´ terminal for protein expression. The plasmid encodes a T7-promotor and a T7-terminator, containing kanamycin resistance, ribosome binding site, *lac*-operator as well as *lac*I-gene. Protein induction was done by isopropylthio-β-galactoside (IPTG).

7.2.7.2 Host cells

***E. coli* TOP 10**: F^-*mc*rA Δ(*mrr-hsd*RMS-*mrc*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7679 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G.

***E. coli* BL21-CodonPlus (DE3)-RIL**: B F^- *omp*T *hsd*S($r_b^-m_b^-$) dcm^+ Tet^r *gal* λ (DE3) *end*A Hte [*arg*U *ile*Y *leu*W Cam^{-1}].

7.2.7.3 TOPO-TA Cloning®

The target DNA fragment was amplified by PCR (see **7.2.4**) and purified (see **7.2.6**). The purified DNA was cloned into Vector pCR4_TOPO. TOPO cloning was set up by using the following reagents: The purified PCR product (4.0 µl), salt solution (1.0 µl) and Vector pCR4_TOPO (1.0 µl). Cloning reaction was performed at RT for 30 min.

7.2.7.4 Transformation

The constructed pCR4_TOPO vector (in 3 µl of cloning reaction mixture, **7.2.7.3**) was applied to competent cell *E. coli* TOP10 (50 µl), mixed by non-pipetting and incubated on ice for 20 min. The mixture was heat-shocked at 42 °C for 20 sec. SOC medium (250 µl) was added immediately. The mixture was incubated at 37 °C, 220 rpm for 1 h and then cultured on LB agar plates containing ampicillin (100 µg/ml). The plates were incubated overnight at 37 °C. Transformation analysis was performed by colony PCR using gene-specific primers for MaPKS and/or M13 forward and M13 reverse (sequences, see Appx **7.1**). PCR products were analyzed by gel electrophoresis (see **7.2.5**).

7.2.7.5 Sub-Cloning of the MaPKS1 in pHis8 expression vector

Amplification of MaPKS1 DNA fragment: The DNA fragment of MaPKS1 without the start methionine was amplified from phagemid pExcell DNA containing MaPKS1 (see **7.2.11**). Specific primers with restriction sites, MaPKS1_BamHI_F (AA GGA TCC GCC AGC CTC CAC GCT CTG) and MaPKS1_Hind III_R (TA AAG CTT TTA GAG TGG TAC GCT GCG) were used (restriction sites underlined). The amplified fragment was cloned into pCR4_TOPO cloning vector. Plasmid DNA was extracted (see **7.2.8**) and sequenced (see **7.2.12**). The pCR4_TOPO_MaPKS1_ex plasmid DNA was later digested with restriction enzymes *Bam* HI and *Hind* III.

***Restriction*:** To construct the expression plasmid of MaPKS1, pHis8_expression plasmid and pCR4_TOPO_MaPKS1_ex-plasmid were restricted with *Bam* HI (G↓GATCC) and *Hind* III (A↓AGCTT).

Restriction reaction with Hind III (10 µl):

5.0 µl	DNA
1.0 µl	NEB Buffer 2
1.0 µl	*Hin*d III
3.0 µl	Sterile H_2O

The reaction mixture was incubated at 37 °C 2 h. Afterwards the enzyme was de-activated at 65 °C for 20 min. The *Hin*d III restricted plasmids were later digested with *Bam* HI.

Restriction reaction with Bam HI (50 µl):

10.0 µl	First step digestion with *Hind* III
5.0 µl	NEB-Bam
2.0 µl	*Bam* HI
0.5 µl	BSA
32.5 µl	Sterile H_2O

The reaction was incubated at 37 °C for 2 h.

Ligation of MaPKS1 fragment to pHis8 expression vector: The restricted DNA fragment of MaPKS1 was gel purified by QIAquick gel extraction kit (see **7.2.6**). The re-stricted pHis8 plasmid was purified from the reaction solution by PCR purification Kit (see **7.2.6**). The concentration of MaPKS1 DNA fragment and pHis8 plasmid was determined by comparison to standard Low DNA Mass ladder on gel. The MaPKS1 DNA fragment was then ligated into pHis8 expression vector to obtain the expression plasmid, pHIS8_MaPKS1.

Ligation reaction (20 µl)

90	fmol	insert
30	fmol	vector
4.0	µl	ligation buffer
1.0	µl	ligase

The reaction was incubated at 16 °C overnight. The constructed pHis8_MaPKS1 plasmids were transformed to competent *E. coli* TOP10 and plated on LB plates containing

kanamycin (50 µg/ml). Plasmid DNA was extracted and purified by minipreparation (see **7.2.8**). The integrity of the insert was verified by sequencing, and the plasmid pHis8_MaPKS1 was used to transform the expression host *E. coli* BL21 CodonPlus (DE3).

7.2.7.6 Transformation of MaPKS1_pHis8 to *E. coli* BL21 CodonPlus (DE3)-RIL

E. coli BL21 CodonPlus (DE3) (30 µl) was thawed on ice. *β*-Mercaptoethanol (2 µl, 1:10 dilution) was added. The mixture was incubated on ice for 10 min and mixed every 2 min. Vector pHis8_MaPKS1 (3 µl of ligation mixture) was added and mixed. The mixture was kept on ice for 30 min and then heat-shocked at 42 ºC for 20 sec. SOC (250 µl) was added to the bacterial mixture. The mixture was incubated at 37 ºC and shaken at 200 rpm for 1 h and plated on LB agar containing kanamycin (50 µg/ml) and chloramphenicol (50 µg/ml). The culture was incubated overnight at 37 ºC. Positive clones were analyzed by colony PCR (primers pHis8_for and pHis8_rev, Appx **7.1**). The plasmid was sequenced again to check the correctness of plasmid construction.

7.2.8 Mini-preparation

Isolation and purification of plasmid DNA were carried out using NucleoSpin Plasmid Kit. The transformed *E. coli* (see **7.2.7**) was allowed to grow overnight in LB medium (5 ml) containing selective antibiotics and were centrifuged. The plasmid DNA was purified from the cell pellets according to the manufacturer's protocol. The plasmid DNA was eluted from the column with 30-50 µl of buffer AE. Quantification and qualification of plasmid DNA were carried out by UV spectroscopy at 260 and 280 nm.

7.2.9 cDNA library of *Musa acuminata* (AAA) Cavendish

***Creation of cDNA library*:** *In-vitro M. acuminata* (AAA) "Cavendish" were treated with jasmonic acid (50 µM). After 24 h, total RNA was extracted (see **7.2.2**). The RNA samples extracted from leaves, petioles and root were pooled (1:1:1). This RNA was used in the creation of a cDNA library by the company Vertis Biotechnologie AG (Freising, Germany). λExCell was used as cloning vector. For transfection, *E. coli* NM522 was used, while for *in vivo* excision, *E. coli* NP66 was used. *E. coli* NM522: *sup*E, *thi*, Δ(*hsd*MS-*mrc*B)5, Δ(*lac*-*pro*AB), F'[*pro*AB, *lac*I^{q}, *lac*ZΔM15]. *E. coli* NP66: *thr*, *leu*, *pro*, *thi*, *bio*, *cI*^{857}*int*$^{-}$*xis*$^{-}$, ΔH1, *lac*ZΔM15, cm^{R}/pJN13[Φ80 repressor, pACYC184 replicon]/pXis[*xis*$^{+}$, str^{R}, spec^{R}, pSC101 replicon]. Phagemid λExCell Not I/EcoR I/CIP. The titer of the primary phage library was 18,000 pfu/µl.

***Amplification of phage library*:** The primary library was amplified by transfection of the phagemid to *E. coli* NM522, which was grown overnight in LB medium at 37 °C and then centrifuged. The pellet was re-suspended in 10 mM $MgSO_4$ to OD_{600} 0.5. Phage suspension (27 µl) was added to *E. coli* NM522 (600 µl) and incubated at 37 °C for 15 min. Pre-warmed NZY-top agar (6.5 ml, 50 °C) was added. The mixture was poured on a pre-warmed NZY agar plate (∅ 14.5 cm, 37 °C), which was incubated at 37 °C for 7 h. After that, SM buffer (8 ml) was applied to the agar surface. The plate was shaken at 4 °C overnight. The SM buffer solution was collected and the extraction repeated (SM buffer, 2 ml). The solutions were pooled, $CHCl_3$ (5% volume of the collected buffer) was added, and centrifuged (500 × g, 10 min). The supernatant was extracted with $CHCl_3$ again. The solution was filtered (0.45 µm) and kept with DMSO (7% v/v) at -80 °C and with 0.3% $CHCl_3$ at 4 °C. The titer (pfu/µl) of this secondary library was checked by the serial dilution method. Diluted phage suspension was grown on LB agar as described above. After the culture was grown at 37 °C for 7 h, the plaque-forming unit (pfu) was counted and the titer (pfu/µl) was calculated.

7.2.10 cDNA library screening

For primary screening of the cDNA libray, *E. coli* NM522 was grown on NZY agar (∅ 14.5 cm) at 37 °C for 7 h. The plates were cooled at 4 °C for 30 min. Then $HybondN^+$ membrane was put on the surface of the plate for 1 min. The membranes were heated at 80 °C for 2 h and then the DNA was cross-linked to the membrane by UV light (UVcross linker). The membranes were washed and pre-hybridized in 2×SSC (0.1% w/v SDS).

***Probes preparation*:** Primers were designed from partial sequences of MaPKS. EST sequences from *Musa* spp were provided by Prof. David G. Heckel, from the Syngenta Banana EST Collection, which was accessed under an MTAagreement between the MPI for Chemical Ecology and Bioversity International (formerly INIBAP, International Network for the Improvement of Banana and Plaintain) http://bananas.bioversityinternational.org/. The EST sequences are stored in the Sputnik database system, maintained by MIPS (Munich Information Center for Protein Sequences), at the Helmholz Zentrum München. To obtain polyketide-synthase-like sequences from *Musa* spp, the Syngenta Banana EST Collection was searched by TBLASTN using the protein sequence of polyketide synthase type III isoform 1 (*Wachendorfia thyrsiflora*), GenBank Accession No. AAW50921.

The DNA fragments were amplified by PCR (see **7.2.4**). The PCR products were purified (see **7.2.6**), cloned into pCR4-TOPO and transformed to competent cell *E. coli* TOP10 (see **7.2.7.3-4**). The plasmids DNA were purified. The MaPKS inserts were verified by sequencing (see **7.2.12**). The plasmid DNAs were later used as templates for amplifying DNA

fragments for hybridization probes. After purification, the DNA was quantified by UV. To create the radioactive labeled probes, 25 ng of the purified DNA fragment were dissolved in sterile water to a total volume of 45 µl. The solution was heated at 95 °C for 3 min. After cooling, the solution was applied to "Ready-to-go DNA labelling bead". [^{32}P]dCTP (5µl) was added, the mixture was incubated at 37 °C for 20 min and was then applied onto ProbeQuant G-50 micro column and centrifuged.

Hybridization: The membranes were pre-hybridized in hybridization buffer (25-50 ml). The radioactive-labelled probe was denatured at 95 °C before being added to the hybridization buffer. Hybridization was done at 50 - 60 °C overnight. Membranes were washed with 2×SSC (0.1% SDS) until their radioactivity reached 100 cts/cm. Membranes were exposed to X-ray film at -80 °C overnight. According to a positive signal on the X-ray film, the plaques were cored with sterile Pasteur pipettes and incubated in SM buffer (500 µl with 20 µl $CHCl_3$). This phage suspension was used in secondary screening.

For secondary screening, the phage suspension obtained from primary screening (100-200 pfu) was plated on NZY agar (∅ 9 cm). Following the procedure described in primary screening, the single positive plaques were collected for *in vivo* excision.

7.2.11 *In vivo* excision

The bacteriophages containing genes of interest were used to infect *E. coli* NP66. This *E. coli* strain can perform the *in vivo* release of pExcell circular phagemid. *In vivo* excision of pExcell is accomplished by recombination between attL and attR; these sites flank the phagemid within the λ Excell DNA. *E. coli* NP66 was grown overnight at 32 °C in 2×YT medium containing spectinomycin (50 µg/ml), chloramphenicol (30 µg/ml) and maltose (0.2% w/v). The overnight culture was diluted (1:5) and grown again under the same conditions to OD_{600} 0.5-0.8, then centrifuged. Cells were re-suspended in NZCYM containing spectinomycin (50 µg/ml), adjusted to OD_{600} 2.0. *E. coli* NP66 (100 µl) was incubated and slightly shaken at 39 °C for 20 min. Phage suspension (100 µl) was added and the samples continued incubating at the same conditions for 20 min. Afterwards, 1 M sodium citrate (200 µl, 22 °C) was added to stop the infection. The pre-warmed 2×YT medium (5 ml, 32 °C) was added. The culture was then incubated and slightly shaken at 32 °C for 1.5 h. This released culture was then grown overnight at 37 °C on LB agar containing ampicillin (100 µg/ml). Colony PCR was done to check the correct clones. The phage-DNA was purified by minipreparation. The MaPKS genes were verified by sequencing.

7.2.12 Sequencing

Sequencing reaction was done using Big-Dye kit. The components of Big-dye® kit are 2.5% $MgCl_2$, polymerase buffer, AmpliTaq DNA polymerase FS, deoxynucleoside triphosphate (dNTP) and dideoxynucleosidetriphosphate (ddNTP) with fluorescence.

Sequencing reaction (10 µl):

Big-dye solution	4 µl
DNA template	300-500 ng
primer (10 pmol/µl)	1 µl

adjusted to 10 µl with sterile H_2O.

Thermo cycle for PCR amplification

Step	1.	denaturation	5 min	96° C
	2.	denaturation	10 sec	96° C
	3.	annealing	20 sec	50° C (or 55° C)
	4.	amplification	4 min	60° C ×34 from step 2.

To rid the PCR products of the excess primers, the reaction mixture was purified using a PCR purification kit (see **7.2.6**) and loaded on a sequencer for sequencing analysis.

7.2.13 Protein expression

E. coli BL21 containing expression plasmid with inserted MaPKS was grown overnight in LB medium containing kanamycin (50 µg/ml) and chloramphenicol (50 µg/ml). The overnight culture was sub-cultured into TB medium with 10x phosphate buffer (start-OD_{600}: 0.06) containing selective antibiotics. After growing to OD_{600} 0.6-0.8 (37 ºC, 220 rpm, 3-4 h), the bacterial culture was cooled to room temperature. To induce protein expression, IPTG was added to a final concentration of 1 mM. The culture was incubated at 22 ºC, 220 rpm, for 4 h. The cells were harvested by centrifugation (4,000 × g, 10 min). The cell pellet was re-suspended in phosphate buffer (25 mM with 150 mM NaCl, pH 7.4) and centrifuged again. The pellet was kept at -80 ºC until being used.

7.2.14 Protein purification

Cell disruption: The pellet was re-suspended in start buffer (25 ml) and disrupted with a French Press machine two times. The homogenate was centrifuged (10,000 x g, 4 ºC, 20 min). The supernatant contained MaPKS proteins.

Start buffer (50 ml):

6.25 ml	8x PO_4 buffer
0.5 ml	2 M imidazole

adjusted to 50 ml with sterile H_2O

Start buffer for cell disruption (25 ml):

25 ml	start buffer
400 µl	RNAse (1 mg/ml)
2mg	DNAse
4.3 mg	PMSF (in 300 µl isopropanol)
6.25 µl	*β*-ME

***Purification with metal chelating chromatography*:** The column (HiTrap® chelating column, 1 ml) was washed with water. $NiSO_4$ (0.1 M, 1 ml) was loaded onto the column. The column was washed again with water and then equilibrated with start buffer (10 ml). The raw extract was loaded onto the column. Then, the column was washed with start buffer (15 ml,). The protein was eluted with elution buffers containing 80 mM, 150 mM and 500 mM imidazole, respectively.

Buffer:	*8×phosphate buffer*	*imidazole (2M, pH 7.4)*
Start buffer (50 ml)	6.25 ml	0.5 ml
80mM imidazole buffer (10 ml)	1.25 ml	0.4 ml
150mM imidazole buffer (10 ml)	1.25 ml	0.75 ml
500mM imidazole buffer (10 ml)	1.25 ml	2.5 ml

The solutions were adjusted to 10 ml with sterile water. *β*-ME (3 mM) was added to all buffers before use.

***Gel filtration**:* To remove imidazole, gel filtration chromatography was performed. The Sephadex™G-35 was first equilibrated with protein buffer (25 ml). The protein fraction was loaded and eluted with protein buffer. Fractions (0.5 ml) were collected and protein was measured at UV 280 nm. The fractions containing proteins (OD~0.8-1.0) were pooled. The protein was frozen with liquid N_2 and kept at -80 °C until it was used. Protein buffer: 100 mM Hepes-KOH buffer (pH 7.0), 100 mM NaCl, 10% glycerine, 2 mM dithiothreitol (DTT)

7.2.15 Protein concentration measurement

Protein concentration was verified by the modified Bradford method (Bio-Rad Protein assay) using albumin as a standard. The protein was diluted in water (total volume 800 µl) to which the protein assay (200 µl) was added and mixed. After 30 min, the absorption was measured at 595 nm.

7.2.16 Enzyme activity assay

MaPKS1 was incubated with [2-^{14}C]malonyl-CoA and the following starter substrates: cinnamoyl-CoA, dihydrocinnamoyl-CoA, coumaroyl-CoA, dihydrocoumaroyl-CoA, caffeoyl-CoA, dihydrocaffeoyl-CoA, feruloyl-CoA, dihydroferuloyl-CoA, benzoyl-CoA and *n*-hexanoyl-CoA. The starter substrates (50 µM) and recombinant MaPKS were added to the reaction buffer (0.1 M Hepes-KOH buffer, pH 7.0) in a total volume of 150 µl. [2-^{14}C]Malonyl-CoA (80 µM, 0.17 Bq nmol^{-1}) was added to start the reaction. The reaction mixture was incubated at 37 ºC. After 3 h, the reaction was quenched by adding 10% acetic acid (10 µl) and then extracted with EtOAc (200 µl × 2). The extracts were pooled and dried in a

vacuum concentrator. The extracts were dissolved with 80% MeOH (20 µl) and analyzed by HPLC-flow scintillation analyzer using *anal. HPLC_system_6*-7 (see Appx **1.1**).

To screen for optimal temperature and pH, dihydrocinnamoyl-CoA and [2-^{14}C]malonyl-CoA were used as substrates. The pH was optimized in two buffer systems: 1. 0.1 M Hepes-KOH buffer, pH 4.5-7.5) and 2. phosphate buffer, pH 5.1-8.0. The optimum temperature was tested by using 0.1 M Hepes-KOH buffer (pH 5.0) at a range of from 25 to 41 ^{o}C. The incubation, extraction and HPLC analysis were done as described above. The activity of the protein was determined by the radioactivity (cts) of the main product. The enzyme activity with different starter-CoA esters was tested again using the optimized pH and temperature conditions.

7.2.17 Software

Sequence analysis: http://www.ebi.ac.uk/Tools/sequence.html, Vector NTI 10 (Invitrogen, Karlsruhe, Germany), DNASTAR 5.0 (DNASTAR Inc., Madison, WI, USA);

ExPasy Translate tool: http://www.expasy.ch/tools/dna.html

Blast from National Institute of Health: http://blast.ncbi.nlm.nih.gov/Blast.cgi

Relationship tree creation: http://www.phylogeny.fr/version2_cgi/index.cgi

7.3 Results

7.3.1 RNA extraction from *in vitro* grown *Musa acuminata*

Total RNA was extracted from non-treated and JA-treated *in vitro*-plants of *M. acuminata* (AAA) "Cavendish" Columbia (see **2.1**). Total RNA was extracted from 100 mg of plant tissue, leaves, petioles and roots separately by Plant RNeasy kit (see **7.2.2**). Leaves provided the most total RNA (~8-10 µg/100 mg leaf material) followed by petioles (~5 µg/100 mg petiole material) and roots (~1-2 µg/100 mg root material). The extracted total RNA, which had a 28S/18S ratio of 1.8-2.0, was further used in cDNA synthesis and amplification of type III PKS or MaPKS (*M. acuminata* polyketide synthase).

7.3.2 Plant type III PKS fragments from *Musa acuminata* (AAA) "Cavendish"

Total RNA was converted to cDNA by reverse transcriptase. Amplification of MaPKS fragment was done by PCR (see **7.2.4**) using degenerate primers K180_oli and FGFG_oli, which were also used in *W. thysiflora* (Brand *et al.*, 2006). A fragment of about 600 bp was obtained. After cloning and transformation (see **7.2.7.3-4**), the plasmid DNA was

prepared (see **7.2.8**) The fragment was named *MaPKS(585)* (see Appx **7.2**). Blastx revealed that *MaPKS(585)* was closely related to plant type III PKS.

With the help of *MaPKS(585)* sequence and the partial sequences of candidate CHS genes of *Musa* (kindly provided by Prof. D. Heckel, see **7.2.10**), specific primers for the sequences were designed and used in PCR amplification. Plasmid DNA was prepared for sequencing reactions as described previously. Five partial DNA sequences were obtained. Blastx (see **7.2.17**) showed that all the sequences were closely related to plant type III PKS. These include *MaPKS_probe585* (391 bp) from primer MaPKS_probe585f/r, *MaPKS_probe055* (301 bp) from primer MaPKS_probe055f/r, *MaPKS_probe587* (376 bp), *MaPKS_probe420* (209 bp) from primer MaPKS_probe420f/r, and *MaPKS_probe084* (365 bp) from primer MaPKS_probe084f/r. Sequences of DNA fragments and primers are shown in Appx **7.1-2**. These DNA fragments were used to prepare probes for cDNA library screening.

7.3.3 Full-length PKS from screening of cDNA library of *M. acuminata*

To study the role of PKS in the phenylphenalenone biosynthetic pathway in *M. acuminata*, a cDNA library was created from the JA-treated (50 µM, 24 h) plant. DNA fragments (see **7.3.2**) were used to prepare hybridization probes to screen for plant type III PKS in the cDNA library. Five full-length genes were obtained. Blastx (see **7.2.17**) revealed that all the isolated genes were type III PKSs. These were named *MaPKS 1-5* (*M. acuminata* polyketide synthase 1-5) obtained by screening with *MaPKS_probe585*, *MaPKS_probe055*, *MaPKS_probe587*, *MaPKS_probe084* and *MaPKS_probe420*, respectively. *MaPKS1* encoded for a protein consisting of 389 amino acids (M_r 43142.79), *MaPKS2*: 389 amino acids (M_r 43079.91), *MaPKS3*: 392 amino acids (M_r 42839.56), *MaPKS4*: 390 amino acids (M_r 42375.72) and *MaPKS5*: 389 amino acids (M_r 41783.06). The full-length sequences including the 5´-non-coding region and the 3´-non-coding region with polyA tail are shown in Appx **7.2.**

Deduced amino acid sequences of *MaPKS1-5* exhibited the type III PKS common characters of the catalytic triad Cys_{164}-His_{303}-Asn_{336}, numbering according to MsCHS (Fig. **7.1**). All the amino acid sequences shared about 60% identity with MsCHS; a typical CHS from *Medicago sativa* except *MaPKS3* that shared about 80%. This information suggested that *MaPKS3* may have a typical CHS function, while *MaPKS1*, *2*, *4* and *5* may have non-typical CHS functions.

```
MaPKS3      ----------MAKLAEIRQSQR--AEGSATVLAIGTATPVNVLYQADYPDYYFRITKSEH 48
MsCHS       ----------MVSVAEIRQAQR--AEGPATIMAIGTANPANCVEQSTYPDFYFKITNSEH 48
Gh2PS       -----MGSYSSDDVEVIREAGR--AQGLATILAIGTATPPNCVAQADYADYYFRVTKSEH 53
MaPKS1      ----------MASLHALRKTQR--TQGPATIMAIGTANPPNLYEQSTYPDYYFRVTNSEH 48
MaPKS2      ----------MASVHPFRKAQR--ARGPATIMAIGTANPPNLYEQSTFPDFYFRVTNSDH 48
WtPKS1      ----------MASTEGIQAYRNNMAEGPATIMAIGTANPPNVVDASTFPDYYWRVTNSEH 50
MaPKS4      ----------MAHINAFPRAQG--ADGPATVLAIGTANPAHFVDQMEYPDFFFRITNAED 48
MaPKS5      ----------MECTNGFRTTQS--ADGPATILAIGTANPSNVIDQSAYPDFYFRVTNSEH 48
CUS         MAPTTTMGSALYPLGEMRRSQR--ADGLAAVLAIGTANPPNCVTQEEIPDFYFRVTNSDH 58

MaPKS3      LT-ELKEKFKRMCDKSMIHKRYMHINEEILKENPNVCAYMA-PSLDARQDIVVVEVPKLG 106
MsCHS       KV-ELKEKFQRMCDKSMIKRRYMYLTEEILKDNPRVCEYMA-PSLAARQDMAVVVVPRLG 106
Gh2PS       MV-DLKEKFKRICEKTAIKKRYLALTEDYLQENPTMCEFMA-PSLNARQDLVVTGVPMLG 111
MaPKS1      KQ-DLKHKFRRMCEKTMVKRRYLHLTEEILKERPKLCSYME-PSFDDRQDIVVEEVPKLA 106
MaPKS2      MP-ELKEKFRRICGKTMIKKRYMHLTEEVLKDKPGMCSYMD-PSLDERQDIVVEEVPRLA 106
WtPKS1      LSPEYRVKLKRICERSSIRKRHLVLTEQLLKENPTLTTYVD-ASYDERQSIVLDAVPKLA 109
MaPKS4      KT-ELKEKFKRICENSAIRKRHMSLTEEILKGNPNLCGYMEQPSFDARQKVVLEAVPKMA 107
MaPKS5      LQ-DLKAKFRRICDKAAIKKRHLYLTEEILRENPSLLAPMA-PSFDARQEIVVTAVPELA 106
CUS         LT-ALKDKFKRICQEMGVQRRYLHHTEEMLSAHPEFVDRDA-PSLDARLDIAADAVPELA 116

MaPKS3      KEAAVKAIKEWGQPESKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVNRFMMYQQGCFA 166
MsCHS       KEAAVKAIKEWGQPKSKITHLIFCTTSGVDMPGADYQLTKLLGLRPYVKRYMMYQQGCFA 166
Gh2PS       KEAAVKAIDEWGLPKSKITHLIFCTTAGVDMPGADYQLVKLLGLSPSVKRYMLYQQGCAA 171
MaPKS1      KEAATKAIKEWGRSKSDITHLVFCSISGIDMPGADYRLAKLLGLPLSVNRIMLYSQACHM 166
MaPKS2      TEAAAKAIEEWGRDKSDVTHLVFCSTSGVDMPGADYRLVKLLGLPLSVNRIMLYSQACHI 166
WtPKS1      CEAAAKAIKEWGRPKTDITHMVVCTGAGVDVPGVDYKMMNLLGLPPTVNRVMLYNVGCHA 169
MaPKS4      AESAEKAIKEWGRPTSDITHLVFCSAAGLDLPGVDYRLVQKLGLPTSVRRVMLYNVGCHA 167
MaPKS5      KEAAAKAIKEWGRPKSDITHLVFCSASGVDMPGSDLQLLKLLGLPMSVSRVMLYNVGCHA 166
CUS         AEAAKKAIAEWGRPAADITHLVVTTNSGAHVPGVDFRLVPLLGLRPSVRRTMLHLNGCFA 176

MaPKS3      GGTVLRMAKDLAENNRGARVLVVCSEITAVTFRGPSESHLDSLVGQALFGDGAAAIIVGA 226
MsCHS       GGTVLRLAKDLAENNKGARVLVVCSEETPVTFRGPSDTHLDSLVGQALFGDGAAALIVGS 226
Gh2PS       GGTVLRLAKDLAENNKGSRVLIVCSEITAILFHGPNENHLDSLVAQALFGDGAAALIVGS 231
MaPKS1      GAAMLRIAKDLAENNKGARVLVVSCEITVLSFRGPDEHDFEALAGQAGFGDGAAAVIVGA 226
MaPKS2      GAQMLRIAKDIAENNKDARVLVVACELNTLIFRGPDERDFLSLAGQAAFADGAAAVIVGA 226
WtPKS1      SGTVLRIAKDLAENNKGARVLVVSSEVSVMFFRGPAEGDVEILLGQALFGDGSAAIIVGA 229
MaPKS4      GASALRLAKDLVENNKGARALVVSSEVNVMFFRGPDDDHFENLIGQALFGDGSAALIVGA 227
MaPKS5      GGTALRVAKDLAENNRGARVLAVCSEITVLSYRGPDAAHMESLIVQALFGDGAAALVVGA 226
CUS         GCAALRLAKDLAENSRGARVLVVAAELTLMYFTGPDEGCFRTLLVQGLFGDGAAAVIVGA 236

MaPKS3      DPDPATEQPIFQLVSASQTILPDSEGAIDGHLREVGLTFHLLKDVPGLISKNIERSLAEA 286
MsCHS       DPIPEIEKPIFEMVWTAHTIAPDSEGAIDGHLREAGLTFHLLKDVPGIVSKNIDKALIEA 286
Gh2PS       GPHLAVERPIFEIVSTDQTILPDTEKAMKLHLREGGLTFQLHRDVPLMVAKNIENAAEKA 291
MaPKS1      DPIQGLEKPIYEIMSATQVTVPESEKAVGGHLREVGLTFHFFNQLPLIIADNIGNSLADA 286
MaPKS2      DPIQGVEKPIFEMMSASQVTVPDCERAVGGHLKEIGLTFHFMNQLPMLIANNLENCLLEA 286
WtPKS1      DPIEGVEKPIFQIFSASQMTLPEGEHLVAGHLRELGLTFHLKPQLPNTVSSNIHKPLKKA 289
MaPKS4      DPVEGVEKPIYEISSASQVMVPESEEMVAGHLREIGLTFHLAKKVPSIVGSNIVRCLQAA 287
MaPKS5      DPVEGVERPIFEVASASQVMLPESAEAVGGHLREVGLTFHLKSQLPTIIASNIEQSLAAA 286
CUS         D-ADDVERPLFEIVSAAQTIIPESDHALNMRFTERRLDGVLGRQVPGLIGDNVERCLLDM 295

MaPKS3      FKPLGISD----WNSIFWIAHPGGPAILDQVEAKLGLEKEKMKATREVLKEYGNMSSACV 342
MsCHS       FQPLNISD----YNSIFWIAHPGGPAILDQVEEKLGLKPEKMKATREVLSEYGNMSSACV 342
Gh2PS       LSPLGITD----WNSVFWMVHPGGRAILDQVERKLNLKEDKLRASRHVLSEYGNLISACV 347
MaPKS1      FKPLGITD----WNEVFWVAHPGNWAIMDAIESKLGLLPEKLSTARHVFAEYGNMQSATV 342
MaPKS2      FKPLGITD----WNEVFWVSHPGNWGIMDAIERKVGLKQEKLRSSRHVFGEYGNMMSATV 342
WtPKS1      FEPLNITD----WNSIFWIVHPGGRAILDQVQEKIGLEENKLDVSRYVLAENGNMMSASV 345
MaPKS4      FAPMGITD----WNELFWIVHTGGRAIIDQVEANAGLAAGKLAPTRHVLSEHGNMQSASV 343
MaPKS5      FAPLGLSD----WNQLFWVVHPGGRAILDQVEARLELHKDRLGATRHVLSEYGNMQSATV 342
CUS         FGPLLGGDGGGGWNDLFWAVHPGSSTIMDQVDAALGLEPGKLAASRRVLSDYGNMSGATV 355

MaPKS3      LFILDEMRKRSAEDGKATTGEGLEWGVLFGFGPGLTVETVVLHSIPIAVH----- 392
MsCHS       LFILDEMRKKSVQAGLKTTGEGLDWGVLFGFGPGLTIETVVLHSVAI-------- 389
Gh2PS       LFIIDEVRKRSMAEGKSTTGEGLDCGVLFGFGPGMTVETVVLRSVRVTAAVANGN 402
MaPKS1      YFVMDEVRKRSVVEGRTTTGDGLQWGVLFGFGPGLSIETVVLRSVPL-------- 389
MaPKS2      LFVMDDVRKRSAAEGRATTGDGLEWGVLSGFGPGLSIETVVLRSVAL-------- 389
WtPKS1      FFIMDEMRKRSAAQGCSTTGEGHEWGVLFGFGPGLSIETVVLHSVPLSI------ 394
MaPKS4      LFIMDEMRKRSAVEGHATTGQGCQWGVLFGFGPGISVETVVLRSFPI-------- 390
MaPKS5      LFILDEMRKRSAAAGQATTGDGLDWGVLLGFGPGLSIETVVLHSVQI-------- 389
CUS         IFALDELRRQRKE--AAAAGEWPELGVMMAFGPGMTVDAMLLHATSHVN------ 402
```

Figure 7.1 Alignment of deduced amino acid sequences of *MaPKS 1-5* with typical MsCHS (*Medicago sativa*, GenBank accession no. L02902, Junghans *et al.*, 1993), Gh2PS (*Gerbera hybrida*, GenBank accession no. CAA86219, Helariutta *et al.*, 1995, 1996), WtPKS1 (*W. thysiflora*, GenBank accession no AY727928, Brand *et al.*, 2006), CUS (*Oryza sativa*, GenBank accession no. AK109558, Katsuyama *et al.*, 2007). The type III PKS catalytic triad Cys_{164}, His_{303} and Asn_{336} was highlighted (the numbering of the amino acid follows MsCHS).

7.3.4 Expression of MaPKS1 recombinant proteins

To study the function of the isolated gene, *MaPKS1* was expressed in *E. coli*. Using MaPKS1_BamHI_F and MaPKS1_Hind III_R primers, a MaPKS1 gene fragment was amplified from pExcell phagemids. The fragment was later cloned into pCR4_TOPO to produce pCR4_MaPKS1_ex-plasmids. The plasmids were then digested with restriction enzymes (*Bam*HI /*Hind*III). TheMaPKS1 gene fragment with restriction sites was ligated to the previously restricted pHis8 to create the expression plasmid, pHis8_MaPKS1 (see **7.2.7.5**). The nucleotide sequence of the expression plasmid was confirmed by sequencing before it was transformed to the expression organism, *E. coli* BL21 Codon Plus (DE3) RIL (*E. coli* BL21 (DE3)_pHis8_MaPKS1).

To express the MaPKS1 recombinant protein on a larger scale, *E. coli* BL21 (DE3)_pHis8_MaPKS1 culture was scaled up to 1 L culture (250 ml×4). After the culture reached OD_{600} of 0.6 to 0.8, it was treated with 1 mM IPTG at 20 ^{o}C for 4 h. Then the cells were harvested, re-suspended in start buffer and disrupted using a French Press machine. The lysate containing *MaPKS1* (protein crude extract) was purified as described in **7.2.14**. Fractions 3-4 (1 ml each) eluted with 500 mM imidazole contained high protein concentrations at about 45 kDa. The calculated Mw of MaPKS1 including His-tag was 45490.19. Figure **7.2** shows the SDS-PAGE of the protein purification. To get rid of imidazole, protein-rich fractions were further purified by gel filtration on Sephadex G-25 and eluted with storage buffer. MaPKS1 was obtained in total 7.2 mg (concentration 3.6 mg/ml).

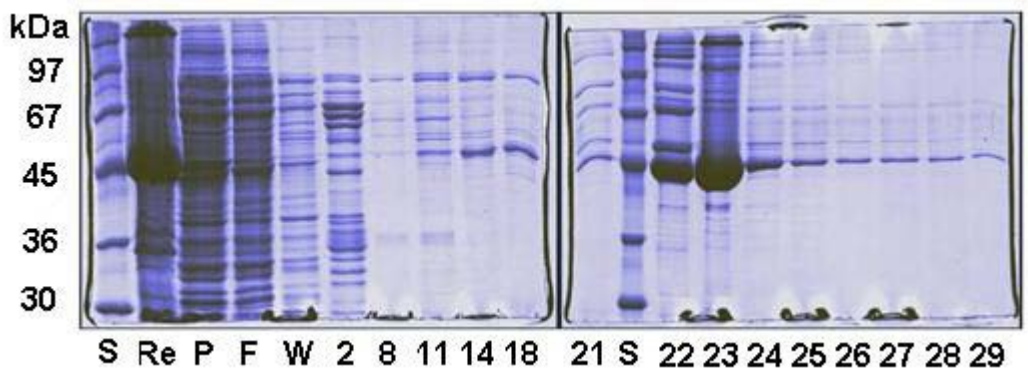


Figure 7.2 SDS-PAGE of a purified MaPKS1 recombinant protein; S = protein standard; Re = raw extract; P = pellet; F = flow-through; W = wash column; 2, 8 = fractions eluted with 80 mM imidazole; 11, 14, 18 = fractions eluted with 150 mM imidazole; 21-29 = fractions eluted with 500 mM imidazole. Fractions 22 and 23 contained a purified MaPKS1 protein.

7.3.5 Activity of recombinant MaPKS1

To determine the activity of recombinant MaPKS1, the protein was incubated with CoA ester starter substrates and [2-^{14}C]malonyl-CoA. The enzymatic products were extracted and analyzed by HPLC using a radio scintillation counter. Optimal pH and incubation temperature were tested using dihydrocinnamoyl-CoA and [2-^{14}C]malonyl-CoA as substrates. The activity of MaPKS1 was determined by the peak area (cts) of the main product. The maximum MaPKS1 activity was found at pH range 5.0 to 5.5 in 0.1 M Hepes-KOH buffer and at pH range 5.5 to 6.5 in phosphate buffer (Fig. **7.3**). The optimum temperature for MaPKS1 activity was screened from 25-40 ^{o}C. Incubation from 35 to 37 ºC gave the maximum activity (Fig. **7.4**). The optimal pH and temperature were used in the enzyme assay. The recombinant MaPKS1 protein was incubated with the phenylpropanoyl-CoA and [2-^{14}C]malonyl-CoA using the optimized conditions (0.1 M Hepes-KOH buffer pH 5; 37 °C; 3 h).

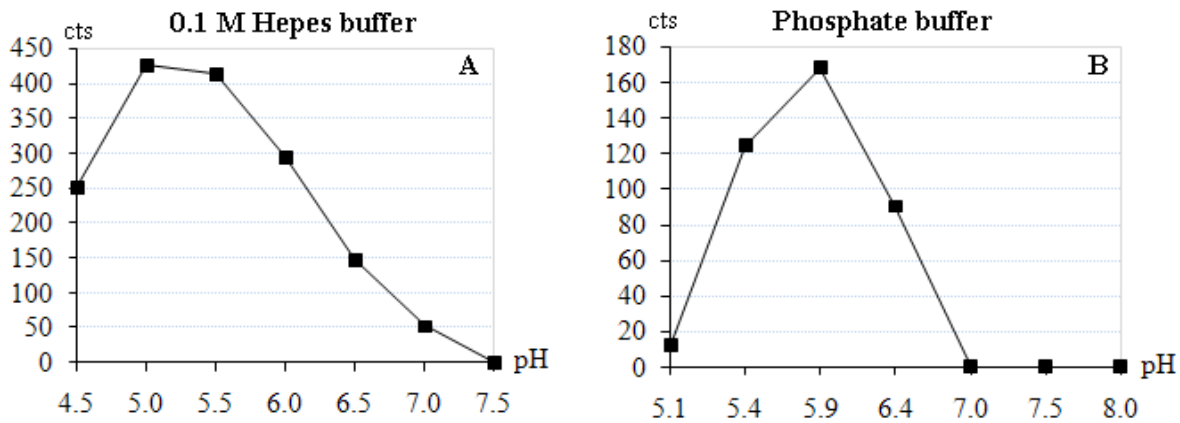
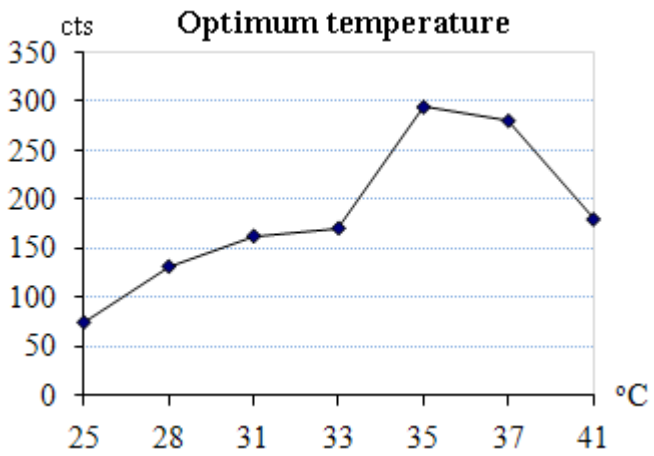


Figure 7.3 Screening for the optimum pH for MaPKS1 activity in 0.1 M Hepes buffer (A) and phosphate buffer (B). Dihydrocinnamoyl-CoA and [2-^{14}C]malonyl-CoA were used as substrates. Enzymatic products were analysed by *anal. HPLC system_6* (see Appx **1.1**).



◄**Figure 7.4** Screening for the optimum temperature for MaPKS1 activity in 0.1 M Hepes buffer (pH 5). Dihydrocinnamoyl-CoA and [2-^{14}C]malonyl-CoA were used as substrates. Enzymatic products were analyzed by *anal. HPLC system_6* (see Appx **1.1**).

Analysis of the enzymatic product using HPLC revealed that MaPKS1 was able to use dihydrocinnamoyl-CoA, coumaroyl-CoA, benzoyl-CoA and aliphatic-CoA esters such as *n*-hexanoyl-CoA. Dihydrocinnamoyl-CoA was the most effective substrate, according to the radioactivity of the product in HPLC analysis (*anal. HPLC system_6* see Appx **1.1**). The enzymatic product of MaPKS1 was preliminarily identified by comparing the R_t with that of WtPKS1 under the HPLC conditions reported in Brand *et al.* (2006) (*anal. HPLC system_7* (see Appx **1.1**). The R_t 29.7 min of the product was identical with that of 6-phenethyl-4-hydroxy-2*H*-pyran-2-one (**68**). The LC-ESI-MSMS spectrum obtained from the 15-fold up-scaled assay using non-radioactive substrates showed an $[M-H]^-$ ion at *m/z* 215.0893 (calc. 215.0708) corresponding to $C_{13}H_{11}O_3$, confirming the product as the pyrone **68** (Fig. **7.5**). MaPKS1 also produced other compounds, which unfortunately were obtained in amounts insufficient to confirm their chemical structures. The compounds could be the products of one condensation step such as benzalacetones or of multiple condensation steps such as chalcones or stilbenes (see Scheme **1.3**).

MaPKS1 was the first type III PKS from *M. acuminata* to be expressed and tested for its activity. The expression of MaPKS2-5 is under investigation.

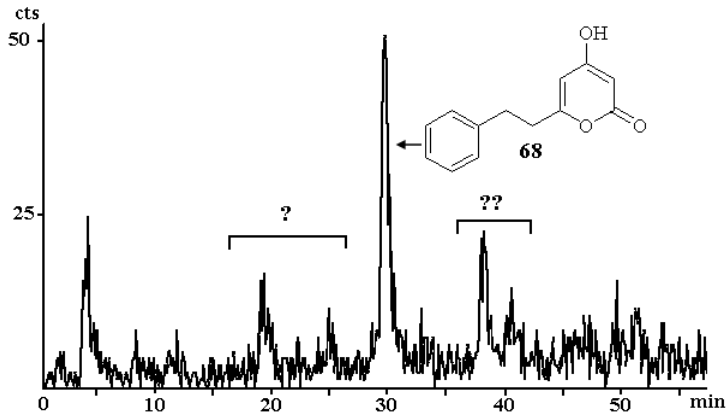


Figure 7.5 HPLC chromatogram of the enzymatic products of MaPKS1. The major product at R_t 29.7 min was identified by its retention times and mass spectra as 6-phenethyl-4-hydroxy-2*H*-pyran-2-one (**68**). ?, ?? = unidentified products.

7.4 Discussion

7.4.1 Full-length type III PKS from *Musa acuminata*

Since Schröder (1997) proposed that CHS-related type III PKS were involved in the biosynthetic of diarylheptanoids and phenylphenalenones, several enzymatic studies of the biosynthesis pathway have been reported. Plant type III PKS were isolated from the diarylheptanoid- or phenylphenalenone-containing species, *W. thysiflora* (Brand, 2005, Brand *et al.*,

2006) and *Curcuma longa* (Katsuyama *et al.*, 2009) as well as from *Oryza sativa*, a species that contains neither diarylheptanoids nor phenylphenalenones (Katsuyama *et al.*, 2007, http://www.biologie.uni-freiburg.de/data/bio2/schroeder). The recombinant proteins of the isolated PKS from those plant species were tested for their activity in the diarylheptanoid/phenylphenalenone biosynthetic pathway. A type III PKS involved in diarylheptanoid/phenylphenalenone biosynthesis in *M. acuminata* has not yet been reported. Using a homology RT-PC based-methods, we isolated candidate genes for this study.

The CHS-like fragments were amplified from *M. acuminata* (AAA) ”Cavendish”. Hybridization probes were created and used to screen for type III PKS from the cDNA library created from JA-treated *M. acuminata*. Five full-length sequences (*MaPKS1* to -*5*) were isolated and reported here for the first time. The deduced amino acid sequences of the isolated genes clearly showed the presence of the catalytic triad Cys-His-Asn, typical of type III PKS (Austin and Noel, 2003).

The identity of deduced amino acid sequences of *MaPKS1-5* with other type III PKS was investigated by creating a relationship tree (Fig. **7.6**). Sequences selected for comparison were the 2-pyrone synthase from *Gerbera hybrida* (Gh2PS, GenBank accession no. CAA86219, Helariutta *et al.*, 1995, 1996), a representative CHS from *Medicago sativa* (MsCHS, L02902, Junghans *et al.*, 1993), a type III PKSs from *W. thyrsiflora* (WtPKS1, AY727928, Brand *et al.*, 2006), curcuminoid synthase from *Oryza sativa* (CUS, AK109558, Katsuyama *et al.*, 2007), diketide-CoA synthase from *Curcuma longa* (DCS, BAH56225, Katsuyama *et al.*, 2009) and curcuminoid synthase from *C. longa* (CURS, BAH56226, Katsuyama *et al.*, 2009).

WtPKS1 was the first isolated type III PKS involved in phenylphenalenone biosynthesis. The enzyme catalyzed the first committed step by condensing phenylpropanoid-CoA with malonyl-CoA to form a diketide intermediate (Brand *et al.*, 2006). Note that enzyme activity assay by WtPKS1 (Brand *et al.*, 2006) resulted in the formation of products derived from one condensation (benzalacetones) and two condensations (pyrones), but neither diarylheptanoid nor phenylphenalenone were found. According to the authors’ argument, the formation of diarylheptanoids could require the functions of a second condensing enzyme, which might be another PKS (Brand *et al.*, 2006, http://www.biologie.uni-freiburg.de/data/bio2/schroeder). This hypothesis has been proven by the isolation of diketide-CoA synthase (DCS) and curcumin synthase (CURS) from *Curcuma longa* (Katsuyama *et al.*, 2009).

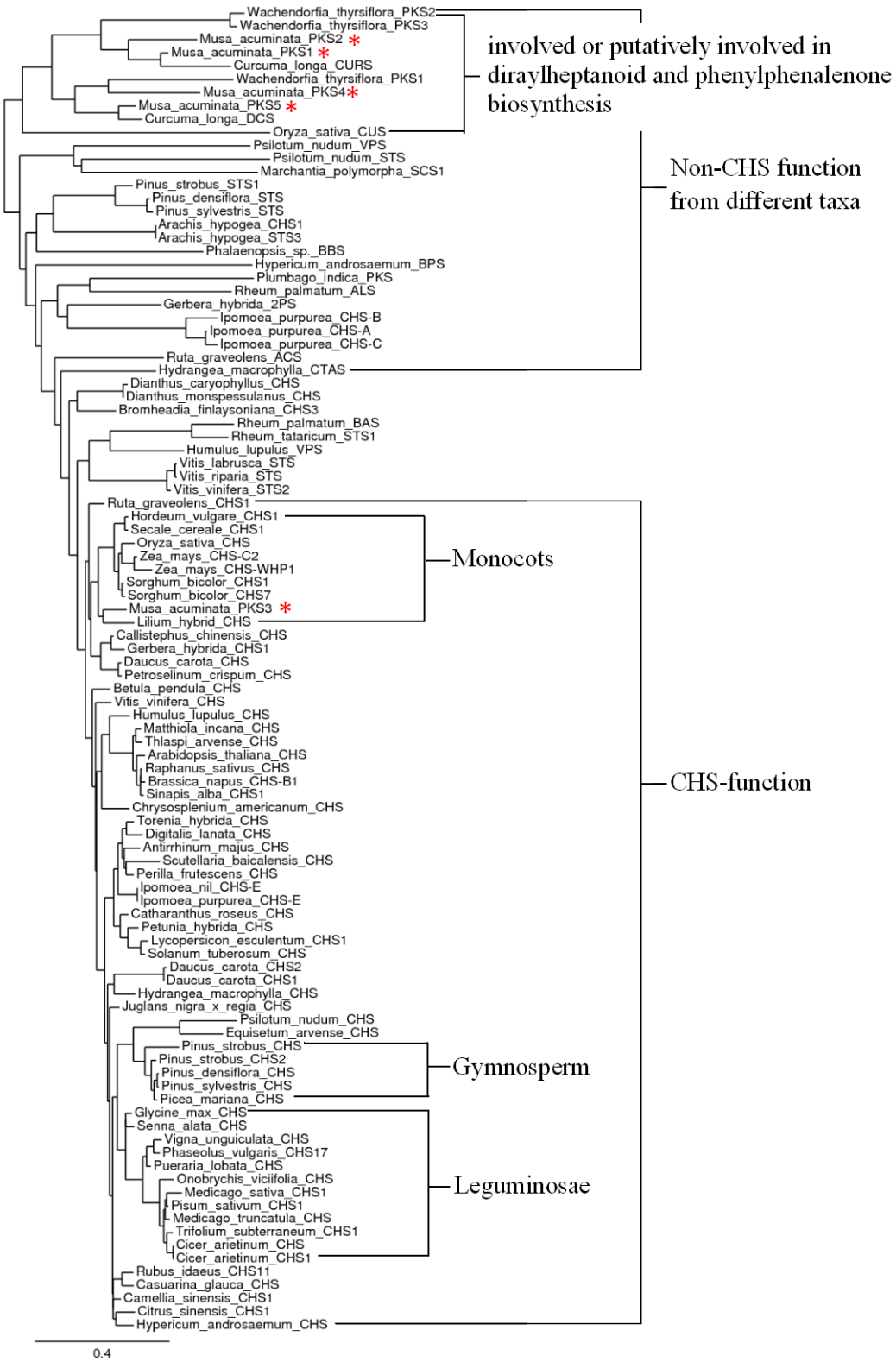


Figure 7.6 Relationship tree created (see **7.2.17**) from 100 selected type III PKSs (Doreeper *et al.*, 2008). The selection criteria focused on typical CHS, and non-CHS, especially from diarylheptanoid- and phenylphenalenone-containing species. Type III PKS from *M. acuminata* are marked with an asterisk.

The relationship tree (Fig. **7.6**) showed that MaPKS3 clustered in a group of PKSs from monocotyledons having typical CHS function, while MaPKS1, 2, 4, and 5 clustered together with type III PKS, that were involved in diarylheptanoid/phenylphenalenone biosynthesis: WtPKS1-3 from *W. thyrsiflora* (Brand, 2005; Brand *et al.*, 2006); CUS from *Oryza sativa*, the enzyme that is able to synthesize curcuminoids in one step (Katsuyama *et al.* 2007); DCS and CURS, the two enzymes that cooperate in curcuminoid biosynthesis from *Curcuma longa* (Katsuyama *et al.* 2009). The clusters suggested that MaPKS 1, 2, 4 and 5 could function differently from typical CHS and may be involved in phenylphenalenone/diarylheptanoid biosynthesis.

Looking more closely at the relationship tree, the sequences from diarylheptanoid-producing plants can be seen clustering into two subgroups. The first group is formed by MaPKS4, MaPKS5 and WtPKS1 together with DCS and the second group by WtPKS2, WtPKS3, MaPKS1, MaPKS2 and CURS. MaPKS4 and MaPKS5 are closely related to DCS and could function in the formation of the diketidyl-CoA, while MaPKS1 and -2, which were closely related to CURS, could condense diketidyl-CoA with the second molecule of phenylpropanoyl-CoA to form diarylheptanoids. The most interesting candidates were MaPKS5 and MaPKS1, which shared 88% and 83% identity in amino acid sequences with DCS and CURS, respectively. These two enzymes could cooperate in diarylheptanoid biosynthesis in *M. acuminata*. Whether or not these type III PKS from *M. acuminata* are able to synthesize diarylheptanoids prompted us to further investigate the activity of the MaPKSs. Unlike MaPKS1, 2, 4 and 5, MaPKS3 clustered with the CHS function enzymes. The enzyme shared approximately 80% of the amino acid sequence with MsCHS. MaPKS3 could be involved in the biosynthesis of flavonoids or stilbenes, which were also found as chemical constituents in *Musa* (Lewis *et al.*, 1999; Hölscher and Schneider, 1996).

7.4.2 Enzyme activity of MaPKS1

MaPKS1 was the first type III PKS from *M. acuminata* expressed in a heterologous system, *E. coli*. The recombinant MaPKS1 used starter CoAs, including phenylpropanoyl-CoA and aliphatic CoA esters, as substrates. It is known that type III PKS can accept a broad range of starter CoAs *in vitro* (Rimando and Baerson, 2006). Due to the weak activity of MaPKS1, only the main enzymatic product from dihydrocinnamoyl-CoA, the best accepted substrate, was identified by HPLC analysis and mass spectrometry. MaPKS1 catalyzed two condensation of dihydrocinnamoyl-CoA with malonyl-CoA yielding 6-phenethyl-4-hydroxy-2*H*-pyran-2-one (**68**). Recently, Katsuyama *et al.* (2009) reported two enzymes involved in the two-step biosynthesis of curcuminoid from *Curcuma longa*. The first enzyme, diketide-CoA synthase (DCS), catalyzed the formation of diketidyl-CoA intermediates from one con-

densation of phenylpropanoid-CoA with malonyl-CoA similar to WtPKS1. The second enzyme, CURS (curcumin synthase), condensed the diketidyl-CoA intermediate with the second phenylpropanoid-CoA, resulting in curcuminoids (Katsuyama *et al.*, 2009).

According to the information from the relationship tree, MaPKS1 is more closely related to CURS (83% identity) than WtPKS1 (60% identity) in amino acid sequences, suggesting MaPKS1 and CURS have similar functions. In contrast to our findings, the results of Katsuyama *et al*. (2009) showed that CURS accepted feruloyl-CoA as the best substrate and did not produce triketide pyrones and benzalacetones. CURS formed high amount of curcuminoids when cinnamoyl diketide-NAC and feruloyl-CoA were used as substrates. After this paper appeared, the activity of MaPKS1 was tested again using cinnamoyl diketide-NAC, a mimic of diketididyl-CoA, and cinnamoyl-CoA as substrates. Again, no diarylheptanoids or phenylphenalenones were detected (data not shown). The temperature and optimal pH of CURS are very different from those of MaPKS. For MaPKS1, optimal conditions were pH 5.0-5.5 at 35-37 ^{o}C, while for CURS; optimal conditions were pH 9 and 50 ^{o}C (Katsuyama *et al.*, 2009). The different conditions of the *in vitro* assay and the physiological environment may explain why MaPKS1 did not produce the final product. The different biosynthetic pathways in *M. acuminata, W. thyrsiflora* and *C. longa* could be another explanation. Whether or not MaPKS1 is involved in diarylheptanoid/phenylphenalenone biosynthesis is still an open question.

As the relationship tree shows (Fig. **7.6**), MaPKS4 and MaPKS 5 were closely related to DCS, while MaPKS1 and MaPKS2 were closely related to CURS, suggesting a two-enzyme system is involved in diarylheptanoid biosynthesis in *M. acuminata* as in *C. longa*. The expression of MaPKS4 and MaPKS5 and corresponding enzyme assays are important direction of future studies.

The chemical constituents of *W. thysiflora, C. longa* and *M. acuminata* also provided important information. From *W. thyrsiflora* root culture, there have been no reports of other type III PKS-derived compounds apart from phenylphenalenones. Therefore, it is likely that the type III PKS from *W. thyrsiflora's* root culture are involved in phenylphenalenone biosynthesis (Brand *et al.*, 2006). In *M. acuminata,* flavonoids and stilbene were found in addition to phenylphenalenones (Lewis *et al.*, 1999; Hölscher and Schneider, 1996). Hence, a part of the plant type III PKSs isolated from *M. acuminata* (e.g. MaPKS3) may function in other biosynthesis pathways and/or there may be some other type III PKS which remained non-isolated. In *Musa* plants, phenylphenalenones are inducible defence compounds produced after elicitation and infection. In this case, studying the gene expression of the isolated MaPKS 1-5 in response to treatment with various types of elicitors may help uncover type III PKS candidates in phenylphenalenone biosynthesis.

Chapter 8 Final discussion and conclusion

Plant secondary metabolites or natural products play many important roles in plants' interaction with their environment. Many natural products function as phytoalexins or phytoanticipins and are involved in plants' resistance to disease. The distribution of natural products is characteristic of biological groups such as families and genera. In the order Zingiberales, the families Zingiberaceae and Musaceae are economically important because they include medicinal plants (Zingiberaceae) and food crops (Musaceae). Diarylheptanoids and phenylphenalenones are characteristic natural products from these families.

Although the Zingiberaceae and Musaceae families are taxonomically related, their evolutionary origin is still unknown. *Alpinia oxymitra* is a candidate ancestor of the Zingiberaceae and Musaceae due to their closely related morphological characters, especially fruits and seeds. In this study, the phytochemistry of *A. oxymitra* was investigated for the first time. The identification of diarylheptanoids or phenylphenalenones from this plant was one of our aims, because the presence of these compounds in *A. oxymitra* would have been a chemotaxonomic indication of their chemical relationship. Using chromatographic and spectroscopic techniques, two known flavonoids and one new natural product of the calamenene-type sesquiterpenes were identified; however, neither diarylheptanoids nor phenylphenalenones were detected. To increase the formation of natural products, *A. oxymitra* was elicited with JA but no significant change in the profiles of natural products was observed. Whether or not *A. oxymitra* is a common ancestor of Zingiberaceae and Musaceae plants, therefore, is still open for investigation. The chemical constituents of *A. zerumbet*, a member of Zingiberaceae, were also investigated. HPLC-SPE-NMR was used as a highly effective method of identifying natural products from small amounts of a rhizome extract from *A. zerumbet*. Again, two main flavonoids were detected but no diarylheptanoids or phenylphenalenones were found. However, the non-detection of diarylheptanoids does not prove their absence. It would be interesting to investigate the phytochemistry of *A. oxymitra* and *A. zerumbet* using a library of candidate compounds as standards for targeted metabolomic profiling. The occurrence of diarylheptanoids and phenylphenalenones should be investigated not only in rhizomes but in all parts of plants, including leaves, flowers and fruits. For the identification of natural products, various elicitors may be applied to plants to stimulate their formation and increase their concentration. The phytochemical studies of *A. oxymitra* and *A. zerumbet* in this work revealed that the two species contain flavonoids as main compounds and are good sources for health-promoting products.

Musa plants originate from southeastern Asia, and *Musa* cultivars are diverse both in terms of genetics and patterns of natural products, especially phenylphenalenones. The concentration and structural diversity of phenylphenalenones in *Musa* correlate to plant's resis-

tance to disease (Otálvaro *et al.* 2002a). Therefore, the identification of phenylphenalenones in *Musa* cultivars from Thailand was a main focus of this work. Fifty-four plants were collected and their phytochemical profiles were investigated. Preliminary screening for natural products in *Musa* plants and *Ensete glauca* was done using TLC. Based on the TLC patterns of the rhizome extracts, plants from different genome groups were selected for further analysis. The selected plants represented two types of profiles of natural products: **1)** complex profiles with high levels of natural products and **2)** simple profiles with low levels of natural products. Using HPLC and spectroscopic methods phenylphenalenones were identified. They were the major secondary metabolites in the rhizomes of *Musa*. Twelve known phenylphenalenones were identified together with a new phenylphenalenone-related compound, 3-hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (**62**). 9-Phenylphenalenones and phenylphenalenones of the condensed dimer type were most frequently found, followed by 4-phenylphenalenones and ring A-oxidized phenylphenalenone-related compounds. The correlation between the occurrence of phenylphenalenones in *Musa* found in this study and the information about plants' resistance to disease reported by Silayoi (2002) was considered. We found that the commercial important cultivars such as *Musa* (AA) "Leb Mue Nang", *Musa* (AAA) "Hom Thong", *Musa* (AAA) "Hochuchu" and *Musa* (ABB) "Nam Wa" contained very low levels of phenylphenalenones. Interestingly, in the wild species, *Enseta glauca*, as well as in the ornamental species, *Musa cocinea* "Rattagatli" 9-phenylphenalenones were the main constituent. Other edible cultivars that contained high level of condensed dimer type phenylphenalenones were *Musa* (AA) "Hom Phol San", "Thong Ngoei", *Musa* (AAB) "Khom", *Musa* (ABB) "Hakmuk" and "Thong Ma Eng". The activity of a variety of 9-phenylphenalenones and 4-phenylphenalenones against pathogenic fungi was previously reported (Luis *et al.*, 1996; Kamo *et al.*, 1998b, 2000; Quiñones *et al.*, 2000; Otálvaro *et al.*, 2007), but the bioactivity of the condensed dimer type phenylphenalenones remains unknown. However, the formation of these compounds was discussed (Otálvaro *et al.*, 2002b). It is likely that 9-phenylphenalenones such as 4´-hydroxyanigorufone isolated from *Anigozanthos flavidus* (Hölscher and Schneider, 1999) are the precursors of the condensed dimer phenylphenalenones (Otálvaro *et al.*, 2002b). In *Musa*, these copounds may be present in lower concentration and under the detection limit.

The phytochemical screening of *Musa* and related species from Thailand has confirmed that phenylphenalenones were the main natural products in these plants. Therefore, the function of phenylphenalenones in *Musa* was investigated next. To understand the ecological function of phenylphenalenones, *in-vitro* plants of *Musa acuminata* were treated with biotic elicitors, *Sporobolomyces salmonicolor* and *Pseudomonas fluorescens* and a non-biotic elicitor, JA. Phenylphenalenones were identified as induced natural products in *Musa*'s roots after they were treated with biotic elicitors or JA. Biotic elicitors induced higher levels of phenyl-

phenalenones than JA did. Seven known phenylphenalenones and a new natural product, 2-(3´,4´-dihydroxyphenyl)-1,8-naphthalic anhydride (**66**), were identified as induced compounds in these experiments. Hydroxyanigorufone (**2**) and 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydrides (**34**) were detected as main phenylphenalenones. Maximum levels of these two compounds accumulated at day 5 after the roots were treated with *S. salmonicolor*. The biotic elicitors used in our study are non-pathogenic strains and the experiments were done on *in-vitro* plants. The responses of *in-vitro* plants of *Musa* may differ from those of *Musa* plants grown in their natural habitat. However, the increase of phenylphenalenones in treated plants which our study found was consistent with the results of previous investigations showing that phenylphenalenones function as phytoalexins and was induced after infection with pathogenic fungi and kanamycin (Luis *et al.*, 1993, 1996; Kamo *et al.*, 1998b, 2000, 2001). The antifungal activity of compounds **2** and **34** was previously reported (Luis *et al.*, 1993, 1996; Kamo *et al.*, 1998b, 2001). The induction of phenylphenalenones by non-pathogenic strains suggested that these strains could be developed for biological pathogen control purposes, i.e. to induce resistance in *Musa* plants.

Interestingly, increasing phenylphenalenone levels were also detected in the culture media of treated plants, suggesting that phenylphenalenones can be secreted from the roots. Although it is not clear that exudation also occurs in the native environment, it can be speculated that phenylphenalenones play a role in underground defense. The frequently reported accumulation of such compounds in the roots and rhizomes of Musaceae and Haemodoraceae supports this suggestion (Luis *et al.*, 1996; Opitz, 2002; Opitz *et al.*, 2003, 2000; Otálvaro, 2004). Therefore, the role of phenylphenalenones as an underground chemical defense merits more detailed study. In addition to the induced phenylphenalenones, 2-(4´-*ß*-glucosyloxyphenyl)-1,8-naphthalic anhydride (**67**), a new phenylphenalenone glucoside was isolated from *M. acuminata*. Phenylphenalenone glucosides are found as constitutive compounds in Haemodoraceae plants (Opitz, 2002; Opitz *et al.*, 2003). Compound **67** was the first compound of this type detected in *Musa*. The compound may be important in the defense and the timing of the formation of its aglycon, the antifungal compound 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**).

In previous reports, hydroxyphenylphenalenone and their *O*-methylated counterpart, methoxyphenylphenalenones such as anigorufone (**2**)/methoxyanigorufone (**3**) and irenolone (**2**)/4´-*O*-methylirenolone (**22**) 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**)/2-(4´-methoxyphenyl)-1,8-naphthalic anhydride (**35**), were detected together in *Musa* after treatment with pathogenic fungi (Kamo *et al.*, 1998b, 2000, 2001). Structural variation by *O*-methylation can change the physiological properties and biological activities of the compounds. Hence *O*-methyl phenylphenalenones and hydroxyphenylphenalenones may function differently. Otálvaro (2004) demonstrated that L-methionine was the origin of the *O*-methyl

group in *O*-methyl phenylphenalenones. The *O*-methyl groups may be introduced to phenylphenalenones in two different steps, either **1)** from the intact *O*-methyl phenylpropanoid precursors or **2)** from L-methionine during direct *O*-methylation of the hydroxyl group. Feeding various phenylpropanoid precursors to *Anigozanthos preissii* revealed that the *O*-methyl groups on the lateral phenyl ring were introduced by incorporating the intact *O*-methyl phenylpropanoid precursors, e.g. ferulic acid (Schmitt *et al.*, 2000). In contrast, the *O*-methyl group on ring A of phenylphenalenones (for ring designation see Fig. **1.3**) are not derived from *O*-methyl phenylpropanoids but are likely provided by L-methionine during the *O*-methylation of the corresponding hydroxyphenylphenalenone. According to this hypothesis, the L-methionine pool might be limiting *O*-methyl phenylphenalenone formation. This biosynthetic aspect of *O*-methyl phenylphenalenones was studied by administering [*methyl*-^{13}C]methionine to *in-vitro M. acuminata*. Elicitation with JA and with $CuCl_2$ was conducted in order to induce phenylphenalenones. The results showed that phenylphenalenones were induced in plants treated with $CuCl_2$ but not in plants treated with JA. The induced phenylphenalenones were mainly anigorufone (**1**) and hydroxyanigorufone (**2**). *O*-Methylirenolone (**22**) was the only *O*-methylphenylphenalenone detected in this experiment. It was found together with irenolone (**21**), its non-methylated hydroxyl structure. The *O*-methyl group in **22** was likely provided by L-methionine during *O*-methylation of **21** because the incorporation of 4´-methoxycinnamic acid was not evident in labelling experiments (Otálvaro, 2004).

Interestingly, *Musa* plants treated with microorganisms produced higher levels of hydroxyphenylphenalenones than of their *O*-methylphenylphenalenone analogues. For example, in *Musa* plants treated with *S. salmonicolor*, the level of hydroxyanigorufone (**2**) was higher than that of 9-(4´-hydroxyphenyl)-2-methoxyphenalen-1-one (**7**). Similar results were previously reported by (Kamo *et al*., 1998b, 2000). *Musa* fruits treated with *Colletotricum musae* showed higher levels of non-methylated phenylphenalenones anigorufone (**1**) and irenolone (**21**) than of methoxyanigorufone (**3**) and 4´-*O*-methylirenolone (**22**), respectively. The different levels of hydroxyphenylphenalenones and *O*-methyl phenylphenalenone in *Mus*a suggest that these two classes of compounds have different functions. Moreover, hydroxyphenylphenalenones are more potent antifungal compounds than are their *O*-methylated derivatives (Otálvaro *et al*., 2007), suggesting that hydroxyphenylphenalenones function in plant defense against pathogens. The accumulation in secretory cavities (Hölscher and Schneider, 2007), which are supposed to function as reservoirs for natural products that defend the plants against chewing insects, supported the assumption that *O*-methyl phenylphenalenones are involved in herbivore deterrence rather than pathogen defense.

An interesting autooxidative process resulting in the conversion of 4´-*O*-methylirenolone (**22**) and isoanigorufone (**20**) to 3-hydroxy-4-methoxyphenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (**40**) and 3-hydroxy-4-methoxyphenyl-1*H*,3*H*-

benzo[*de*]isochromen-1-one (**62**), respectively, was observed and a plausible mechanism has been proposed. As related phenyl-benzoisochromenones are known from Haemodoraceae and Musaceae plants and some of them were isolated from *M. acuminata* in this work, enzymatic formation also seems to be possible. Thus, more detailed studies of the mechanisms of oxidative conversion of phenylphenalenones should be undertaken.

The biosynthesis of diarylheptanoids and phenylphenalenones from two molecules of phenylpropanoids and one molecule of malonate has been established by labelling experiments (Hölscher and Schneider, 1995a, b; Schmitt and Schneider 1999; Schmitt *et al.*, 2000; Kita *et al*., 2008). In addition to the biosynthetic pathways using precursor feeding experiments, we studied biosynthetic enzymes. The type III PKS were thought to be involved in diarylheptanoid/phenylphenalenone biosynthesis (Schröder, 1997). Later this hypothesis was proven by enzymatic studies in *Wachendorfia thyrsiflora* (Haemodoraceae) (Brand *et al.*, 2006), *Oryza sativa* (Poaceae) (Katsuyama *et al*., 2007) and *Curcuma longa* (Zingiberaeae) (Katsuyama *et al.*, 2009). Based on these studies, diarylheptanoids were biosynthesized via two different enzymatic systems, which in the following discussion were named systems A and B. **System A**: One enzyme performs condensations of two phenylpropanoyl-CoAs with malonyl-CoA resulting in the final product. The enzyme curcuminoid synthase (CUS) isolated from *O. sativa,* in which diarylheptanoids or phenylphenalenones were not detected, belongs to system A. **System B**: The co-function of two enzymes catalyzes two different steps; the first enzyme forms the diketide intermediate by condensing a phenylpropanoyl-CoA and malonyl-CoA. Then another type III PKS condenses the diketide intermediate with the second phenylpropanoid to form diarylheptanoids. System B was found in *C. longa* to produce curcuminoids (Katsuyama *et al.*, 2009). In *C. longa*, two distinct enzymes, diketide-CoA synthase (DCS) and curcumin synthase (CURS), are involved in curcuminoid biosynthesis. DCS catalyzes the formation of the diketide, which is then condensed with a second phenylpanoyl-CoA by CURS to biosynthesize curcumin. In *W. thyrsiflora*, a protein operating by system B is supposed to catalyze the formation of diarylheptanoid intermediates, which undergo further biosynthetic steps towards phenylphenalenones (Brand *et al*., 2006). Using RT-PCR based methods, five candidate type III PKS were isolated from *M. acuminata*. They were named "*Musa acuminata* polyketide synthases" and numbered consecutively (*MaPKS1-5)*. The relationship tree created from the deduced amino acid sequences of *MaPKS1-5* and type III PKS from different species including the above-mentioned proteins from *W. thyrsiflora, C. longa* and *O. sativa* revealed that phenylphenalenone biosynthesis in *M. acuminta* seems to belong to enzyme system B, where two proteins co-function. *MaPKS 4* and *5* are likely to be involved in the formation of a diketide intermediate, as the deduced amino acid sequences clustered to WtPKS1 and DCS. The deduced amino acid sequences of *MaPKS1* and *2* clustered with CURS and therefore may be involved in the condensation of the inter-

mediate with the second phenylpropanoid to form the diarylheptanoid. MaPKS1 was the first expressed type III PKS in this study. Under *in vitro* assay conditions, the recombinant MaPKS1 accepted various phenylpropanoid-CoAs as starter substrates and produced pyrones by condensing phenylpropanoid-CoA with two malonyl-CoAs instead of diarylheptanoids or phenylphenalenones. The differences between the physiological conditions and the conditions used in the assay may explain why pyrones were formed instead of the supposed product of MaPKS1. Derailment products were found as the products of type PKS III *in vitro* conditions (Schröder, 2000; Austin and Noel, 2003). Future enzymatic studies of MaPKS1-5 will help clarify phenylphenalenone biosynthesis in *M. acuminata*.

In this study chromatographic and spectroscopic methods were used to establish the phytochemical data of plants from Zingiberaceae and Musaceae. In Musaceae, phenylphenalenones were found as main natural products and the role of phenylphenalenone in *Musa* as induced phytoalexins were confirmed. Type III PKS, which are candidate enzymes of the biosynthetis diarylheptanoids and phenylphenalenones in *Musa* were isolated. The results of this work are an important basis in further studies to develop disease resistant *Musa* cultivars.

Chapter 9 Summary

Musa acuminata (banana) and related species were studied with the respect to phytochemical composition, biosynthesis, inducibility, and the oxidative conversion of phenylphenalenones and related natural products. Isolation, purification and identification of natural products, especially diarylheptanoids and phenylphenalenones, were conducted in order to understand the chemical relationship between Zingiberaceae and Musaceae and to investigate the correlation between the occurrence of phenylphenalenones and disease resistance in Musaceae. Using TLC, HPLC, 1D and 2D NMR and MS techniques, flavonoids were identified as major natural products in *Alipinia zerumbet* and *A. oxymitra*. In *A. oxymitra*, a new clamenene-type sesquiterpene was additionally detected. Phenylphenalenones were identified as major natural products in *Musa* and *Ensete glauca*, including a new benzoisochromenone derivative. Evidence of correlation of the occurrence and level of phenylphenalenones and reported resistance has been provided. Auto-oxidation of 4-phenylphenalenones to phenylbenzoisochromenones was observed under acidic condition and a mechanistic model of this conversion has been proposed.

The function of phenylphenalenones in *M. acumina* was studied. The treatment of *in-vitro* plants of *M. acuminata* with *Sporobolomyces salmonicolor* and *Pseudomonas fluorescens* or JA strongly stimulated the formation of phenylphenalenone formation. Eight metabolites were induced in response to elicitation, suggesting their function as phytoalexins. More phenylphenalenones accumulated in roots than in leaves and were also detected in plants' culture media. The induced metabolites included a new natural product, 2-(3´,4´-dihydroxyphenyl)-1,8-naphthalic anhydride. Another natural product, 2-(4'-glucosyloxyphenyl-1,8-napthalic anhydride, is the first phenylphenalenone glucoside identified in *Musa*. The discovery of this compound is interesting for the timing and formation of phenylphenalenones in *Musa*.

The biosynthesis of *O*-methyl phenylphenalenones in *M. acuminata* was studied by feeding [*methyl*-^{13}C]methionine to plants elicited with JA and with $CuCl_2$. $CuCl_2$ was able to induce the formation of phenylphenalenones including *O*-methyl phenylphenalenones. The *O*-methyl group in 4´*O*-methylirenolone was likely provided by L-methionine during the *O*-methylation of irenolone.

Using RT-PCR-based methods, five type III PKS were identified in *M. acuminata*, namely *MaPKS1-5* (*Musa acuminata* polyketide synthase 1-5). Due to sequence identity with enzymes reported from other plants, four are likely to be involved in diarylheptanoid biosynthesis. Under *in vitro* conditions, the recombinant MaPKS1 showed type III PKS function. Using phenylpropanoid-CoA's as starter substrates, the protein performed two condensations

to produce diketides and released them as pyrones. This preliminary study provides the basis for future biosynthetic study of phenylphenalenones in *Musa*.

Chapter 10 Zusammenfassung

Musa acuminata (Bananen) und verwandte Pflanzen wurden in Bezug auf ihre phytochemischen Komponenten, deren Biosynthese und Induzierbarkeit, sowie hinsichtlich von an der Biosynthese beteiligten Enzymen und der oxidativen Umwandlung von Phenylphenalenonen untersucht. Einige Naturstoffe, speziell Diarylheptanoide und Phenylphenalenone, wurden isoliert, gereinigt und identifiziert um die phytochemische Verwandtschaft zwischen Zingiberaceae and Musaceae zu verstehen und die Beziehungen zwischen dem Vorkommen von Phenylphenalenonen und der Resistenz von *Musa* gegenüber Infektionen zu untersuchen. Mit Hilfe der TLC, HPLC, 1D und 2D NMR und MS-Methoden wurden Flavonoide als Hauptnaturstoffe in *Alpinia oxymitra* und *A. zerumbet* identifiziert. In *A. oxymitra* wurde eine neue Verbindung vom Typ der Calamanen-Sesquiterpene gefunden. In *Musa* und *Ensete glauca* wurden Phenylphenalenone als Hauptnaturstoffe identifiziert, darunter ein neues Phenylbenzoisochromenon. Es wurde ein Zusammenhang zwischen dem Vorkommen und der Konzentration von Phenylphenalenonen einerseits und der Resistenz von *Musa*-Pflanzen andererseits festgestellt. Unter sauren Bedingungen wurde Autoxidation von 4-Phenylphenalenonen zu Phenylbenzoisochromenonen beobachtet und es wurde ein Modell des Mechanismus dieser Umwandlung vorgeschlagen.

Die Funktion der Phenylphenalenone in *M. acuminata* wurde ebenfalls studiert. Die Behandlung von *in vitro*-Pflanzen mit *Sporobolomyces salmonicolor* und *Pseudomonas fluorescens* oder Jasmonsäure stimuliert die Bildung von Phenylphenalenonen. Acht Verbindungen dieses Typs wurden durch Elicitierung induziert. Diese Ergebnisse haben gezeigt dass Phenylphenalenonene in *Musa* als Phytoalexine fungieren. Wurzeln enthielten höhere Konzentrationen an Phenylphenalenonen als die Blätter und auch in den Kulturmedien wurden Phenylphenalenone gefunden. Unter den induzierten Verbindungen befand sich eine neue Verbindung, 2-(3´,4´-Dihydroxyphenyl)-1,8-naphthalinanhydrid. Eine andere neue Verbindung, 2-(4´–*ß*-Glucosyloxyphenyl)-1,8-naphthalinanhydrid, gehört zu den Phenylphenalenonenglucosiden. Es ist die erste Verbindung dieses Typs aus *Musa*. Die Entdeckung dieser Verbindung ist sehr interessant und ihr Metabolismus könnte Information über den Zeitpunkt der Bildung von Phenylphenalenone in *Musa* liefern.

Die Biosynthese der *O*-methylierten Phenylphenalenone in *M. acuminata* wurde durch Fütterung von [*methyl*-^{13}C]Methionin an mit JA oder $CuCl_2$ elicitierten Pflanzen untersucht. $CuCl_2$ konnte die Bildung der Phenylphenalenone und der *O*-methylierten Phenylphenalenonen induzieren. Die *O*-Methylgruppe von *O*-Methylirenolon stammt vom L-Methionine und wird wahrscheinlich durch *O*-Methylierung auf Irenolon übertragen.

Mit Hilfe von RT-PCR Methoden wurden fünf Typ III PKS, *MaPKS1-5* (*Musa acuminata* Polyketidsynthase) aus *M. acuminata* identifiziert. Wegen der Ähnlichkeit der Se-

quenzen von *MaPKS1-5* mit Typ III PKS von anderen Pflanzen lässt sich vermuten, dass vier davon an der Diarylheptanoid-Biosynthese beteiligt sind. Unter *in vitro*-Bedingungen weist rekombinante MaPKS1 Typ III PKS-Aktivität auf. Werden Phenylpropanoid-CoA Ester als Starter-Substrate eingesetzt, führt das Protein zwei Kondensationsreaktionen mit Malonyl-CoA aus und produziert Diketide, die als Pyrone freigesetzt werden. Diese vorläufigen Untersuchungen stellen die Grundlage für die zukünftige Untersuchung der Phenylphenalenone-Biosynthese in *M. acuminata* dar.

References

Alvarez, E., Francisco, C. G., and Freire, R., **Salazar, J. A., Suarez, E. and Betancor, C.** (1986). Regioselectivity of the reaction of 3-ketosteroids with potassium superoxide. J. Chem. Soc., Perkin Trans. 1 **8**, 1523 – 1526.

Austin, M. B., and Noel, A. J. P. (2003). The chalcone synthase superfamily of type III polyketide synthases. Nat. Prod. Rep. **20**, 79-110.

Bais, H. P., Walker, T. S., Schweiyer, H. P., and Vivanco, J. M. (2002). Root specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of sweer basil (*Ocimum basilicum* L.). Plant Physiol. Biochem. **40**, 983-995.

Beckert, C., Horn, C., Schnitzler, J. P., Lehning, A., Heller, W., and Veit, M. (1997). Styrylpyrone biosynthesis in *Equisetum arvense*. Phytochemistry **44**, 275-283.

Behura, S., and Srivastava, V. K. (2004). Essential oils of leaves of *Curcuma* species. J. Ess. Oil Res. **16**, 109-110.

Bick, I. R. C., and Blackman, A. J. (1973). Haemodorin - phenalenone pigment. Aust. J. Chem. **26**, 1377-1380.

Binks, R. H., Greenham, J. R., Luis, J. G., and Gowen, S. R. (1997). A phytoalexin from roots of *Musa acuminata* var Pisang sipulu. Phytochemistry **45**, 47-49.

BorejszaWysocki, W., and Hrazdina, G. (1996). Aromatic polyketide synthases purification, characterization, and antibody development to benzalacetone synthase from raspberry fruits. Plant Physiol. **110**, 791-799.

Boyd, A. (1992). Musopsis N. Gen.: A banana-like leaf genus from the early tertiary of eastern north Greenland. Am. J. Bot. **79**, 1359-1367.

Brand, S., Hölscher, D., Schierhorn, A., Svatos, A., Schröder, J., and Schneider, B. (2006). A type III polyketide synthase from *Wachendorfia thyrsiflora* and its role in diarylheptanoid and phenylphenalenone biosynthesis. Planta **224**, 413-428.

Brand, S. (2005). Pflanzliche Polyketidsynthase des Type III in *Wachendorfia thyrsiflora* (Haemodoraceae). Ph. D. Thesis, Faculty of biology and pharmacy, University of Jena, Germany.

Broadbent, D., Mabelis, R. P., and Spencer, H. (1976). C-Acetylphloroglucinols from *Pseudomonas fluorescens*. Phytochemistry **15**, 1785-1785.

Chattopadhyay, I., Biswas, K., Bandyopadhyay, U., and Banerjee, R. K. (2004). Turmeric and curcumin: biological actions and medicinal applications. Curr. Sci. **87**, 1325-1325.

Cheong, J. J., and Choi, Y. D. (2003). Methyl jasmonate as a vital substance in plants. Trends Genet. **19**, 409-413.

Cooke, R.G., and Segal, W. (1955). Colouring matters of Australian plants .4. Haemocorin - a unique glycoside from *Haemodorum corymbosum* Vahl. Aust. J. Chem. **8**, 107-113.

Cooke, R. G., and Dagley, I. J. (1979). Coloring matters of Australian plants .21. naphthoxanthenones in the Haemodoraceae. Aust. J. Chem. **32**, 1841-1847.

Cooke, R. G., and Edwards, J. M. (1981). Naturally occurring phenalenones and related compounds. Fortschr Chem Org Naturst **40**, 153-190.

Dahlgren, R., and Clifford, H. T. (1982). The Monocotyledons: a comparative study, Academic Press, London.

De Ascensao, A., and Dubery, I. A. (2000). Panama disease: cell wall reinforcement in banana roots in response to elicitors from *Fusarium oxysporum* f. sp cubense race four. Phytopathology **90**, 1173-1180.

Del Rio, J. C., Jimenez-Barbero, J., Chavez, M. I., Politi, M., and Gutierrez, A. (2006). Phenylphenalenone type compounds from the leaf fibers of abaca (*Musa textilis*). J. Agri. Food Chem. **54**, 8744-8748.

Dereeper A., Guignon V, Blanc G., Audic S., Buffet S., Chevenet F., Dufayard J. F., Guindon S., Lefort V., Lescot M., Claverie J.M., Gascuel O. (2008)**.** Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. **36** No. suppl_2 W465-W469.

DellaGreca, M., Molinaro, A., Monaco, P., and Previtera, L. (1992a). Dimeric phenalene metabolites from *Eichhornia crassipes*. Tetrahedron **48**, 3971-3976.

DellaGreca, M., Lanzetta, R., Molinaro, A., Monaco, P., and Previtera, L. (1992b). Phenalene Metabolites from *Eichhorniacrassipes*. Bioorg. Med. Chem. Lett. **2**, 311-314.

DellaGreca, M., Previtera, L. and Zarrelli, A. (2008) Revised structures of phenylphenalene derivatives from *Eichhornia crassipes*. Tetrahedron Lett. **49**, 3268-3272.

Dixon, R.A. (2001). Natural products and plant disease resistance. Nature **411**, 843-847.

Dong, H., Chen, S. X., Xu, H. X., Kadota, S., and Namba, T. (1998). A new antiplatelet diarylheptanoid from *Alpinia blepharocalyx*. J. Nat. Prod. **61**, 142-144.

Dora, G., Xie, X. Q., and Edwards, J. M. (1993). 2 Novel phenalenones from *Dilatris viscosa*. J. Nat. Prod. **56**, 2029-2033.

Eckermann, S., Schröder, G., Schmidt, J., Strack, D., Edrada, R.A., Helariutta, Y., Elomaa, P., Kotilainen, M., Kilpelainen, I., Proksch, P., Teeri, T.H., and Schroder, J. (1998). New pathway to polyketides in plants. Nature **396**, 387-390.

Edwards, J. M., and Weiss, U. (1974). Pigments of *L. tinctoria* Ell .5. phenalenone pigments of root system of *Lachnanthes tinctoria*. Phytochemistry **13**, 1597-1602.

Elzaawely, A. A., Xuan, T. D., Koyama, H., and Tawata, S. (2007). Antioxidant activity and contents of essential oil and phenolic compounds in flowers and seeds of *Alpinia zerumbet* (Pers.) BL Burtt. & RM Sm. Food Chem. **104**, 1648-1653.

Farmer, E. E., and Ryan, C. A. (1990). Interplant communication airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc. Natl. Acad. Sci. USA **87**, 7713-7716.

Ferrer, J. –L., Austin, M.B., Stewart, C. Jr., Noel, J. P. (2008), Structure and finction of enzymes involved in the biosynthesis of phenylpeopanoids. Plant Physiol. Biochem. **46**, 356-370.

Friedrich, W. L., and Koch, B. E. (1970). Comparison of fruits and seeds of fossil *Spirematospermum* with those of living *Cenolophon*. Bull. geol. Soc. Denmark **20**, 192-195.

Giang, P. M., Son, P. T., Matsunami, K., and Otsuka, H. (2005). New diarylheptanoids from *Alpinia pinnanensis*. Chem. Pharm. Bull. **53**, 1335-1337.

Grayer, R. J., and Kokubun, T. (2001). Plant-fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants. Phytochemistry **56**, 253-263.

Gundlach, H., Muller, M. J., Kutchan, T. M., and Zenk, M. H. (1992). Jasmonic acid is a signal transducer in elicitor induced plant bell cultures. Proc. Natl. Acad. SCi. USA **89**, 2389-2393.

Habsah, M., Amran, M., Mackeen, M. M., Lajis, N.H., Kikuzaki, H., Nakatani, N., Rahman, A. A., **Ghafar, and Ali, A.M.** (2000). Screening of Zingiberaceae extracts for antimicrobial and antioxidant activities. J. Ethnopharmacol. **72**, 403-410.

Harborne, J. B. (1993). Advances in chemical ecology. Nat. Prod. Rep. **10**, 327-348.

Harborne, J. B. (1999). Recent advances in chemical ecology. Nat. Prod. Rep. **16**, 509-523.

Harmon, A. D., Edwards, J .M., and Highet, R. J. (1977). The biosynthesis of 2, 5, 6 tri hydroxy-9-phenyl phenalenone by *Lachnanthes tinctoria* incorporation of carbon-13 phenyl alanine. Tetrahedron Lett. **51**, 4471-4474.

Hartmann, T. (1996). Diversity and variability of plant secondary metabolism: a mechanistic view. Entomol. Exp. Appl. **80**, 177-188.

Hartmann, T. (1999). Chemical ecology of pyrrolizidine alkaloids. Planta **207**, 483-495.

Hartmann, T. (2007). From waste products to ecochemicals: fifty years research of plant secondary metabolism. Phytochemistry **68**, 2831-2846.

Helariutta, Y., Elomaa, P., Kotilainen, M., Griesbach, R. J., Schröder, J., and Teeri, T. H. (1995). Chalcone synthase-like genes active during corolla development are differentially expressed and encode enzymes with different catalytic properties in Gerberahybrida (Asteraceae). Plant Mol. Biol. **28**, 47-60.

Helariutta, Y., Kotilainen, M., Elomaa, P., Kalkkinen, N., Bremer, K., Teeri, T. H., and Albert, V. A. (1996). Duplication and functional divergence in the chalcone synthase gene family of Asteraceae: Evolution with substrate change and catalytic simplification. Proc. Natl. Acad. Sci. USA **93**, 9033-9038.

Herderich, M., Beckert, C., and Veit, M. (1997). Establishing styrylpyrone synthase activity in cell free extracts obtained from gametophytes of *Equisetum arvense* L by high performance liquid chromatography tandem mass spectrometry. Phytochem. Anal. **8**, 194-197.

Heslop-Harrison, J. S., and Schwarzacher, T. (2007). Domestication, genomics and the future for banana. Ann. Bot. **100**, 1073-1084.

Hirai, N., Ishida, H., and Koshimizu, K. (1994). A phenalenone type phytoalexin from *Musa acuminata*. Phytochemistry **37,** 383-385.

Hölscher, D., and Schneider, B. (1998). Phenylphenalenones from *Ensete ventricosum*. Phytochemistry (Oxford) **49**, 2155-2157.

Hölscher, D., and Schneider, B. (1999). HPLC-NMR analysis of phenylphenalenones and a stilbene from *Anigozanthos flavidus*. Phytochemistry **50**, 155-161.

Hölscher, D., and Schneider, B. (1995a). A diarylheptanoid intermediate in the biosynthesis of phenylphenalenones in *Anigozanthos preissii*. J. Chem. Soc., Chem. Commun., 525-526.

Hölscher, D., and Schneider, B. (1995b). The Biosynthetic origin of the central one-carbon unit of phenylphenalenones in *Anigozanthos preissii*. Nat. Prod. Lett. **7**, 177-182.

Hölscher, D., and Schneider, B. (1996). A resveratrol dimer from *Anigozanthos preisii* and *Musa* Cavendish. Phytochemistry **43**, 471-473.

Hölscher, D., and Schneider, B. (1997). Phenylphenalenones from root cultures of *Anigozanthos preissii*. Phytochemistry **45**, 87-91.

Hölscher, D., and Schneider, B. (2000). Phenalenones from *Strelitzia reginae*. J. Nat.Prod. **63**, 1027-1028. **Hölscher, D., and Schneider, B.** (2005). The biosynthesis of 8-phenylphenalenones from *Eichhornia crassipes* involves a putative aryl migration step. Phytochemistry **66**, 59-64.

Hölscher, D., and Schneider, B. (2007). Laser microdissection and cryogenic nuclear magnetic resonance spectroscopy: an alliance for cell type-specific metabolite profiling. Planta **225**, 763-770.

Ibrahim, R.K., Bruneau, A., and Bantignies, B. (1998). Plant *O*-methyltransferases: molecular analysis, common signature and classification. Plant Mol. Biol. **36**, 1-10.

Ingham, J. L. (1973). Disease resistance in higher plants. The concept of pre-infectional and post-infectional resistanceSFX]. Phytopathologische Zeitschrift (J. Phytopathol.) **78**, 314-335.

Jang, D. S., Park, E. J., Hawthorne, M. E., Vigo, J. S., Graham, J. G., Cabieses, F., Santarsiero, B. D., Mesecar, A. D., Fong, H. H. S., Mehta, R. G., Pezzuto, J. M., and Kinghorn, A. D. (2002). Constituents of *Musa x paradisiaca* cultivar with the potential to induce the phase II enzyme, quinone reductase. J. Agri.Food Chem. **50**, 6330-6334.

Jasril, Mooi, L. Y., Lajis, N. H., Ali, A. M., Sukari, M. A., Rahman, A. A., Othman, A. G., Kikuzaki, H., and Nakatani, N. (2003). Antioxidant and antitumor promoting activities of the flavonoids from *Hedychium thyrsiforme*. Pharm. Biol. **41**, 506-511.

Jiang, H. L., Xie, Z. Z., Koo, H. J., McLaughlin, S. P., Timmermann, B. N., and Gang, D. R. (2006). Metabolic profiling and phylogenetic analysis of medicinal *Zingiber* species: tools for authentication of ginger (*Zingiber officinale* Rosc.). Phytochemistry **67**, 1673-1685.

Jitsaeng, K., De-Eknamkul, W., and Schneider, B., (2009). Flavonoids and a new calamenene-type sesquiterpene from rhizomes of *Alpinia oxymitra* K. Schum. (Zingiberaceae). Rec. Nat. Prod. **3**, 110-113.

Joshi, S., Chanotiya, C. S., Agarwal, G., Prakash, O., Pant, A.K., and Mathela, C. S. (2008). Terpenoid compositions, and antioxidant and antimicrobial properties of the rhizome essential oils of different *Hedychium* species. Chem. Biodivers. **5**, 299-309.

Junghans, H., Dalkin, K., and Dixon, R.A. (1993). Stress responses in alfalfa (*Medicago sativa* L) .15. Characterization and expression patterns of members of a subset of the chalcone synthase multigene family. Plant Mol. Biol. **22**, 239-253.

Kamo, T., Kato, N., Hirai, N., Tsuda, M., Fujioka, D., and Ohigashi, H. (1998a). A biosynthetic intermediate of phytoalexins in banana fruits. Phytochemistry **49**, 1617-1621.

Kamo, T., Kato, N., Hirai, N., Tsuda, M., Fujioka, D., and Ohigashi, H. (1998b). Phenylphenalenone-type phytoalexins from unripe Bungulan banana fruit. Biosci. Biotech. Biochem. **62**, 95-101.

Kamo, T., Hirai, N., Tsuda, M., Fujioka, D., and Ohigashi, H. (2000). Changes in the content and biosynthesis of phytoalexins in banana fruit. Biosci. Biotech. Biochem. **64**, 2089-2098.

Kamo, T., Hirai, N., Iwami, K., Fujioka, D., and Ohigashi, H. (2001). New phenylphenalenones from banana fruit. Tetrahedron **57**, 7649-7565.

Katsuyama, **Y.**, **Matsuzawa, M., Funa**, **N.**, **and Horinouchi**, **S.** (2007). *In vitro* synthesis of curcuminoids by Type III Polyketide Synthase from *Oryza sativa***.** J. Biol. Chem. **282**, 37702-37709.

Katsuyama, **Y.**, **Kita**, **T.**, **Funa**, **N.**, **and Horinouchi**, **S**. (2009). Curcuminoid biosynthesis by two Type III polyketide synthases in the herb *Curcuma longa*. J. Biol. Chem. **284**, 11160-11170.

Kavino, M., Harish, S., Kumar, N., Saravanakumar, D., and Samiyappan, R. (2008). Induction of systemic resistance in banana (*Musa* spp.) against banana bunchy top virus (BBTV) by combining chitin with root-colonizing *Pseudomonas fluorescens* strain CHA0. Eur. J. Plant Pathol. **120**, 353-362.

Khan, N., and Mukhtar, H. (2007). Tea polyphenols for health promotion. Life Sci. **81**, 519-533.

Kim, M. S., Kim, C., Jo, D. H., and Ryu, Y. W. (1999). Effect of fungal elicitor and heavy metals on the production of flavonol glycosides in cell cultures of *Ginkgo biloba*. J. Microbiol. Biotech. **9**, 661-667.

Kita T., Imai, S., Sawada, H., Kumagai, H., and Seto, H. (2008) The biosynthetic pathway of curcuminoid in turmeric (*Curcuma longa*) as revealed by ^{13}C-labelled precursors. Biosci. Biotech. Biochem. **72,** 1789-1798.

Kitdamrongsont K, Pothavorn P, Swangpol S, Wongniam S, Atawongsa K, Svasti J, and J., S. (2008). Anthocyanin composition of wild bananas in Thailand. J. Agric. Food Chem. **56**, 10853–10857.

Knapp, F. F., and Nicholas, H. J. (1969). Sterols and triterpenes of banana pulp. J. Food Sci. **34**, 584-586.

Kodama, O., Yamada, A., Yamamoto, A., Takemoto, T., and Akatsuka, T. (1988). Induction of phytoalexins with heavy metal ions in rice leaves. J. Pestic. Sci. **13**, 615-617.

Kress, W. J. (1990). The phylogeny and classification of the Zingiberales. Ann. Mo. Bot. Gard. **77**, 698-721.

Kuster, R. M., Mpalantinos, M. A., de Holanda, M.C., Lima, P., Brand, E. T., and Parente, J. P. (1999). GC-MS determination of kava-pyrones in *Alpinia zerumbet* leaves. J. High Resolut. Chrom. **22**, 129-130.

Lahlou, S., Interaminense, L. F. L., Leal-Cardoso, J. H., and Duarte, G. P. (2003). Antihypertensive effects of the essential oil of *Alpinia zerumbet* and its main constituent, terpinen-4-ol, in DOCA-salt hypertensive conscious rats. Fund. Clin. Pharmaco. **17**, 323-330.

Lewinsohn, E., and Gijzen, M. (2009). Phytochemical diversity: the sounds of silent metabolism. Plant Sci. **176**, 161-169.

Lewis, D. A., Fields, W. N., and Shaw, G. P. (1999). A natural flavonoid present in unripe plantain banana pulp (*Musa sapientum* L. var. *paradisiaca*) protects the gastric mucosa from aspirin-induced erosions. J. Ethnopharmacol. **65**, 283-288.

Lila, M.A. (2004). Anthocyanins and human health: an *in vitro* investigative approach. J. Biomed. Biotech. **5**, 306-313.

Luis, J. G., Echeverri, F., Quinones, W., Brito, I., Lopez, M., Torres, F., Cardona, G., Aguiar, Z., Pelaez, C., and Rojas, M. (1993). Irenolone and emenolone - 2 new types of phytoalexin from *Musa paradisiaca*. J. Org. Chem. **58**, 4306-4308.

Luis, J.G., Fletcher, W.Q., Echeverri, F., and Grillo, T.A. (1994). Phenalenone-type phytoalexins from *Musa acuminata* synthesis of 4-phenylphenalenones. Tetrahedron **50**, 10963-10970.

Luis, J. G., Fletcher, W. Q., Echeverri, F., Grillo, T. A., Perales, A., and Gonzalez, J. A. (1995). Intermediates with biosynthetic implications in de-novo production of phenylphenalenone-type phytoalexins by *Musa acuminata* revised structure of emenolone. Tetrahedron **51**, 4117-4130.

Luis, J. G., Quinones, W., Echeverri, F., Grillo, T.A., Kishi, M. P., GarciaGarcia, F., Torres, F., and Cardona, G. (1996). Musanolones: four 9-phenylphenalenones from rhizomes of *Musa acuminata*. Phytochemistry **41**, 753-757.

Ma, X. Q., and Gang, D. R. (2006). Metabolic profiling of turmeric (*Curcuma longa* L.) plants derived from *in vitro* micropropagation and conventional greenhouse cultivation. J. Agri. Food Chem. **54**, 9573-9583.

Ma, J. P., Jin, X. L., Yang, L., and Liu, Z. L. (2004). Diarylheptanoids from the rhizomes of *Zingiber officinale*. Phytochemistry **65**, 1137-1143.

Manchester, S. R., and Kress, J. W. (1993). Fossil bananas (Musaceae): *Ensete oregonense* Sp. nov. from the eocene of western north america and its phytogeographic significance. Am. J. Bot. **80**, 1264-1272.

Marín, D.H., Romero, R.A., and Guzman, M. (2003). Black sigtoka: an increasing threat to banana cultivation. Plant Dis. **87**, 208-222.

Medeiros, J. R., Campos, L. B., Mendonca, S. C., Davin, L. B., and Lewis, N. G. (2003). Composition and antimicrobial activity of the essential oils from invasive species of the Azores, *Hedychium gardnerianum* and *Pittosporum undulatum*. Phytochemistry **64**, 561-565.

Morrissey, J. P., and Osbourn, A. E. (1999). Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbiol. Mol. Biol. Rev. **63**, 708-724.

Müller, K. O., and Börger, H. (1940). Experimentelle Untersuchungen über die Phytophthora-Resistenz der Kartoffel. Arbeiten aus der Biologischen Reichsanstalt für Land- und Forstwirtschaft, Berlin-Dahlem **23**, 189-231.

Ndungo, V., Eden-Green, S., Blomme, G., Crozier, J., and Smith, J. J. (2006). Presence of banana x*anthomonas* wilt (*Xanthomonas campestris* pv. musacearum) in the Democratic Republic of Congo (DRC). Plant Pathol. **55**, 294-294.

Nishizawa, M., Inoue, A., Sastrapradja, S., and Hayashi, Y. (1983). (+)-8-Hydroxycalamenene - a fish-poison principle of *Dysoxylum acutangulum* and *Dysoxylum alliaceum*. Phytochemistry **22**, 2083-2085.

Noel, J. P., Dixon, R. A., Pichersky, E., Zubieta, C., Ferrer, J. L., (2003). Strucutural functional, and evolutionary basis of methylation in plant small molecules. Rec. Adv. Phytochem. **37**, 37-58.

Nuehoff, V., Arnold, N., Taube, D., and Ehrhard, W. (1988). Improved satining of protein in poly acrylamide gels including isoelectric-focusing gels with clear background at nanogram sensitivity using cooomassie brilliant blue G-250 and R-250. Electrophoresis **9**, 255-262.

Opitz, S., Otalvaro, F., Echeverri, F., Quinones, W., and Schneider, B. (2002). Isomeric oxabenzochrysenones from *Musa acuminata* and *Wachendorfia thyrsiflora*. Nat. Prod. Lett. **16**, 335-338.

Opitz, S., and Schneider, B. (2002). Organ specific analysis of phenylphenalenone-related compounds in *Xiphidium caeruleum*. Phytochemistry **61**, 819-825.

Opitz, S. (2002). Phenylphenalenones and related phenolic pigments of the Haemodoraceae: Structure, biosynthesis and accumulation patterns in *Xiphidium caeruleum* and *Wachendorfia thysiflora*. Ph.D. thesis. Faculty of biology and pharmacy, University of Jena, Germany.

Opitz, S., Schnitzler, J.P., Hause, B., and Schneider, B. (2003). Histochemical analysis of phenylphenalenone-related compounds in *Xiphidium caeruleum* (Haemodoraceae). Planta **216**, 881-889.

Orjeda, G. (1998). Evaluation of *Musa* germplasm for resistance to Sikatoga disease and *Fusarium* wilt, INIBAP technical guidelines 3 (Rome): PGRI,Monpellier: INIBAP: Wageningen: CTA).

Otálvaro, F., Echeverri, F., Quinones, W., Torres, F., and Schneider, B. (2002a). Correlation between phenylphenalenone phytoalexins and phytopathological properties in *Musa* and the role of a dihydrophenylphenalene triol. Molecules **7**, 331-340.

Otálvaro, F., Gorls, H., Holscher, D., Schmitt, B., Echeverri, F., Quinones, W., and Schneider, B. (2002b). Dimeric phenylphenalenones from *Musa acuminata* and various Haemodoraceae species. crystal structure of anigorootin. Phytochemistry **60**, 61-66.

Otálvaro, L. F. (2004). Strucutral, phytopathological and biosynthesis aspects of phenylphenalenones from *Musa*. Ph.D. thesis. Factulty of exacts and natural sciences, Medellin, Columbia.

Otálvaro, F., Nanclares, J., Vasquez, L.E., Quinones, W., Echeverri, F., Arango, R., and Schneider, B. (2007). Phenalenone-type compounds from *Musa acuminata* var. "Yangambi km 5" (AAA) and their activity against *Mycosphaerella fijiensis*. J. Nat. Prod. **70**, 887-890.

Pascual-Villalobos, M.J., and Rodriguez, B. (2007). Constituents of *Musa balbisiana* seeds and their activity against *Cryptolestes pusillus*. Biochem. Syst. Ecol. **35**, 11-16.

Ploetz, R.C. (2000). Panama disease: a classic and destructive disease of banana. Plant Health Progress. doi:10.1094/PHP-2000-1204-01-HM.

Pourcel, L., Routaboul, J.M., Cheynier, V., Lepiniec, L., and Debeaujon, I. (2007). Flavonoid oxidation in plants: from biochemical properties to physiological functions. Trends Plant Sci. **12**, 29-36.

Prasain, J. K., Tezuka, Y., Li, J. X., Tanaka, K., Basnet, P., Dong, H., Namba, T., and Kadota, S. (1997). Six novel diarylheptanoids bearing chalcone or flavanone moiety from the seeds of *Alpinia blepharocalyx*. Tetrahedron **53**, 7833-7842.

Prasain, J. K., Tezuka, Y., Li, Y. X., Tanaka, K., Basnet, P., Dong, H., Namba, T., and Kadota, S. (1998). Novel diarylheptanoids from the seeds of *Alpinia blepharocalyx*: Revised structure of calyxin A. J. Chem. Res. (S), 22-23.

Price, N.S. (2006). The banana burrowing nematode, *Radopholus similis* (Cobb) Thorne, in the Lake Victoria region of East Africa: its introduction, spread and impact. Nematology **8**, 801-817.

Qin, B., Shao, Z.Y., Zeng, W., Wang, H.Q., and Zhu, D.Y. (2006). Musellactone, a new lactone from *Musella lasiocarpa*. J. Chin. Chem. Soc. **53**, 475-478.

Quiñones, W., Escobar, G., Echeverri, F., Torres, F., Rosero, Y., Arango, V., Cardona, G., and Gallego, A. (2000). Synthesis and antifungal activity of *Musa* phytoalexins and structural analogs. Molecules **5**, 974-980.

Rajappan, K., Vidhyasekaran, P., Sethuraman, K., and Baskaran, T. L. (2002). Development of powder and capsule formulations of *Pseudomonas fluorescens* strain Pf-1 for control of banana wilt. Z. Pflanzenk. Pflanzens.-J. Plant Dis. Prot. **109**, 80-87.

Rakwal, R., Tamogami, S., Kodama O. (1996). Role of jasmonic acid as a signaling molecule in copper chloride-elicited rice phytoalexin production. Biosci. Biotech . Biochem. **60**, 1046-1048.

Reeder, R. H., Muhinyuza, J. B., Opolot, O., Aritua, V., Crozier, J., and Smith, J. (2007). Presence of banana bacterial wilt (*Xanthomonas campestris* pv. musacearum) in Rwanda. Plant Pathol. **56**, 1038-1038.

Rhodes, M. J. C. (1994). Physiological Rrles for secondary metabolites in plants - some progress, many outstanding problems. Plant Mol. Biol. **24**, 1-20.

Rimando, A. M., and Baerson, S. R. (2006). Polyketides: biosynthesis, biological activity, and genetic engineering, Oxford University Press.

Roje, S. (2006). S-Adenosyl-L-methionine: beyond auniversal methyl group donor. Phytochemistry **67**, 1618-1698.

Ruangsuttapha, S., Eimert, K., Schroder, M. B., Silayoi, B., Denduangboripant, J., and Kanchanapoom, K. (2007). Molecular phylogeny of banana cultivars from Thailand based on HAT-RAPD markers. Genet. Resour. Crop Ev. **54**, 1565-1572.

Saensouk, S. (2006). Taxonomy and Bioligy of the genus *Alpinia* Roxb. (Zingiberaceae) in Thailand. PhD. Thesis. Faculty of Sciences, Khon Kaen University, Thailand.

Salmoun, M., Braekman, J. C., Ranarivelo, Y., Rasamoelisendra, R., Ralambomanana, D., Dewelle, J., Darro, F., and Kiss, R. (2007). New calamenene sesquiterpenes from *Tarenna madagascariensis*. Nat. Prod. Res. **21**, 111-120.

Saravanan, T., Bhaskaran, R., and Muthusamy, M. (2004a). *Pseudomonas fluorescens* induced enzymological changes in banana roots (Cv. Rasthali) against *Fusarium* wilt disease. Plant Pathol. J. **3**, 72-80.

Saravanan, T., Muthusamy, M., and Marimuthu, T. (2004b). Effect of *Pseudomonas fluorescens* on *Fusarium* wilt pathogen in banana rhizosphere. J. Biol. Sci. **4**, 192-198.

Schmitt, B., and Schneider, B. (1999). Dihydrocinnamic acids are involved in the biosynthesis of phenylphenalenones in *Anigozanthos preissii*. Phytochemistry **52**, 45-53.

Schmitt, B., Holscher, D., and Schneider, B. (2000). Variability of phenylpropanoid precursors in the biosynthesis of phenylphenalenones in *Anigozanthos preissii*. Phytochemistry **53**, 331-337.

Schröder, J. (1997). A family of plant-specific polyketide synthases: facts and predictions. Trends Plant Sci. 2, 373-378.

Schröder J (2000) The family of chalcone synthase-related proteins: functional diversity and evolution. Recent Adv Phytochem **34**, 55–89.

Sharrock, S. (1997). The banana and its relatives. INIBAP annual report, 52-55.

Shen, B. (2000). Biosynthesis of aromatic polyketides.Curr. Top. Chem. **209**, 1-51.

Silayoi, B., and Babpraserth, C. (1983). Banana genetic resource exploration in Thailand. Report submitted to IBGR, Kasetsart Universiy, Bangkok, Thailand.

Silayoi, B. (2002). Banana. Kasetsart University Press. Bangkok, Thailand.

Simmonds, N. W., and Shepherds, K. (1955). The taxonomy and origin of the cultivated bananas. J. Linn. Soc. Bot. **55**, 302-312.

Simmonds, N. W., (1982). Bananas. Longman: London.

Stahl, E. (1888). Pflanyen und Schnecken, Biologishce Studie über die Schutzmitel der Pflanzen gegen Schneckenfrass. Jena. Zeitschr. Naturwiss. **22**, 657-684.

Staunton, J., and Weissman, K. J. (2001). Polyketide biosynthesis: a millennium review. Nat. Prod. Rep. **18**, 380-416.

Sun, Y., Matsubara, H., Kitanaka, S., and Yasukawa, K. (2008). Diarylheptanoids from the rhizomes of *Alpinia officinarum*. Helv. Chim. Acta. **91**, 118-123.

Sy, L.K., and Brown, G.D. (2002). The mechanism of the spontaneous autoxidation of dihydroartemisinic acid. Tetrahedron **58**, 897-908.

Tao, Q. F., Xu, Y., Lam, R. Y. Y., Schneider, B., Dou, H., Leung, P. S., Shi, S. Y., Zhou, C. X., Yang, L. X., Zhang, R. P., Xiao, Y. C., Wu, X. M., Stockigt, J., Zeng, S., Cheng, C. H. K., and Zhao, Y. (2008). Diarylheptanoids and a monoterpenoid from the rhizomes of *Zingiber officinale*: antioxidant and cytoprotective properties. J. Nat. Prod. **71**, 12-17.

Tebayashi S, Ishihara A., and Iwamura, M. 2001. Elicitor-induced changes in isoflavonoid metabolism in red clover roots. J. Exp. Bot. **52**, 681-689.

Tezuka, Y., Gewali, M.B., Ali, M.S., Banskota, A.H., and Kadota, S. (2001). Eleven novel diarylheptanoids and two unusual diarylheptanoid derivatives from the seeds of *Alpinia blepharocalyx*. J. Nat. Prod. **64**, 208-213.

Thomas, R. (1961). Studies in biosynthesis of fungal metabolites .O. biosynthesis of fungal perinaphthenones. Biochem. J. **78**, 807-813.

Thomas, R. (1971). The biosynthesis of the plant phenalenone haemocorin. Chem. Commun. **14**, 739-740.

Thomas, R. (1973). The biosynthesis of phenalenones. Pure Appl. Chem. **34**, 515.

Thomas, J. E., Iska-Caruna, M. -L., and Jones, D. R. (1994). *Musa* disease Fact No 4, banana bunchy top disease. INIBAP annual report.

Thomashow, L. S., and Weller, D. M. (1988). Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological-control of *Gaeumannomyces graminis* var *tritici*. J. Bacteriol. **170**, 3499-3508.

Urban, M., Sarek, J., Klinot, J., Korinkova, G., and Hajduch, M. (2004). Synthesis of A-seco derivatives of betulinic acid with cytotoxic activity. J. Nat. Prod. **67**, 1100-1105.

Urban, M., Sarek J, Tislerova I, Dzubak P, Hajduch M. (2005). Influence of esterification and modification of A-ring in a group of lupane acids on their cytotoxicity. Bioorg. Med. Chem.**13**, 5527-5535

Van Etten, H. D., Matthews, D. E., and Matthews, P. S. (1989). Phytoalexin detoxification: importance for pathogenicity and practical implications. Ann. Rev. Phytopathol. **27**, 143-164.

Walker, T. S., Harsh, P. B., Grotewold, E., and Vivanco, J. M. (2003). Root exudation and rhizosphere biology. Plant Physiol. **132**, 44-51.

Wang, L. Y., Zhang, M., Zhang, C. F., and Wang, Z. T. (2008). Diaryl derivatives from the root tuber of *Curcuma longa*. Biochem. Syst. Ecol. **36**, 476-480.

Wu, D., and Kress, J. W. (2000). Musaceae. Flora of China **24**, 297-313.

Xu, H. X., Hui, D., and Sim, K. Y. (1995). The isolation of a new labdane diterpene from the seeds of *Alpinia Zerumbet*. Nat. Prod. Lett. **7**, 29-34.

Zhao, J., Zheng, S. H., Fujita, K., and Sakai, K. (2004). Jasmonate and ethylene signalling and their interaction are integral parts of the elicitor signalling pathway leading to beta-thujaplicin biosynthesis in *Cupressus lusitanica* cell cultures. J. Exp. Bot. **55**, 1003-1012.

Zhu, J.P., Islas-Gonzalez, G., and Bois-Choussy, M. (2000). Recent progress in isolation, bioactivity evaluation and total synthesis of diarylheptanoids. Org. Prep. Proced. Int. **32**, 505-546.

Appendices

1. Chromatographic methods

1.1 Analytical HPLC

Anal. HPLC system_1). Column: LiChrospher® RP18 (5 µm); gradient 0 min: 5% MeCN in 0.1% TFA-H_2O, 60 min: 95% MeCN in 0.1% TFA-H_2O, 65 min: 100% MeCN, 70 min: 5% MeCN in 0.1% TFA-H_2O; flow rate 1 ml/min; UV 254 nm; sample preparation: 5 mg/ml in MeOH; injection volume 50 µl.

Anal. HPLC system_2). Column: LiChrospher® RP18 (5 µm); gradient 0 min: 30% MeCN in 0.1% TFA-H_2O, 60 min: 70% MeCN in 0.1% TFA-H_2O, 62 min: 95% MeCN in 0.1% TFA-H_2O, 67 min: 95% MeCN in 0.1% TFA-H_2O, 70 min: 30% MeCN in 0.1% TFA-H_2O; flow rate 1 ml/min; UV 254 nm; sample preparation: 10 mg/ml in MeOH; injection volume 25 µl.

*Anal. HPLC system_3).*Column: LiChrospher® RP18 (5 µm); gradient 0 min: 30% MeCN in 0.1% TFA-H_2O, 45 min: 70% MeCN in 0.1% TFA-H_2O, 50 min: 100% MeCN, 55 min: 30% MeCN in 0.1% TFA-H_2O; flow rate 1 ml/min; UV 254 nm; sample preparation: 10 mg/ml in MeOH; injection volume 15 µl.

Anal. HPLC system_4). Column: CC250/3 Nucleosil 100-3 C18 (3 µm); gradient 0 min: 35% MeCN in 0.1% TFA-H_2O, 45 min: 45% MeCN in 0.1% TFA-H_2O, 50 min: 100% MeCN, 55 min: 35% MeCN in 0.1% TFA-H_2O; flow rate 0.4 ml/min; UV 254 nm; sample preparation: 0.4 mg/ml in MeOH with 3-phenylpropionic acid (Sigma) (1 mg/ml) as internal standard; injection volume 10 µl.

Anal. HPLC system_5). Column: CC250/3 Nucleosil 100-3 C18 (3 µm); gradient 0 min: 35% MeCN in 0.1% TFA-H_2O, 60 min: 70% MeCN in 0.1% TFA-H_2O, 65 min: 100% MeCN, 70 min: 35% MeCN in 0.1% TFA-H_2O; flow rate 0.4 ml/min; UV 254 nm; sample preparation: 1 mg/ml in MeOH with pyrene (0.1 mg/ml) as internal standard; injection volume 10 µl.

Anal. HPLC system_6). Column: Supelcosil LC18, 250 × 2.1 mm; gradient 0 min: 0% MeCN in 0.1% TFA-H_2O, 40 min: 50% MeCN in 0.1% TFA-H_2O, 45 min: 95% MeCN in 0.1% TFA-H_2O; 50 min: 0% MeCN in 0.1% TFA-H_2O; flow rate 0.25 ml/min. injection volume 15 µl.

Anal. HPLC system_7). Column: Supelcosil LC18, 250 × 2.1 mm; gradient 0 min: 10% MeCN in 0.1% TFA-H_2O, 40 min: 50% MeCN in 0.1% TFA-H_2O, 45 min: 95% MeCN in 0.1% TFA-H_2O; 50 min: 10% MeCN in 0.1% TFA-H_2O; flow rate 0.25 ml/min; injection volume 15 µl.

1.2 Semi-preparative HPLC

Semiprep. HPLC system_1). Column: LiChrospher® RP18 (5 µm); gradient 0 min: 20% MeCN in 0.1% TFA-H_2O, 60 min: 50% MeCN in 0.1% TFA-H_2O, 65 min: 100% MeCN, 70 min: 20% MeCN in 0.1% TFA-H_2O; flow rate 1 ml/min.

Semiprep. HPLC system_2). Column: LiChrospher® RP18 (5 µm); isocratic 0 min: 45% MeCN in 0.1% TFA-H2O, 40 min: 45% MeCN in 0.1% TFA-H_2O, 50 min: 100% MeCN, 55 min: 45% MeCN in 0.1% TFA-H_2O; flow rate 1 ml/min.

Semiprep. HPLC system_3). Column: LiChrospher® RP18 (5 µm); gradient 0 min: 35% MeCN in 0.1% TFA-H2O, 60 min: 50% MeCN in 0.1% TFA-H_2O, 65 min: 100% MeCN, 70 min: 35% MeCN in 0.1% TFA-H2O; flow rate 1 ml/min.

Semiprep. HPLC system_4). Column: LiChrospher® RP18 (5 µm); gradient 0 min: 40% MeCN in 0.1% TFA-H_2O, 40 min: 50% MeCN in 0.1% TFA-H_2O, 50 min: 100% MeCN, 55 min: 40% MeCN in 0.1% TFA-H_2O; flow rate 1.2 ml/min.

Semiprep. HPLC system_5). Column: CC 250/2 Nucleosil 100-3 C18); gradient 0 min:35% MeCN in H_2O, 50 min:50% MeCN in H_2O, 55 min: 100% MeCN, 60 min: 35% MeCN in H_2O; flow rate 0.25 ml/min.

Semiprep. HPLC system_6). Column: CC Nucleosil 100-3 C18, isocratic eluent 30% MeCN in H2O, 60 min, flow rate 0.2 ml/min.

*semiprep. HPLC system_7).*Column: LiChrospher® RP18 (5 µm); gradient 0 min: 30% MeCN in 0.1% TFA-H_2O, 45 min: 70% MeCN in 0.1% TFA-H_2O, 50 min: 100% MeCN, 55 min: 30% MeCN in 0.1% TFA-H_2O; flow rate 1 ml/min.

semiprep. HPLC system_8). Column: CC250/3 Nucleosil 100-3 C18 (3 µm); gradient 0 min: 35% MeCN in 0.1% TFA-H_2O, 45 min: 45% MeCN in 0.1% TFA-H_2O, 50 min: 100% MeCN, 55 min: 35% MeCN in 0.1% TFA-H_2O; flow rate 0.4 ml/min.

semiprep. HPLC system_9). Column: CC250/3 Nucleosil 100-3 C18 (3 µm); gradient 0 min: 35% MeCN in 0.1% TFA-H_2O, 60 min: 70% MeCN in 0.1% TFA-H_2O, 65 min: 100% MeCN, 70 min: 35% MeCN in 0.1% TFA-H_2O; flow rate 0.4 ml/min.

1.3 Preparative HPLC

Prep. HPLC system_1). Gradient 0 min: 10% MeCN in 0.1% TFA-H_2O, 40 min: 30% MeCN in 0.1% TFA/H_2O, 45 min: 100% MeCN, 50 min: 10% MeCN in 0.1% TFA-H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_2). Isocratic conditions from 0-60 min: 10% MeCN in 0.1% TFA/H_2O, then gradient 65 min: 100% MeCN, 70 min: 10% MeCN in 0.1% TFA/H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_3). Gradient 0 min: 30% MeCN in H_2O, 40 min: 55% MeCN in H_2O, 45 min: 100% MeCN, 50 min: 30% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_4). Gradient 0 min: 40% MeCN in H_2O, 40 min: 55% MeCN in H_2O, 45 min: 100% MeCN, 50 min: 40% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_5). Gradient 0 min: 50% MeCN in H_2O, 60 min: 70% MeCN in H_2O, 65 min: 100% MeCN, 70 min: 70% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_6). gradient 0 min: 60% MeCN in H2O, 60 min: 80% MeCN in H_2O, 65 min: 100% MeCN, 70 min: 80% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_7). Gradient 0 min: 20% MeCN in H_2O, 60 min: 75% MeCN in H_2O, 65 min: 100% MeCN, 70 min: 20% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_8). Gradient 0 min: 45% MeCN in H_2O, 60 min: 90% MeCN in H_2O, 65 min: 100% MeCN, 70 min: 45% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_9). Gradient 0 min: 20% MeCN in H_2O, 60 min: 100% MeCN, 65 min: 100% MeCN, 70 min: 20% MeCN in H2O; flow rate 3.5 ml/min.

Prep. HPLC system_10). Gradient 0 min: 30% MeCN in H_2O, 60 min: 85% MeCN in H2O, 65 min: 100% MeCN, 70 min: 30% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_11). Gradient 0 min: 30% MeCN in H_2O, 60 min: 70% MeCN in H_2O, 65 min: 100% MeCN, 70 min: 30% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_12). Gradient 0 min: 60% MeCN in H_2O, 60 min: 100% MeCN in H_2O, 65 min: 100% MeCN, 70 min: 60% MeCN in H_2O; flow rate 2.8 ml/min.

Prep. HPLC system_13). Gradient 0 min: 25% MeCN in H_2O, 60 min: 85% MeCN in H_2O, 65 min: 100% MeCN, 70 min: 25% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_14). Gradient 0 min: 40% MeCN in H_2O, 60 min: 100% MeCN in H_2O, 65 min: 100% MeCN, 70 min: 40% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_15). Gradient 0 min: 5% MeCN in H_2O, 60 min: 95% MeCN in H_2O, 65 min: 100% MeCN, 70 min: 5% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_16). Gradient 0 min: 30% MeCN in H_2O, 60 min: 60% MeCN in H_2O, 65 min: 100% MeCN, 70 min: 30% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_17). Gradient 0 min: 20% MeCN in H_2O, 60 min: 55% MeCN in H_2O, 65 min: 100% MeCN, 70 min: 20% MeCN in H_2O; flow rate 3.5 ml/min.

Prep. HPLC system_18). Gradient 0 min: 30% MeCN in H_2O, 50 min: 50% MeCN in H_2O, 55 min: 100% MeCN, 60 min: 30% MeCN in H_2O; flow rate 2.8 ml/min.

1.4 HPLC-SPE-NMR

HPLC_SPE_NMR system 1). Column: LiChrospher® RP18 (5µm); gradient 0 min: 20% MeCN in 0.1% TFA-H_2O, 40 min: 60% MeCN in 0.1% TFA-H_2O, 4 min: 100% MeCN, 70 min: 5% MeCN in 0.1% TFA-H_2O; flow rate 1 ml/min; UV 254 nm; sample preparation: 5 mg/ml in MeOH; injection volume 50 µl.

2. List of collected Musa and related species from Thailand

***Musa* (AA group):**

"Kluai Homchampa" (1), " Kluai Pa Pli Laung" (2), "Kluai Homchan" (3), "Kluai Wan Tab Maew" (4), "Kluai Sa" (5), "Kluai Leb Mu Nang" (6), "Kluai Nam Thai" (7), "Kluai Hom Phol San" (8), "Kluai Thong Ngoei" (9), "Kluai Khai Thong Raung" (10);

***Musa* (AAA group):**

"Kluai Hom Thong" (11), "Kluai Hom Hochuchu" (Cavendish) (12), "Kluai Khiew" (13), "Kluai Nak Daeng" (14), "Kluai Khai Pratabong" (15), "Kluai Hom Thong Plok" (16)

***Musa* (AAB group):**

"Kluai Nam Phad" (21), "Kluai Roi Wi" (22), "Kluai Nam" (23), "Kluai Khom" (24), "Kluai Nom Sao" (25), "Kluai Cornplaintain" (26), "Kluai New Mu Nang" (27), "Kluai Sam Dauen" (28)

***Musa* (ABB group):**

"Kluai Nom Mi" (29), "Kluai Nang Paya" (30), "Kluai Hak Muk" (31), "Kluai Som" (32), "Kluai Teeb" (33), "Kluai Nam Wa" (34), "Kluai Nam Wa Khom" (35), "Kluai Nam Wa Dam" (36), "Kluai Thong Ma Eng" (37)

***Musa* (ABBB group):**

"Kluai Theparos" (38)

***Musa* (BBB group):**

"Kluai Leb Chang Gud" (39), "Kluai Pama Haek Kuk" (40), "Kluai Thep Panom" (Inabaniko) (41), "Kluai Hin" (42)

Wild Balbisiana group:

Musa balbisiana Colla. "Kluai Tani" (43), *Musa* "Kluai Tani Dam" (44)

Wild Callimusa:

Musa coccinea "Kluai Rattagatli" (17), *Musa. gracillis* "Kluai Sau Pran" (18)

Wild Rhodochlamys:

Musa ornata Roxb. "Kluai Bao Si Chompu" (19), *Musa* "Kluai Bua Si Maung" (20); **Wild Itinerans:** *Musa itinerans* Cheeseman. "Kluai Hok" (45)

Wild Ensete:

Ensete superba Roxb. "Kluai Pha" (46), *E. glauca* "Kluai Naul" (54)

Wild banana from other countries:

"Abisecnia" (47), "Karas" (48)

Unknown genome group:

Musa **"**Kluai Hom Nong Bau Lampu" (49), "Kluai Hom Sri Saket" (50), "Kluai Keeb Ma" (51), "Kluai Daeng Hawaii" (52), "Kluai Deang Israel" (53).

3. Spectroscopic data of the isolated compounds

Anigorufone (1)

UV (MeCN-H_2O): λ_{max} 205, 234, 276, 343 nm; ^{1}H NMR (500 MHz, acetone-d_6 TMS as an internal standard): δ 8.41 (1H, d, J = 8.3 Hz, H-7), 8.10 (1H, d, J_1 = 8.0 Hz, J_2 = 1.0 Hz, H-6), 7.90 (d, J_1 = 7.2 Hz, J_2 = 1.0 Hz, H-4) , 7.70 (1H, dd, J_1 = 7.2 Hz, J_2 = 8.0 Hz, H-5), 7.63 (1H, d, J = 8.3 Hz, H-8), 7.37-7.50 (5H, m, H-2´- 6´), 7.20 (1H, s, H-3).

Hydroxyanigorufone (2)

UV (MeCN-H_2O): λ_{max} 220, 282, 362 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.37 (1H, d, J = 8.3 Hz, H-7), 8.06 (1H, dd, J_1 = 8.2 Hz, J_2 = 1.0 Hz, H-6), 7.85 (1H, dd, J_1 = 7.3 Hz, J_2 = 1.0 Hz, H-4), 7.67 (1H, dd, J_1 = 8.2 Hz, J_2 = 7.3 Hz, H-5), 7.64 (1H, d, J = 8.3 Hz, H-8),

7.28 (2H, d, J = 8.7 Hz, H-2´/6´), 7.16 (1H, s, H-3), 6.92 (2H, d, J = 8.7 Hz, H-3´/5´); EIMS: *m/z* (rel. int. %) 288 $[M]^+$ (58), 287 $[M-H]^+$ (100), $C_{19}H_{12}O_3$.

2-Methoxy-9-phenyl-1*H*-phenalen-1-one (or methoxyanigorufone) (3)
UV (MeCN-H_2O): λ_{max} 203, 260, 367 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.34 (1H, d, J = 8.3 Hz, H-7), 8.05 (1H, dd, J_1 = 8.2 Hz, J_2 = 0.9 Hz, H-6), 7.86 (1H, dd, J_1 = 7.1 Hz, J_2 = 0.9 Hz, H-4), 7.67 (1H, dd, J_1 = 8.3 Hz, J_2 = 7.1 Hz, H-5), 7.58 (1H, d, J = 8.3 Hz, H-8), 7.33-7.45 (5H, m, H-2´/6´, H-3´/5´ and H-4´), 7.15 (1H, s, H-3), 3.85 (3H, s, 2-OCH_3); EIMS: *m/z* (rel. int. %) 286 $[M]^+$ (70), 285 $[M-H]^+$ (100), $C_{20}H_{14}O_2$.

9-(4´-Hydroxyphenyl)-2-methoxyphenalen-1-one (7)
UV (MeCN-H_2O): λ_{max} 205, 264, 371, 418 nm; ^{1}H NMR (400 MHz, acetone-d_6, TMS as an internal standard): δ 8.29 (1H, d, J = 8.4 Hz, H-7), 8.02 (1H, dd, J_1 = 8.1 Hz, J_2 =1.0 Hz, H-6), 7.84 (1H, dd, J_1 = 7.4 Hz, J_2 = 1.0 Hz, H-4), 7.64 (1H, dd, J_1 = 8.1 Hz, J_2 = 7.4 Hz, H-5), 7.60 (1H, d, J = 8.4 Hz, H-8), 7.24 (2H, d, J = 8.7 Hz, H-2´/6´), 7.13 (1H, s, H-3), 6.88 (2H, d, J = 8.7 Hz, H-3´/5´), 3.86 (3H, s, 4´-OCH_3); EIMS: *m/z* (rel. int. %): 302 $[M]^+$ (64), 301 $[M-H]^+$ (100), $C_{20}H_{14}O_3$.

Isoanigorufone (20)
UV (MeCN-H_2O): λ_{max} 205, 277, 340, 382 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.72 (1H, dd, J_1 = 7.4, J_2 = 1.3 Hz, H-9), 8.48 (1H, dd, J_1 = 8.1, J_2 = 1.3 Hz, H-7), 8.14 (1H, d, J = 8.3 Hz, H-6), 7.94 (1H, dd, J_1 = 8.1 Hz, J_2 = 7.4 Hz, H-8), 7.66 (1H, d, J = 8.6 Hz, H-5), 7.50-7.64 (5H, m, H-2´/6´, H-3´/5´ and H-4´), 7.14 (1H, s, H-3).

Irenolone (21)
UV (MeCN-H_2O): λ_{max} 249, 321 nm; ^{1}H NMR (500 MHz, acetone-d_6,TMS as an internal standard): δ 8.71 (1H, dd, J_1 = 7.3 Hz, J_2 = 1.3 Hz, H-9), 8.45 (1H, dd, J_1 = 8.0 Hz, J_2 = 1.3 Hz, H-7), 8.10 (1H, d, J = 8.5 Hz, H-6), 7.91 (1H, dd, J_1 = 7.3 Hz, J_2 = 8.0 Hz, H-8), 7.64 (H, d, J = 8.5 Hz, H-5), 7.40 (2H, d, J = 8.8 Hz, H-2´/6´), 7.24 (1H, s, H-3), 7.08 (2H, d, J = 8.8 Hz, H-3´/5´); EIMS: *m/z* (rel. int. %) 288 $[M]^+$ (100), 287 $[M-H]^+$ (37), $C_{19}H_{12}O_3$.

4´-*O*-Methylirenolone (22)
UV (MeCN-H_2O): λ_{max} 208, 239, 263, 369 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.71 (1H, dd, J = 7.2, 1.2 Hz, H-9), 8.46 (1H, dd, J = 8.0, 1.2 Hz, H-7), 8.11 (1H, d, J = 8.4 Hz, H-6), 7.91 (1H, dd, J = 8.0, 7.2 Hz, H-8), 7.65 (1H, d, J = 8.4 Hz, H-5), 7.50 (2H, d, J = 8.8 Hz, H-2´/6´), 7.16 (2H, d, J = 8.8 Hz, H-3´/5´), 7.21 (1H, s, H-3), 3.92 (3H, s, 4´-OCH_3); HR-ESIMS *m/z* (rel. int. %): 301.0865 $[M-H]^-$ (98), (calc. for $C_{20}H_{13}O_3$ 301.0865), corresponding to the molecular formula $C_{20}H_{14}O_3$.

2-Phenyl-1,8-naphthalic anhydride (33)
UV (MeCN-H_2O): λ_{max} 241, 257 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.65 (1H, dd, J_1 = 7.3 Hz, J_2 = 1.3 Hz, H-7), 8.58 (1H, dd, J_1 = 8.2 Hz, J_2 = 1.3 Hz, H-5), 8.54 (1H, d, J = 8.5 Hz, H-4), 7.97 (1H, dd, J_1 = 8.2 Hz, J_2 = 7.3 Hz, H-6), 7.75 (1H, d, J = 8.5 Hz, H-3), 7.45-7.51 (5H, m, H-2´/6´, H-3´/5´ and H-4´); EIMS *m/z* (rel. int. %): 274 $[M]^+$ (87), 273 $[M-H]^+$ (100); $C_{18}H_{10}O_3$.

2-(4´-Hydroxyphenyl)-1,8-naphthalic anhydride (34)
UV (MeCN-H_2O): λ_{max} 209, 240, 345 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): □8.63 (1H, dd, J = 7.4, 1.2 Hz, H-7), 8.54 (1H, dd, J = 8.2, 1.2 Hz, H-5), 8.50 (1H, d, J = 8.5 Hz, H-4), 7.76 (1H, d, J = 8.5 Hz, H-3), 7.40 (2H, d, J = 9.2 Hz, H-2´/6´), 6.96 (2H, d, J = 9.2 Hz, H-3´/5´);; EIMS *m/z* (rel. int. %): 290.1 $[M]^+$ (100), 289.1 $[M-H]^+$ (47); $C_{18}H_{10}O_4$.

3-Hydroxy-4-(4´-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (39)
UV (MeCN-H_2O): λ_{max} 228, 250, 278, 326 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.42 (1H, dd, J_1 = 7.2 Hz, J_2 = 1.1 Hz, H-9), 8.35 (1H, dd, J_1 = 8.2 Hz, J_2 = 1.1 Hz, H-7),

8.14 (1H, d, J = 8.5 Hz, H-6), 7.78 (1H, dd, J_1 = 8.2 Hz, J_2 = 7.2 Hz, H-8), 7.63 (1H, d, J = 8.5 Hz, H-5), 7.50 (2H, d, J = 8.6 Hz, H-2´/6´), 7.15 (1H, d, J = 5.4 Hz, 3-OH), 7.01 (2H, d, J = 8.6 Hz, H-3´/5´), 6.69 (1H, d, J = 5.4 Hz, H-3); HR-ESIMS: *m/z* (rel. int. %) 291.0653 $[M-H]^-$ (45); (calc. for $C_{18}H_{11}O_4$: 291.0663), corresponding to the molecular formula $C_{18}H_{12}O_4$.

3-Hydroxy-4-(4´-methoxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (40)

UV (MeCN-H_2O): λ_{max} 226, 249, 275, 325, 347 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.43 (1H, dd, J = 7.4, 1.2 Hz, H-9), 8.35, (1H, dd, J = 8.2, 1.2 Hz, H-7), 8.15, (1H, d, J = 8.6 Hz, H-6), 7.78 (1H, dd, J = 8.2, 7.4 Hz, H-8), 7.63 (1H, d, J = 8.6 Hz, H-5), 7.60 (2H, d, J = 9.1 Hz, H-2´/6´), 7.10 (2H, d, J = 9.1 Hz, H-3´/5´), 6.68 (1H, s, H-3), 3.89 (3H, s, 4´-OCH_3); HR-ESIMS *m/z* (rel. int. %): 305.0817 $[M-H]^-$ (98), (calc. for $C_{19}H_{13}O_4$ 305.0814), corresponding to the molecular formula $C_{19}H_{14}O_4$.

Anigorootin (47)

UV (MeCN-H_2O): λ_{max} 239, 285, 379 nm; ^{1}H NMR (400 MHz, acetone-d_6, TMS as an internal standard): δ 8.16 (2H, d, J= 8.4 Hz , H-4/11), 7.83 (2H, d, J = 8.9 Hz, H-3/10), 7.42 (2H, d, J= 8.4 Hz, H-5/12), 7.40-7.50 (10H, m, H-2´-6´/2″-6″), 7.03 (2H, d, J= 8.9 Hz, H-2/9), 4.19 (4H, 2H, s, H-7b/ 14b).

4´-Hydroxyanigorootin (48)

UV (MeCN-H_2O) λ_{max} 220, 282, 362 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.16 (1H, d, J = 8.4 Hz) and, 8.11 (1H, d, J = 8.3 Hz): H-4 and H-11, 7.82 (1H, d, J = 8.8 Hz) and 7.79 (1H, d, J = 8.9 Hz): H-3 and H-10, 7.40-7.51 (5H, m, H-2″-6″), 7.42 (1H, d, J = 8.4 Hz) and 7.40 (1H, d, J = 8.3 Hz): H-5 and H-12, 7.31 (2H, d, J = 8.7 Hz, H-2´/6´), 7.02 (1H, d, J = 8.8 Hz) and 6.99 (1H, d, J = 8.9 Hz): H-2 and H-9, 6.96 (2H, d, J = 8.7 Hz, H-3´/5´), 4.18 (2H, t, J = 5.3 Hz) and 4.17 (2H, t, J = 5.3 Hz): H-7b and H-14).

4´,4´´-Dihydroxyanigorootin (49)

UV (MeCN-H_2O): λ_{max} 230, 297, 371 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.11 (2H, d, J = 8.5 Hz, H-4/11), 7.79 (2H, d, J = 8.8 Hz, H-3/10), 7.41 (2H, d, J = 8.5 Hz, H-5/12), 7.31 (4H, d, J = 8.5 Hz, H-2´/6´ and H-2″/6″), 6.99 (2H, d, J = 8.8 Hz, H-2/9), 6.96 (4H, d, J = 8.5 Hz, H-3´/5´, H-3″/5″), 4.16 (2H, s, H-7b/14b).

Epicatechin (57)

^{1}H NMR (500 MHz, DMSO-d_6, TMS as an internal standard): δ 9.08 (1H, s, 5-OH), 8.87 (1H, s, 7-OH), 8.77 (1H, s, 4´-OH), 8.69 (1H, s, 3´-OH), 6.88 (1H, d, J = 1.8 Hz, H-2´), 6.66 (1H, d, J = 8.1 Hz, H-5´), 6.63 (1H, dd, J_1= 8.1 Hz, J_2 = 1.8 Hz, H-6´), 5.88 (1H, d, J = 2.3 Hz, H-6), 5.70 (1H, d, J = 2.3 Hz, H-8), 4.72 (1H, brs, H-2), 4.64 (1H, d, J = 4.5 Hz, 3-OH), 3.99 (1H, m, H-3), 2.66 (1H, dd, J_1 = 16.2 Hz, J_2 = 4.6 Hz, H-4a), 2.46 (1H, dd, J_1 = 16.2 Hz, J_2 = 3.5 Hz, H-4b); ^{13}C-NMR (125 MHz, DMSO-d_6, TMS as an internal standard): δ 156.0 (C-5), 155.7 (C-7), 155.3 (C-8a), 143.8 (C-3´), 144.0 (C-4´), 130.0 (C-1´), 117.7 (C-6´), 114.7 (C-2´), 114.5 (C-5´), 97.9 (C-4a), 94.9 (C-6), 93.8 (C-8), 78.0 (C-2), 64.6 (C-3), 28.1 (C-4); HR-ESIMS: *m/z* (rel. int. %): 289.0713 $[M-H]^+$ (98) (calc. for $C_{15}H_{13}O_6$ 289.0712), corresponding to the molecular formula $C_{15}H_{14}O_6$.

Galloepicatechin (58)

^{1}H NMR (500 MHz, DMSO-d_6, TMS as an internal standard): δ 9.07 (1H, s, 5-OH), 8.86 (1H, s, 7-OH), 8.67 (2H, s, 3´/5´-OH), 7.91 (1H, s, 4´-OH), 6.36 (2H, s, H-2´/6´), 5.87 (1H, d, J = 2.3 Hz, H-6), 5.70 (1H, d, J = 2.3 Hz, H-8), 4.64 (1H, brs, H-2), 4.58 (1H, d, J = 4.6 Hz, 3-OH), 3.97 (1H, m, H-3), 2.65 (1H, d, J_1 = 16.3 Hz, J_2 = 4.6 Hz, H-4a), 2.46 (1H, dd, J_1 = 16.3 Hz, J_2 = 3.5 Hz, H-4b); ^{13}C-NMR (125 MHz, DMSO-d_6, TMS as an internal standard): δ 156.1 (C-5), 155.9 (C-7), 155.1 (C-8a), 141.6 (C-4´), 145.2 (C-3´,5´), 129.7 (C-1´), 105.4 (C-2´,6´), 98.4 (C-4a), 94.4 (C-6), 93.5 (C-8), 77.3 (C-2),

64.8 (C-3), 28.1 (C-4); HR-ESIMS: *m/z* (rel. int. %): 305.0663 $[M-H]^+$ (98) (calc. for $C_{15}H_{13}O_7$ 305.0661), corresponding to the molecular formula $C_{15}H_{14}O_7$.

Pinocembrin (60)
^{1}H NMR (500 MHz, MeCN-d_3, TMS as an internal standard): δ 12.09 (1H, s, 5-OH), 7.97 (1H, brs, 7-OH), 7.49 (2H, m, H-2´/6´), 7.44 (2H, m, H-3´/5´), 7.39 (1H, m, H-4´), 5.96 (1H, d, J = 2.2 Hz, H-6), 5.94 (1H, d, J = 2.2 Hz, H-8), 5.51 (1H, dd, J_1 = 12.7 Hz, J_2 = 3.2 Hz, H-2), 3.17 (1H, dd, J_1 = 17.2 Hz, J_2 = 12.7 Hz, H-3a), 2.80 (1H, dd, J_1 = 17.2 Hz, J_2 = 3.2 Hz, H-3b); HR-ESIMS: *m/z* (rel. int. %): 255.0660 $[M-H]^-$ (98) (calc. for $C_{15}O_4H_{11}$ 255.0657), corresponding to the molecular formula $C_{15}O_4H_{12}$.

Izalpinin (61)
^{1}H NMR (500 MHz, MeCN-d_3, TMS as an internal standard): δ 6.30 (1H, s, 5-OH), 8.04 (2H, m, H-2´/6´), 7.55 (3H, m, H-3´/5´ and H-4´), 6.25 (1H, d, J = 2.1 Hz, H-6), 6.46 (1H, d, J = 2.1 Hz, H-8), 3.83 (3H, s, 7-OCH_3); ESIMS: *m/z* (rel. int. %): 283 $[M-H]^-$ (98), corresponding to the molecular formula $C_{16}O_5H_{12}$.

2-Phenylnaphthalene-1,8-dicarboxylic acid (63)
UV (MeCN-H_2O): λ_{max} 223, 244, 312 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.53 (1H, dd, J = 7.3, 1.3 Hz, H-7), 8.47 (1H, dd, J = 8.4, 1.3 Hz, H-5), 8.25 (1H, d, J = 8.5 Hz, H-4), 7.88 (1H, dd, J = 8.4, 7.3 Hz, H-6), 7.58 (1H, d, J = 8.5 Hz, H-3), 7.40-7.47 (5H, m, H-2´–6´); HR-ESIMS: *m/z* (rel. int. %) $[M-H]^-$ 291.0653 (100), (calc. for $C_{18}H_{11}O_4$ 291.0657), corresponding to the molecular formula $C_{18}H_{12}O_4$.

8-(Methoxycarbonyl)-2-phenylnaphthalene-1-carboxylic acid (64)
UV (MeCN-H_2O): λ_{max} 209,241, 288 nm; ^{1}H NMR (500 MHz, acetone-d_6, TMS as an internal standard): δ 8.28 (1H, d, J = 8.2, 1.3 Hz, H-5), 8.26 (1H, d, J = 8.5 Hz, H-4), 8.04 (1H, dd, J = 7.2, 1.1 Hz, H-7), 7.71 (1H, dd, J = 8.2, 7.2 Hz, H-6), 7.65 (1H, d, J = 8.5 Hz, H-3), 7.46 (3H, m, H-3´,4´,5´), 7.29 (2H, m, H-2´,6´), 3.72 (3H, s, 8-$COOCH_3$).

4. Autoxidation of 4-phenylphenalenones

The co-eluted 4´-*O*-methylirenolone (**22**) and isoanigoruofne (**20**) were converted to 3-hydroxy-4-(4´-methoxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**40**) and 3-hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (**62**) by autoxidation when the mixture was subjectted again onto to the HPLC column under acidic condition (Fig. **A1.)**

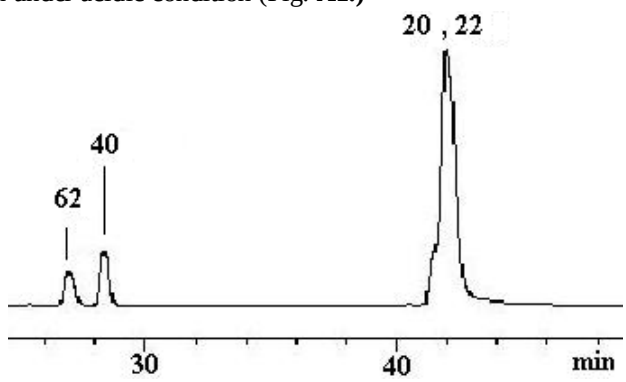


Figure A1. HPLC chromatogram shows the peak of co-eluted 4´-O-methylirenolone (**22**) and isoanigorufone (**20**) (*anal. HPLC system_5*, see App **1.1**). 3-Hydroxy-4-(4´-methoxyphenyl)-1*H*,3*H*-benzo[*de*]isochromen-1-one (**40**) and 3-hydroxy-4-phenyl-1*H*,3*H*-benzo[*de*]isochromen-1-one (**62**) were detected as oxidative products of **22** and **20**, respectively.

5. Quantification of phenylphenalenones

5.1 Quantification of phenylphenalenones in response to biotic elicitors and JA

To quantify hydroxyanigorufone (**2**) and 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) levels in *Musa acuminata* in response to elicitation with JA and treatment with *Sporobolomyces salmonicolor* the calibration curves were created. The methanolic solution of compounds **2** and **34** were prepared in five different concentrations. Pyrene (0.05mg/ml) was added as an internal standard. Calibration curves were created from concentration vesus peak area ratio of the analyte normalized to the internal standard (Table **A1.**). The calibration curves are shown in Fig **A2**.

Table A1. Concentration and peak area ratio of hydroxyanigorufone **(2)** and 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) to create calibration curves determine by *anal. HPLC system_3* (see Appx **1.1**).

Hydroxyanigorufone (**2**)		2-(4´-Hydroxyphenyl)-1,8-naphthalic anhydride (**34**)	
Conc.(mg/ml)	Ratio[1)]	Conc. (mg/ml)	Ratio[1)]
0.005	0.043	0.05	0.051
0.01	0.100	0.10	0.115
0.02	0.174	0.20	0.165
0.05	0.406	0.40	0.302
0.08	0.594	0.80	0.630

[1)]Ratio of peak area (integral) of peak area of analyte: internal standard

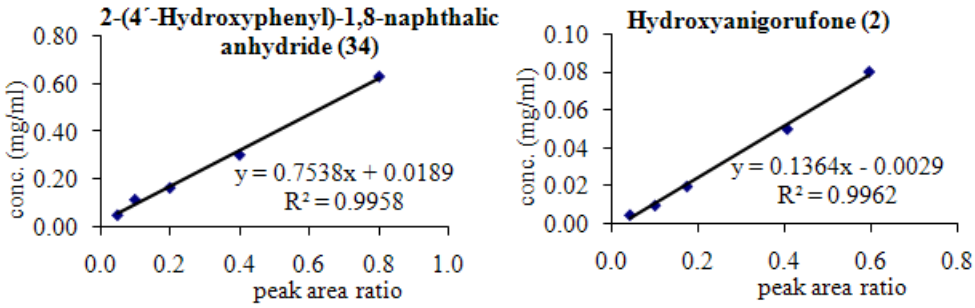


Figure A2. Calibration curves used to determine hydroxyanigorufone (**2**) and 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) levels in *M. acuminata* in response to elicitation with JA and treatment with *S. salmonicolor*.

5.2 Content of phenylphenalenones in response to biotic elicitor and JA

Content of hydroxyanigorufone (**2**) and 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) in *M. acuminata* after challenged with *S. salmomicolor* were determined by HPLC anaylsis (see Chapter **5**). Contents of phenylphenalenones (mg/g dry root) were calculated from calibration curve in (Table **A2.** and **A3.**).

Table A2. Content of hydroxyanigorufone (**2**) in *S. salmonicolor*-treated *M. acuminata* and control plants, determined by HPLC analysis (*anal. HPLC system_2*, see Appx **1.1**)

Day after treatment	Hydroxyanigorufone (**2**) (mg/g dry root)					
	Control			*S. salmonicolor*		
	Plant A	Plant B	mean	Plant A	Plant B	mean
day 1	0.5	1.1	0.8	0.1	0.7	0.4
day 3	0.2	0.1	0.1	1.0	1.3	1.2
day 5	0.2	0.3	0.2	0.9	1.6	1.2
day 7	0.1	0.1	0.1	0.6	0.9	0.8

Table A3. Content of 2-(4´-hydroxyphenyl)-1,8-naphthalic anhydride (**34**) in *S. salmonicolor*-treated *M. acuminata* and control plants, determined by HPLC analysis (*anal. HPLC system_2*, see Appx **1.1**)

Day after treatment	2-(4´-Hydroxyphenyl)-1,8-naphthalic anhydride (**34**) (mg/g dry root)					
	Control			*S. salmonicolor*		
	Plant A	Plant B	mean	Plant A	Plant B	mean
day 1	7.1	10.4	8.8	5.9	8.8	7.4
day 3	5.7	7.6	6.6	64.4	56.6	60.5
day 5	7.9	4.7	6.3	93.6	101.3	97.4
day 7	3.3	6.4	4.8	49.2	78.0	63.6

5.3 Quantification of phenylphenalenones in feeding experiments

To quantify anigorufone (**1**), hydroxyanigorufone (**2**), irenolone (**21**) and 4´-O-methylirenolone (**22**) levels in *Musa acuminata* in feeding experiments (Chapter **6**) the calibration curves were created. The methanolic solution of compounds **1**, **2**, **21** and **22** were prepared in four to five different concentrations. Pyrene (0.1mg/ml) was added as an internal standard for analyzing compound **21** and **22**, while 3-phenylpropionic acid (1mg/ml) was added as an internal standard forcompounds **1** and **2**. Calibration curves were created from concentration vesus peak area ratio of the analyte normalized to the internal standard (Table **A4.**). The calibration curves are shown in Fig **A3**.

Table A4. Concentrations of phenylphenalenones and peak area ratio used to create standard curves. Analytical conditions: irenolone (**21**) and hydroxyanigorufone (**2**), see *anal. HPLC system_4* (**2.2.1**); anigorufone (**1**) and 4´-*O*-methylirenolone (**22**), see *anal. HPLC system_5* (**2.2.1**)

Irenolone (**21**)		Hydroxyanigorufone (**2**)		Anigorufone (**1**)		4´-*O*-Methylirenolone (**22**)	
Conc. (mg/ml)	Ratio[1)]	Conc. (mg/ml)	Ratio[1)]	Conc. (mg/ml)	Ratio[1)]	Conc. (mg/ml)	Ratio[1)]
0.01	0.14	0.0050	0.23	0.01	0.14	0.01	0.25
0.02	0.33	0.0075	0.32	0.03	0.38	0.02	0.56
0.04	0.58	0.01	0.42	0.06	0.95	0.04	1.01
0.06	0.94	0.02	0.95	0.09	1.42	0.08	2.23
0.10	1.65	0.04	1.65	0.12	1.84	-	-

[1)] Ratio of peak area (integral) of peak area of analyte: internal standard

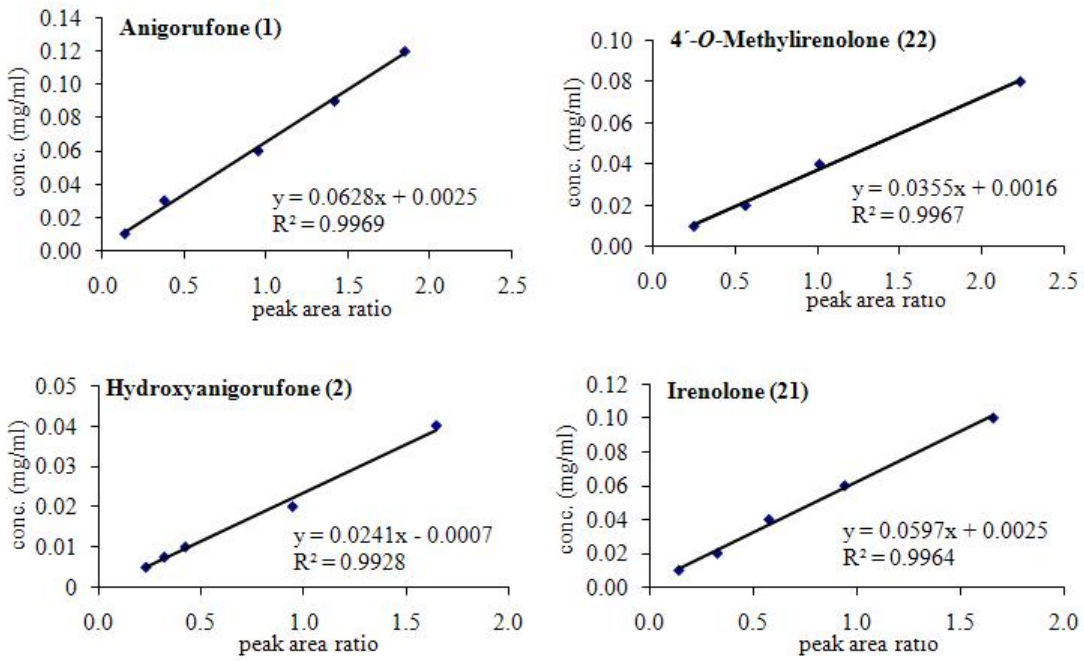


Figure A3. Calibration curves used to determine phenylphenalenone levels in *M. acuminata.*

5.4 Content of phenylphenalenones in feeding experiments

Content of hydroxyanigorufone (**2**), irenolone (**21**)**,** anigorufone (**1**) and 4´-*O*-methylirenolone (**22**), in *M. acuminata* were determined by HPLC analysis.). Contents of phenylphenalenones (mg/g dry root) were calculated from calibration curve in (Table **A5.**).

Table A5. Content of phenylphenalenone type compounds; hydroxyanigorufone (**2**), irenolone (**21**)**,** anigorufone (**1**) and 4´-*O*-methylirenolone (**22**), in *M. acuminata*, determined by HPLC analysis

Treatments	**Phenylphenalenones content (µg/g dry root)**			
	2	**21**	**1**	**22**
Control 1	**	<25	**	***
Control 2	**	<25	**	***
[*methyl*-^{13}C]Methionine 1	45.4	>25	21.1	26.4
[*methyl*-^{13}C]Methionine 2	91.4	>25	123.7	19.7
[*methyl*-^{13}C]Methionine+$CuCl_2$ 1	72.8	>25	**	31.0
[*methyl*-^{13}C]Methionine+$CuCl_2$ 2	154.3	>25	87.1	46.1
[*methyl*-^{13}C]Methionine+JA 1	**	<25	**	***
[*methyl*-^{13}C]Methionine+JA 2	132.9	>25	**	38.4
$CuCl_2$ 1	**	<25	**	***
$CuCl_2$ 2	209.4	>25	205.4	75.5
JA 1	**	<25	**	***
JA 2	**	<25	**	***

Note: Levels of phenylphenalenones were determined by standard curve methods created from authentic phenylphenalenone-type compounds. Hydroxyanigorufone (**2**) was analysed from fractions F03 with *anal. HPLC system_4* (see Appx **1.1**). Anigorufone (**1**) and 4´-*O*-methylirenolone (**22**) were analysed from fraction F04s with *anal. HPLC system_5* (see Appx **1.1**), ** = lower than 20 µg/g dried root; *** = lower than 15 µg/g dried root

6. List of media and buffers

6.1. Media

MS media (1l):

Solution MS1	20 ml
Solution MS2	20 ml
Solution MS3	20 ml
Sucrose	30 g

Adjusted to 1 l with de-ionized water and to pH 5.8 and the autoclaved to sterility

Solution MS 1 (1l)

NH_4NO_3	82.5 g
KNO_3	95.0 g
H_3BO_3	310 mg
$MnSO_4.4H_2O$	1.115 g
$ZnSO_4.\ 7H_2O$	430 mg
KI	41.5 mg
$Na_2Mo_4\ .2H_2O$	12.5 mg
$CuSO_4\ .5\ H_2O$	1.25 g
$CoCl_2\ .6H_2O$	1.25 g
$MgSO_4\ .7H_2O$	18.5 g
KH_2PO_4	8.5 g

Adjusted to 1 l with de-ionized water

Solution MS 2 (1l)

$CaCl_2\ .2H_2O$ 22 g

Dissolved in 1 l of de-ionized water

Solution MS 3 (1l)

$FeSO_4.\ 7H_2O$	1.39 g
Na_2-DETA	1.866 g

Dissolved in 1 l of de-ionized water

2x YT Broth (1l):

NaCl	10 g
Yeast extract	10g
Tryptone	16g

Adjusted to 1 l with de-ionized water, to pH 7.5 with NaOH and then autoclaved to sterility.

NZYM (1 l):

NZ amine	10 g
NaCl	5 g
Yeast extract	5 g
Casamino acids	1 g
$MgSO_4.\ 7H_2O$	2 g

Adjusted to 1 l with de-ionized water, to pH 7.5 with NaOH and then autoclaved to sterility.

LB agar (1 l):

NaCl	10 g
Tryptone	10 g
Yeast extract	5 g
Agar	20 g

Adjusted to 1 l with de-ionized water, to pH 7.0 with 5N NaOH and then autoclaved to sterility.

NZY broth (1 l):

NaCl	5 g
MgSO4.7H2O	2 g
Yeast extract	2 g
Casein hydrolysate	10 g
Agar	15 g

Adjusted to 1 l with de-ionized water, to pH 7.5 with NaOH and then autoclaved to sterility.

NZY agar (1 l):

NZY broth	1 l
Agar	15 g

Autoclaved to sterility

NZY top agar (1 l):

NZY broth	1 l
Agarose	7 g

6.2 Buffers

20x SSC buffer (1 l):

NaCl	175.3 g
Sodium citrate	88.2 g
De-ionized water	800 ml
Adjust pH to 7.0 with NaOH	
Add de-ionized water to 1 L	

50x TAE:

Tris base	242 g
Acetic acid	57.1 ml
0.5 M EDTA	100 ml
Adjusted to pH 8.5 with KOH	
Adjusted to 1 L with H_2O and adjusted	

SM buffer (1 l):

NaCl	5.8 g
$MgSO_4$. $7H_2O$	2 g
1 M Tris- HCl (pH 7.5)	50 ml
2% w/v gelatin	5 ml
De-ionized water to 1 l	

8×phosphate buffer (100 ml):

$Na_2HPO_4.2H_2O$	1.42 g
$NaH_2PO_4.2H_2O$	1.11 g
NaCl	23.38g
The solution was adjusted to pH 7.4	

7. Sequences

7.1. Primers

List of primer sequences used in PCR amplification of MaPKS fragments as well as the universal primers used in sequencing reaction and colony PCR are shown as follows:

	5´-	-3´
K180_oli	GCIAA(AG)GA(CT)ITI GCI GA(AG)AA(CT)AA(CT)AAIGG	
FGFG_oli	CC(AT)GG(AT)CCGAA(GT)CCGAA(GT)AG(GT)AC(AT)CCC	
M13reverse	CAG GAA ACA GCT ATG AC	
M13 forward	GTA AAA CGA CGG CCA G	
T7 universal	TAA TAC GAC TCA CTA TAG GG	
T3 universal	ATT AAC CCT CAC TAA AGG GA	
pHis8_for	CGA AAT TAA TAC GAC TCA C	
pHis8_rev	CTT CCT TTC GGG CTT TGT TAG	
MaPKS_KJ1_r	CCT GAC CTT CCA CTT CTT CAA CC	
MaPKS_KJ1_f	GCT TAA TTA GAG TGGTAC GCT GCG	
MaPKS_94909_2r	AGT GCT GCA TCC ATC CCT CTA GC	
MaPKS_94909_2r	GTA TGG TCT GCC AGC TTC ATC AC	
MaPKS_104055_2r	GAA CGA GGT CTT CTG GGT TTC G	
MaPKS_104055_2f	TAG CGG CTA AGA ACC CTA GAG AGG	
MaPKS_145587_1r	AAG ATg AAG GCA ACG CGG GAG G	
MaPKS_145587_1f	TTT CTC CGA CGC CTT CAG CC	
MaPKS_102084_1r	CTT CGG CAG CAA CAT C CAT CGT	
MaPKS_102084_1f	GGG AAT CGG ACT AGA TCG GGA AG	
MaPKS_132420_1r	CAC AAG GAT CGC CTC GGG	
MaPKS_132420_1f	TCA GAG TGT GTC GTT GAC GGG	

7.2. *MaPKS*

The information of the isolated *MaPKS1-5* including the 5´-non coding region, the ORF and the 3´-non coding region with poly A tail are shown in Table **A6.**

Table A6. *MaPKS1-5* full-length genes including 5´-non coding and 3´-noncoding with poly A tail region

Full-length	5´-non coding	ORF with start and stop codons	3´-non coding + poly A tail
MaPKS, 11390 bp	33 bp	1170 bp, 389 amino acid, (M_r 43142.79)	187 bp
MaPKS2, 1560 bp	178 bp	1170 bp, 389 amino acid (M_r 43079.91)	212 bp
MapKS3, 1464 bp	72 bp	1179 bp, 392 amino acid (M_r 42839.56)	213 bp
MaPKS4, 1465 bp	119 bp	1173 bp, 390 amino acid (M_r 42375.72)	173 bp
MaPKS5, 1496 bp	147 bp	1170 bp, 389 amino acid (M_r 41783.06)	179 bp

The full sequences of the *MaPKS1-5* are shown as follows. The the open reading frames of the *MaPKS1-5* were highlight in grey.

MaPKS1

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TGGACCTTGGCTGTCACTCAGTTGGCCACACTAATGGCCAGCCTCCACGCTCTGCGCAAGACACAGAGGACTCAGGGTCCGGCCACCATCATGG
CCATCGGCACCGCCAACCCTCCCAACCTCTACGAACAGAGCACCTATCCCGACTACTACTTCCGCGTCACCAACTCTGAGCACAAGCAGGATCT
CAAGCACAAGTTTCGGCGCATGTGCGAGAAGACGATGGTCAAACGGCGCTACCTGCATCTCACCGAGGAGATCCTCAAGGAGAGACCAAAACTG
TGCTCCTACATGGAGCCATCCTTCGACGACCGGCAGGACATCGTGGTGGAGGAGGTGCCCAAGCTGGCCAAGGAAGCCGCCACCAAGGCCATCA
AGGAGTGGGGCCGCTCCAAGTCCGACATCACCCACTTGGTCTTCTGCTCCATCAGCGGCATCGACATGCCCGGCGCCGACTACCGTCTTGCAAA
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CGGCAACATGCAGAGCGCCACCGTGTATTTCGTGATGGATGAGGTGAGGAAGCGGTCGGTGGTGGAGGGGCGGACGACCACCGGGGATGGACTG
CAGTGGGGGGTGCTCTTTGGGTTTGGACCGGGGCTCAGCATCGAGACTGTCGTGCTCCGCAGCGTACCACTCTAATTAAGCTACTTTTTTTCAG
CGATAATACCGCGCACAAATATTACGCAGTCGTGTGTGATGTTCTTCTTTGATCTCCATGCTGTTGGTTCTGCAAGGCATATGTAACTTGTCTA
TTCTTTTTGTAAGATCAAGAAAAAGGAAAGAAACGATGCAAATTCTCCAAAAAAAAAAAAAAAAAAAAAAAAAA
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MaPKS2

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ATACGCCAGCTATTTAGGTGACACTATAGAATACAGGCCTCGAGGGCCGAATTCCTGGACCTTGGCTGTCACTCAGTTGATCCTGCGTTCGCTA
ACTTTACTGCTGCTTTAATCCATTCATTCCTCGCTTGATCTCCTTCTGGTGCTCGCTGCTCCTTCTCCTCCCAGAATCGTGAAGATGGCCAGCG
TCCATCCATTCCGCAAGGCCCAAAGAGCACGAGGTCCGGCCACCATCATGGCCATCGGGACGGCCAATCCTCCCAACCTCTACGAGCAGAGCAC
TTTCCCCGACTTCTACTTTCGGGTCACCAACTCCGACCACATGCCGGAACTCAAGGAAAAGTTCCGCCGCATCTGTGGGAAGACCATGATCAAG
AAGCGGTACATGCACCTCACGGAGGAGGTCCTCAAGGATAAACCAGGGATGTGCTCCTACATGGACCCGTCTCTGGACGAGCGACAGGACATCG
TGGTGGAGGAGGTGCCGAGGCTCGCCACGGAAGCCGCCGCCAAGGCCATCGAAGAGTGGGGCCGCGACAAGTCCGACGTCACCCACTTGGTCTT
CTGCTCCACCAGCGGCGTCGACATGCCCGGCGCCGACTACCGCCTGGTCAAGCTCCTCGGTCTCCCCCTATCCGTCAACCGCATCATGCTCTAC
AGCCAGGCCTGCCACATCGGCGCCCAGATGCTCCGCATCGCCAAGGACATCGCCGAGAACAACAAGGACGCCCGTGTGCTCGTGGTGGCCTGCG
AGCTCAACACGCTCATCTTCCGAGGCCCAGACGAGCGCGACTTCCTGAGCCTCGCAGGCCAAGCCGCATTTGCGGATGGAGCAGCAGCGGTCAT
CGTCGGCGCGGATCCAATCCAAGGCGTCGAGAAGCCCATCTTCGAGATGATGTCTGCTTCACAGGTGACGGTACCGGACTGCGAAAGGGCGGTC
GGAGGCCATCTCAAGGAGATCGGTCTCACCTTCCATTTCATGAACCAGCTCCCCATGCTCATCGCCAACAACTTGGAGAACTGCCTCCTGGAGG
CGTTCAAGCCATTAGGCATCACCGACTGGAACGAGGTCTTCTGGGTCTCTCACCCGGGGAACTGGGGAATCATGGACGCCATCGAGAGGAAGGT
GGGGCTCAAGCAGGAGAAGCTCCGCTCGTCGAGGCACGTGTTCGGTGAGTACGGGAACATGATGAGCGCCACGGTTCTCTTCGTGATGGACGAT
GTGAGGAAGCGGTCGGCGGCGGAGGGGCGGGCGACAACCGGCGATGGGTTGGAGTGGGGAGTGCTCTCCGGCTTTGGACCGGGCCTCAGCATCG
AGACCGTGGTGCTTCGCAGCGTCGCCCTCTAATAGGGTTTCTCACCTCCAAAATTATCAAAAAAAAATTTCTTCTTTCTTTTCGCATTCTTTCC
TATTTGAATTTGTAAGAGAATAATGTAAACTACATAACGTTGTCGATGCATATACCTTTTTCTTTTCTTTTTTTGTATTTCCGATTTTTAACAG
TGTTTGACTGATATAAGAAAAAAATATCAACCCAACAAAAAAAAAAAAAAAAAAAAA
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MaPKS3

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CAGTTGATCTCCGGACCGAACCACCAACTCTTCGATCGACCTAGTACTTGGTCCTACTCCCACTTCTAGCTCATGGCCAAGTTGGCGGAGATCA
GGCAATCGCAGAGGGCAGAGGGTTCGGCGACGGTGCTCGCCATCGGCACCGCCACCCCTGTCAACGTCTTGTACCAGGCCGACTACCCAGACTA
CTACTTCCGCATCACCAAGAGCGAGCACCTCACTGAACTCAAGGAAAAGTTCAAGAGAATGTGTGACAAATCGATGATCCACAAACGTTACATG
CACATCAACGAGGAGATCCTGAAGGAGAACCCTAACGTCTGCGCGTACATGGCGCCGTCGCTGGACGCTCGGCAGGATATCGTGGTGGTGGAAG
TGCCGAAGCTAGGCAAGGAGGCGGCTGTCAAGGCCATCAAGGAATGGGGGCAGCCCGAGTCCAAGATCACCCATCTCGTCTTCTGCACCACCAG
CGGCGTCGACATGCCTGGCGCCGACTACCAGCTTACCAAACTGCTTGGCCTCCGACCCTCCGTCAACCGGTTCATGATGTACCAGCAGGGCTGC
TTCGCGGGCGGCACGGTGCTCCGCATGGCCAAGGACCTGGCCGAGAATAACCGCGGCGCCCGGGTGCTCGTGGTCTGTTCCGAGATCACCGCTG
TCACCTTCCGCGGGCCCTCGGAGTCCCACCTCGACAGCCTTGTCGGGCAGGCCCTGTTCGGCGACGGCGCCGCGGCCATCATCGTCGGCGCTGA
CCCCGACCCCGCCACAGAGCAGCCGATTTTCCAGCTCGTCTCCGCCAGCCAGACCATCCTCCCCGACTCCGAGGGCGCCATCGACGGCCACCTA
CGGGAGGTCGGCCTCACCTTCCATCTGCTCAAGGACGTGCCGGGCCTCATCTCCAAGAACATCGAGCGGAGCCTGGCGGAGGCGTTCAAGCCGC
TGGGGATCAGCGACTGGAACTCCATATTCTGGATCGCGCACCCCGGCGGGCCGGCGATCCTCGACCAGGTGGAGGCCAAGCTCGGCTTGGAGAA
GGAAAAGATGAAGGCAACGCGGGAGGTGCTGAAAGAGTACGGGAACATGTCGAGCGCCTGCGTGCTGTTCATCCTGGACGAGATGAGGAAGCGG
TCGGCCGAGGACGGGAAGGCGACCACCGGCGAGGGGCTTGAGTGGGGCGTCCTCTTCGGGTTCGGTCCCGGCCTCACGGTGGAGACCGTGGTCT
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ATCTAGTGTTATGTTCTATGGTGCTTAATAAACTCCGTTTCCTCAGACAAAAAA
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MaPKS4

AATACCCTCCTCGGGGCCGAATTCTTGGACCTTGGCTGTCACTCAGTTGGCCCCTTCATCGACATCCTTCTTCAGGCGATACGCAGTGACCATT
CAGCGTATCCAATCCATCCCCAAAGATGGCACACATCAACGCCTTCCCGAGGGCGCAGGGCGCCGACGGCCCGGCTACGGTCCTGGCCATCGGC
ACCGCCAATCCTGCACATTTCGTGGATCAGATGGAATATCCTGACTTCTTCTTCCGAATCACCAACGCAGAGGACAAGACGGAGCTCAAAGAGA
AGTTTAAGCGGATATGTGAGAATTCGGCCATCAGGAAGCGGCACATGTCCCTGACGGAGGAGATCCTCAAGGGGAACCCCAATCTGTGCGGGTA
CATGGAGCAGCCGTCGTTCGACGCGCGGCAGAAGGTGGTGCTGGAGGCAGTGCCAAAGATGGCGGCGGAGTCGGCCGAGAAGGCCATCAAGGAG
TGGGGTCGCCCCACGTCGGACATCACACATCTGGTCTTCTGCTCCGCCGCCGGGTTGGACCTCCCGGGCGTCGACTACCGCCTGGTCCAGAAGC
TCGGCCTCCCCACCTCCGTCCGCCGCGTGATGCTCTACAACGTGGGCTGCCACGCCGGCGCCTCGGCGCTCCGGTTGGCCAAGGACCTCGTCGA
GAACAACAAGGGCGCCCGCGCGTTGGTGGTCTCCTCCGAGGTGAACGTCATGTTCTTCCGTGGCCCCGACGACGACCACTTCGAGAACCTCATC
GGCCAGGCCCTTTTCGGCGACGGCTCGGCGGCGCTCATCGTCGGCGCTGACCCGGTCGAGGGGGTTGAGAAGCCCATCTACGAGATCAGCTCGG
CGTCGCAAGTGATGGTGCCGGAGAGCGAGGAGATGGTGGCGGGGCACCTGAGGGAGATCGGCCTGACCTTCCACCTCGCGAAGAAGGTCCCCTC
CATCGTCGGCAGCAACATCGTGCGGTGCCTGCAGGCGGCGTTCGCGCCGATGGGCATCACGGACTGGAACGAGCTGTTCTGGATCGTGCACACG
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ACATGCAGAGCGCGTCGGTGCTGTTCATCATGGACGAGATGCGGAAGCGATCGGCGGTGGAGGGCCACGCCACCACCGGACAGGGCTGCCAGTG
GGGGGTGCTCTTCGGCTTCGGCCCGGGCATCTCCGTGGAGACCGTCGTCCTCCGCAGCTTCCCGATCTAGTCCGATTCCGTCCGGTTCATGTCG
TAGTATGGGTTGGAAATGTATGTCCAACTAAGTAAACCATCGTAACATGTATGTATGTATATATGCATGCATGTATTTCTTTAGAGACTTTGCC
GCTTCTATAAATATAATAAATTATATTGGATCGATTAAAAAAAAAAAAAAAAAAAA

MaPKS5

TTAGGTGACACTATAGAATACAGGCCTCGAGGGCCGAATTCCTGGACCTTGGCTGTCACTCAGTTGGGCATCCATCCTCGCCAAGAGTCTACTC
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CGGCCCGGCGACGATCTTGGCCATCGGCACCGCGAACCCCAGCAACGTCATCGACCAGAGCGCTTATCCTGACTTCTACTTTCGGGTCACCAAC
TCGGAGCACTTGCAGGATCTCAAAGCCAAGTTCAGACGCATCTGTGACAAGGCGGCGATCAAGAAGCGGCACTTGTATCTGACAGAGGAGATCC
TGAGGGAGAACCCGAGCCTGTTGGCTCCCATGGCGCCCTCCTTCGACGCGCGTCAGGAGATCGTGGTGACCGCAGTGCCGGAGCTGGCCAAGGA
GGCCGCCGCCAAGGCCATCAAGGAGTGGGGGCGCCCCAAGTCGGACATCACCCACCTCGTCTTCTGCTCCGCCAGCGGCGTCGACATGCCCGGT
TCCGATCTCCAGCTCCTCAAGCTCCTTGGCCTGCCCATGTCCGTCAGCCGCGTCATGCTCTACAACGTCGGCTGCCACGCCGGCGGCACGGCGC
TCCGCGTCGCCAAGGACCTCGCGGAGAACAACCGCGGCGCCCGTGTGCTTGCCGTCTGCTCCGAGATCACCGTGCTCTCCTACCGCGGCCCCGA
CGCCGCCCACATGGAGAGCCTCATCGTGCAGGCCCTATTCGGCGACGGGGCGGCGGCGCTCGTCGTGGGGGCGGACCCGGTCGAGGGGGTCGAA
AGGCCCATCTTCGAGGTGGCGTCGGCGTCGCAGGTGATGCTCCCGGAGAGCGCGGAGGCGGTGGGGGGCCACCTCCGGGAGGTCGGCCTCACCT
TCCACCTCAAGAGCCAGCTGCCGACCATCATCGCCAGCAACATCGAGCAGAGCCTGGCGGCGGCGTTCGCGCCGCTCGGCCTGTCGGACTGGAA
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CCGACCACTGTTCATACTTATTGTTATCGGTGGCAGAATACTTTTACTTGTTTTCGCGTAAAAAAAAAAAAAAAAAAAAAAAA

The partial MaPKS sequneces used in cDNA library screening to obtain the full-length MaPKS are shown as follows:

MaPKS(585)

accgaagccgaataggacaccccactgcagtccatccccggtagtcgcccgcccctccaccaccgaccgcttcctcacctcatccatcacgaaa
tacacggtggcgctctgcatgttgccgtactcggcgaatacatgccgtgctgtgctgagcttctccggcaggagccccagcttggactcgatgg
cgtccatgatggcccaatttcccgggtgcgccacccagaacacctcgttccagtcggtgatgcccaagggcttgaatgcgtccgcgaggctgtt
cccgatgttgtcggcgatgatcatgggcagctggttgaagaagtggaaggtcaggccgacctcccggaggtggcctcccacggccttctcgctc
tccggcaccgttacttgcgtggccgacattatctcgtagatcggcttctccaggccttggatcggatcggcacccacgatgaccgcagctgctc
cgtcaccgaaccccgcttggccagcgagggcctcgaagtcgtgctcgtccgggcctcggaagctgaggacggtgatctcgtcatggacagccac
gagcacacgagcccccttatt

MaPKS_probe585

GCTTAATTAGAGTGGTACGCTGCGGAGCACGACAGTCTCGATGCTGAGCCCCGGTCCAAACCCAAAGAGCACCCCCCACTGCAGTCCATCCCCG
GTGGTCGTCCGCCCCTCCACCACCGACCGCTTCCTCACCTCATCCATCACGAAATACACGGTGGCGCTCTGCATGTTGCCGTACTCGGCGAATA
CATGCCGTGCTGTGCTGAGCTTCTCCGGCAGGAGCCCCAGCTTGGACTCGATGGCGTCCATGATGGCCCAATTTCCCGGGTGCGCCACCCAGAA
CACCTCGTTCCAGTCGGTGATGCCCAAGGGCTTGAATGCGTCCGCGAGGCTGTTCCCGATGTTGTCGGCGATGATCAGGGGCAGCTGGTTGAAG
AAGTGGAAGGTCAGG

MaPKS_probe055

GAACGAGGTCTTCTGGGTTTCGCACCCGGGGAACTGGGGGATCATGGACGCCATCGAGGCCAAGCTGGCGCTCCAGAAGGGGAAGCTCCGCTCC
TCGAGACATGTGTTCAGCGAGTACGGGAACATGATGGGCGCCACCGTTCTCTTCGTCATGGACGACGTCAGGAAGCGGTCGACGGTCGAGGAGC
GGACAACCACCGGCGACGGGCTCGAGTGGGGATTCCTCTGCGCCTTTGGACCTGGGCTGTCCATTGAGACTCTGGTGCTCCACAGTGTACCTCT
CTAGGGTTCTTAGCCGCTA

MaPKS_probe587

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ATAGCAGTAGCCACACAACCAGTTACAGGGAGATTCACGTGATGGAAGGAAAAGCTTGCGTCAGTGAGCAGCGATGGGGATGCTGTGCAAGACC
ACGGTCTCCACCGTGAGGCCGGGACCGAACCCGAAGAGGACGCCCCACTCAAGCCCCTCGCCGGTGGTCGCCTTCCCGTCCTCGGCCGACCGCT
TCCTCATCTCGTCCAGGATGAACAGCACGCAGGCGCTCGACATGTTCCCGTACTCTTTCAGCACCTCCCGCGTTGCCTTCATCTT

MaPKS_probe420

CCTTAGTGTGTCGTTGACGGGTGTCTGAGATCCCGCAAGTGCCTCGATCCGTTTAGGGGTTTGCTTGATATGGAAGACTAGATTTGCACGCTGT
GGAGGACGACGGTCTCGATGGAGAGGCCCGGGCCGAAGCCGAGGAGCACGCCCCAGTCGAGACCGTCGCCGGTGGTGGCTTGGCCCGCCGCCGC
CGACCGCTTCCGCATCTCGTCCAGGATGAAGAGGACCGTGGCGCTCTGCATGTTTCCGTACTCGCTGAGCACGTGGCGAGTCGCCCCGAGGCGA
TCCTTGTG

MaPKS_probe084

CTTCATCGTCGGCAGCAACATCGTGCGGTGCCTGCAGGCGGCGTTCGCGCCGATGGGCATCACGGACTGGAACGAGCTGTTCTGGATCGTGCAC
ACGGGCGGACGGGCCATCATCGACCAGGTGGAGGCCAATGCGGGGCTGGCGGCGGGGAAGCTGGCGCCGACGAGGCACGTGCTTAGTGAGCACG
GGAACATGCAGAGCGCGTCGGTGCTGTTCATCATGGACGAGATGCGGAAGCGATCGGCGGTGGAGGGCCACACCACCACCGGACAGGGCTGCCA
GTGGGGGGTGCTCTTCGGCTTCGGCCCGGGCATCTCCGTGGAGACCGTCGTCCTCCGCAGCTTCCCGATCTAGTCCGATT

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Curriculum Vitae

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2003-present Ph. D. student at Max Planck Institute for Chemical Ecology and Friedrich-Schiller University, Jena, Germany.

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1998-present Lecturer, Division of Pharmaceutical Chemistry and Pharmaceutical technology, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Thailand

Publications

Jitsaeng, K., and Schneider, B. (2007). Phenylphenalenones in elicited *Musa acuminata* (Musaceae). Journal of Pharmacy and Pharmacology **59**, A61-A61. (Conference paper)

Jitsaeng, K., De-Eknamkul, W., and Schneider, B., (2009). Flavonoids and a new calamenene-type sesquiterpene from rhizomes of *Alpinia oxymitra* K. Schum. (Zingiberaceae). Record of Natural Products **3**, 110-113.

Otálvaro, L, **Jitsaeng, K**., Munde, T. Echeverri F., Quiñones, W., and Schneider, B. O-methylation of inducible phenylphenalenones in *Musa acuminata* and *Wachendorfia thyrsiflora.* In review.

Jitsaeng, K. and Schneider, B. Flavonoid glycosides and 2-phenyl-1,8-naphthalic anhydride glucoside from *Musa acuminata* (Musaceae). In preparation.

Jitsaeng, K., Munde, T. and Schneider, B. Structure assignment of 2-phenyl-1,8-naphthalic anhydride phytoalexins by ^{1}H NMR spectroscopy of their *O*-methyl derivates. In preparation.

Oral presentations

Jitsaeng, K., Phillips, M., and Schneider, B. (2006) Study of response in transcriptional level of in vitro *Musa acuminata* to elicitation by JA. 4th Biannual symposium, International Max Planck Research School, Jena, Germany.

Jitsaeng, K. and Schneider B. (2007) Phenylphenalenones in *Musa acuminata* defense. 6th Biannual symposium, International Max Planck Research School, Jena, Germany.

Poster presentations

Jitsaeng, K., and Schneider, B. (2005) Phenylphenalenones as pathogen responsive compound in *Musa acuminata*. 3rd Biannual symposium, International Max Planck Research School, Jena, Germany.

Jitsaeng, K., Phillips, M. and Schneider, B. (2006) Transcriptional analysis of a chalcone synthase in *Musa acuminata* in response to elicitation by jasmonic acid. 5th Biannual symposium, International Max Planck Research School, Jena, Germany.

Jitsaeng, K., and Schneider, B. (2007) Phytochemical studies of phenylphenalenones in *Musa acuminata* and *Ensete glauca* (Musaceae) from Thailand. Future trends in phytochemistry - PSE young scientists symposium, Gargnano, Italy.

Jitsaeng, K., Phillips, M. and Schneider, B. (2007) Phenylphenalenones in *Musa acuminata* defense. 23rd International Society of Chemical Ecology Annual meeting, Jena, Germany.

Jitsaeng, K., and Schneider, B. (2007) Phenylphenalenones in elicited *Musa acuminata* (Musaceae). British pharmaceutical conference and exhibition, Manchester, Great Britain.

Selbständigkeiterklarung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, das ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzen Hilfsmittel und Quellen angegeben habe. Alle Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben, sind in der Danksagung genannt. Es wurde weder Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerden weder als Prüfungsarbeit für eine staatliche order andere wissenschaftliche Prüfung noch als Dissertation an der anderen Hochschule eigereichet.

Kusuma Jitsaeng

List of presentations and publications

Publications

Jitsaeng, K., and Schneider, B. (2007). Phenylphenalenones in elicited *Musa acuminata* (Musaceae). Journal of Pharmacy and Pharmacology **59**, A61-A61. (Conference paper)

Jitsaeng, K., De-Eknamkul, W., and Schneider, B., (2009). Flavonoids and a new calamenene-type sesquiterpene from rhizomes of *Alpinia oxymitra* K. Schum. (Zingiberaceae). Record of Natural Products **3**, 110-113.

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Jitsaeng, K. and Schneider, B. Flavonoid glycosides and 2-phenyl-1,8-naphthalic anhydride glucoside from *Musa acuminata* (Musaceae). In preparation.

Jitsaeng, K., Munde, T. and Schneider, B. Structure assignment of 2-phenyl-1,8-naphthalic anhydride phytoalexins by ^{1}H NMR spectroscopy of their *O*-methyl derivates. In preparation.

Oral presentations

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Jitsaeng, K. and Schneider B. (2007) Phenylphenalenones in *Musa acuminata* defense. 6th Biannual symposium, International Max Planck Research School, Jena, Germany.

Poster presentations

Jitsaeng, K., and Schneider, B. (2005) Phenylphenalenones as pathogen responsive compound in *Musa acuminata.* 3rd Biannual symposium, International Max Planck Research School, Jena, Germany.

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Jitsaeng, K., and Schneider, B. (2007) Phytochemical studies of phenylphenalenones in *Musa acuminata* and *Ensete glauca* (Musaceae) from Thailand. Future trends in phytochemistry - PSE young scientists symposium, Gargnano, Italy.

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Jitsaeng, K., and Schneider, B. (2007) Phenylphenalenones in elicited *Musa acuminata* (Musaceae). British pharmaceutical conference and exhibition, Manchester, Great Britain.