



HOKKAIDO UNIVERSITY

Title	Study on Prenyltransferases Responsible for Indole Diterpene Biosynthesis
Author(s)	劉, 成偉
Degree Grantor	北海道大学
Degree Name	博士(工学)
Dissertation Number	甲第11481号
Issue Date	2014-03-25
DOI	https://doi.org/10.14943/doctoral.k11481
Doc URL	https://hdl.handle.net/2115/55397
Type	doctoral thesis
File Information	Liu_Chengwei.pdf



Study on Prenyltransferases Responsible for Indole Diterpene Biosynthesis

(インドールジテルペンの生合成に関与するプレニル基転
移酵素に関する研究)

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ABSTRACT

Indole diterpene compounds, which are produced by filamentous fungi, are derived from polyprenyl diphosphates and tryptophan or its derivatives. They show diverse chemical structures and biological activities. In this study, I characterized the prenyltransferases responsible for biosynthesis of indole diterpenes.

In the chapter 2, I carried out in vitro analysis of *paxC* and *paxD*, which are located at the paxilline biosynthetic gene cluster with recombinant enzymes. PaxC catalyzed a transfer of geranylgeranyl diphosphate (GGDP) to both indole and indole 3-glycerol phosphate to give geranylgeranyl indole (GGI). PaxD transfers dimethylallyl diphosphate (DMAPP) to paxilline to yield regularly 21,22-diprenylated paxilline.

In the chapter 3, a putative prenyltransferase, *atmD*, which is located in the aflatrems biosynthetic gene cluster in *Aspergillus flavus* and encodes an enzyme with 32% amino acid identity to PaxD, was characterized using recombinant enzyme. When paxilline and DMAPP were used as substrates, two major products and a trace of minor product were formed. The structures of the two major products were determined to be reversely mono-prenylated paxilline at either the 20- or 21-position. I also investigated the substrate specificities of AtmD and PaxD. Both the enzymes accepted paspaline, which is an intermediate of paxilline biosynthesis, and catalyzed a regular mono-prenylation of paspaline at either the 21- or 22-position though the reverse prenylation was observed

with paxilline. The results suggested that the fungal indole diterpene prenyltransferases have the potential to alter their position and regular/reverse specificities for prenylation and can be applicable for synthesis of industrially useful compounds.

In the chapter 4, three prenyltransferases, the coding genes of which are located in a putative indole diterpene biosynthetic gene cluster in *Phomopsis amygdali*. Recombinants of the three putative prenyltransferase genes were overexpressed in *E. coli* and the purified enzymes were used for in vitro analysis. One (AmyG, an ortholog of PaxG) was suggested to be a geranylgeranyl diphosphate (GGDP) synthase. Other two enzymes were confirmed to be prenyltransferases catalyzing a transfer of GGDP to indole/indole-3-glycerol phosphate (AmyC) and a regular di-prenylation at the 20,21-positions of paxilline (AmyD).

TABLE OF CONTENTS

TITLE PAGE.....	i
ABSTRACT.....	ii
LIST OF TABLES.....	ix
LIST OF FIGURES.....	x
LIST OF SCHEMES.....	xiv
CHAPTER	
1 General introduction.....	1
1.1 Fungal secondary metabolites.....	2
1.2 Gene cluster for fungal secondary metabolites.....	4
1.3 Regulation of fungal secondary metabolite production.....	6
1.3.1 Culture medium regulation	
1.3.2 Chemical epigenetics	
1.3.3 Transcription factor regulation	
1.3.4 Co-cultivation regulation	
1.4 Heterologous expression.....	11
1.5 Indole diterpene.....	12
1.6 Prenylated indole derivatives produced by fungi.....	14
1.6.1 Prenyltransferases	
1.6.2 Prenyltransferases acting on indole compounds	
1.6.3 Prenylated indole-diterpenes	
2 Functional analysis of PaxC and PaxD in the paxilline biosynthetic gene cluster.....	20
2.1 Introduction.....	21
2.2 Materials and Methods.....	26
2.2.1 General.....	26
2.2.2 Strain.....	26

2.2.3	DNA, RNA isolation and cDNA synthesis.....	27
2.2.4	Cloning, expression and purification of TtIGPS/PaxC/PaxD.....	27
2.2.5	Chemical synthesis CdRP.....	29
2.2.6	Enzyme reaction with TtIGPS.....	29
2.2.7	Enzyme reaction with PaxC.....	30
2.2.7.1	Kinetic analysis of PaxC	
2.2.8	In vitro assay of PaxD.....	31
2.2.8.1	Kinetic parameters of PaxD	
2.2.9	LC/ESI-MS analytical conditions.....	32
2.2.10	Structural analysis of reaction product.....	32
2.3	Results.....	34
2.3.1	Synthesis of indole-3-glycerol phosphate (IGP).....	34
2.3.1.1	Preparation of TtIGPS	
2.3.1.2	Enzyme reaction with TtIGPS and CdRP	
2.3.1.3	Brief summary	
2.3.2	Functional analysis of PaxC.....	37
2.3.2.1	Preparation of PaxC	
2.3.2.2	In vitro assay of PaxC with GGDP and IGP/indole/tryptophan	
2.3.2.3	In vitro assay of PaxC with FDP and IGP/indole/tryptophan	
2.3.2.4	Metal dependency of PaxC	
2.3.2.5	Kinetic analysis of PaxC	
2.3.2.6	LC/ESI-MS analysis of byproduct formed from IGP by PaxC	
2.3.3	Functional analysis of PaxD.....	44
2.3.3.1	Preparation of PaxD	
2.3.3.2	In vitro assay of PaxD	
2.3.3.3	LC/ESI-MS analysis of reaction product formed from paxilline and DMAPP by PaxD	
2.3.3.4	NMR analysis of reaction product formed from paxilline and DMAPP by PaxD	
2.3.3.5	Prenylated paxillines in culture broth of <i>P. paxilli</i>	
2.3.3.6	Fragmentation analysis of product by LC/ESI-MS	
2.3.3.7	Biochemical characterization of PaxD.....	54

2.4	Discussion.....	60
3	Regio- and mode- specificities of AtmD and PaxD.....	63
3.1	Introduction.....	64
3.2	Materials and Methods.....	65
3.2.1	Strain.....	65
3.2.2	PCR amplification and purification of AtmD.....	65
3.2.3	In vitro assay of AtmD.....	66
	3.2.3.1 Kinetic parameters of AtmD for Paxilline and DMAPP	
	3.2.3.2 Kinetic parameters of AtmD for Paspaline and DMAPP	
	3.2.3.3 Kinetic parameters of PaxD for Paspaline and DMAPP	
3.2.4	LC/ESI-MS analytical conditions by Phenyl column.....	67
3.2.5	Structural analysis of reaction product.....	67
3.2.6	Accession number.....	71
3.3	Results.....	72
3.3.1	Gene cluster.....	72
	3.3.1.1 PCR amplification of <i>atm</i> genes in <i>A. flavus</i> NBRC 4295	
	3.3.1.2 Search for indole diterpene-related compound in culture broth of <i>A. flavus</i> NBRC 4295	
3.3.2	Functional analysis of AtmD.....	75
	3.3.2.1 Overexpression and purification of AtmD	
	3.3.2.2 In vitro assay of AtmD	
	3.3.2.3 LC/ESI-MS analysis of reaction product formed from paxilline and DMAPP by AtmD	
	3.3.2.4 NMR analysis of reaction product formed from paxilline and DMAPP by AtmD	
	3.3.2.5 Biochemical characterization of AtmD.....	81
3.3.3	Substrate specificity.....	85
	3.3.3.1 Analysis of reaction product formed from paspaline and DMA PP by AtmD.....	86
	3.3.3.2 Analysis of reaction product formed from paspaline and DMAPP by PaxD.....	90

3.3.3.3	Substrate specificities of AtmD and PaxD (1).....	94
3.3.3.4	Substrate specificities of AtmD and PaxD (2).....	97
3.4	Discussion.....	99
4	Characterization of prenyltransferases in silent indole diterpene biosynthetic gene cluster in <i>Phomopsis amygdali</i>	101
4.1	Introduction.....	102
4.2	Materials and Methods.....	104
4.2.1	Transformations.....	104
4.2.2	Oligonucleotides used for PCR amplification.....	106
4.2.3	Purification of enzyme.....	106
4.2.4	Culture conditions of <i>P. amygdali</i> (17 different conditions).....	107
4.2.5	Culture conditions with 5-Azacytidine and SAHA.....	109
4.2.6	In vitro assay of AmyG.....	109
4.2.7	In vitro assay of AmyC/D.....	110
4.2.7.1	Kinetic analysis of AmyD.....	
4.2.8	Structural analysis of reaction product.....	111
4.2.9	Accession numbers.....	111
4.3	Results.....	112
4.3.1	Identification of putative indole diterpene Gene cluster.....	112
4.3.2	Attempt to identify a compound governed by the cluster.....	113
4.3.2.1	Optimization of culture medium and conditions.....	
4.3.2.2	Utilization of histone deacetylase-inhibitor or DNA methylation inhibitor.....	
4.3.2.3	Activation of transcriptional regulators.....	
4.3.2.4	Brief summary.....	
4.3.3	cDNA preparation by heterologous expression in <i>Aspergillus oryzae</i>	115
4.3.4	Functional analysis of AmyG.....	116
4.3.4.1	Overexpression and purification of AmyG.....	
4.3.4.2	In vitro assay of AmyG.....	
4.3.5	Functional analysis of AmyC.....	121
4.3.5.1	Overexpression and purification of AmyC.....	

4.3.5.2 In vitro assay of AmyC	
4.3.6 Functional analysis of AmyD.....	125
4.3.6.1 Overexpression and purification of AmyD	
4.3.6.2 In vitro assay of AmyD	
4.3.6.3 NMR analysis of reaction product formed from paxilline and DMAPP by AmyD	
4.3.6.4 Biochemical characterization of AmyD	
4.3.6.5 Substrate specificity of AmyD	
4.3.7 Functional analysis of AmyJ.....	143
4.3.7.1 Heterologous expression of AmyJ	
4.4 Discussion.....	146
5 Conclusion.....	148
References.....	151
Acknowledgements.....	168

LIST OF TABLES

Table

1.1 Secondary metabolite gene clusters in fungal.....	5
2.1 Predicted functions of <i>pax</i> genes identified in <i>P. paxilli</i>	23
2.2 Primers used for <i>ttIGPS/paxC/paxD</i> amplifications.....	27
2.3 Kinetic parameter of PaxC.....	41
3.1 PCR primers used for amplifications of each <i>atm</i> gene in <i>A. flavus</i> NBRC 4295...	65
3.2 Oligonucleotides used for construction of expression AtmD.....	65
3.3 Predicted functions of <i>atm</i> genes identified in <i>A. flavus</i> NRRL6541.....	73
4.1 Oligonucleotides used for construction of expression plasmids.....	106
4.2 Predicted functions of <i>amy</i> genes identified in <i>P. amygdali</i>	112
4.3 Sequence identities among AmyG/PaxG/AtmG/LtmG.....	116
4.4 Sequence identities among AmyC/PaxC/AtmC/LtmC.....	121
4.5 Sequence identities among AmyD/PaxD/AtmD/LtmE.....	125

LIST OF FIGURES

Figure

1.1 Epigenetic remodeling of fungal secondary metabolite.....	7
1.2 Targeted cluster activation through overexpression of <i>apdR</i> gene.....	8
1.3 Physical interaction of <i>A. nidulans</i> with <i>S. hygrosopicus</i>	10
1.4 Chemical diversity of indole diterpene.....	13
1.5 Prenyltransferases acting on different positions of indole compounds.....	17
1.6 Prenylated indole diterpene; structure and biological activity.....	19
2.1 Paxilline biosynthetic gene cluster in <i>Penicillium paxilli</i>	22
2.2 Heterologous expression of paxilline gene cluster in <i>A.oryzae</i>	24
2.3 SDS-PAGE analysis of purified TtIGPS.....	35
2.4 HPLC analysis of reaction products formed from CdRP by TtIGPS.....	35
2.5 SDS-PAGE analysis of purified PaxC.....	37
2.6 HPLC analysis of reaction products formed from IGP/indole/tryptophan with GGDP by PaxC.....	38
2.7 HPLC analysis of reaction products formed from IGP/indole/tryptophan with FDP by PaxC.....	39
2.8 Fonction of DDxxD motif in PaxD.....	40
2.9 HPLC profiles of PaxC reaction to investigate an effect of Mg ²⁺ ions.....	40
2.10 LC-MS profiles of PaxC reaction product.....	43
2.11 Alignment of amino acid sequences of PaxD and AtmD constructed by CLUSTALW.....	44
2.12 SDS-PAGE and gel filtration chromatography of purified PaxD.....	45
2.13 HPLC analysis of the reaction products formed from paxilline and DMAPP with PaxD.....	46
2.14 Total ion chromatogram of products formed by in vitro assay without and with PaxD.....	47
2.15 LC/ESI-MS analysis of the prenylated paxilline formed by PaxD reaction.....	48
2.16 HPLC analysis of prenylated paxilline accumulated in culture broth of <i>P. paxilli</i>	51

2.17 LC/ESI-MS analysis of prenylated paxilline accumulated in culture broth of <i>P. paxilli</i>	52
2.18 Fragmentation analysis of products by LC/MS.....	53
2.19 Alignment of amino acid sequences of PaxD and fungal prenyl transferases.....	55
2.20 Effects of temperature on PaxD activity.....	56
2.21 Effects of pH on PaxD activity.....	57
2.22 Effects of metal ions on PaxD activity.....	58
2.23 Hanes-Woolf plots for calculation of K_m and k_{cat} values of PaxD.....	59
3.1 Aflatrem biosynthesis gene cluster in <i>A. flavus</i> NRRL6541.....	72
3.2 PCR amplifications of each of <i>atm</i> gene in <i>A. flavus</i> NBRC 4295.....	73
3.3 LC/ESI-MS analysis of compound accumulated in culture broth of <i>A. flavus</i> NBRC 4295.....	74
3.4 Alignment of amino acid sequences of CAP53937 and AB778117.....	75
3.5 SDS-PAGE and gel filtration chromatography of Purified AtmD.....	76
3.6 HPLC analysis of reaction products formed from paxilline and DMAPP by AtmD.....	77
3.7 LC/ESI-MS analysis of reaction products formed from paxilline and DMAPP by AtmD.....	78
3.8 Key HMBC, H-H COSY, and NOESY correlations of 20 or 21 mono-prenylated paxilline.....	80
3.9 Effects of temperature on AtmD activity.....	81
3.10 Effects of pH on AtmD activity.....	82
3.11 Effects of metal ions on AtmD activity.....	83
3.12 Hanes-Woolf plots for calculation of K_m and k_{cat} values of AtmD for paxilline and DMAPP.....	84
3.13 HPLC analysis of reaction products formed from paspaline and DMAPP by AtmD.....	86
3.14 LC/ESI-MS analysis of the reaction products formed from paspaline and DMAPP by AtmD.....	87
3.15 Key HMBC, H-H COSY, and NOESY correlations of 21 and 22 mono-prenylated paxilline.....	88

3.16 Hanes-Woolf plots of AtmD for paspaline and DMAPP.....	89
3.17 HPLC analysis of reaction products formed from paspaline and DMAPP by PaxD.....	90
3.18 LC/ESI-MS analysis of reaction products formed from paspaline and DMAPP by PaxD.....	91
3.19 Hanes-Woolf plots of PaxD for paspaline and DMAPP.....	92
3.20 HPLC analysis of reaction products formed from FI and DMAPP by AtmD and PaxD.....	94
3.21 LC/ESI-MS analysis of reaction products formed from FI and DMAPP by AtmD and PaxD.....	95
3.22 Key HMBC, H-H COSY, and NOESY correlations of 5- or 6- prenylated FI.....	96
3.23 HPLC analysis of reaction products formed from GGI and DMAPP by AtmD and PaxD.....	97
3.24 LC/ESI-MS analysis of reaction products formed from GGI and DMAPP by AtmD.....	98
4.1 Comparison <i>pax</i> and <i>amy</i> gene cluster.....	103
4.2 <i>amyG</i> nucleotide sequence including intron regions.....	116
4.3 Alignment of amino acid sequences of AmyG, PaxG, AtmG and LtmG.....	117
4.4 SDS-PAGE analysis of purified AmyG.....	118
4.5 HPLC profiles of the AmyG reaction products.....	120
4.6 <i>amyC</i> nucleotide sequence including intron regions.....	121
4.7 Alignment of amino acid sequences of AmyC, PaxC, AtmC and LtmC.....	122
4.8 SDS-PAGE analysis of purified AmyC.....	123
4.9 HPLC profiles of the AmyC reaction products.....	124
4.10 <i>amyD</i> nucleotide sequence including intron regions.....	125
4.11 SDS-PAGE analysis of purified AmyD.....	126
4.12 Gel filtration chromatography of AmyD.....	127
4.13 HPLC and LC/ESI-MS analysis of reaction products formed from paxilline and DMAPP by AmyD.....	129
4.14 Key HMBC, COSY, and NOESY correlations of 20,21-prenylated paxilline.....	130
4.15 Effects of temperature on AmyD activity.....	131

4.16 Effects of pH on AmyD activity.....	132
4.17 Effects of divalent metal ions on AmyD activity.....	133
4.18 Hanes-Woolf plots of AmyD for paxilline and DMAPP.....	134
4.19 HPLC analysis of reaction products formed from paspaline and DMAPP by AmyD.....	135
4.20 LC/ESI-MS analysis of reaction products formed from paspaline and DMAPP by AmyD.....	137
4.21 HPLC analysis of the closely related compounds formed from paspaline and DMAPP by AmyD with XBridge™ Phenyl Column.....	138
4.22 HPLC and LC/ESI-MS analysis of reaction products formed from FI and DMAPP by AtmD and AmyD.....	140
4.23 HPLC and LC/ESI-MS analysis of reaction products formed from GGI and DMAPP by AtmD and AmyD.....	142
4.24 <i>amyJ</i> nucleotide sequence including intron regions.....	143
4.25 Alignment of amino acid sequences of AmyJ and LtmJ.....	144

LIST OF SCHEMES

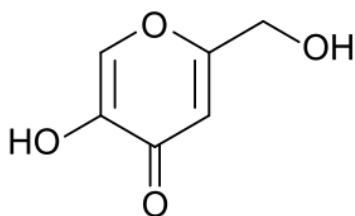
Scheme	
2.1 Biosynthetic pathway of paxilline.....	24
2.2 Reaction catalyzed by IGPS.....	34
2.3 Chemical synthesis of CdRP and its conversion into IGP by TtIGPS.....	36
2.4 Proposed reaction mechanism of PaxC.....	42
2.5 Reaction catalyzed by PaxD with paxilline and DMAPP.....	50
2.6 Summary of reactions catalyzed by PaxC and PaxD.....	62
3.1 Predict reaction products formed from paxilline and DMAPP by AtmD.....	79
3.2 AtmD-catalyzed reaction.....	80
3.3 Reaction products formed from paspaline and DMAPP by AtmD and PaxD.....	93
3.4 Reaction products formed from FI and DMAPP by AtmD and PaxD.....	95
3.5 Reaction product formed from GGI and DMAPP by AtmD.....	98
3.6 Summary of reactions catalyzed by AtmD and PaxD.....	100
4.1 AmyG-catalyzed reaction.....	120
4.2 AmyC-catalyzed reaction.....	124
4.3 AmyD-catalyzed reaction with paxilline and DMAPP.....	128
4.4 Predicted reactions catalyzed by AmyD with paspaline and DMAPP.....	138
4.5 Predicted reactions catalyzed by AmyD with FI and DMAPP.....	139
4.6 Predicted reactions catalyzed by AmyD with GGI and DMAPP.....	141
4.7 Predicted AmyJ-catalyzed reaction.....	145
4.8 Summary of reactions catalyzed by AmyG, AmyC and AmyD.....	147

Chapter 1

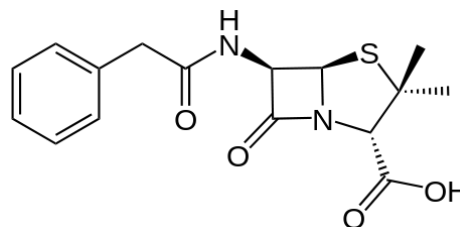
General introduction

1.1 Fungal secondary metabolites

The chemical activities of fungi have a long history. Many fungi produce antibiotics of varying efficiency because of the competitive environment in which they live. A long time ago, there were records of the use of fungi; for example, the fungus *Aspergillus oryzae* has been used in Japan to produce the fermentation of rice to make sake. Kojic acid (5-Hydroxy-2-hydroxymethyl-4-pyrone) was isolated from this fungi and its structure was established in 1924 (1). Penicillin, a β -lactam antibiotic, was isolated from *Penicillium* species in 1928 by Fleming (2, 3) and is historically significant because it is the first drugs that were effective against many previously serious diseases.



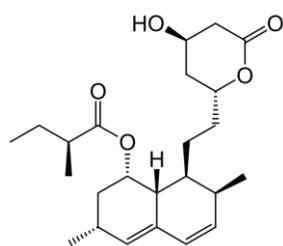
kojic acid



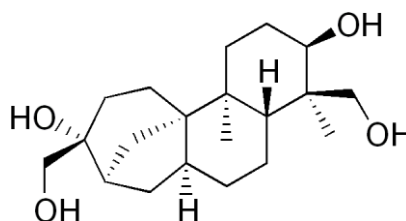
penicillin G

In recent years, many important compounds with useful pharmaceutical activity have been isolated from the fungi. These include the statins such as lovastatin from *Penicillium citrium*, which is used for an inhibition of cholesterol biosynthesis (4). Aphidicolin from *Cephalosporium aphidicola* was reported in 1972 (5). This metabolite

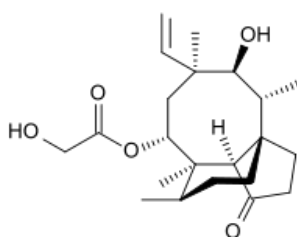
is a potent inhibitor of DNA polymerase (6-8) and a potential tumor inhibitor and anti-viral agent (9). Pleuromutilin was first described in 1951 from *Pleurotus mutilus* (10) and its derivatives are antibacterial drugs that inhibit protein synthesis in bacteria (11, 12). Cyclosporins were discovered in 1969 as metabolites of the fungus *Tolypocladium inflation* (13) and are widely used as an immunosuppressant agent (14, 15).



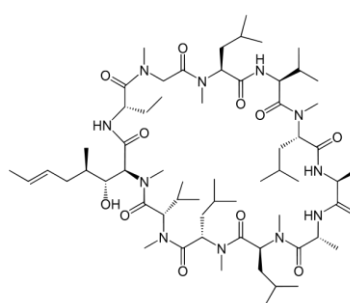
lovastatin



aphidicolin



pleuromutilin



cyclosporine

1.2 Gene cluster for fungal secondary metabolites

Gene clusters contain two or more genes that participate in biosynthesis of a related metabolic pathway (16). They involve contiguous enzymatic and often regulatory gene devoted to the production of a metabolite chemical class. Gene clusters are well known in prokaryotes in the form of operon. In which clustered gene with related biochemical functions are transcribed by a single promoter as a single mRNA (e.g. the lac operon). In fungi, which have more complex genomes than bacteria, they have no operons but constitute gene clusters composed of the individually transcribed gene.

The functional categories of genes found in fungi include ergot alkaloids (EA), indole diterpene (ID), polyketide synthase (PKS), nonribosomal peptide synthase (NRPS), PKS-NRPS (Hybrid), and nonreduced polyketide synthase (NR-PKS) (Table 1.1).

Table 1.1 Secondary metabolite gene clusters in fungal.

species	metabolite	cluster			function
		type	size(kb)	number	
<i>Aspergillus. fumigatus</i>	fumigaclavine C (17)	EA	22	10	inhibiting TNF-alpha production (18)
	fumitremorgin (19)	NRPS	28	9	BCRP (20, 21)
	gliotoxin (22)	NRPS	81.5	12	antiviral (23, 24)
<i>Aspergillus. nidulans</i>	sterigmatocystin (25)	NR-PKS	60	25	carcinogen (26)
	F9775A/B (27)	PKS	14	5	cathepsin K inhibitors (28)
	terrequinone A (29)	NRPS	12	5	antitumor (30)
<i>Neosartorya fischeri</i>	acetylaszonalenin (31)	NRPS	12.8	3	
<i>Aspergillus. flavus</i>	aflatrem (32)	ID	23	8	tremorgen (33)
	α -cyclopiazonic acid (34)	PKS-NRPS	15	3	inhibitor of Ca ²⁺ -ATPase (35)
<i>Lyngbya majuscula</i>	lyngbyatoxin (36)	NRPS	11.3	4	skin irritants (37)
<i>Penicillium.Paxilli</i>	paxilline (38)	ID	22.3	6	tremorgen (39)
<i>Chaunopycnis alba</i>	Terpendole E (40)	ID	16	7	kinesin Eg5 inhibitor (40, 41)
<i>Epichloe festucae</i>	lolitrems (42)	ID	~35	10	tremorgen (43)

ID= indole diterpene; EA= ergot alkaloids;

PKS=polyketide synthase; NRPS=non ribosomal peptide synthetase;

PKS-NRPS= (Hybrid); NR-PKS= nonreduced polyketide synthase.

BCRP=Breast cancer resistance protein.

1.3 Regulation of fungal secondary metabolite production

The growth of fungi is dependent on the chemical composition and environmental. In the laboratory, fungus can be grown on “solid” or “liquid” medium under aerated condition with static, shake and stirred fermentations. Different cultivation conditions affect not only the rate of growth but also the production of the metabolites. Indeed, genome projects have revealed that fungi have many secondary metabolite biosynthetic gene clusters, but many of them are not normally expressed. To activate the silent gene cluster, genetic engineering is very important.

1.3.1 Culture medium regulation

A typical synthetic medium contains a carbon source, normally a sugar, a nitrogen source such as an ammonium salt, phosphate, magnesium, and a potassium salt often also providing sulfate and chloride ions together with the salts of trace elements. In laboratory, common media Czapek-Dox medium or Potato Dextrose Broth (44) were used. Many enzymes require metal ions as a functional component; for example, some prenyltransferases (not all) require Mg^{2+} (45) and iron is particularly important in the cytochrome P450 oxidases (46), which are involved in the biosynthesis of many fungal metabolites. For this reason, trace metal ion is often supplied for growing fungi.

1.3.2 Chemical epigenetics

Histone modifications and DNA methylation operate in tandem to bring about changes in chromatin status and gene expression or silencing in fungi and other organisms (Fig 1.1). Recently, the uses of epigenetic modifying agents to induce changes in fungal secondary metabolism are reported (47). Keller et al. reported that a treatment of *Alternaria alternata* and *Penicillium expansum* with histone deacetylase (HDAC) inhibitors resulted in the increased production of secondary metabolites (48). In *A. niger*, the addition of the HDAC inhibitor, suberoylanilidehydroxamic acid (SAHA), generated the production of nygerone A (49). Chung et al added a DNA methylation inhibitor, azacytidine to culture broth of *Aspergillus sydowii* to produce a new bisabolane-type sesquiterpenoid (7S)-(+)-7-O-methylsydonol (50).

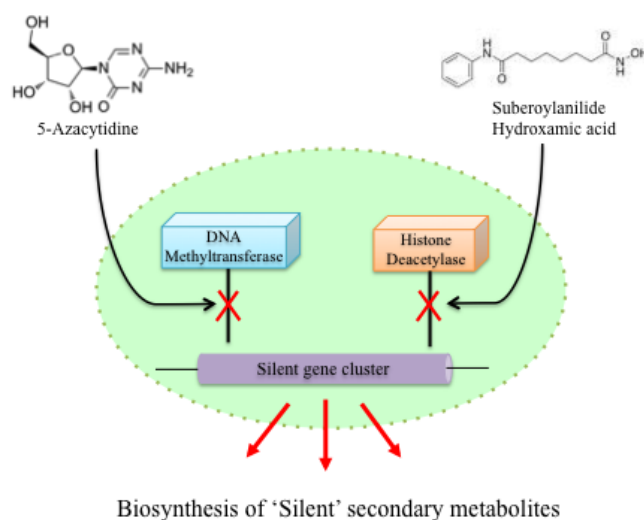


Figure 1.1 Epigenetic remodeling of fungal secondary metabolite.

1.3.3 Transcription factor regulation

As mentioned above, genome projects have revealed that fungi have many secondary metabolite biosynthetic gene clusters, but many of them are not normally expressed. Besides to the uses of epigenetic modifying agents, a modification of transcriptional regulator has also been reported to wake up the silent genes (51). Many (but not all) secondary metabolite gene clusters contain a class of zinc binuclear transcription factor genes (Zn_2Cys_6), which are unique in fungi such as *apdR* in *A. nidulans*. Overexpression of a regulatory gene could lead to activation of a silent gene cluster and resulted in production of a new metabolite, aspyridones A/B (52) (Fig1.2).

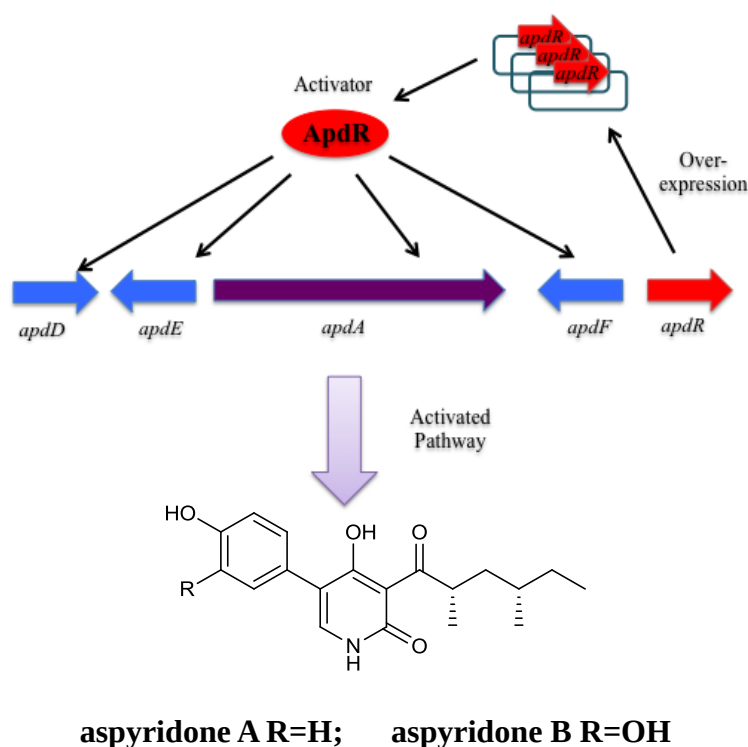
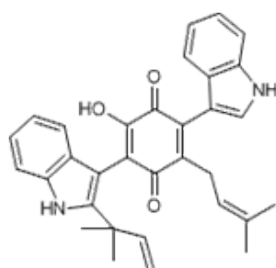


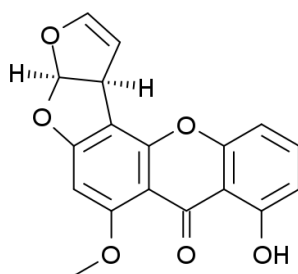
Figure 1.2 Targeted cluster activation through overexpression of *apdR* gene.

LaeA, a global regulator of secondary metabolism, is a nuclear protein identified in *A. nidulans* (53, 54) and its orthologs were identified in *A. fumigatus*, *A. terreus*, *A. flavus* (55) and the other genres (56-59). A deletion mutant of LaeA in *A. nidulans*, eliminated the production of the antitumor compound terrequinone A (29).



terrequinone A

The eukaryotic basic leucine zipper (**bZIP**) transcription factors also uncovered new global regulators of secondary metabolism. It was able to enhance secondary metabolite production in *A. nidulans* (60). Overexpression of RsmA (a like bZIP protein) enabled to greatly increase sterigmatocystin yield in *Aspergillus nidulans* (61).



Sterigmatocystin

1.3.4 Co-cultivation regulation

The functional role of natural products in the environment is largely unknown. Bacteria and fungi co-inhabit a wide variety of environments, such as in soil, plant, insect, and the gut (62). Schroeckh et al reported a microarray technology to monitor the selective induction of silent gene cluster through bacterial-fungal interactions (27). They co-cultivated *A. nidulans* with 58 of actinomycetes and identified a bacterium (*Streptomyces rapamycinicus*) that selectively triggered the expression of silent genes encoding the PKS genes for orsellinic acid, a typical lichen metabolite lecanoric acid, and the cathepsin K inhibitors F-9775A/B (Fig1.3).

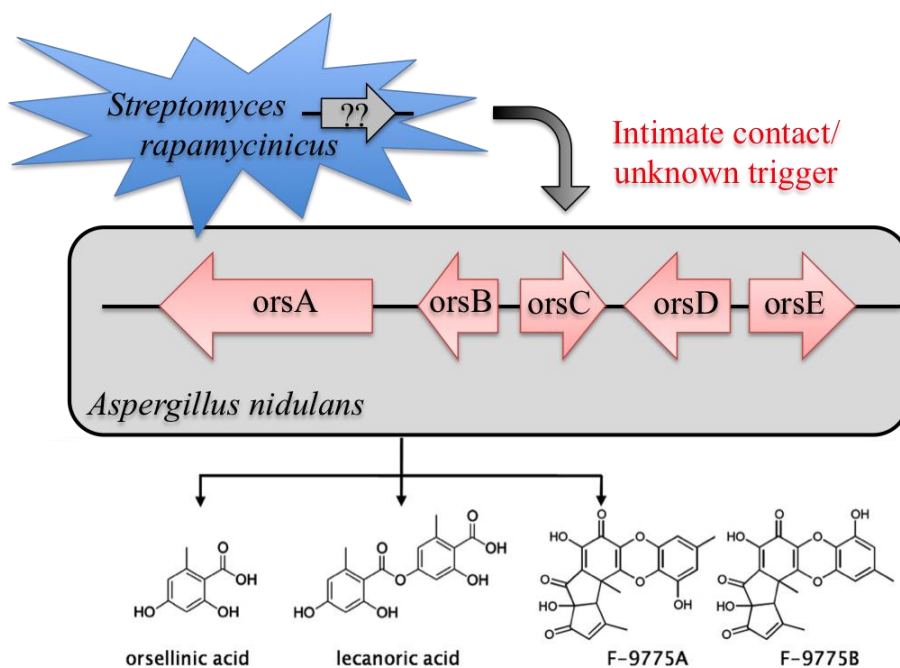
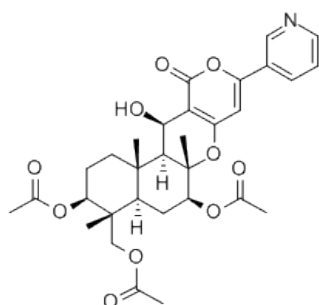


Figure 1.3 Physical interaction of *A. nidulans* with *S. hygroscopicus*

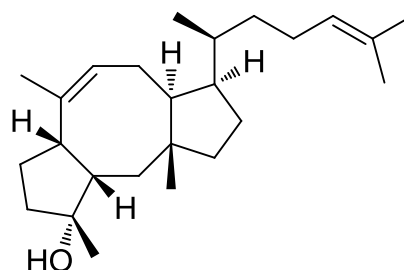
1.4 Heterologous expression

Transference of whole gene clusters to another host organism, which does not contain the related genes are also useful. However, this approach has some limitations, including the difficulty of transferring large genes and gene clusters, as well as finding a suitable host. Nevertheless, researchers succeeded in reconstituting up to five steps of the biosynthesis of the meroterpenoid pyripyropene A from *A. fumigatus*, a potent inhibitor of acyl-coenzyme A cholesterol acyltransferase, using *A. oryzae* M-2-3 as a host (63).

Very recently, Tagami et al carried out heterologous expression of paxilline biosynthetic genes (*paxG*, *paxC*, *paxM*, *paxB*, *paxP*, and *paxQ*) in *Aspergillus oryzae* NSAR1. Transformation of each gene into *A. oryzae* in stepwise manner enabled to isolate the biosynthetic intermediates and achieve the total biosynthesis of paxilline (64). By using this method, identification of ophiobolin F synthase gene from *A. clavatus* was also reported (65).



pyripyropene A



ophiobolin F

1.5 Indole diterpene

Indole diterpene compounds are a large and structurally diverse group of fungal secondary metabolites, many of which are potent tremorgenic mammalian mycotocins (66). These metabolites have a common core structure comprising of geranylgeranyl diphosphate (GGDP) and an indole skeleton that is most likely derived from indole-3-glycerol phosphate (67), which is an intermediate of tryptophan biosynthesis (68). Administration of geranylgeranyl indole (GGI) revealed that is a common precursor of indole diterpenes (69).

The indole diterpene can be divided into two classes (70), the paxilline-like compounds and those lacking the paxilline-type core (Fig 1.4A). The paxilline-like compounds were derived from paspaline (71). This group included the paxilline (72), penitrems (73), janthitrems (74, 75), lolitrems (76), aflatrem (77), and shearinines (78, 79). The structural diversity observed within this group of metabolites is achieved by additional prenylations, different patterns of ring substitutions, and different ring stereochemistry.

The other group includes the nodulisporic acid (80, 81) and thiersinines (82). Moreover, a number of distinctive skeletal variations have been identified in this group such as radarins (83), emindoles (84, 85) and nominine (86), in which bicyclic or tricyclic structures are attached to the indole unit at only one position (Fig 1.4B).

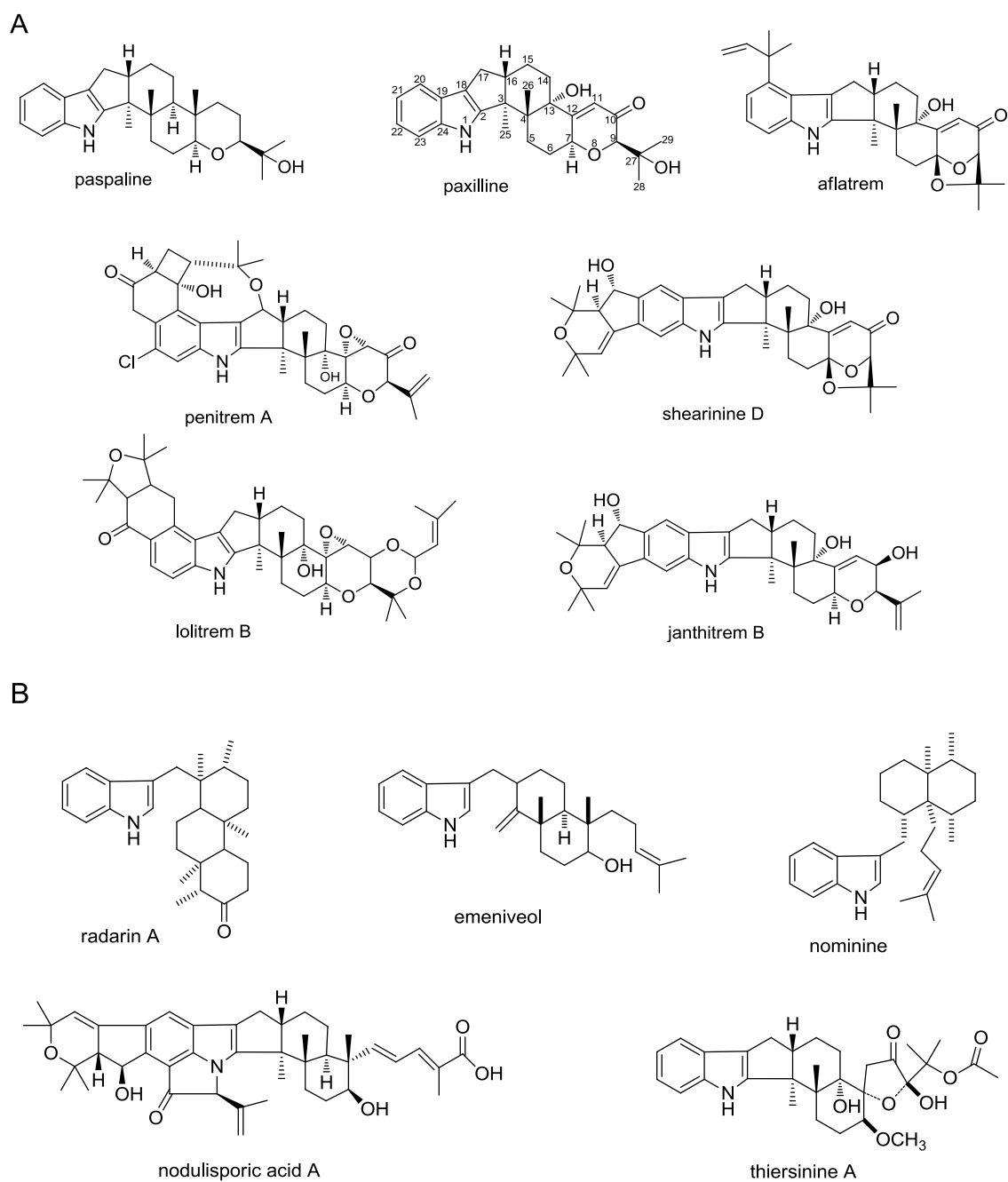


Figure 1.4 Chemical diversity of indole diterpene. (A) Paxilline-like group. (B) Other group.

1.6 Prenylated indole derivatives produced by fungi

Prenylated indole alkaloids are hybrid natural products derived from prenyl diphosphates and tryptophan or its precursors and widely distributed in filamentous fungi. These compounds represent a group of natural products with diverse chemical structures and biological activities. Significant progress on their biosynthesis has been achieved in recent years by identification of biosynthetic gene clusters from genome sequences and by molecular biological and biochemical investigations (87). In addition, a series of prenylated indole derivatives have been produced by enzymatic synthesis using overproduced and purified enzymes (88).

1.6.1 Prenyltransferases

Prenyltransferases attach isoprenoid moieties derived from allylic isoprenyl diphosphates such as dimethylallyl diphosphate (DMAPP; C5), geranyl diphosphate (GPP; C10) farnesyl diphosphate (FPP; C15), and geranylgeranyl diphosphate (GGDP; C20) to acceptor compounds and give rise to diversity of secondary metabolites in plants, fungi, and bacteria (89) .

The known prenyltransferases can be phylogenetically classified into three groups (89). The first one includes the soluble aromatic prenyltransferases identified mainly in *Streptomyces* strains. CloQ, which participates in clorobiocin biosynthesis, was the first

example (90). Based on sequence similarity with CloQ, several enzymes with similar activities have been reported (45, 91, 92). Of these, the crystal structure of NphB, which is essential for the biosynthesis of naphterpin, was solved and found to contain an architecturally new $\alpha\beta\beta\alpha$ secondary structure element (45). Interestingly, some enzymes from this group were shown to catalyze the transfer of prenyl diphosphate to flavonoids in addition to THN (tetrahydroxynaphthalene), a genuine substrate (45, 93). Very recently, enzymes belonging to this class have been identified in several fungi (94).

The second group includes soluble indole prenyltransferases identified mostly in fungi. Dimethylallyl tryptophan synthases, such as DmaW from *Claviceps fusiformis* (95) and FgaPT1 from *Aspergillus fumigates* (96) are representatives. More than ten enzymes of this class have been reported (97-108). Recently, an indole prenyltransferase, which is phylogenetically positioned between soluble aromatic prenyltransferases and fungal indole prenyltransferases, has been identified in a *Streptomyces strain* (109, 110).

The third group contains membrane-bound prenyltransferases identified in various organisms. 4-Hydroxybenzoate polyprenyltransferase responsible for the biosynthesis of ubiquinones and plant naphthoquinone shikonin are examples (111, 112). Recently, flavonoid-specific prenyltransferases have been also identified in plants (113, 114).

1.6.2 Prenyltransferases acting on indole compounds

Until the end of January 2012, indole prenyltransferases with regioselectivity for N-1, C-2, C-3, C-4, C-5, C-6, and C-7 have been identified and characterized in detail (115). These enzymes catalyzed the transfer of prenyl moieties onto nitrogen or carbon atoms at the indole ring resulting in formation of “regularly” or “reversely” prenylated derivatives. Moreover, some enzyme showed broad substrate specificity. CymD from the marine actinobacterium *Salinispora arenicola* catalyzed the reverse prenylation at the indole nitrogen of l-tryptophan (116). C-2-prenylations were observed for FtmPT1 from *A. fumigatus* (98). FgaPT1 catalyzed the reverse prenylation of fumigaclavine A at C-2 position (96). A prenylation of C-3 position of cyclic dipeptides by AnaPT (108) and CdpC3PT (104), both of which were from a fungal *Neosartorya fischeri* was reported. FgaPT2 / DmaW from the different fungus catalyzed the regular prenylation of l-tryptophan at C-4 (17, 95, 117). 5-DMATS from *Aspergillus clavatus* catalyzed prenylations of l-tryptophan and its derivatives at C-5 position (115). IptA from a soil bacterium *Streptomyces sp.* was reported to prenylate l-tryptophan at C-6 (110). 7-DMATS from *A. fumigatus* at C-7 (100) (Fig 1.5) was shown to be indole prenyltransferases with different prenylation positions.

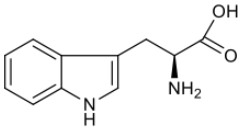
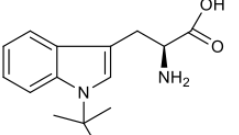
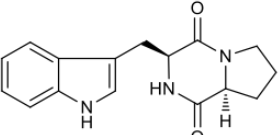
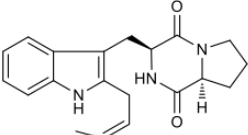
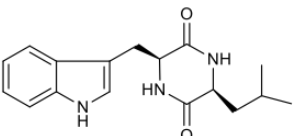
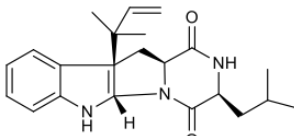
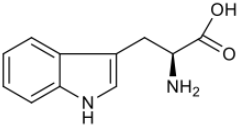
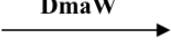
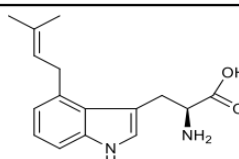
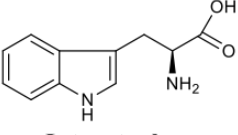
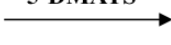
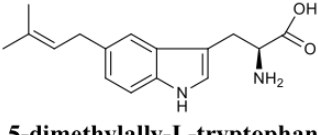
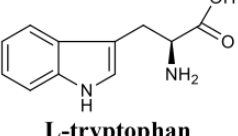
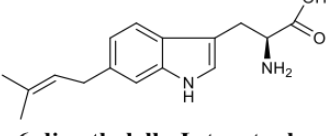
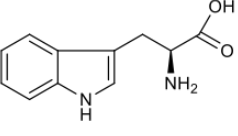
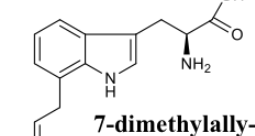
Prenylation position	Substrate	Enzyme	Product
N1	 L-tryptophan	 CymD	 N-prenylated-L-tryptophan
C2	 Brevianamide F	 FtmPT1	 Tryprostatin B
C3	 Cyclo-LTrp-L-Leu	 CdpC3PT	 C3-beta-prenylated Cyclo-LTrp-L-Leu
C4	 L-tryptophan	 FgaPT2 DmaW	 4-dimethylallyl-L-tryptophan
C5	 L-tryptophan	 5-DMATS	 5-dimethylallyl-L-tryptophan
C6	 L-tryptophan	 IptA	 6-dimethylallyl-L-tryptophan
C7	 L-tryptophan	 7-DMATS	 7-dimethylallyl-L-tryptophan

Figure 1.5 Prenyltransferases acting on different positions of indole compounds.

1.6.3 Prenylated indole-diterpenes

Prenylated indole-diterpenes are structurally and functionally diverse group of secondary metabolites produced by filamentous fungi. Lolitrems, paspalitrem A/B (118), aflatrem/ β -aflatrem, janthitrems, penitrems, and terpendoles are representatives. These compounds affect neuroreceptors and neurotransmitter release mechanisms in the central and peripheral nervous systems (119-121). Penitrems were reported to have beneficial anti-insectan effects (122). Lolilline and the tremorgenic lolitrems A/B were identified in *Neotyphodium*-infected plants *Lolium perenne* and *Festuca* sp. Although both compounds have very similar structures, lolilline has no tremorgenic activity (123). These compounds are also known to inhibit the α -subunit of the large conductance calcium-activated potassium channels known as BK, maxi-K channels (71, 124-127). Other biological activities associated with indole-diterpenes are inhibition of mammalian acyl-CoA: cholesterol O-acyltransferase (128-131) and mitotic kinesin (41).

On the other hand, shearinine D and 21-isopenteylpaxilline from *Penicillium* strains have biological activities to induce apoptosis in human leukemia HL-60 (79) cells and anti H1N1 virus activity (132), respectively. Nodulisporic acid A was isolated from an endophytic fungus *Nodulispororium* sp, which is potent anti-flea activity (133, 134). In summary, prenylated indole-diterpenes show variety biological activities (Fig 1.6).

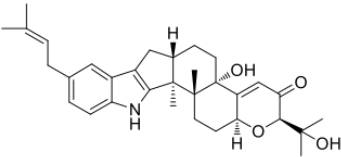
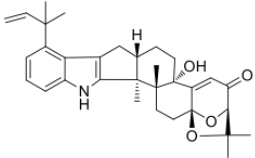
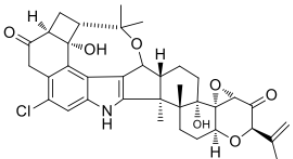
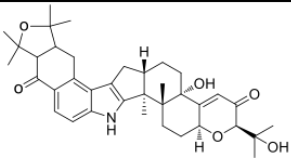
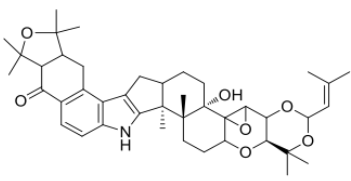
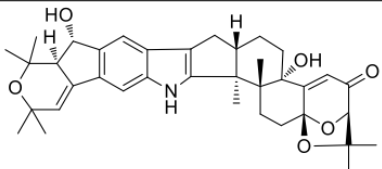
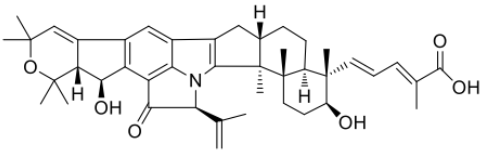
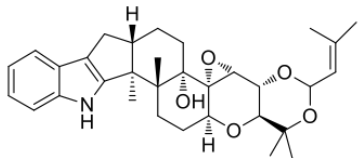
Compound	Structure	Biological activity	Species
21-isopentenyl paxilline		anti-H1N1 virus activity	<i>Penicillium camemberti</i>
aflatrem		tremorgen	<i>Aspergillus flavus</i>
Penitrem A		Anti-insect	<i>Penicillium crustosum</i>
lolilline		Non-tremorgen	<i>Acremonium lolii</i>
Lolitre B		Tremorgen increase intestinal motility inhibit BK channels	<i>Epichloë festucae</i> .
Shearinine D		Apoptosis in human leukemia HL-60	<i>Penicillium janthinellum</i>
Nodulisporic Acid A		Anti-flea activity	<i>Nodulisporium sp</i>
Terpendole C		inhibition ACAT	<i>Albophoma yamanashiensis</i>

Figure 1.6 Prenylated indole diterpene; structure and biological activity.

Chapter 2

Functional analysis of PaxC and PaxD in the paxilline biosynthetic gene cluster

2.1 Introduction

Isoprenoid compounds found in nature with over 50,000 known examples contain industrially useful compounds such as flavors, antibiotics and plant hormones, among others (135-137). In some cases, isoprenoids are attached to other moieties, such as polyketide (138), indole/tryptophan (88, 139), (iso)flavonoid (140, 141), and phenazine moieties (142-144). Prenylated indole compounds, most of which are produced by filamentous fungi are examples of this type (87, 88). These compounds are derived from polyprenyl diphosphates and tryptophan or its derivatives and show diverse chemical structures and biological activities (11, 124, 134). Among prenylated indole compounds, indole-diterpenes are unique from the following viewpoints: geranylgeranyl diphosphate (GGDP) is used as a prenyl donor in contrast to other prenylated indoles, for which dimethylallyl diphosphate (DMAPP) is the usual donor, and indole/indole-3-glycerol phosphate is used as the prenyl acceptor instead of tryptophan and its derivatives (145, 146).

Pioneering studies of biosynthetic genes and enzymes for indole diterpenes were carried out by Scott et al. with *Penicillium paxilli*, a paxilline producer. They first identified 21 clustered genes (Fig 2.1 and Table 2.1) in a genomic locus (38, 147). They later showed that *paxG*, *paxM*, *paxB*, and *paxC* were required for paxilline biosynthesis by expression of these genes in a mutant deleted for the *pax* gene cluster (67). Paxilline

shown to be successively oxidized by a cytochrome P450 gene, *paxP*, to give 13-desoxypaxilline, followed by an additional oxidation by *paxQ* to give paxilline (148). Very recently, Tagami et al have clarified the detailed function of each of these *pax* genes (*paxG*, *paxC*, *paxM*, *paxB*, *paxP*, and *paxQ*) by heterologous expression (Fig 2.2), in vitro assay with recombinant enzymes and biotransformation (64). They confirmed that a transformant of *Aspergillus oryzae* carrying *paxG* and *paxC* produced geranylgeranyl indole (GGI). Introduction of additional *paxM*, *paxB*, and *paxP/paxQ* genes into the transformant resulted in the production of mono-epoxide GGI, paspaline, and paxilline, respectively. However, GGI's real substrate and PaxD function remain unclear (Scheme 2.1).

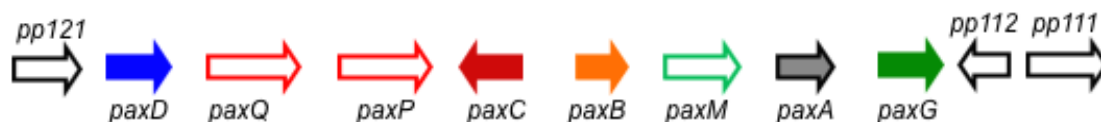


Figure 2.1 Paxilline biosynthetic gene cluster in *Penicillium paxilli*.

Table 2.1 Predicted functions of *pax* genes identified in *P. paxilli*.

<i>pax</i> genes	Protein (accession No.)	Amino acid (no.)	Function
<i>pp101</i>	ADO29923	402	FAD-dependent Oxidoreductase
<i>pp102</i>	ADO29924	508	β -1,3- glucanosyltransferase
<i>pp103</i>	ADO29925	103	Unknown
<i>pp104</i>	ADO29926	477	Zn(II)2Cys6 transcription factor
<i>pp105</i>	ADO29927	684	Zn(II)2Cys6 transcription factor
<i>pp106</i>	ADO29928	345	Unknown
<i>pp107</i>	ADO29929	385	NADH oxidoreductase
<i>pp108</i>	ADO29930	543	Capsule associated protein
<i>pp109</i>	ADO29931	175	Acetyltransferase
<i>pp110</i>	ADO29932	818	Unknown
<i>pp111</i>	AAK11533	478	Transporter (MFS)
<i>pp112</i>	AAK11532	291	DUF829-Conserved Protein(unknown)
<i>paxG</i>	AAK11531	371	GGDP synthase
<i>paxA</i>	ADO29933	356	Integral membrane protein
<i>paxM</i>	AAK11530	477	FAD-dependent monooxygenase
<i>paxB</i>	ADO29934	243	Cyclase
<i>paxC</i>	AAK11529	317	Prenyltransferase
<i>paxP</i>	AAK11528	515	Cytochrome P450 monooxygenase
<i>paxQ</i>	AAK11527	512	Cytochrome P450 monooxygenase
<i>paxD</i>	AAK11526	438	Prenyltransferase
<i>pp121</i>	ADO29935	418	FAD-binding oxidoreductase

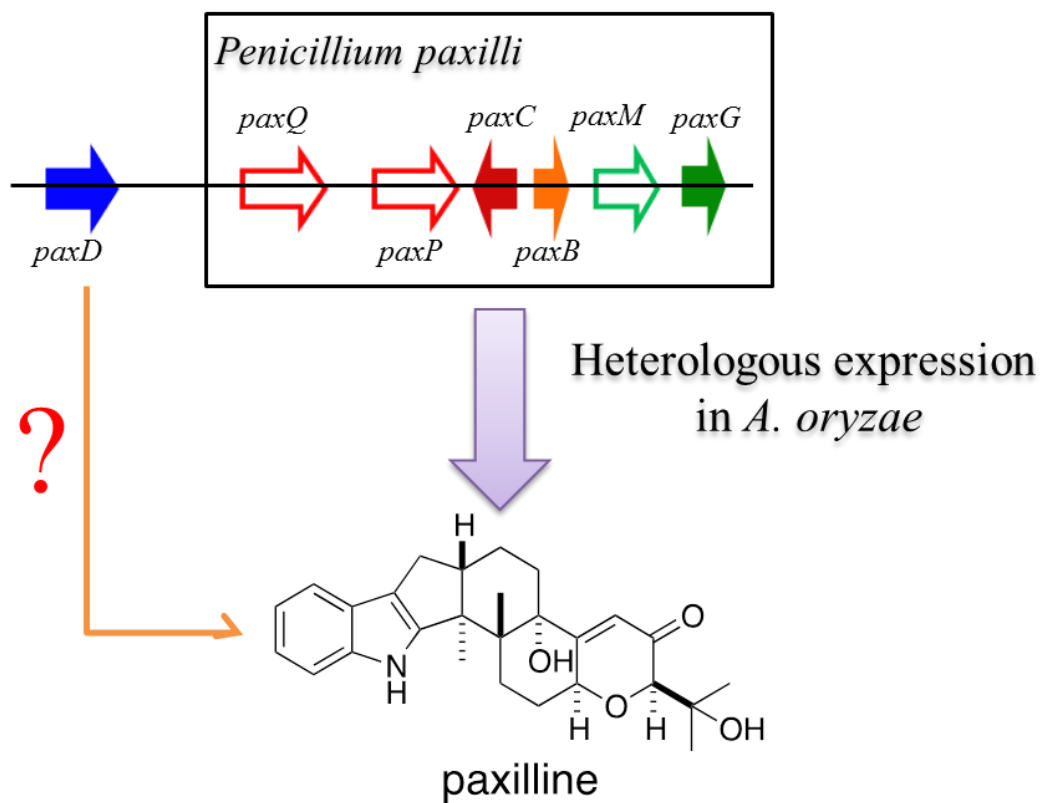
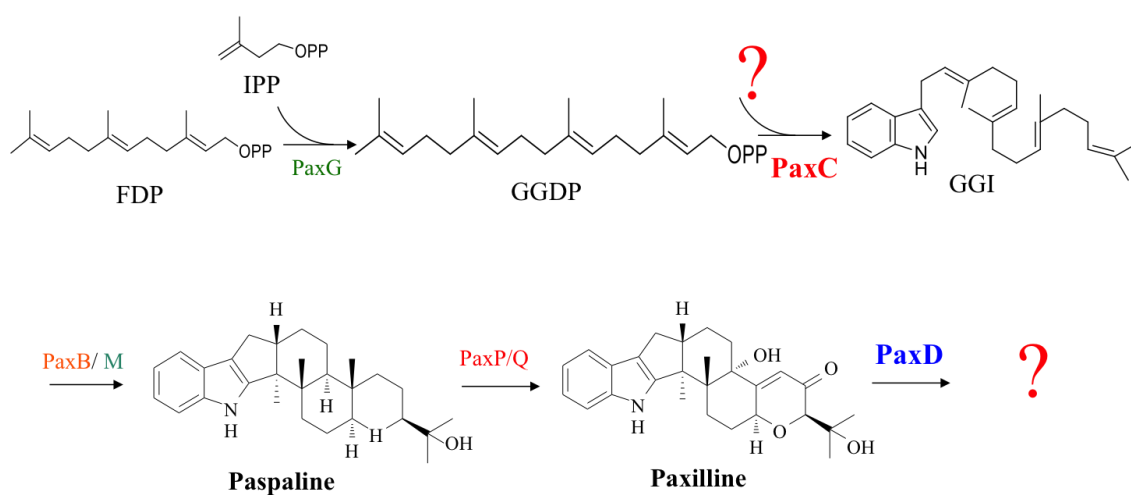


Figure 2.2 Heterologous expression of paxilline gene cluster in *A.oryzae*.



Scheme 2.1 Biosynthetic pathway of paxilline.

In this chapter, I carried out an in vitro analysis using recombinant PaxC and confirmed that the enzyme could utilize both indole and indole-3-glycerol phosphate to yield GGI. Moreover, I focused on the putative prenyltransferase gene *paxD*, which is located next to the *paxQ* gene and examined whether it participates in the biosynthesis of paxilline derivatives (Fig 2.2). The PaxD was overexpressed in *E. coli* and purified enzyme was used for in vitro analysis. When paxilline and DMAPP were used as the substrates, one major and one minor product, which were identified as di-prenyl paxilline and mono-prenyl paxilline by liquid chromatography–mass spectrometry analysis, were formed. The structure of the major product was determined to be regularly 21,22-diprenylated paxilline. Traces of both products were detected in culture broth of *P. paxilli* by liquid chromatography mass spectrometry analysis. In this chapter, the details of these experiments are described.

2.2 Materials and Methods

2.2.1 General

Sequence analysis of PCR fragments was performed by the dideoxy chain termination method with an automatic DNA sequencer (Li-Cor, model 4000L). Cell disruption was performed with an Ultrasonic Disruptor (TOMY, UD-200). Analysis of the samples during protein purification was performed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were visualized by using Coomassie brilliant blue staining. Protein concentration was determined by the Bradford method with bovine serum albumin as a standard. Plasmids from *E. coli* were prepared using a Qiagen plasmid kit. All restriction enzymes, T4 ligase, and calf intestinal alkaline phosphatase were obtained from Toyobo (Osaka, Japan), and used according to the manufacturer's instructions. Transformation of *E. coli* with plasmid DNA by electroporation was performed under standard conditions and a BTX ECM 600 electroporation system (Biotechnologies and Experimental Research).

2.2.2 Strain

Penicillium paxilli ATCC26601 was grown in 100 ml of medium containing 3.4% Czapek-Dox, and 0.5% Yeast extract at 25°C for 6 days in a reciprocal shaker (125 strokes/min).

2.2.3 DNA, RNA isolation and cDNA synthesis

The mycelia were transferred to a 50 ml plastic tube and mixed with 20 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1% SDS, 0.1% NaCl). After incubation at 80°C for 10 min, followed by cooling, the lysate was treated with Proteinase K (Sigma Aldrich, 1 mg/ml) and RNase (Takara Bio Inc., 0.1 mg/ml) at 30°C for 1 hr. The DNA was then extracted with chloroform-phenol and precipitated with isopropanol. After washing with 70% ethanol, the DNA was resuspended in TE buffer. For the preparation of cDNA, the mycelia, which were cultivated by the same method as described above, were filtered using a plastic filter and dried on paper towel. Then, they were frozen in liquid nitrogen and homogenized with a SK-mill (Tokken Inc., Japan). The total RNA of the strain was isolated using the TRIzol® reagent (Invitrogen, US) according to the manufacturer's protocol. The fragments containing the 5' or 3'-termini of cDNA were obtained using the SMART™ RACE cDNA Amplification Kit (Clontech, US).

2.2.4 Cloning, expression and purification of TtIGPS/PaxC/PaxD

Table 2.2 Primers used for *ttIGPS/paxC/paxD* amplifications.

Amplified genes	Restriction sites used	Sequence (5' to 3')	Vectors and Host
TtIGPS	<i>NheI</i> <i>HindIII</i>	F: TAGCTAGCATGCGGCCCGACCTTTCCCG R: GCAAGCTTTCCTACAAGCTGGCGGAGGG	pET21b BL21
PaxC	<i>SphI</i> <i>PstI</i>	F: AAGCATGCATGGGCGTAGCAGGGAGCGGAGTTCTTTAC R: AACTGCAGTCATGCCTTCAGGTCAAGCTTCTCCTCCCG	pQE30 M15
PaxD	<i>KpnI</i> <i>PstI</i>	F: TTGGTACCATGCAAAGCGATGAGTTGCAAATCCCACC R: TTCTGCAGCTATGCCTTGAAAAGCTTAGGAGCCCATAC	pQE30 M15

The transformant harboring pQE30-*paxC/M15*, pQE30-*paxD/M15*, and pET21b-*TtIGPS/BL21* was separately grown in L-broth supplemented with 100 µg/mL ampicillin at 37°C until optical density (OD)₆₀₀ reached 0.5, and then isopropyl β-D-thiogalactopyranoside (0.5 mM) was added to the culture, followed by additional cultivation at 18°C (pQE30-*paxC/M15* and pQE30-*paxD/M15*) or 25°C (pET21b-*TtIGPS/BL21*) for 18 hours. The harvested cells were suspended in a buffer containing 50 mM tris (hydroxymethyl) aminomethane (Tris)-HCl (pH 8.0), 0.5 M NaCl, and 10 mM imidazole, and then disrupted by sonication. The debris was removed by centrifugation, and the supernatant was applied on a Ni²⁺-nitrilotriacetic acid agarose column (Qiagen) equilibrated with buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 10 mM imidazole. The column was washed with equilibration buffer and then eluted with a buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 0.5 M imidazole. The eluent was desalted using an Amicon apparatus (Millipore, Bedford, MA, USA).

All recombinant enzymes were subjected to SDS-PAGE analysis to confirm their molecular sizes and purified enzymes were used for in vitro assay. The recombinant PaxD was then subjected to gel filtration chromatography. HiLoad 26/60 Superdex 75 pg gel-filtration column (Amersham Biosciences) to estimate its subunit structure. A buffer containing 50 mM Tris-HCl (pH 8.0) and 10 mM NaCl was used for elution. The

retention volume of PaxD was compared with those of the marker proteins. The markers applied to the column were aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa) (GE Healthcare, Little Chalfont, UK).

2.2.5 Chemical synthesis CdRP

The previously reported chemical synthesis protocols were used (149) for CdRP synthesis: 0.87 mmol of anthranilic acid (Aldrich) were dissolved in 1.07 mL isopropanol and mixed with 0.43 mmol of R5P (Sigma) dissolved in 553 μ L of water. The reaction mixture was kept in the dark at room temperature overnight. Reaction was then cooled at 4 °C for 1h, after which two layers were formed. The aqueous phase was removed and the remnant dark yellow oil was washed with chloroform and extract with methanol. After desiccation, CdRP was kept at –20 °C.

2.2.6 Enzyme reaction with TtIGPS

The assay mixture for TtIGPS contained, in a final volume of 100 μ L, 0.5 mM CdRP, 50 mM Tris–HCl (pH 8.0), and a suitable amount of TtIGPS. This mixture was incubated at 60°C for 15min, and the reaction was stopped by the addition of 100 μ L methanol. The products were analyzed by high performance liquid chromatography (HPLC). Analytical conditions were follow as; SeQuantTM ZIC-HILIC column (150 $\times$ 2.1 mm), 5 μ m, (Merck); mobile phase of acetonitrile in water (0 to 25 min, 90% to 20%

acetonitrile; 25 to 35 min, 20%; 35 to 50 min, 90%); flow rate, 1.0 mL/min; detection, 278 nm.

2.2.7 Enzyme reaction with PaxC

The assay conditions are as follows; a reaction mixture (final volume, 100 μ L) containing 50 mM of Tris-HCl (pH 8.0), 500 μ M of substrates (IGP, tryptophan, or indole), 500 μ M of GGDP, 500 μ M of $MgCl_2$ and 10 μ M of PaxC was incubated at 30 °C for overnight. The reaction was stopped by the addition of methanol (100 μ L). The supernatant was directly analyzed by HPLC. The analytical conditions were as follow; column, Merck Mightysil RP-18GP column (250 mm x 4.6 mm) (Kanto Chemicals, Tokyo, Japan); mobile phase, acetonitrile in water (0 to 35 min, 0 to 100%; 35 to 60 min, 100%); flow rate, 1.0 mL/min; detection, 278 nm.

2.2.7.1 Kinetic analysis of PaxC

The assay for determination of the kinetic parameters of PaxC for IGP contained 50 mM of Tris-HCl (pH 8.0), 500 μ M of $MgCl_2$, 2.5 mM of GGPP, 1 μ M of PaxC, and 10 μ M to 1 mM of IGP in a final volume of 100 μ L. The mixture was incubated at 30°C for 10 min. When the concentration of indole 3-glycerol phosphate was fixed at 2.5 mM, the concentration of GGPP was varied from 2 μ M to 100 μ M. The same assay mixture except for the concentration (100 μ M to 1.5 mM) was used for indole.

2.2.8 In vitro assay of PaxD

The assay mixture contained, in a final volume of 100 μ L, 0.25 mM of paxilline, 0.5 mM of DMAPP, 50 mM Tris–HCl (pH 8.0), and a suitable amount of the PaxD. This mixture was incubated at 30°C for overnight and the reaction was stopped by the addition of 100 μ L of methanol. The products were analyzed by HPLC. The analytical conditions were as follow; Merck Mightisil RP-18GP Aqua column (250 mm $\times$ 4.6 mm) (Kanto Chemicals, Japan); mobile phase of acetonitrile in water (0 to 25 min, 70% AcCN; 25 to 40 min, 70 to 100%; 40 to 50 min, 100%); flow rate, 1.0 mL/min; detection, 230 nm.

2.2.8.1 Kinetic parameters of PaxD

The assay was linear with respect to protein concentration up to 1 μ g for 30 min incubation and no substrate inhibition was observed with paxilline and DMAPP up to 1.0 mM of each of the substrate. The assays for determination of the kinetic parameters of paxilline contained, in a final volume of 100 μ L, 50 mM Tris–HCl (pH 8.0), 0.5 mM DMAPP, 0.2 μ g of the PaxD, and 20 μ M to 0.5 mM paxilline. When the concentration of paxilline was fixed at 0.25 mM, the concentration of DMAPP was varied from 0.1 μ M to 20 μ M. This mixture was incubated at 30°C for 20 min.

2.2.9 LC/ESI-MS analytical conditions

LC/ESI-MS (Waters ACQUITY UPLC equipped with SQD2) was used. The analytical conditions were as follows:

PaxC reaction products; column, ZIC-HILIC column (150 mm x 2.1 mm); column temperature, 40°C; detection, positive mode; mobile phase following conditions: 90% acetonitrile (containing 0.1% formic acid) for 8 min, a linear gradient from 90% to 10% acetonitrile for 9 min and 10% acetonitrile for 13 min at a flow rate of 0.3 mL/min. Cone voltage (30 V).

PaxD reaction products; column, Waters ACQUITY UPLC C18 column (2.1 × 50 mm); column temperature, 40°C; detection, positive mode; mobile phase, 0.1% formic acid : acetonitrile = 30:70 at 5 min, and a linear gradient to 50:50 for an additional 30 min; flow rate, 0.3 ml/min. Cone voltage (30 V).

Fragment detection; column, Waters ACQUITY UPLC C18 column (2.1 × 50 mm); column temperature, 40°C; detection, positive mode; mobile phase, 0.1% formic acid: acetonitrile = 30:70 at 5 min, and a linear gradient to 50:50 for an additional 30 min; flow rate, 0.3 ml/min. Cone voltage (50 V).

2.2.10 Structural analysis of reaction product

The reaction product formed from paxilline and DMAPP by PaxD was fractionated

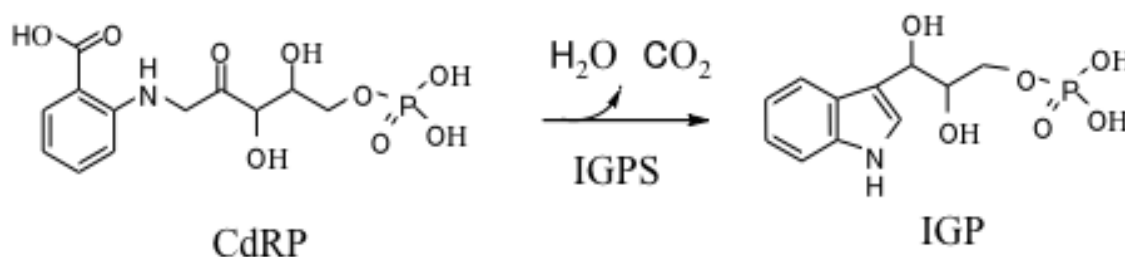
with a preparative HPLC. ^1H - and ^{13}C -NMR spectra were recorded on Bruker AMX-500 spectrometer. NMR δ_{H} (CDCl_3 , 500 MHz) 1.02 (s, 3H), 1.28 (s, 3H), 1.29 (s, 3H), 1.30 (s, 3H), 1.43 (dd, $J = 4.6, 13.2$ Hz, 1H), 1.65 (d, $J = 12.7$ Hz, 1H), 1.71 (s, 3H), 1.74 (s, 6H), 1.75 (s, 3H), 1.78 (m, 1H), 1.88 (m, 1H), 2.02 (m, 1H), 2.06 (m, 1H), 2.32 (m, 1H), 2.41 (dd, $J = 11.0, 13.0$ Hz, 1H), 2.71 (dd, $J = 6.2, 13.0$ Hz, 1H), 2.79 (m, 1H), 2.82 (m, 1H), 3.39 (d, $J = 6.9$ Hz, 4H), 3.72 (d, $J = 1.8$ Hz, 1H), 4.85 (t, $J = 9.2$ Hz, 1H), 5.29 (t, $J = 6.9$ Hz, 1H), 5.30 (t, $J = 6.9$ Hz, 1H), 5.88 (d, $J = 1.7$ Hz, 1H), 7.10 (s, 1H), 7.21 (s, 1H), 7.56 (s, 1H). NMR δ_{C} (CDCl_3 , 125 MHz) 16.1, 17.8, 17.9, 19.7, 20.9, 24.2, 25.8 (x2), 26.6, 27.3, 28.0, 28.4, 31.8, 32.0, 34.4, 43.2, 49.4, 50.7, 72.4, 72.6, 77.4, 83.3, 111.4, 117.1, 118.4, 119.6, 123.4, 123.8, 124.2, 130.0, 131.4, 131.6, 132.8, 138.8, 151.1, 168.2, 199.3. High resolution (HR)-ESIMS: calcd. for $\text{C}_{37}\text{H}_{50}\text{NO}_4$ $[\text{M}+\text{H}]^+$: 572.3734, found: 572.3754.

2.3 Results

2.3.1 Synthesis of indole-3-glycerol phosphate (IGP)

IGP is an intermediate of tryptophan biosynthesis (68). As mentioned above, it was suggested to be the origin of the indole skeleton to produce geranylgeranyl indole (GGI) biosynthesis. Since it was not commercially available, I first tried synthesizing it by the two steps: synthesis of CdRP (1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate) from anthranilic acid and ribose-5-phosphate (R5P) and then decarboxylation of CdRP by IGPS (indole-3-glycerol phosphate synthase) to generate IGP (Scheme 2.2).

CdRP was prepared by the previously reported (149) chemical protocols as described in 2.2.5.



Scheme 2.2 Reaction catalyzed by IGPS.

2.3.1.1 Preparation of TtIGPS

Recombinant IGPS from *Thermus thermophilus* HB27 (TtIGPS) (150) was prepared and used for the conversion of CdRP into IGP. The purity of the recombinant enzyme was checked by SDS-PAGE. His-tagged TtIGPS recombinant enzyme was successfully expressed in a soluble form. The obtained recombinant TtIGPS, which had a calculated molecular mass of 27 kDa, was subjected to SDS-PAGE analysis to confirm its

molecular size and purity (Fig. 2.3).

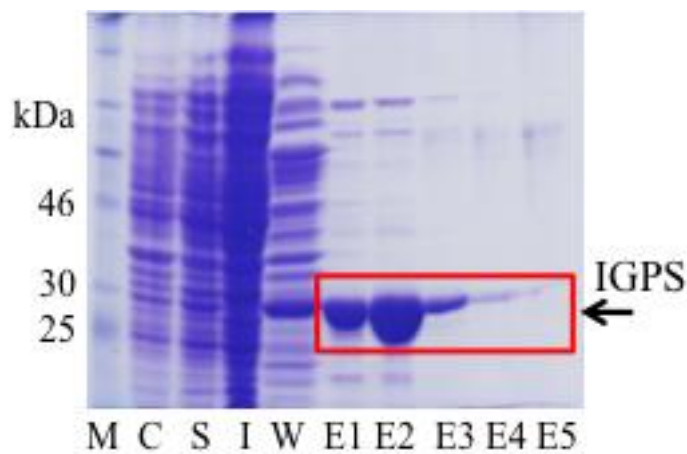


Figure 2.3 SDS-PAGE analysis of the purified TtIGPS: (M, marker; C, cell; S, soluble; I, insoluble; W, washing; E, elution fraction)

2.3.1.2 Enzyme reaction with TtIGPS and CdRP

The recombinant TtIGPS was incubated with CdRP and the product was analyzed by HPLC. One major product was specifically detected (Fig 2.4). After purification, the structure was confirmed to be IGP by NMR.

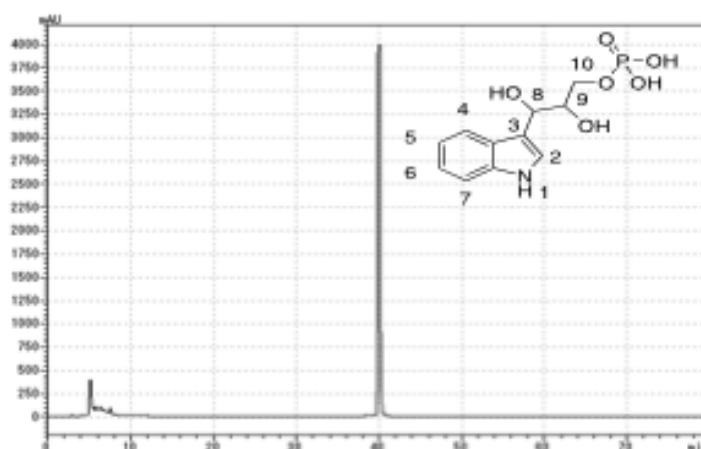
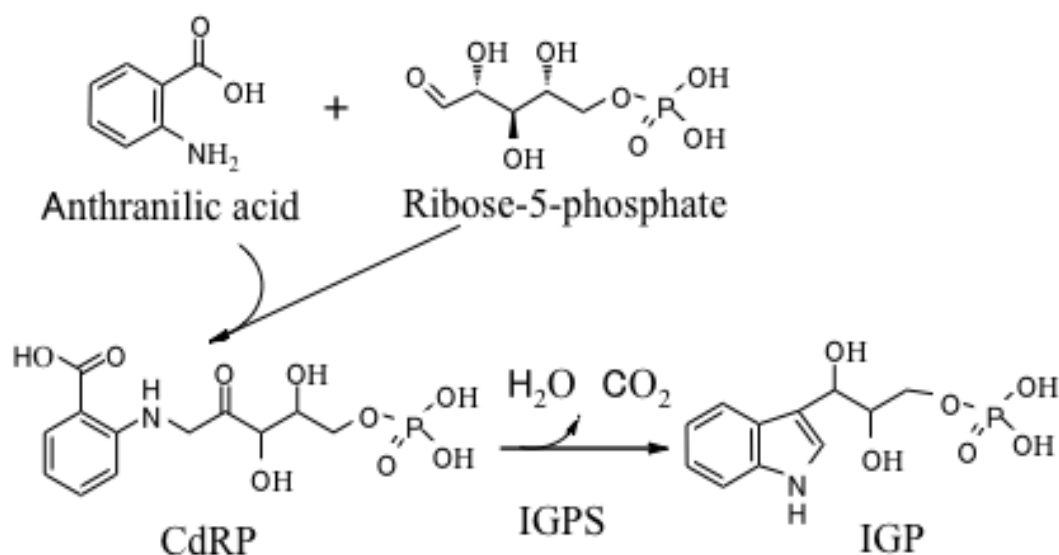


Figure 2.4 HPLC analysis of reaction product formed from CdRP by TtIGPS.

2.3.1.3 Brief summary

The TtIGPS from *Thermus thermophilus* HB27 was used for the preparation of IGP. Since the strain is extremely thermophilic with an optimal growth temperature of about 65 °C (151), the reaction was done at 65 °C for 15min. The enzyme catalyzed a ring closure reaction of the substrate to produce IGP through a sequence of condensation, decarboxylation, and dehydration (Scheme 2.3) (152).



Scheme 2.3 Chemical synthesis of CdRP and its conversion into IGP by TtIGPS.

2.3.2 Functional analysis of PaxC

2.3.2.1 Preparation of PaxC

cDNA of *paxC* was amplified by the rapid amplification of cDNA ends (RACE) method based on the nucleotide sequence of the cDNA reported by Scott et al (38).

His-tagged PaxC recombinant enzyme was successfully expressed in a soluble form. The obtained recombinant PaxC, which had a calculated molecular mass of 36 kDa, was subjected to SDS-PAGE analysis to confirm its molecular size and purity (Fig 2.5).

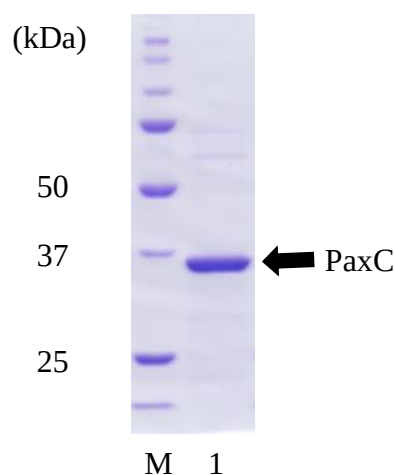


Figure 2.5 SDS-PAGE analysis of purified PaxC. Lane M: Marker, Lane 1: PaxC.

2.3.2.2 In vitro assay of PaxC with GGDP and IGP/indole/tryptophan

Recombinant PaxC was incubated with GGDP and indole, IGP, or tryptophan. By HPLC analysis, one major product was specifically detected, which eluted at the same retention time with that of standards 3-geranylgeranyl indole (GGI) when IGP and GGDP were used as the substrates. Moreover, a minor peak was also detected at the same retention time of GGI with indole and GGDP. In contrast, no GGI was detected in

the reaction with tryptophan (Fig 2.6). The results clearly showed that PaxC was a GGI synthase.

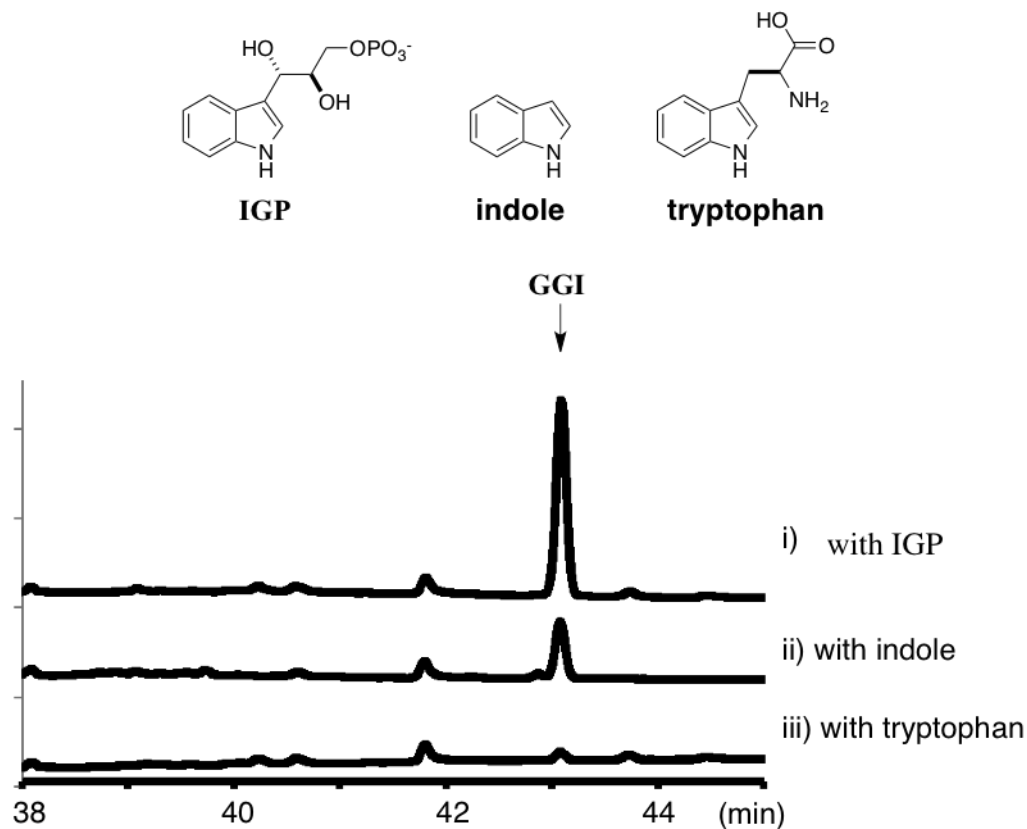


Figure 2.6 HPLC analysis of reaction products formed from IGP/indole/tryptophan with GGDP by PaxC; i) with IGP; ii) with indole; iii) with tryptophan.

2.3.2.3 In vitro assay of PaxC with FDP and IGP/indole/tryptophan

Next, I used FDP as the prenyl donator. When IGP and FDP were incubated with PaxC, one specific peak was detected, which eluted at the same retention time with that of standard farnesyl indole (FI). Moreover, a minor peak was also detected at the same retention time with indole. However, tryptophan was not utilized (Fig 2.7). The results showed that PaxC reacted on not only GGDP but also on FDP.

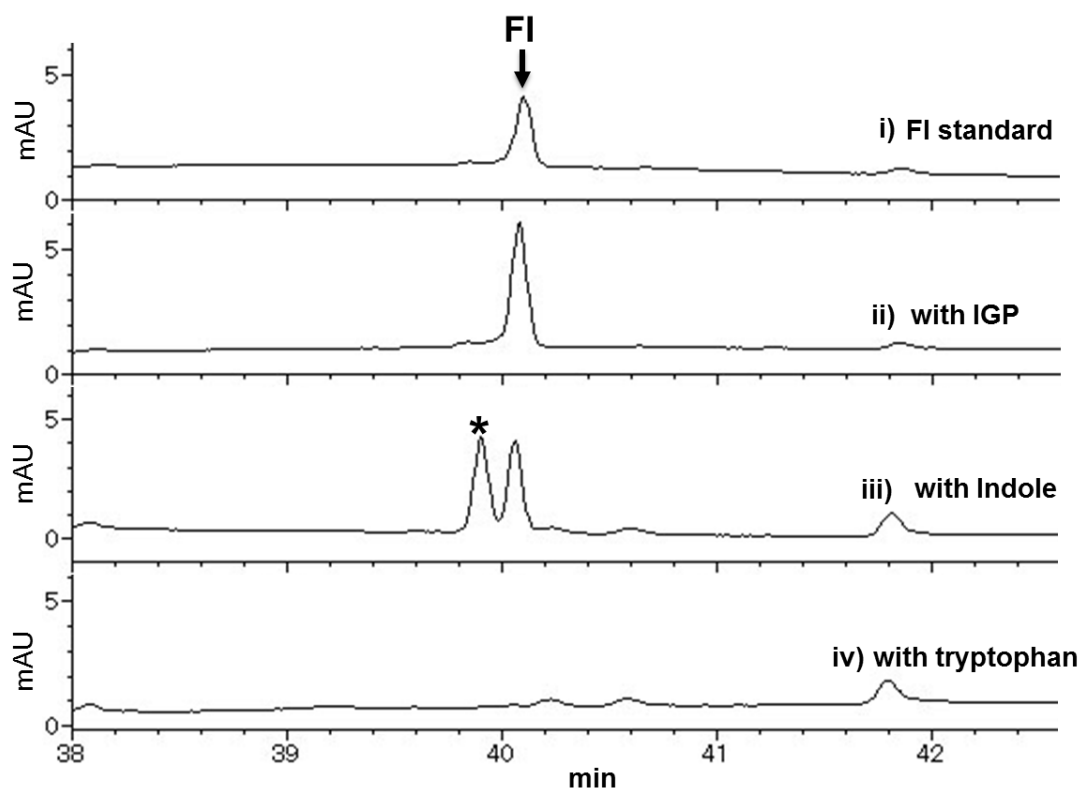


Figure 2.7 HPLC analysis of reaction products formed from IGP/indole/tryptophan with FDP by PaxC. Reaction products formed from IGP (ii), indole (iii), and tryptophan (iv) with farnesyl diphosphate as a prenyl donor by PaxC were analyzed together with standard (i). Asterisk indicates an unknown product.

3.3.2.4 Metal dependency of PaxC

Some polyprenyl diphosphate synthases have the two conserved DDxxD motifs that represent the binding sites for the allylic and the homoallylic substrate, respectively (45). Through analysis PaxC amino acid sequence, I could find one DDxxD motif (Fig. 2.8) and the effect and dependence of Mg^{2+} ions on PaxC activity were tested. Consequently, PaxC was found to require Mg^{2+} (Fig 2.9).

10 20 30 40 50 60
 MG VAGSGVLY FLFNNVPSR FWLKKTLQIG TENPEGITGY ECPYEYLRKS YGKHHWAAVF

 70 80 90 100 110 120
 DKLSPNLQNE DPAKYRMVLE TMDVIHLCLM MV **DDISD** GSE YRKGKPAAHK IYGAPETANR

 130 140 150 160 170 180
 AYYRVTQILA QTATEFPRLS PWLMTDLRDI LEGQDMSLVW RRDGVNGFPG TASERTAAYK

 190 200 210 220 230 240
 RMVLLKTGGL FRLLGHTLE NNSMDEAFST LGWHSQLQND CKNVYSSEYA KMKGVVAEDL

 250 260 270 280 290 300
 LNREMTYPIV LALDASGGHW VEAALKSPSR RNVGNALKII QCDYVRDVCM AELARSGAPV

 310 320
 KEWLKLWKRE EKLDLKA

Figure 2.8 Function of DDxxD motif in PaxD

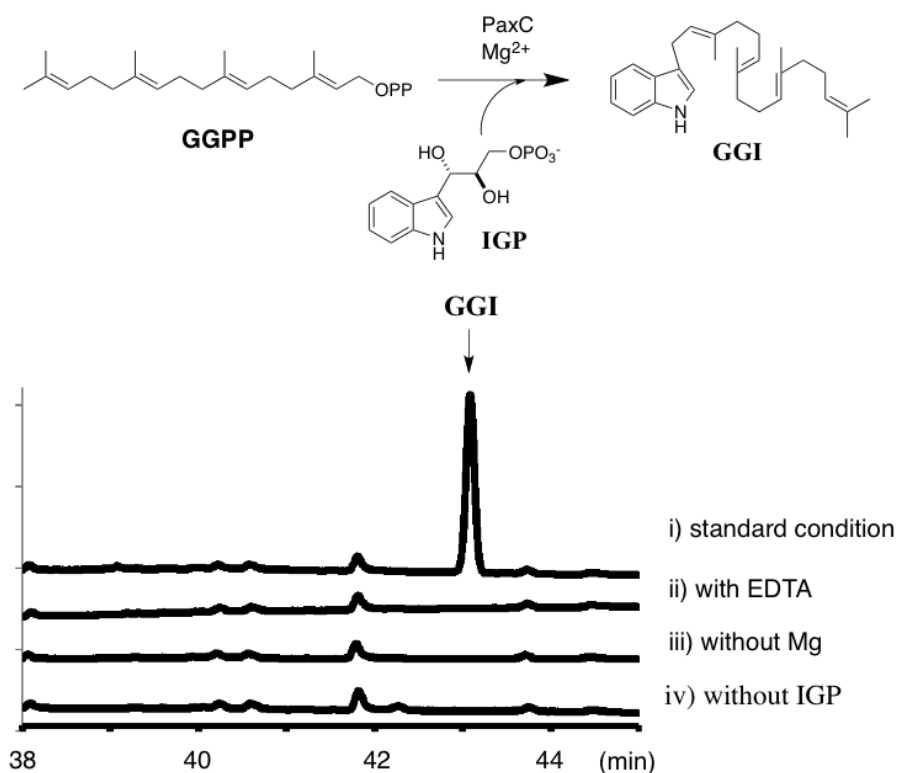


Figure 2.9 HPLC profiles of PaxC reaction to investigate an effect of Mg²⁺ ions; i) with Mg²⁺ standard conditions; ii) with EDTA; iii) without Mg²⁺; iv) without IGP.

2.3.2.5 Kinetic analysis of PaxC

Triplicate sets of enzyme assays were performed and the Hanes-Woolf plot was used for estimation of kinetic constants. The kinetic parameters suggested that IGP and GGDP are preferred substrate for PaxC (Table 2.3).

Table 2.3 Kinetic parameters of PaxC.

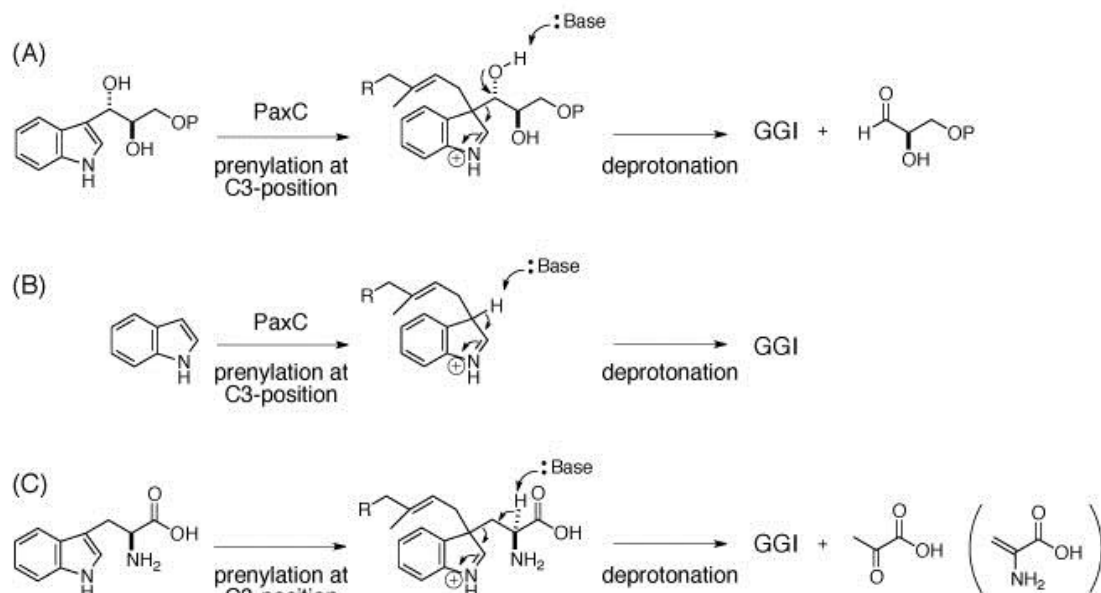
Kinetic parameters of PaxC

substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1}\text{mM}^{-1}$)
IGP	166 $\pm$ 9	4.7$\pm$0.06	28
GGDP (IGP)	17 $\pm$ 2		278
indole	606 $\pm$ 47	2.2$\pm$0.1	3.6
GGDP (indole)	11 $\pm$ 0.1		192.9

substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1}\text{mM}^{-1}$)
IGP	581 $\pm$ 26	0.69$\pm$0.02	1.2
FDP (IGP)	42 $\pm$ 3		16
indole	1208 $\pm$ 37	1.3$\pm$0.02	1.1
FDP (indole)	115 $\pm$ 9		11

2.3.2.6 LC/ESI-MS analysis of byproduct formed from IGP by PaxC.

LC-MS analysis revealed that PaxC reaction accompanied the formation of glyceraldehyde 3-phosphate (G3P) as a byproduct (Fig 2.10). The result indicated that PaxC catalyzed geranylgeranylation at C3 position of GGI followed by elimination of glyceraldehyde 3-phosphate (Scheme 2.4).



Tagami et al. *J Am Chem Soc.* 2013.**135**.1260.

Scheme 2.4 Proposed reaction mechanism of PaxC with (A) IGP, (B) indole, and (C) tryptophan.

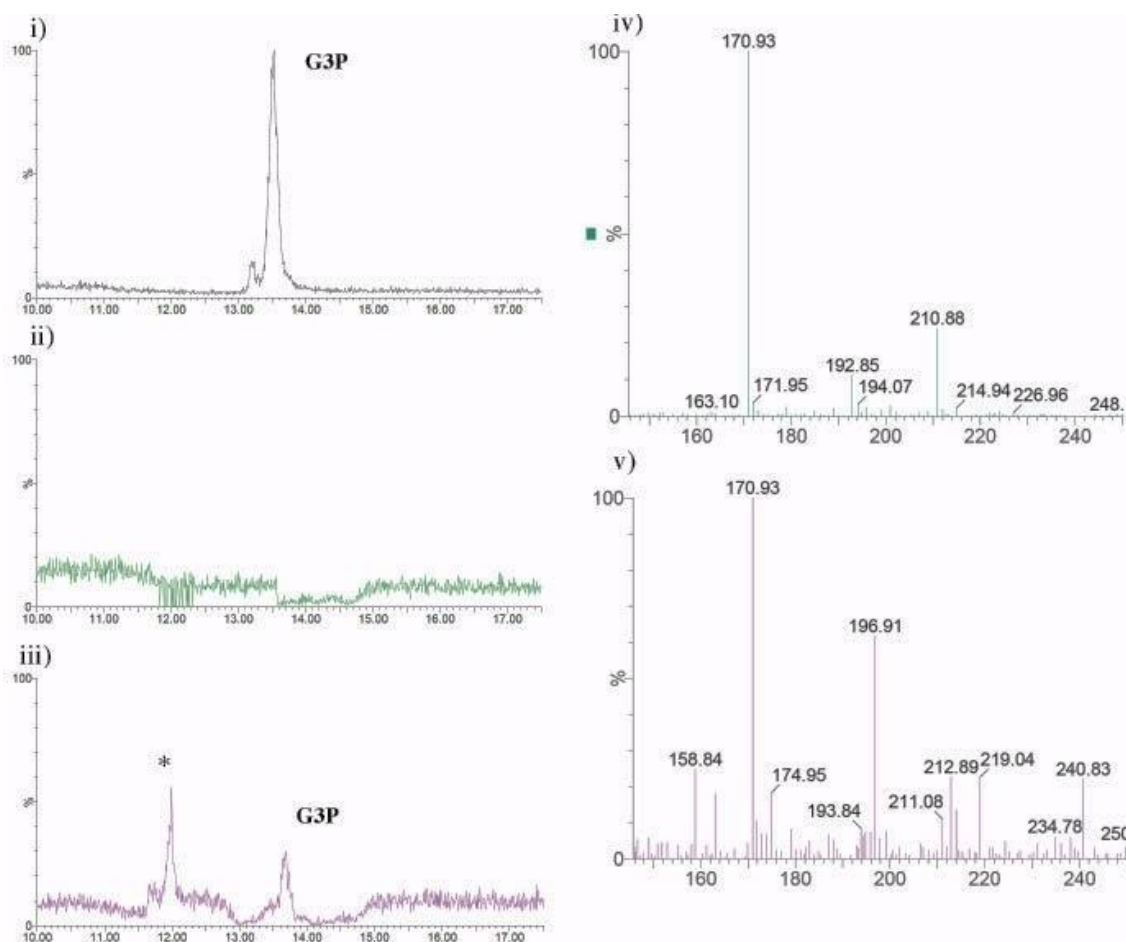


Figure 2.10 LC-MS profiles of PaxC reaction product {Selected ion of m/z 171 $[M+H]^+$ was used to detect the glyceraldehyde 3-phosphate (G3P)}: i) authentic G3P, ii) reaction product without PaxC, iii) reaction product in standard conditions (IGP, GGPP, Mg^{2+}). MS spectra of : iv) authentic G3P, and v) PaxC reaction product. * impurity

2.3.3 Functional analysis of PaxD

2.3.3.1 Preparation of PaxD

cDNA of *paxD* was amplified by the rapid amplification of cDNA ends (RACE) method based on the nucleotide sequence of the cDNA reported by Scott et al (38). (GenBank; AAK11526). The predicted gene product consisted of 438 amino acids as previously reported. In a BLAST search, the most homologous enzyme to PaxD was the *atmD* product (32% amino acid identity Fig 2.11), which is located in the aflatrem biosynthetic gene cluster in *Aspergillus flavus* although its function remains unclear (32).

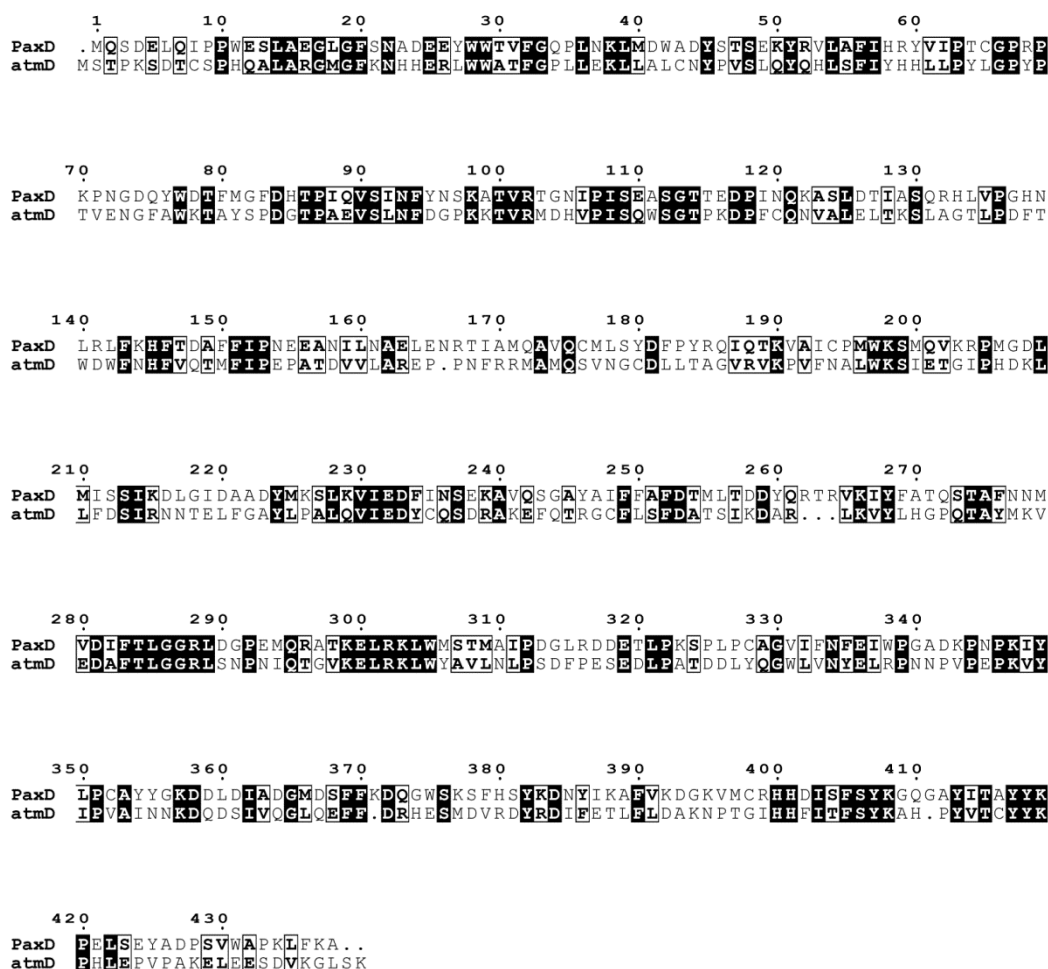


Figure 2.11 Alignment of amino acid sequences of PaxD (GenBank; AAK11526) and AtmD (GenBank; CAP53937) constructed by CLUSTALW.

His-tagged PaxD recombinant enzyme was successfully expressed in a soluble form. The obtained recombinant PaxD, which had a calculated molecular mass of 47 kDa, was subjected to SDS-PAGE analysis to confirm its molecular size and purity (Fig 2.12a). The recombinant enzyme was then subjected to gel filtration chromatography to estimate its subunit structure (Fig 2.12b). The result suggested that PaxD forms a homo-dimer structure.

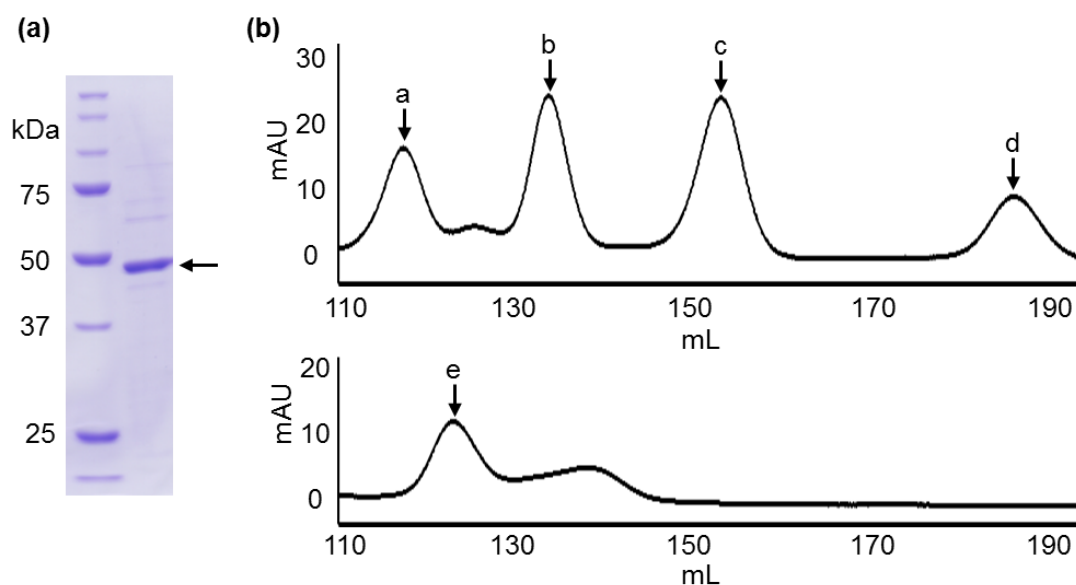


Figure 2.12 SDS-PAGE and gel filtration chromatography of purified PaxD. (a) molecular mass markers (lane 1) and purified PaxD (lane 2). (b) elution profiles of the standard proteins [aldolase (a, 158 kDa), albumin (b, 67 kDa), ovalbumin (c, 43 kDa) and chymotrypsinogen A (d, 25 kDa); top] and purified PaxD (bottom, e)

2.3.3.2 In vitro assay of PaxD

Since *paxD* is located in the *pax* gene cluster, I thought that PaxD might catalyze a prenylation of paxilline. The purified recombinant PaxD was therefore incubated with paxilline and DMAPP. By HPLC analysis, one major product (Fig 2.13 peak B) and a minor product (Fig 2.13 peak A) were specifically detected.

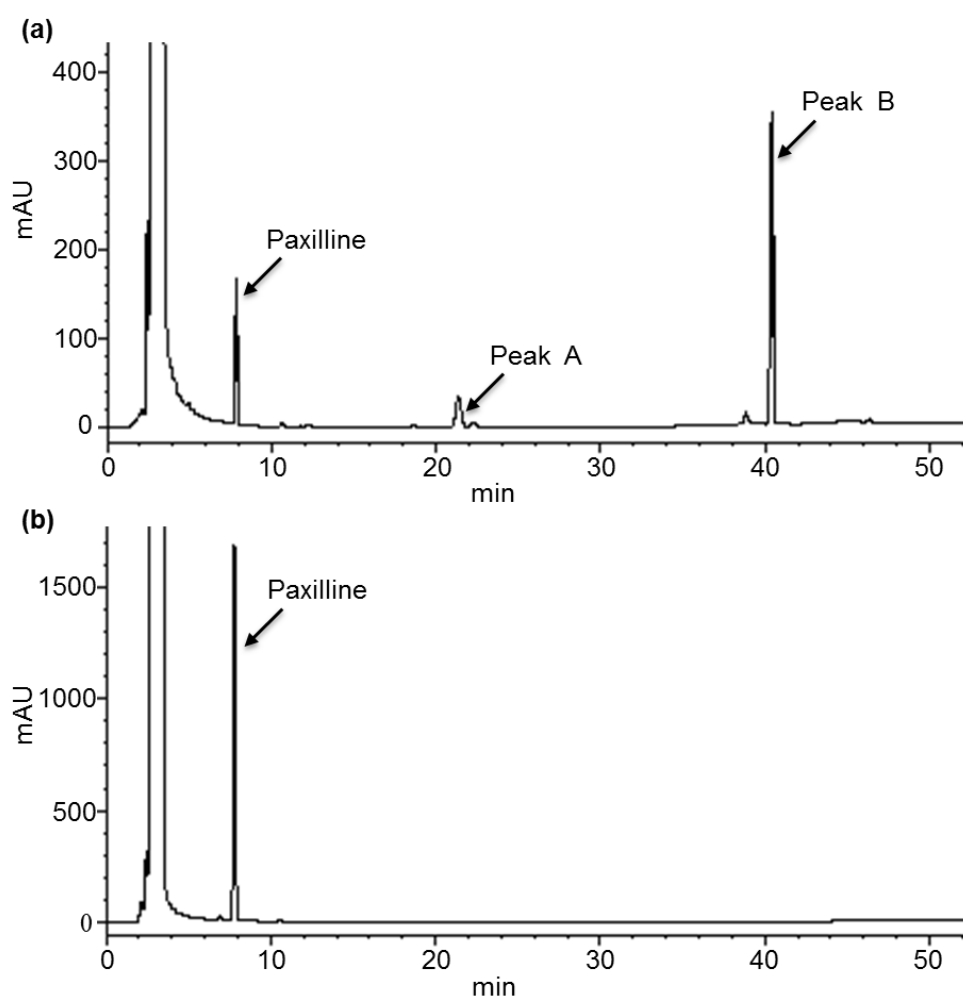


Figure 2.13 HPLC analysis of the reaction products formed from paxilline and DMAPP with PaxD. The reaction products formed with (a) and without (b) PaxD.

2.3.3.3 LC/ESI-MS analysis of reaction product formed from paxilline and DMAPP by PaxD

Total ion chromatograms obtained by LC/ESI-MS analysis showed two specific peaks (Peak A and B) with molecular masses corresponding to mono-prenylated and di-prenylated paxilline (Fig 2.14).

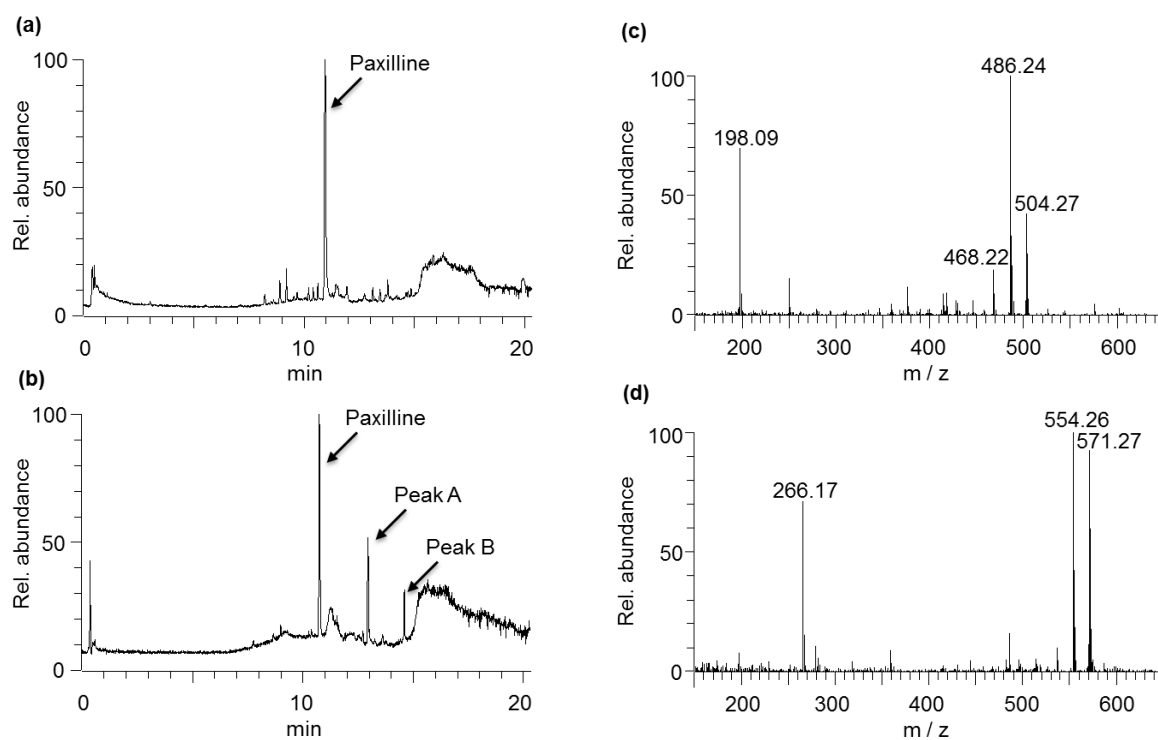


Figure 2.14 Total ion chromatogram of products formed by in vitro assay without (a) and with (b) PaxD and mass spectra of peak A (c) and peak B (d) are shown.

Moreover, selected ion chromatograms and their mass spectra strongly suggested that one product was mono-prenylated paxilline and the other one was di-prenylated paxilline (Fig 2.15 a-d).

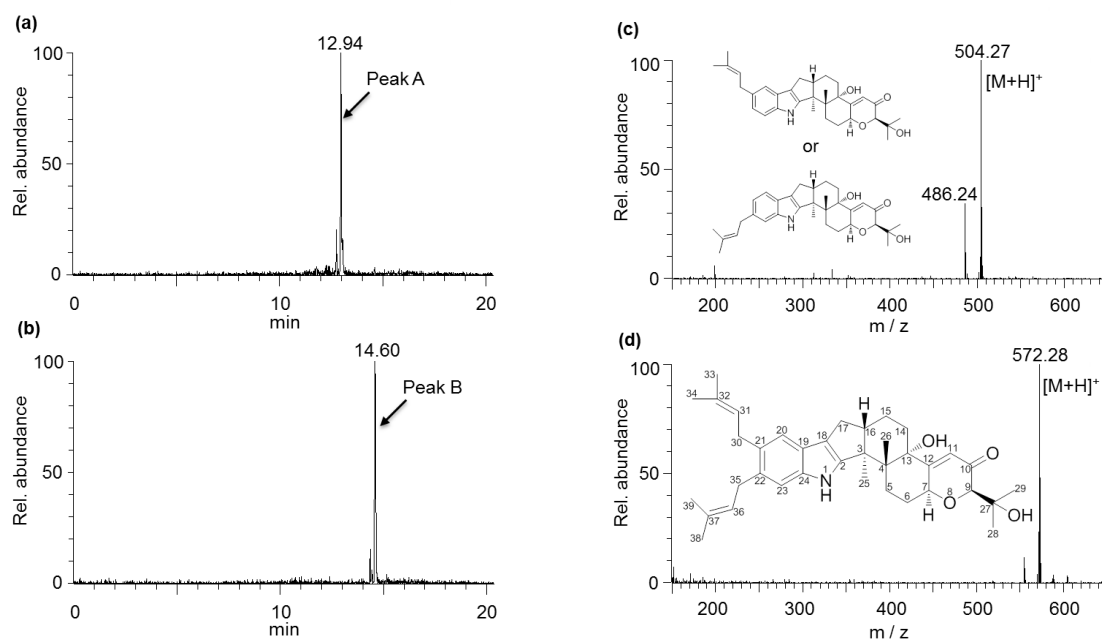
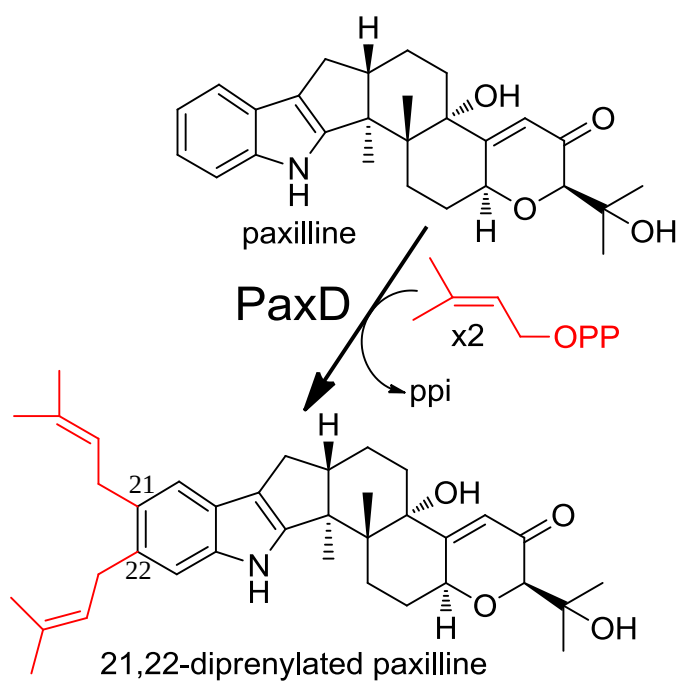


Figure 2.15 LC/ESI-MS analysis of the prenylated paxilline formed by PaxD reaction; (a, b) Selected ion chromatograms; (c, d) spectra; (a, c) for mono-prenylated paxilline and (b, d) di-prenylated paxilline. Structures of 21,22-diprenylated paxillin and 21- or 22-prenylated paxilline are also shown.

2.3.3.4 NMR analyzed of reaction product formed from paxilline and DMAPP by PaxD

Since the yield of the minor product was low, I determined the exact structure of the major product. HR-ESIMS of the major product (peak B) indicated the molecular formula $C_{37}H_{49}NO_4$, supporting production of di-prenylated paxilline. ^1H -NMR spectra showed new signals for regular prenyl moieties at $\delta = 5.30$ (t, $J = 6.9$ Hz, 1H), $\delta = 5.29$ (t, $J = 6.9$ Hz, 1H), and $\delta = 3.39$ (d, $J = 6.9$ Hz, 4H) concomitant with two singlet signals ($\delta = 7.21$ and $\delta = 7.10$) without *o*- and *m*-coupling, indicating the substitution of the prenyl moieties at C-21 and C-22 positions on the indole moiety. Finally, extensive NMR data analysis, including ^1H -NMR, ^{13}C -NMR, hetero-nuclear single quantum coherence (HSQC), hetero-nuclear multiple quantum coherence (HMBC), correlation spectroscopy (COSY), and nuclear overhauser effect spectroscopy (NOESY) proved the structure as 21,22-diprenylated paxilline.

Since fungal dimethylallyl tryptophan synthases usually catalyze the formation of mono-prenylated compounds as the main product, and di-prenylated compounds were reported to be produced as minor products by a few enzymes such as AnaPT and 7-DMATS (153), the reaction catalyzed by PaxD is unique (Scheme 2.5).



Scheme 2.5 Reaction catalyzed by PaxD with paxilline and DMAPP

2.3.3.5 Prenylated paxillines in culture broth of *P. paxilli*

Since PaxD catalyzed the prenylation of paxilline *in vitro*, I examined whether PaxD functioned *in vivo* by searching for mono-prenylated paxilline and 21,22-diprenylated paxilline in culture broth of the paxilline producer.

The strain *P. paxilli* was cultivated under the standard condition. The supernatant of the culture broth obtained by centrifugation was extracted with ethyl acetate and evaporated *in vacuo* (154). The sample thus obtained was analyzed by HPLC. The *in vitro* reaction product was also analyzed as a control. In the sample of culture broth, one specific peak (40.5min) was detected, which eluted at the same retention time with that of standard 21,22-diprenylated paxilline (Fig 2.16).

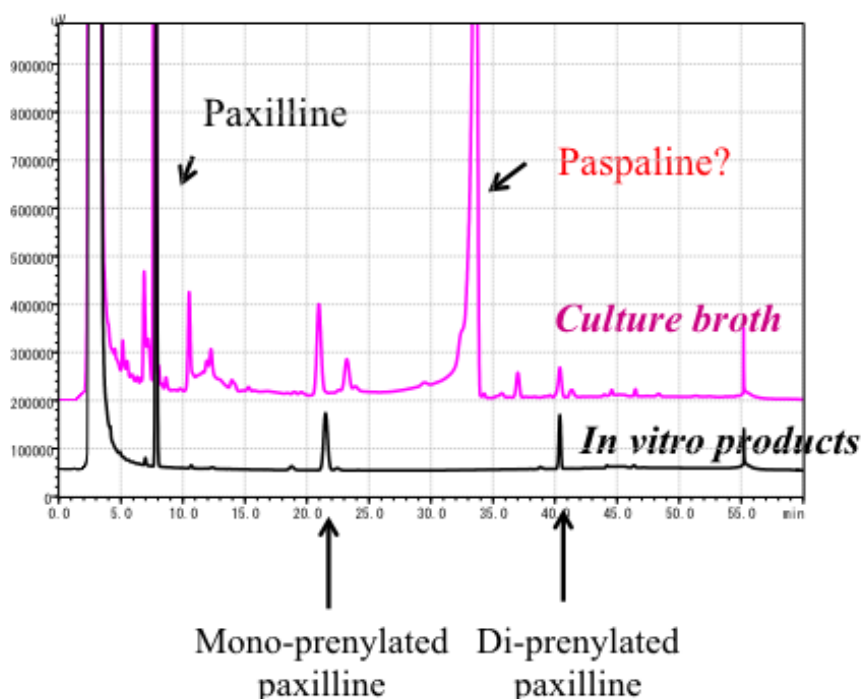


Figure 2.16 HPLC analysis of prenylated paxilline accumulated in culture broth of *P. paxilli*.

The sample was also analyzed by LC/ESI-MS. The selected ion chromatogram, with peak a molecular mass of 504.27 and 572.32, which corresponded to $[M+H]^+$ of mono-prenylated paxilline and di-prenylated paxilline, were detected at the same retention time (Peak C/D) as that of the product obtained by the in vitro assay (Fig. 2.17).

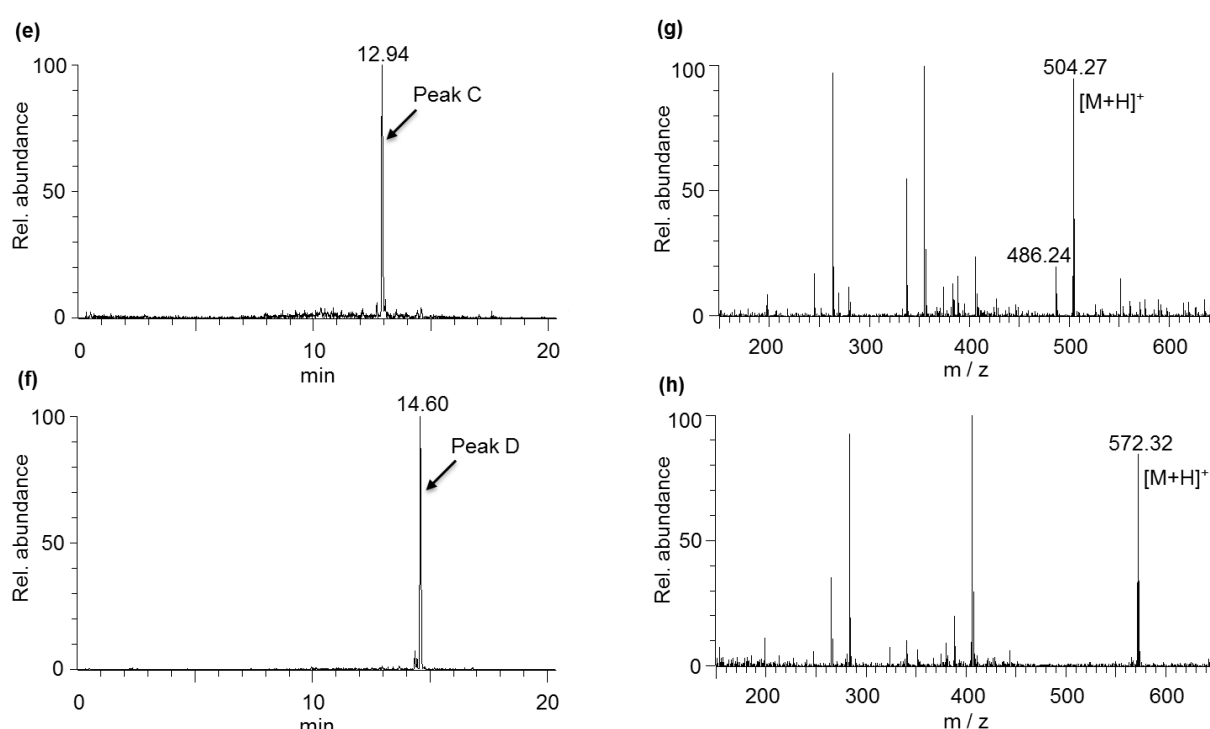


Figure 2.17 LC/ESI-MS analysis of the prenylated paxilline accumulated in culture broth of *P. paxilli* (e-h): Selected ion chromatograms (e, f), and mass spectra (g, h); for mono-prenylated paxilline (e, g), and di-prenylated paxilline (f, h) in samples from culture broth of *P. paxilli*.

2.3.3.6 Fragmentation analysis of product by LC/ESI-MS

The product's fragmentations were also analyzed (Fig. 2.18). A specific fragment spectrum of 198.09 was detected, which had been observed as a fragment of paspalitrem

A by LC/ESI-MS analysis. Since the structures of the A to E rings of paspalitrem A and 21-prenylated paxilline are the same (155), this spectrum (198.09) was suggested to result from the same fragmentation as that of paspalitrem (Fig 2.18e). For di-prenylated paxilline, a peak with the molecular mass of 21,22-prenylated paxilline (572.32) was detected in the same manner as that of the in vitro sample. A fragment spectrum of 266.20, which probably resulted from the same fragmentation pattern as that of mono-prenylated paxilline, was also observed (Fig 2.18f). These results suggested that both the mono- and di-prenylated paxillines existed in culture broth of *P. paxilli* and that the final fermentation product governed by the *pax* gene cluster in *P. paxilli* might be the prenylated paxillines rather than paxilline, though their productivities were quite low.

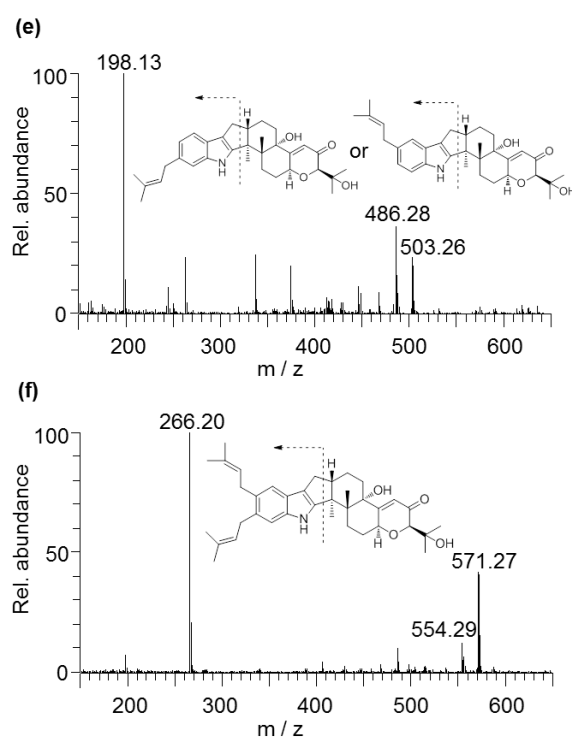


Figure 2.18 Fragmentation analysis of products by LC/MS: Mass spectra fragmentations obtained from selected ion chromatograms of products accumulated in culture broth are also shown (**e** and **f**) together with probable fragment principles.

2.3.3.7 Biochemical characterization of PaxD

2.3.3.7.1 Substrate specificity

The substrate specificity of the PaxD enzyme was investigated. For the prenyl acceptor, compounds related to indole diterpene biosynthesis were used. I also examined several cyclo-dipeptides and hydroxynaphthalenes as substrates including benzyl D-glucopyranoside, phenyl α -D-glucopyranoside, β -naphthyl α -D-glucopyranoside, naringenin-7-glucoside, polydatin, cyclodipeptides (cyclo-L-Trp-L-Tyr, cyclo-L-Pro-L-Tyr, cyclo-L-His-L-Phe, cyclo-L-Phe-L-Pro, cyclo-L-Phe-L-Trp, cyclo-L-Phe-L-Leu, all of which were kindly provided by Dr. H. Kanzaki of Okayama University, Japan), hydroxynaphthalenes (1-naphthol, 1,3-dihydroxynaphthalene, 2,6-dihydroxynaphthalene, 2,7-dihydroxynaphthalene, 3,7-dihydroxy-2-naphtholic acid), indole, L-tryptophan and L-tyrosine because these compounds were reported to be utilized by many fungal prenyltransferases (88). However, no products were formed. I next examined the substrate specificity of the prenyl donors. Aside from DMAPP, geranyl diphosphate, farnesyl diphosphate, and GGDP were examined. However, no products were formed with paxilline as the prenyl acceptor.

PaxD did not react with cyclodipeptides and other compounds examined. The results are in good agreement with the low amino acid sequence similarities between PaxD and fungal prenyltransferases that accept cyclo-dipeptides and hydroxynaphthalenes (17–22% identities, Fig 2.19).

(a)

```

1          10          20          30          40
PaxD      ....MQSDEL.....QIPPWESLAEGLGFSNADEEYWTVFGQPTLNKLM
CdpC3PT   ....MTVSS...TAVEASAPCAEMG.....HDIPYRTSRSMIFANLDQYQYWHQIGPVLKML
CdpNPT    .MDGEMTASPPDISACDTSAYDEQGTGSGSQSQAIPKDIAYHTKALLFPPDIDQYQYWHHVPAMLAKML
CtrpPT    ....MTTYTSL.KGSDRPQTSQD...GQPYDVLSKYLRFPDFDQDQWQNTAPMLLKLL
AnaPT     MSPLSMQTDSVQGTAKNSLETNGTSDND.....QQLPWKVLSKSLGLPTIEQEQYVNLNTAPYFNLL
FgaPT2    .....MKAANAS.....SAEAYRVLSRAFRFDNEDQKLWWHSTAPMFAKML
FtmPT1    .....MPPAPPDQKPCQHL.....QPAPYRALSESLEFGSDEERWWHSTAPILSRLL

50          60          70          80          90          100
PaxD      DWADYSTSEKVRVLAFTHRYVIFTCGPRPKPNGDQ....YWDTFMGFDHTPTQVSNIFYNSKATVRTGN
CdpC3PT   VDGEYSIHRQVEYLLSLFAHLLIIPKIGFPFSPGRD....IYRCLLGNIGGPFELSONFORLGSTARLAF
CdpNPT    VDGEYSIHRQVEYLLSLFAHLLIIPKIGFPFSPGRD....VYRCTLGGN.MTVELSONFORSGSTTRIAF
CtrpPT    SQCKYNNVHQYQYHLCYGLHVIPFGPWPDIQRSK....LYKSVLSGLSELFSONFTKTKTVRMGF
AnaPT     IQCGYDVHQYQYLAFYHRRHVLPVIGPFIRSSAEA....NYISGFSAEGYPMELSVNYQASKATVRLGC
FgaPT2    ETANYTTPCQYQYLITYKECVIPPSIGCYPTNS....APRWLSILTRYGTPELSNLCSN..SIVRYTF
FtmPT1    ISSNYDVQYQYVLSYLRHLVLPALGYPQRPDPETGIIATQWRSGMVLTGLPIEFSNNVAR..ALIGTV

110         120         130         140         150         160         170
PaxD      IPTSEASGTTEDPINQKASLDTASORHLVPGHNLRLKFKHFTDAFFTPNEEANILNAELENRTIAMQAV
CdpC3PT   EPTSYLASTSADPFNRHAVHATLAELRMTGSATVLELHHALAADLTLTDR....NEQLLTAELAKTNW
CdpNPT    EPWRYQASVGHDRFNRTSVNAFFSQLQLLVKSVNIELHLLSEHLTLTAKDERNLNEQLTKYLLTNFQV
CtrpPT    EPTTFIASTSRDVCNRHCLGKVLNRPKOLDIKLDLQLYHQLVNEVSLTDQEE....NTLLERAILLDDEPA
AnaPT     EPVGEFACTSDPFMNQFMTREVLGRISRLDPTFDLRLFDFDSQFSLTTS....EANLAASKLTKQR
FgaPT2    EPQNQHTGTDKDPFNTHAIWESLQHLLPLEKSIDLEWFRHFKHDLTLNSEEES....AFLAHNDRLVGGTI
FtmPT1    DVTADSGTACDPFNTRPKVYLETAARLLPGVDLTREFETELVITKAE....AVLQANPDLFRSPW

180         190         200         210         220         230
PaxD      QCMLSYDFPYR..QIQTKVAICPMWKSMQVKRPMDGLMISSIKDLG..IDAADYMKSLKVIEDFINSEK
CdpC3PT   KSQILLALDLN..KTGITVKEYFPALKAAATGHSVAELCFSAIRKVD..VQGRLAAPCKAIEAHMQR..Q
CdpNPT    KTQYVVALDLR..KTGIVAKEYFFPGKCAATGQTSGNACFGAIRAVD....KDGHLDSLCQLIEAHQ..Q
CtrpPT    KSQSLLALDFN..KDDVTVKLYLPQLKSLATGIPRTOLMFSAVRNVD....KTRAFSESMNMIEEYFTSVR
AnaPT     RQSKVIAFDLKDGAIIPKAYFFLKGKSLASGIPVQDVAFNAIESIAPKQIESPLRVLRTFTKLFSKPT
FgaPT2    RTQNKLALDLK..DGRFALKTYIYPALKAVVTGKTIHELVFGSVRRLAV..REPRILPLNMIEEYIRSRG
FtmPT1    KSQILLALMDLQSGSTVLVKAYFYPQKSAVTGRSTEDLVNAIRKV..REGRFETQLANLQRYIERRR

240         250         260         270         280
PaxD      AVCSGAY....AIFFAFDMLTDDYQRTRVKIYFATQSTAFNNMVDIFTLGGR
CdpC3PT   TQTDDIH....FLSVDLVEPG...TTRFKLYLMELEVTLAKLEEHWTGGT
CdpNPT    SKIDDA....FLCCDLVDPA...HTRFKVYIADPLVTLARAEHHWTGGT
CtrpPT    ANAAPY....WISCDLVEPN...KTRFKYIALFQVNFENAVSMWTGNR
AnaPT     VTSDVF....ILAVDCIVPE...KSRIKLYVADSQLSLATLREFWTLGGS
FgaPT2    SKSTAS....PRLVSCDLTSPA...KSRIKLYLLEQMVSLEAMEDLWTGGT
FtmPT1    RGLHVPGVTADKPPATAADKAFDACSFFPHLSTDLVEPG...KSRVKEYASERHVNLQQMEDIWTGGT

290         300         310         320         330         340         350
PaxD      LDGPEMRQATKELLRKLMTMAIPDGLRDDETLPKSPLP....CAGVIFNFETWPGA..DKFNPKIYLPC
CdpC3PT   LTDKETRGLQMIRELWV.DLEIVDGKRAEQRPSLPG..DPLSIVPPFMNYEITPGQ..PLPKPFYFP
CdpNPT    LTDEDAAVGLEIRGLWS..ELGIIQ..PLEPSAMMEKG....LLPIMLNYETMPGA..RLPKPLYMP
CtrpPT    ITDPETRGLAVRDLWN..SFDIQDGLREQNNRPGNPG..DPSNIVPMLFNLELLPGR..AYDQPKYFP
AnaPT     VTDSATMKGLEIAEELWR.ILQYDDAVCSHSN....MDQLPLVVNYELSSGS..ATPQPLYLP
FgaPT2    RRDASTLEGLSLVRELWD.LIQLSPGLKSYPAPYLPLG.VIPDERLPLMANFTLHQND..PVPQQYFT
FtmPT1    RRDPDALRGLELRHFWA..DLQMREGYYTMPRRGFCELGKSSAGFEAPMMFHELDGSQSPFPQQYVC

360         370         380         390         400         410
PaxD      AYYGKDDLDIADGMDSEFKDQGWSKSFHSYKDNYIKAFVKD..GKVMCRHHDISFSSYRG..QGAYITAYYK
CdpC3PT   LLCIPELKIANVLASEFERHGMHDLARVYPENLQSYYPGDLAIATDRQAWLSISYTEEKGBYLTMYYH
CdpNPT    LLCIPETKIARIMTASEFORHDMPECAEVFMENLOAYYEGKNLEEATRYQAWLSFAYTKEKGBYYSIYYF
CtrpPT    TTGMNDLDVAKVMVEEFKRHGLHEHAQSYIDNLASYVPHMDLNDCTDLQAWISFSYSDATGEYITYYH
AnaPT     LHGRNDEAMANALTKSEWDYLGWKGLAAQYKKDLYANNPCRNLAETTTVQRWVAFSVTESGGAYLTVYFH
FgaPT2    TTGMNDMAVADALTTFEFERRGWSEMARTYETTLKSYYPHADHDKNLYLHAYISFSYRD..RTBYLSVYLQ
FtmPT1    VFGMNSRKLVEGLLTTEYYRRVGWEEMASHYQANFIANYPDEDFEKAAHLCAYVSFAYKN..GGAYVTYNH

420         430
PaxD      PELSEYADPSVWAPKLFKA.....
CdpC3PT   .....
CdpNPT    WPE.....
CtrpPT    .....
AnaPT     AVGGMKGNL.....
FgaPT2    SFTGDWAVANLSESKVKCQDAACQPTALPPDLSTKGVYYSGLH
FtmPT1    SFNP....VGDVSFNP.....

```

(b) Sequence identities / %

Enzyme	FtmPT1	FgaPT2	AnaPT	CtrpPT	CdpNPT	CdpC3PT	PaxD
PaxD	18	19	18	22	17	19	100
CdpC3PT	28	29	29	40	48	100	
CdpNPT	23	25	26	33	100		
CtrpPT	26	29	29	100			
AnaPT	23	24	100				
FgaPT2	29	100					
FtmPT1	100						

Figure 2.19 Alignment of amino acid sequences of PaxD and fungal prenyl transferases.

2.3.3.7.2 Temperature effect

The temperature dependency of the enzyme activity was investigated using paxilline and DMAPP as the substrates.

The optimal temperature was 40°C (Fig 2.20)

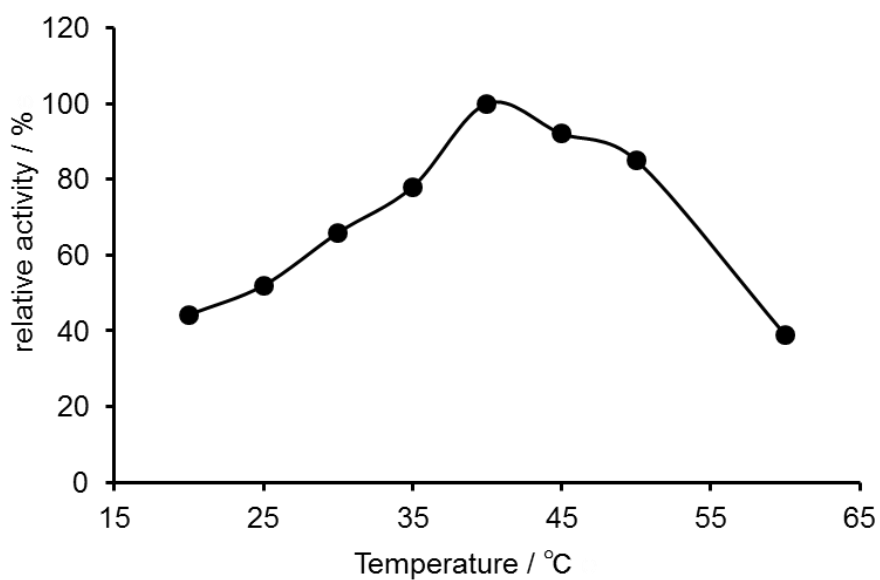


Figure 2.20 Effects of temperature on PaxD activity. The assay mixture contained, in a final volume of 100 μ L, 0.25 mM of paxilline, 0.5 mM of DMAPP, 50 mM Tris-HCl (pH 8.0), and 0.2 μ g of PaxD. This mixture was incubated at each temperature for 20 min.

2.3.3.7.3 pH-rate effect

The pH effect on the enzyme activity was investigated using paxilline and DMAPP as the substrates at pH range from 5.0 to 10, employing a mixed buffer system with Citrate buffer (pH 5.0~6.0), MES buffer (pH 6.0~7.0), Tris-HCl buffer (pH 7.0~9.0) and borate-NaOH buffer (pH 9.0~10.0).

The optimal pH was 8.0 (Fig 2.21)

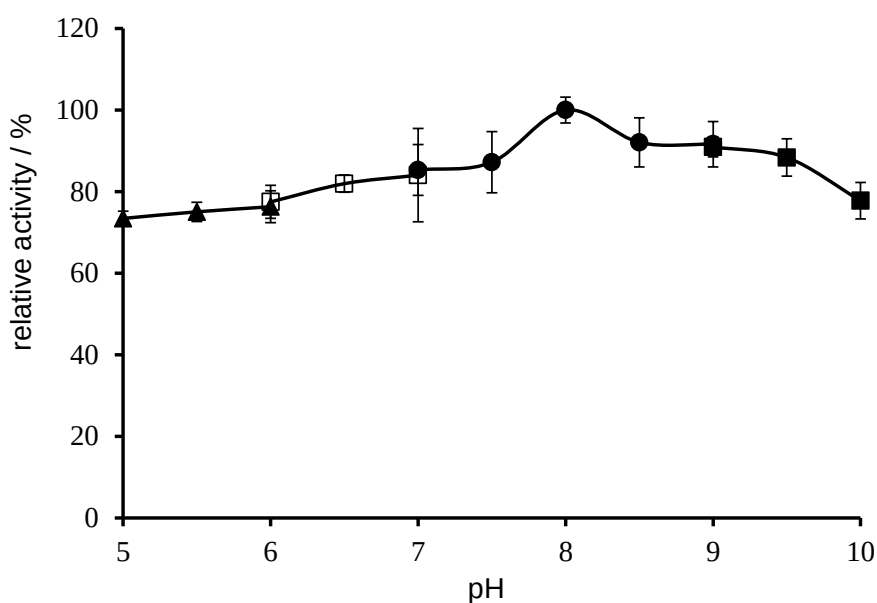


Figure 2.21 Effects of pH on PaxD activity. The assay was conducted at a pH range from 5.0 to 10, with 50 mM citrate (solid triangles), 50 mM MES (open squares), 50 mM Tris-HCl (solid circles), and 50 mM borate-NaOH buffer (solid squares). The assay mixture and condition were the same as those described. This mixture was incubated at 30°C for 20 min.

2.3.3.7.4 Metal dependency

Although PaxD lacked a (N/D)DXXD motif, which is known to be required for binding of divalent cations (156, 157), some prokaryotic prenyltransferases without the motif require divalent cations for activity (90, 91). Therefore, the effects and dependence of divalent metal ions on the PaxD activity were tested at a concentration of 5 mM (Fig 2.22). The enzyme showed similar activity regardless of the presence of 5 mM EDTA, suggesting that it did not require Mg^{2+} for its activity. In contrast, the PaxD activity was decreased to less than 50% of the control experiment (without divalent cations) by the addition of Co^{2+} , Cu^{2+} , and Zn^{2+} .

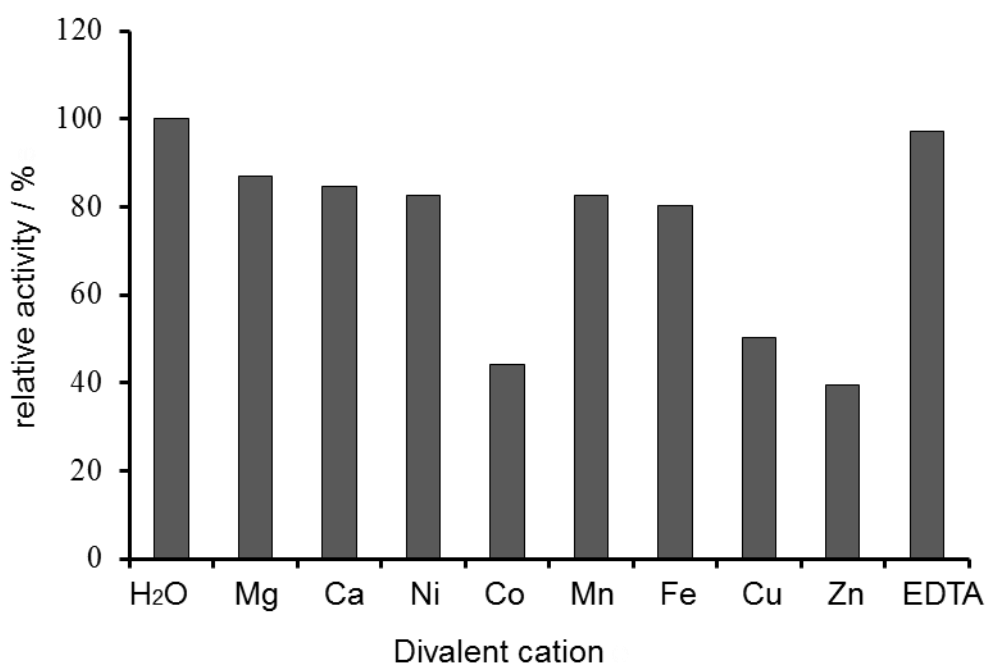


Figure 2.22 Effects of metal ions on PaxD activity. Each of divalent cations (5 mM), or 5 mM EDTA was added to the reaction mixture. The mixture was incubated at 30°C for 20 min.

2.3.3.7.5 Kinetic parameters

The kinetic parameters of PaxD were investigated. The enzyme reaction followed Michaelis-Menten kinetics. By using Hanes-Woolf plots, the K_m values were calculated as $106.4 \pm 5.4 \mu\text{M}$ for paxilline, a plausible substrate for PaxD, and $0.57 \pm 0.02 \mu\text{M}$ for DMAPP. The k_{cat} values were calculated as $0.97 \pm 0.01/\text{sec}$ (Fig 2.23).

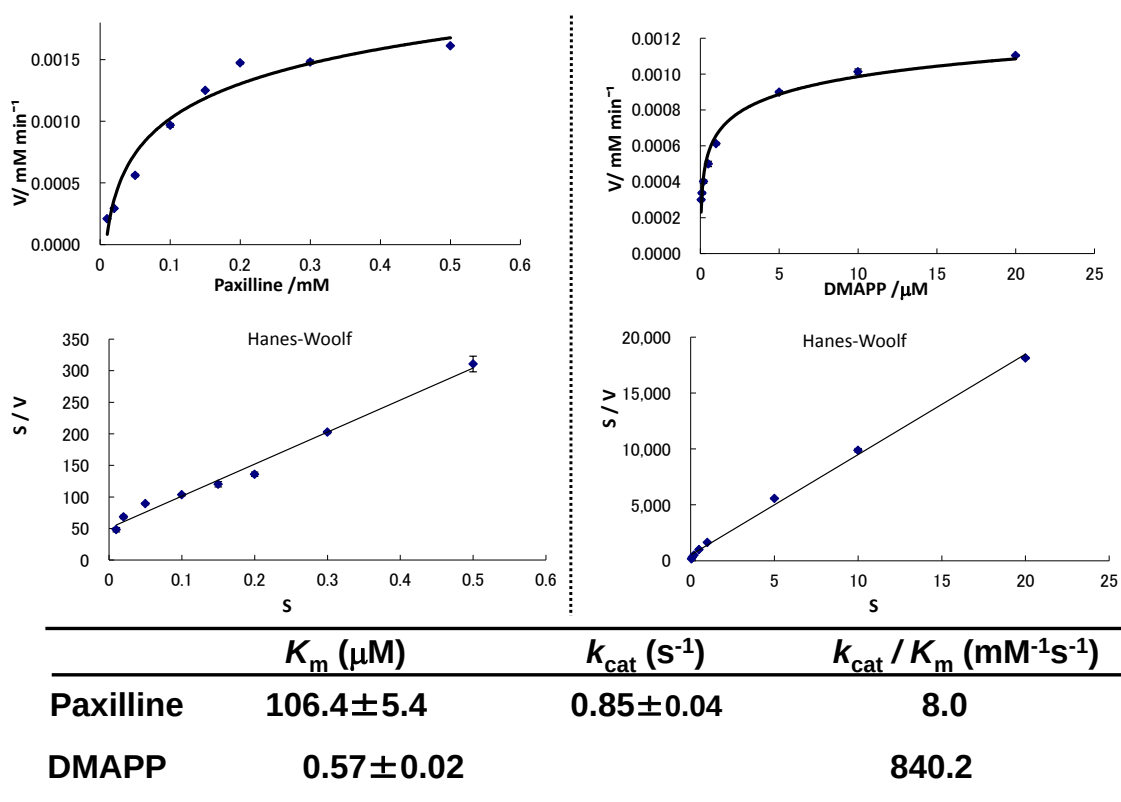


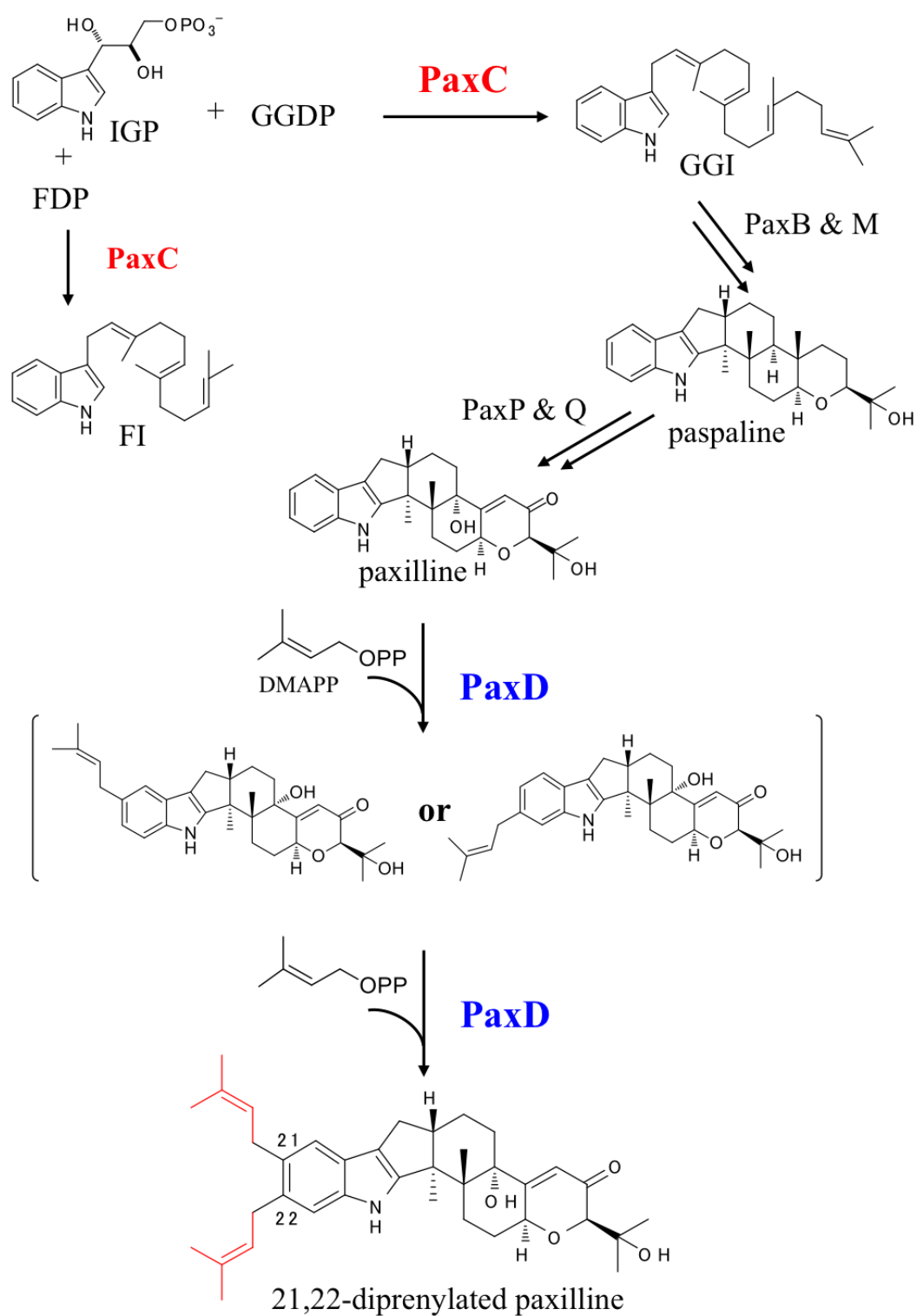
Figure 2.23 Hanes-Woolf plots for calculation of K_m and k_{cat} values of PaxD

2.4 Discussion

In this study, I confirmed that PaxC catalyzed the transfer of GGDP into IGP/indole for the first time to synthesize GGI. Based on the calculated k_{cat}/K_m value of PaxC, the true substrate was suggested to be IGP. I also demonstrated that the *paxD* encoding a prenyltransferase catalyzed a successive attachment of DMAPP to positions 21 and 22 of paxilline (Scheme 2.6). Since cDNA of the *paxD* gene was efficiently obtained and the prenylated paxillines existed in culture broth of *P. paxilli*, *paxD* gene was functionally expressed in vivo, showing that *paxD* is a member of the *pax* gene cluster. However, the estimated amounts of the prenylated paxillines were quite low when compared to paxilline. This would explain why there were no reports of the prenylated paxillines after the discovery of paxilline. The calculated k_{cat}/K_m value of PaxD for paxilline was $9.2 \text{ S}^{-1} \text{ mM}^{-1}$. This value is slightly lower than that of PaxC ($28.2 \text{ S}^{-1} \text{ mM}^{-1}$ for indole-3-glycerol phosphate). The low catalytic activity of PaxD might be one of the reasons for the accumulation of paxilline in culture broth of *P. paxilli*.

In the in vitro assay with PaxD, two products (Fig 2.13, peaks A and B) were detected with paxilline and DMAPP as the substrates. I could not determine the structure of the minor compound because of the low yield. However, considering that the products formed by PaxD were 21,22-diprenylated paxilline and mono-prenylated paxilline and that the fragment spectrum (198.09), which had been shown to result from

fragmentation in the C ring of paspalitrem A (155), was also detected by LC/ESI-MS analysis (Fig 2.18), the mono-prenylated compound was suggested to be either 21-prenylated paxilline or 22-prenylated paxilline. In that case, PaxD catalyzes a step-wise di-prenylation via a mono-prenylated intermediate. Since fungal dimethylallyl tryptophan synthases usually catalyze the transfer of one molecule of DMAPP, the di-prenylation catalyzed by PaxD is unique. At this stage, I am not able to estimate the differences in the reaction mechanism between mono-prenylation and di-prenylation. However, comparisons of a crystal structure of PaxD with those of fungal enzymes catalyzing mono-prenylation, such as FtmPT1 (158), might give me a clue.



Scheme 2.6 Summary of reactions catalyzed by PaxC and PaxD

Chapter 3

Regio- and mode- specificities of AtmD and PaxD

3.1 Introduction

In the chapter 2, I characterized *paxD*, which is located next to *paxQ* in the paxilline biosynthetic gene cluster and has weak similarities to fungal prenyltransferase genes. Recombinant PaxD catalyzed the successive regular attachment of DMAPP to positions 21 and 22 of paxilline to form di-prenylated paxilline via a mono-prenylated paxilline intermediate. A Blast search showed that the most homologous enzyme to PaxD is *atmD* product (32% amino acid identity), which is located in the aflatrem biosynthetic gene cluster in *A. flavus* (32). However, aflatrem and β -aflatrem, which are paxilline-related compounds, are reversely mono-prenylated at the 20- and 21-position, respectively. Therefore, I examined whether AtmD catalyzes the reverse prenylation to produce these compounds or regular di-prenylation like PaxD. During the study, more importantly and surprisingly, I found that AtmD and PaxD accepted paspaline, an intermediate of paxilline biosynthesis that has a similar structure to paxilline, and that both enzymes unexpectedly showed different position and regular/reverse specificities to those with paxilline. Moreover, both AtmD and PaxD accepted farnesyl indole (FI) to yielded regular mono-prenylated FI. These results suggested that fungal indole diterpene prenyltransferases could be applicable for synthesis of important compound including bioactive compounds.

3.2 Materials and methods

3.2.1 Strain

Aspergillus flavus NBRC 4295 was grown in 100 ml of medium containing 2.4% potato dextrose broth at 30°C for 3 days in a reciprocal shaker (125 strokes/min). Preparation of genomic DNAs, RNA and cDNA were described in the materials and methods 2.2.

3.2.2 PCR amplification and purification of AtmD

Table 3.1 PCR primers used for amplifications of each *atm* gene in *A. flavus* NBRC 4295.

Primers

Gene	Sequence 5'-3'
<i>atmG</i>	F: ATGATTTCAGGTGTGCCCCGATCGTTGGAAGG R: TCACAATAAAAACGCTAAGATATCATAGATACC
<i>atmC</i>	F: ATGGGCTTCTTTTCATGACTTCCTCTCTCGGCCTAC R: TCATTTCTCGCTCTTCATGTCCATCTTTTCATTCC
<i>atmM</i>	F: ATGTGTGATAAAGATCGCTTCAAAGTGATTATTG R: TTA ~~CT~~ CAACCGTTTTAGAAACCAGTGAATTACTCC
<i>atmD</i>	F: TTGCATGCATGTCCACTCCCAAGTCGGATACATGC R: ATCTGCAGCTACTTGGAAAGCCCCCTTCACATCTGAC
<i>atmQ</i>	F: GTCTGGTAGAGTACCAGCCAACCT R: CACCATTCCCTTTCATATCCGGGAAC
<i>atmB</i>	F: ATGGACGGATTGGCTCATCACAGGCCCCAGC R: CTAGACTGTCTTCCGATCGGAATCGTGCCCAA
<i>atmA</i>	F: CTCGTTCTACATGATGTACATCTCGC R: GGATGAGTCTGAATTTGTTCGATCTC
<i>atmP</i>	F: GTAGAGAATGGTTCCATGTTGTTGG R: ACATCCACAGAGGTATTGGGAACG

Table 3.2 Oligonucleotides used for construction of expression AtmD.

Amplified genes	Restriction sites	Sequence (5' to 3')	Vectors /Host
AtmD	<i>SphI</i> <i>PstI</i>	F: TTGCATGCATGTCCACTCCCAAGTCGGATACATGC R: ATCTGCAGCTACTTGGAAAGCCCCCTTCACATCTGAC	pQE30 /M15

Cell cultivation and protein purification were described in the 2.2.

3.2.3 In vitro assay of AtmD

The assay mixture for AtmD contained, in a final volume of 100 μ L, 0.25 mM of prenyl acceptor, 0.5 mM of DMAPP, 50 mM Tris–HCl (pH 8.0), and a suitable amount of recombinant enzyme. This mixture was incubated at 30°C for overnight and the reaction was stopped by the addition of 100 μ L methanol. The products were analyzed and purified by HPLC. Analytical conditions were as follow; Merck Mightisil RP-18GP Aqua column (250 mm $\times$ 4.6 mm) (Kanto Chemicals, Japan); mobile phase of acetonitrile in water (0 to 25 min, 70% AcCN; 25 to 40 min, 70 to 100%; 40 to 50 min, 100%); flow rate, 1.0 mL/min; detection, 230 nm.

3.2.3.1 Kinetic parameters of AtmD for Paxilline and DMAPP

The assay was linear with respect to protein concentration up to 1 μ g for 30 min incubation and no substrate inhibition was observed with paxilline and DMAPP up to 1.0 mM of each of the substrate. The assay mixture contained, in a final volume of 100 μ L, 50 mM Tris–HCl (pH 8.0), 0.5 mM DMAPP, 0.5 μ g of the AtmD, and 0.5 μ M to 0.1 mM paxilline. When the concentration of paxilline was fixed at 0.25 mM, the concentration of DMAPP was varied from 0.02 μ M to 10 μ M. This mixture was incubated at 30°C for 10 min.

3.2.3.2 Kinetic parameters of AtmD for Paspaline and DMAPP

The assay mixture contained, in a final volume of 100 μ L, 50 mM Tris–HCl (pH 8.0),

0.5 mM DMAPP, 0.5 μ g of the AtmD, and 0.01 μ M to 1 mM paspaline. When the concentration of paspaline was fixed at 0.25 mM, the concentration of DMAPP was varied from 0.01 μ M to 1.5 mM. This mixture was incubated at 30°C for 20 min.

3.2.3.3 Kinetic parameters of PaxD for Paspaline and DMAPP

The assays mixture contained, in a final volume of 100 μ L, 50 mM Tris-HCl (pH 8.0), 0.5 mM DMAPP, 0.5 μ g of the PaxD, and 0.1 μ M to 0.5 mM paspaline. When the concentration of paspaline was fixed at 0.25 mM, the concentration of DMAPP was varied from 0.01 μ M to 0.1 mM. This mixture was incubated at 30°C for 20 min.

3.2.4 LC/ESI-MS analytical conditions by Phenyl column

LC/ESI-MS (Waters ACQUITY UPLC equipped with SQD2) was used. The analytical conditions were as follows; column, Waters ACQUITY UPLC C18 column (2.1 $\times$ 50 mm) or Waters ACQUITY UPLC BEH Phenyl 1.7 μ m column (2.1 $\times$ 50 mm); column temperature, 40°C; detection, positive mode; mobile phase, 0.1% formic acid : acetonitrile = 30:70 at 5 min, and a linear gradient to 50:50 for an additional 30 min; flow rate, 0.3 ml/min. Cone voltage (30 V).

3.2.5 Structural analysis of reaction product

All compounds structural analysis NMR spectra were recorded on Bruker AMX-500

spectrometer.

Reversely 20-prenylated paxilline

NMR δ_{H} (CDCl_3 , 500 MHz) 1.03 (s, 3H), 1.28 (s, 3H), 1.30 (s, 3H), 1.33 (s, 3H), 1.48 (m, 1H), 1.52 (s, 3H), 1.53 (s, 3H), 1.65 (m, 1H), 1.76 (m, 1H), 1.87 (m, 1H), 2.01 (m, 1H), 2.03 (m, 1H), 2.33 (m, 1H), 2.55 (dd, $J = 11.2, 13.1$ Hz, 1H), 2.77 (m, 1H), 2.78 (m, 1H), 2.88 (dd, $J = 6.0, 13.1$ Hz, 1H), 3.73 (s, 1H), 4.87 (m, 1H), 4.88 (d, $J = 17.5$ Hz, 1H), 5.00 (d, $J = 10.7$ Hz, 1H), 5.88 (s, 1H), 6.26 (dd, $J = 10.7, 17.5$ Hz, 1H), 7.01-7.07 (m, 1H) X2, 7.22 (d, $J = 7.6$ Hz, 1H), 8.00 (brs, 1H). NMR δ_{C} (CDCl_3 , 125 MHz) 16.0, 19.6, 20.8, 24.2, 26.5, 27.9, 28.5, 29.5, 29.7, 33.5, 34.1, 41.6, 43.2, 48.9, 49.9, 72.5, 72.7, 77.5, 83.2, 110.3, 111.7, 116.8, 117.0, 119.5, 120.2, 123.7, 140.3, 140.4, 149.4, 151.0, 168.5, 199.4. HR-ESIMS: $[\text{M}+\text{H}]^+$ (calcd: 504.3108, found: 504.3110).

Reversely 21-prenylated paxilline

NMR δ_{H} (CDCl_3 , 500 MHz) 1.05 (s, 3H), 1.30 (s, 3H), 1.32 (s, 3H), 1.35 (s, 3H), 1.47 (s, 3H) X2, 1.48 (m, 1H), 1.69 (m, 1H), 1.82 (m, 1H), 1.92 (m, 1H), 2.08 (m, 1H) X2, 2.35 (m, 1H), 2.47 (dd, $J = 11.0, 13.1$ Hz, 1H), 2.77 (dd, $J = 6.0, 13.1$ Hz, 1H), 2.83 (m, 1H), 2.87 (m, 1H), 3.75 (d, $J = 2.1$ Hz, 1H), 4.87 (m, 1H), 5.04 (dd, $J = 1.4, 10.7$ Hz, 1H), 5.11 (dd, $J = 1.4, 17.4$ Hz, 1H), 5.91 (d, $J = 2.1$ Hz, 1H), 6.11 (dd, $J = 10.7, 17.4$ Hz, 1H), 7.13 (dd, $J = 1.8, 8.6$ Hz, 1H), 7.26 (d, $J = 8.6$ Hz, 1H), 7.43 (brs, 1H), 7.79 (s, 1H). NMR δ_{C} (CDCl_3 , 125 MHz) 16.2, 19.6, 20.9, 24.2, 26.5, 27.2, 27.9, 28.5, 28.7 X2, 34.3,

41.0, 43.2, 49.5, 50.7, 72.5, 72.6, 77.5, 83.2, 109.9, 111.1, 115.4, 117.4, 119.5, 119.7, 124.8, 138.0, 140.2, 149.0, 151.9, 168.4, 199.4. HR-ESIMS: [M+H]⁺ (calcd: 504.3108, found: 504.3100).

Regularly 21-prenylated paspaline

NMR δ_{H} (CDCl₃, 500 MHz) 0.87 (s, 3H), 1.01 (s, 3H), 1.12 (s, 3H), 1.13 (m, 1H), 1.17 (s, 3H), 1.19 (s, 3H), 1.45 (m, 1H) X2, 1.46 (m, 1H), 1.60 (m, 1H), 1.68 (m, 1H), 1.65-1.70 (m, 1H), 1.69 (m, 1H), 1.73 (s, 3H), 1.75 (s, 3H), 1.72-1.80 (m, 1H), 1.83 (m, 1H), 1.95 (m, 1H), 2.30 (dd, $J = 10.7, 13.2$ Hz, 1H), 2.65 (dd, $J = 6.4, 13.2$ Hz, 1H), 2.74 (m, 1H), 3.02 (dd, $J = 4.0, 11.9$ Hz, 1H), 3.21 (dd, $J = 2.9, 12.1$ Hz, 1H), 3.40 (d, $J = 7.5$ Hz, 1H), 5.38 (m, 1H), 6.91 (dd, $J = 1.4, 8.2$ Hz, 1H), 7.20 (d, $J = 8.2$ Hz, 1H), 7.21 (s, 1H), 7.61 (brs, 1H). NMR δ_{C} (CDCl₃, 125 MHz) 12.7, 14.5, 17.8, 19.9, 21.9, 22.0, 23.7, 24.7, 25.3, 25.8, 26.1, 27.5, 33.9, 34.5, 36.6, 37.7, 40.0, 46.4, 48.7, 53.0, 71.9, 84.7, 85.7, 111.2, 117.5, 118.0, 121.2, 124.7, 125.3, 131.3, 133.1, 138.5, 151.1. HR-ESIMS: [M+H]⁺ (calcd: 490.3680, found: 490.3675).

Regularly 22-prenylated paspaline

NMR δ_{H} (CDCl₃, 500 MHz) 0.88 (s, 3H), 1.04 (s, 3H), 1.12 (s, 3H), 1.13 (m, 1H), 1.17 (s, 3H), 1.19 (s, 3H), 1.43 (m, 1H) X2, 1.48 (m, 1H), 1.58 (m, 1H), 1.68 (m, 1H) X4, 1.73 (s, 3H) X2, 1.76 (m, 1H), 1.78 (m, 1H), 1.83 (m, 1H), 1.95 (m, 1H), 2.29 (dd, $J = 10.7, 13.2$ Hz, 1H), 2.64 (dd, $J = 6.2, 13.2$ Hz, 1H), 2.74 (m, 1H), 3.02 (dd, $J = 3.6,$

11.7 Hz, 1H), 3.21 (dd, $J = 2.7, 12.0$ Hz, 1H), 3.41 (d, $J = 7.2$ Hz, 1H), 5.37 (m, 1H), 6.90 (brd, $J = 8.2$ Hz, 1H), 7.32 (d, $J = 8.0$ Hz, 1H), 7.09 (brs, 1H), 7.59 (brs, 1H). NMR δ_C (CDCl₃, 125 MHz) 12.7, 14.5, 17.8, 19.9, 21.9, 22.0, 23.7, 24.6, 25.3, 26.1 X2, 27.6, 33.9, 34.5, 36.6, 37.7, 40.0, 46.4, 48.7, 53.0, 71.9, 84.7, 85.7, 110.8, 118.1, 118.2, 120.5, 123.3, 124.2, 131.7, 134.3, 140.5, 150.3. HR-ESIMS: [M+H]⁺ (calcd: 490.3680, found: 490.3703).

5 or 6- prenylated FI

NMR δ_H (CDCl₃, 500 MHz) 1.60 (s), 1.68 (s), 1.75 (s), 1.76 (s), 1.90-2.20 (m), 3.44 (m, H1'' and H1'), 5.09 (m), 5.12 (m), 5.38 (m), 5.43 (m), 6.86 (brs, 1H, H2-**14**), 6.89 (brs, 1H, H2-**13**), 6.94 (brd, $J = 8.1$ Hz, 1H, H5-**14**), 7.00 (brd, $J = 8.2$ Hz, 1H, H6-**13**), 7.13 (s, 1H, H7-**14**), 7.24 (d, $J = 8.2$ Hz, 1H, H7-**13**), 7.36 (s, 1H, H4-**13**), 7.48 (d, $J = 8.1$ Hz, 1H, H4-**14**), 7.74 (s, 1H, NH-**14**), 7.77 (s, 1H, NH-**13**). NMR δ_C (CDCl₃, 125 MHz) 16.0, 16.1, 17.7, 17.8, 24.0, 24.1, 25.7, 25.8, 26.6, 26.7, 26.8, 34.5 (C1''-**13**), 34.6 (C1''-**14**), 39.7 (x2), 110.2 (C7-**14**), 110.8 (C7-**13**), 115.8 (C3-**13**), 116.0 (C3-**14**), 118.0 (C4-**13**), 118.9 (C4-**14**), 120.2 (C5-**14**), 120.6 (C2-**14**), 121.4 (C2-**13**), 122.8 (C6-**13**), 123.0 (x2), 124.1, 124.3, 124.4, 124.6, 125.7 (C3a-**14**), 127.8 (C3a-**13**), 131.3, 131.5, 131.9, 132.5 (C5-**13**), 135.0, 135.1 (C7a-**13**), 135.5 (x2), 135.8 (C6-**14**), 137.0 (C7a-**14**). HR-ESIMS: [M+H]⁺ (calcd: 390.3155, found: 390.3121).

3.2.6 Accession number

The nucleotide sequence of cDNA of *atmD* was submitted to the DDBJ/GenBankTM/EBI Data Bank with accession number, AB778117.

3.3 Results

3.3.1 Gene cluster

Aflatrem is a member of indole diterpene and its biosynthetic gene cluster had been identify in the *A. flavus* NRRL6541 by Scott et al. The genes for aflatrem biosynthesis were scattered at two discrete loci; the first, ATM1, is telomere proximal on chromosome 5 and contains a cluster of three genes, *atmG*, *atmC*, and *atmM*, and the second, ATM2, is telomere distal on chromosome 7 and contains five genes, *atmD*, *atmQ*, *atmB*, *atmA*, and *atmP* (fig 3.1) (32). This cluster is highly similar to paxiline biosynthesis gene cluster (Table 3.3).

In this study, the strain *A. flavus* NBRC 4295 was used for the preparation of cDNA of *atmD* since I could not obtain *A. flavus* NRRL6541.

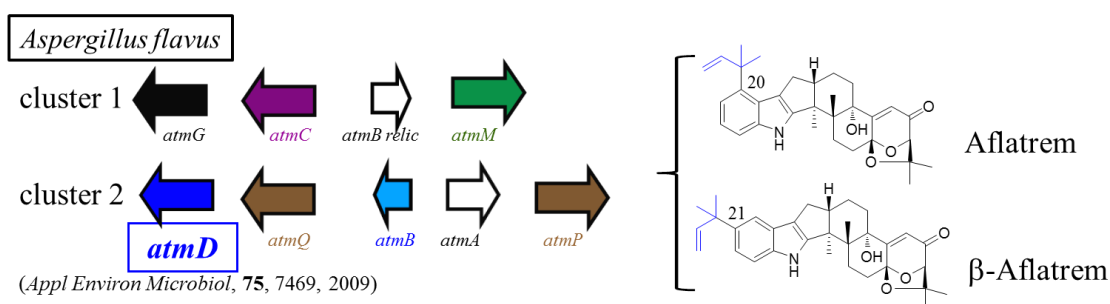


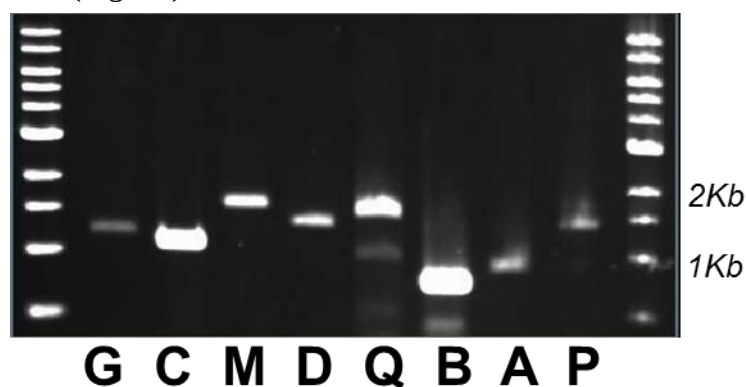
Figure 3.1 Aflatrem biosynthesis gene cluster in *A. flavus* NRRL6541.

Table 3.3 Predicted functions of *atm* genes identified in *A. flavus* NRRL6541.

<i>pax</i> genes	Protein (accession No.)	ID (%)	Function
ATM1			
<i>AtmG</i>	<i>paxG</i> AAK11531	51	GGDP synthase
<i>AtmC</i>	<i>paxC</i> AAK11529	69	Prenyltransferase
<i>AtmM</i>	<i>paxM</i> AAK11530	60	FAD-dependent Monooxygenase
ATM2			
<i>AtmB</i>	<i>paxB</i> ADO29934	62	Cyclase
<i>AtmA</i>	<i>paxA</i> ADO29933	33	Integral membrane protein
<i>AtmP</i>	<i>paxP</i> AAK11528	64	Cytochrome P450 monooxygenase
<i>AtmQ</i>	<i>paxQ</i> AAK11527	60	Cytochrome P450 monooxygenase
<i>AtmD</i>	<i>paxD</i> AAK11526	32	Prenyltransferase

3.3.1.1 PCR amplification of *atm* genes in *A. flavus* NBRC 4295

The presence of the aflatrem biosynthetic gene cluster in the genome was confirmed by PCR with specific primers (Table 3.1), which were designed based on the sequences of each *atm* gene in *A. flavus* NRRL6541. All *atm* genes can be amplification in *A. flavus* NBRC 4295 (Fig 3.2).

**Figure 3.2** PCR amplifications of each of *atm* gene in *A. flavus* NBRC 4295.

3.3.1.2 Search for indole diterpene-related compound in culture broth of *A. flavus*

NBRC 4295

Then production aflatrem-related compounds by the strain NBRC 4295 was examined. By analysis of culture broth with LC/ESI-MS, two specific peaks were detected by the selected ion chromatograms with 502 $[M+H]^+$, which corresponded to $[M+H]^+$ of aflatrem (Fig 3.3), suggesting that the strain NBRC 4295 produced aflatrem.

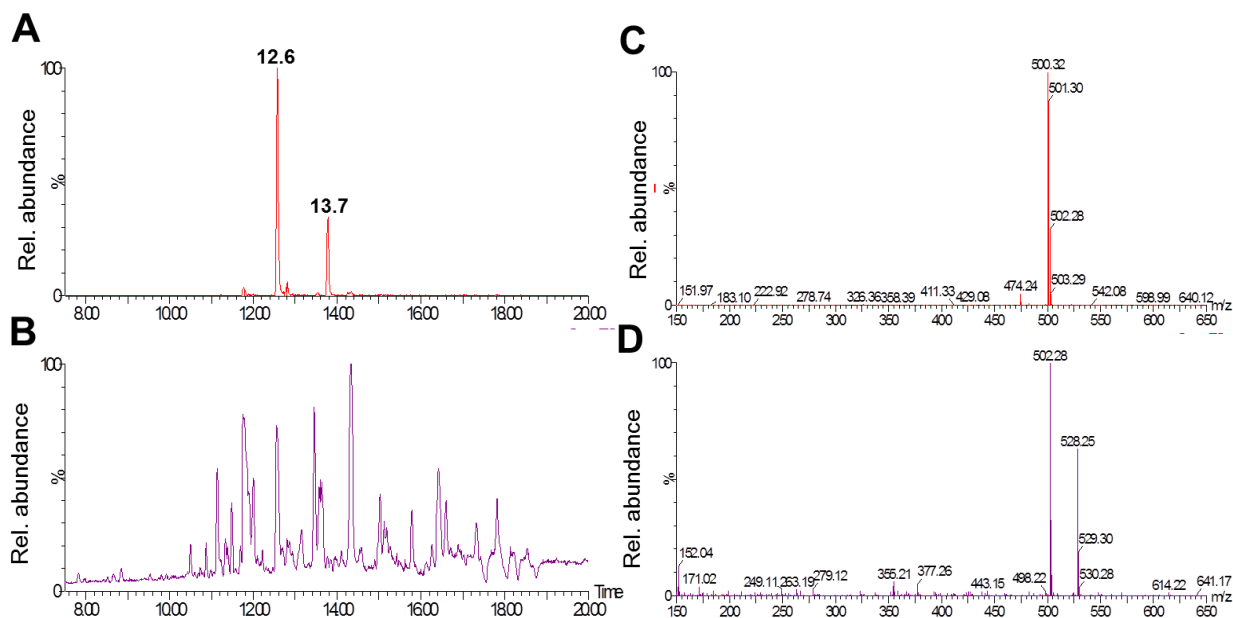


Figure 3.3 LC/ESI-MS analysis of compound accumulated in culture broth of *A. flavus* NBRC 4295.

A) Selected ion chromatograms (m/z 502 $[M+H]^+$); B) Total ion chromatogram;
C) Mass spectra of the peak of 12.6 min in A; D) mass spectra of the peak of 13.7 min in A.

3.3.2 Functional analysis of AtmD

cDNA of *atmD* was amplified based on the nucleotide sequence of the cDNA reported by Scott et al (32). (GenBank; CAP53937). In the strain *A. flavus* NBRC 4295, a predicted gene product was consisted of 435 amino acids (GenBank; AB778117) and had 96% amino acid identity with CAP53937 (Fig 3.4).

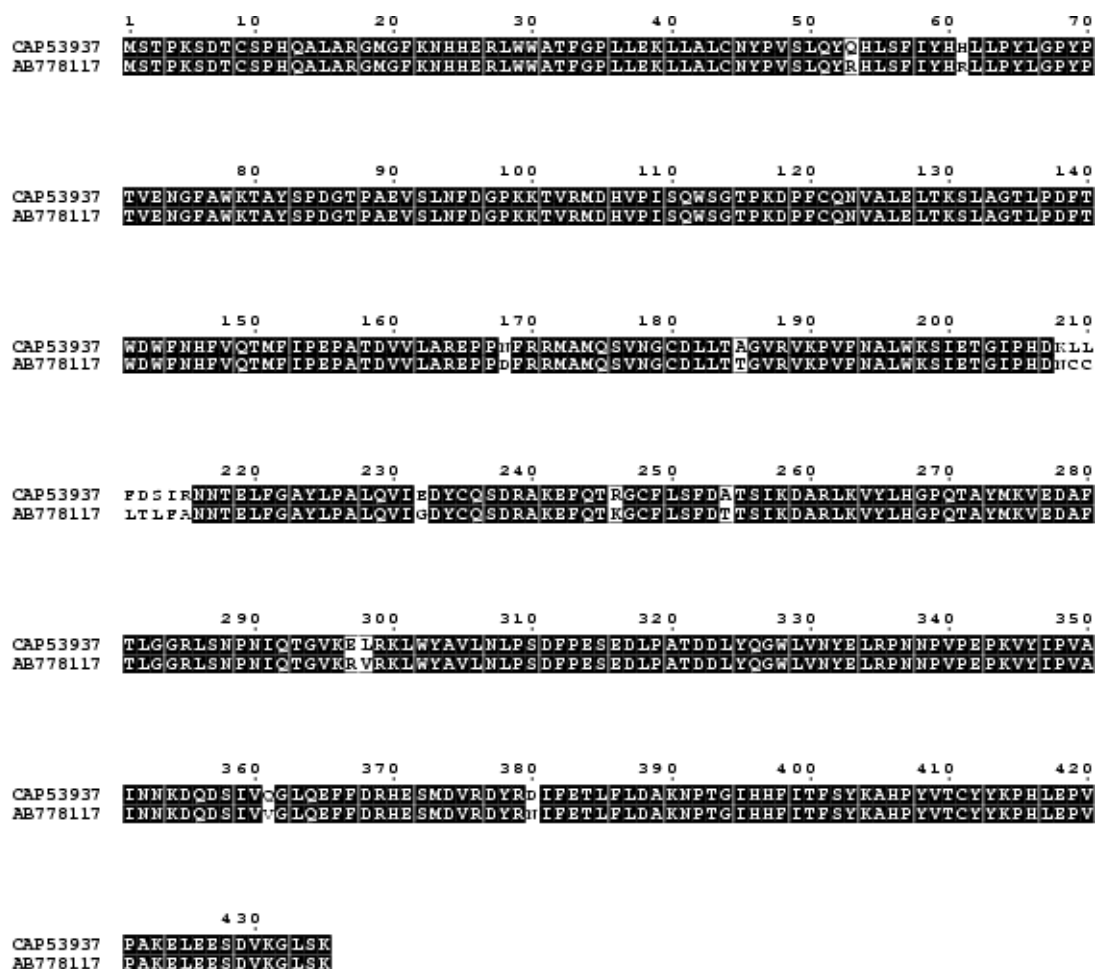


Figure 3.4 Alignment of amino acid sequences of CAP53937 and AB778117.

3.3.2.1 Overexpression and purification of AtmD

The AtmD cDNA was cloned into the pQE30 vector for protein expression in *E. coli* M15. His-tagged AtmD recombinant enzyme was successfully expressed in a soluble form. The obtained recombinant AtmD, which had a calculated molecular mass of 46 kDa, was subjected to SDS-PAGE analysis to confirm its molecular size and purity (Fig 3.5A). The recombinant enzyme was then subjected to gel filtration chromatography to estimate its subunit structure. As shown in Fig 3.5B, a peak with a calculated molecular mass of 95 kDa was detected, suggesting that AtmD forms a homo-dimer similar to PaxD.

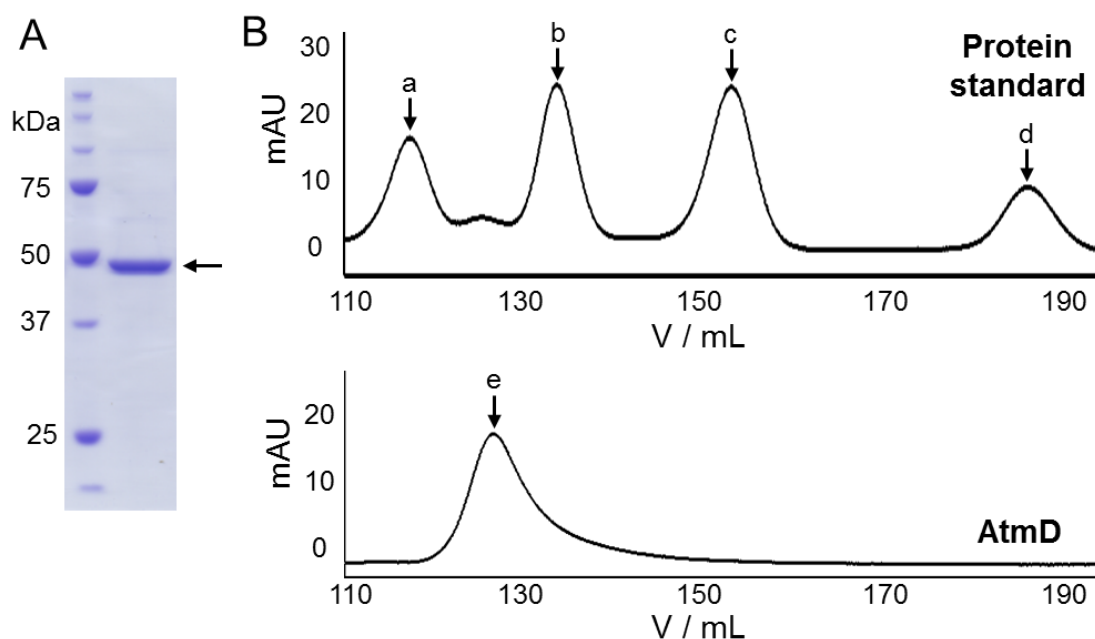


Figure 3.5 SDS-PAGE and gel filtration chromatography of Purified AtmD. (A) molecular mass markers (lane 1) and purified AtmD (lane 2). (B) elution profiles of the standard proteins [aldolase (a, 158 kDa), albumin (b, 67 kDa), ovalbumin (c, 43 kDa) and chymotrypsinogen A (d, 25 kDa); top] and purified AtmD (bottom, e)

3.3.2.2 In vitro assay of AtmD

For in vitro assay, I used commercially available paxilline as a prenyl acceptor, which has a similar structure to paspalinine, a probable intrinsic substrate of AtmD (32). After the recombinant AtmD was incubated with paxilline and DMAPP, reaction products were analyzed by HPLC. Two major products and a trace of minor product were specifically detected (Fig 3.6).

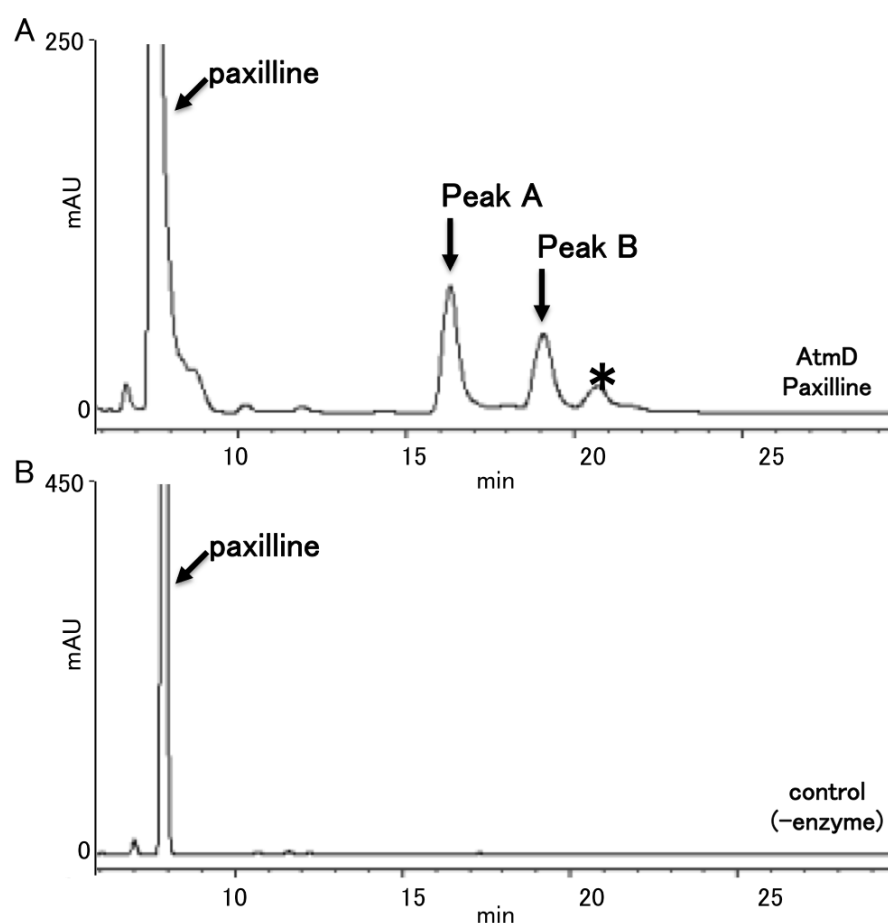


Figure 3.6 HPLC analysis of the reaction products formed from paxilline and DMAPP with (A) and without (B) AtmD.

3.3.2.3 LC/ESI-MS analysis of reaction product formed from paxilline and DMAPP by AtmD

The products formed by in vitro reaction was analyzed by LC/ESI-MS. Total ion chromatograms showed three specific peaks with molecular masses corresponding to mono-prenylated paxilline. Moreover, selected ion chromatograms and their mass spectra strongly suggested that all products were mono-prenylated paxilline (Fig 3.7, Scheme 3.1). Since the yield of the minor product was low, the exact structures of the two major products were analyzed.

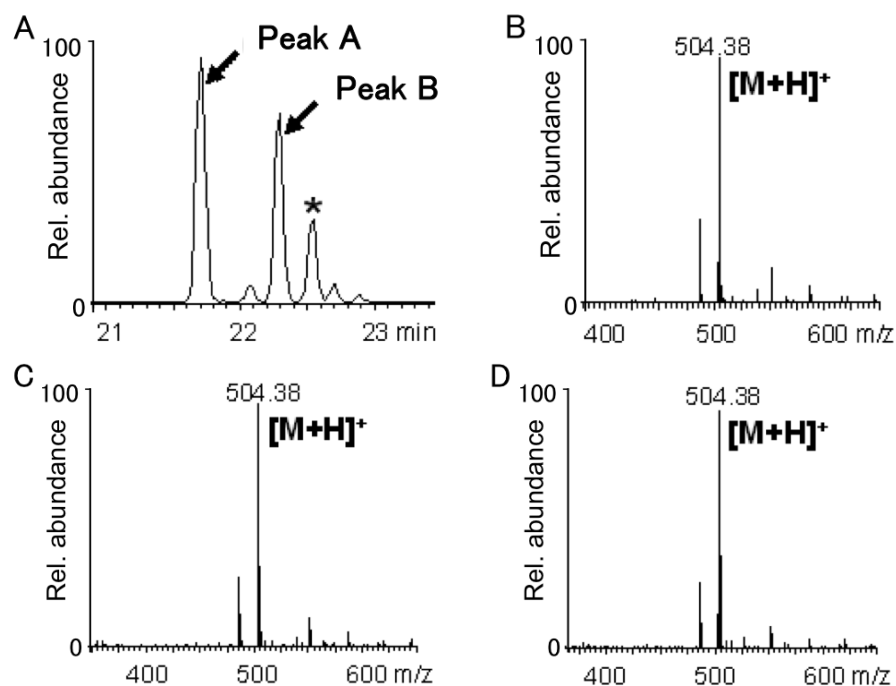
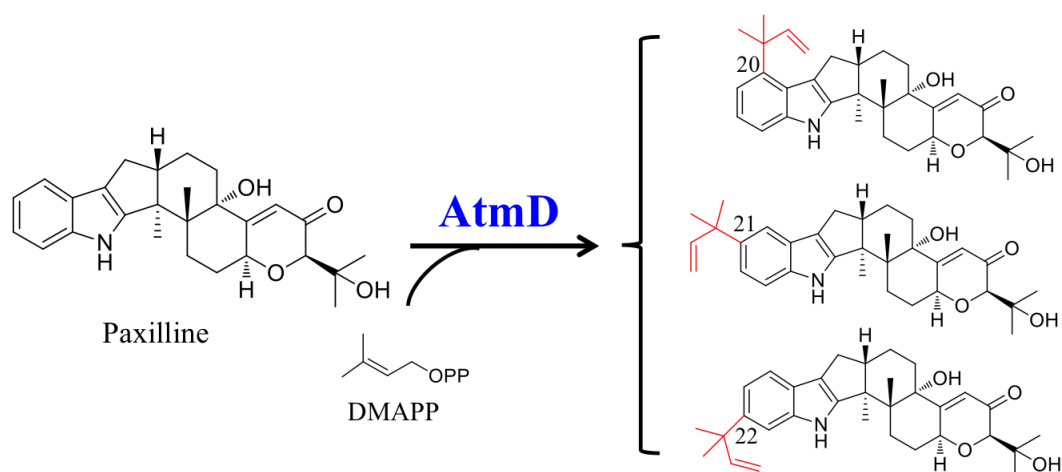


Figure 3.7 LC/ESI-MS analysis of reaction products formed from paxilline and DMAPP by AtmD. Selected ion chromatograms (A) and mass spectra of Peak A (B), Peak B (C) and the minor product indicated by asterisks in A (D) are shown.



Scheme 3.1 Predict reactions formed from paxilline and DMAPP by AtmD.

3.3.2.4 NMR analysis of reaction product formed from paxilline and DMAPP by AtmD

The exact structures of the two major products (Peak A and B) were analyzed. The ^1H -NMR spectra of the one major product (Peak A) showed new signals for a reversely prenylated moiety at $\delta = 4.88$ (d, 1H), $\delta = 5.00$ (d, 1H), $\delta = 6.26$ (dd, 4H), 1.52 (s, 3H), and 1.53 (s, 3H). Subsequently, extensive NMR data analysis, including COSY, HSQC, HMBC, and NOESY, proved the structure was reversely mono-prenylated paxilline at position 20 (Fig 3.8). The ^1H -NMR spectra of other major product (Peak B) showed new signals assigned to be a reversely prenylated moiety at $\delta = 5.04$ (dd, 1H), $\delta = 5.11$ (dd, 1H), $\delta = 6.11$ (dd, 1H), and 1.47 (s, 6H). Extensive NMR data analysis, including COSY, HSQC, HMBC, and NOESY, proved the structure was reversely mono-prenylated paxilline at position 21 (Fig 3.8).

3.3.2.5 Biochemical characterization of AtmD

3.3.2.5.1 Temperature effect

The biochemical properties of the enzyme were investigated using paxilline and DMAPP as the substrates. The temperature dependency of the enzyme activity was investigated at range from 20 to 60°C.

The optimal temperature was 50°C (Fig 3.9)

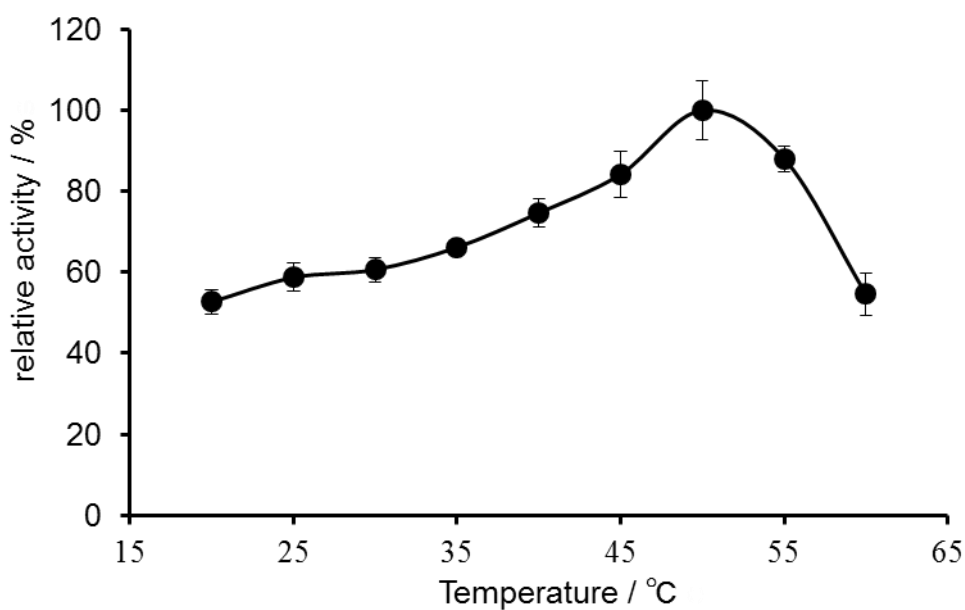


Figure 3.9 Effects of temperature on AtmD activity. The assay mixture contained, in a final volume of 100 μ L, 0.25 mM of paxilline, 0.5 mM of DMAPP, 50 mM Tris-HCl (pH 8.0), and 0.2 μ g of AtmD. This mixture was incubated at each temperature for 10 min.

3.3.2.5.2 pH-rate effect

The pH effect of the enzyme was investigated using paxilline and DMAPP as the substrates, at different pH range from 5.0 to 10, employing a mixed buffer system with citrate buffer (pH 5.0~6.0), MES buffer (pH 6.0~7.0), Tris-HCl buffer (pH 7.0~9.0) and borate-NaOH buffer (pH 9.0~10.0).

The optimal pH was 7.0 (Fig 3.10)

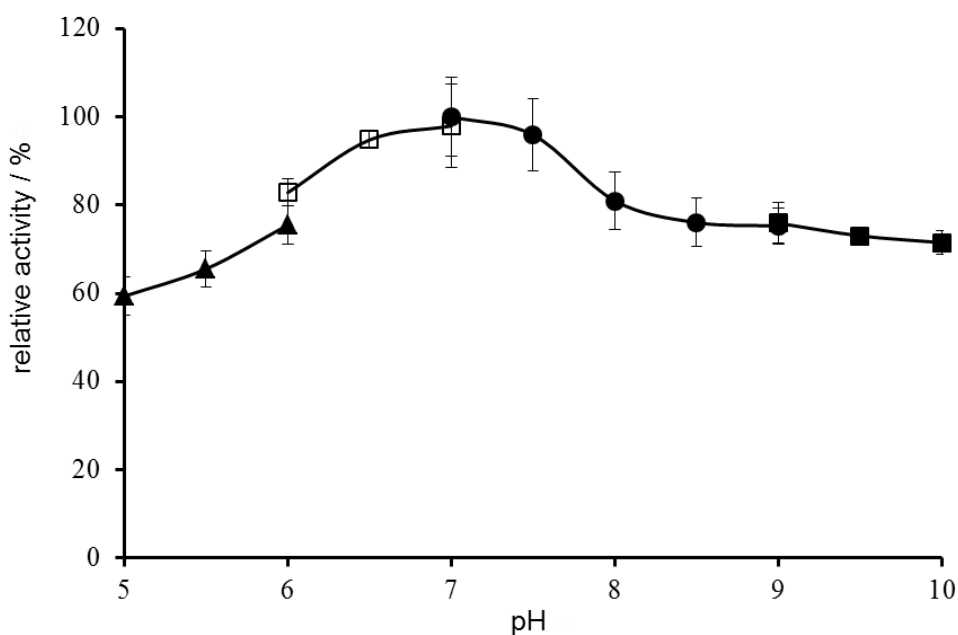


Figure 3.10 Effects of pH on AtmD activity. The assay was conducted at a pH range from 5.0 to 10, with 50 mM citrate (solid triangles), 50 mM MES (open squares), 50 mM Tris-HCl (solid circles), and 50 mM borate-NaOH buffer (solid squares). The assay mixture and condition were the same as those described. This mixture was incubated at 30°C for 10 min.

3.3.2.5.3 Metal dependency of AtmD

Although AtmD lacked a (N/D)DXXD motif, which is required for binding of divalent cations, some prokaryotic prenyltransferases without the motif require divalent cations for activity. Therefore, the effect and dependence of divalent metal ions on the AtmD activity was tested at a concentration of 5 mM (Fig 3.11). The enzyme showed sufficient activity regardless of the presence of 5 mM of EDTA, suggesting that the enzyme did not require Mg^{2+} for its activity. In contrast, Cu^{2+} and Zn^{2+} significantly inhibited the activity.

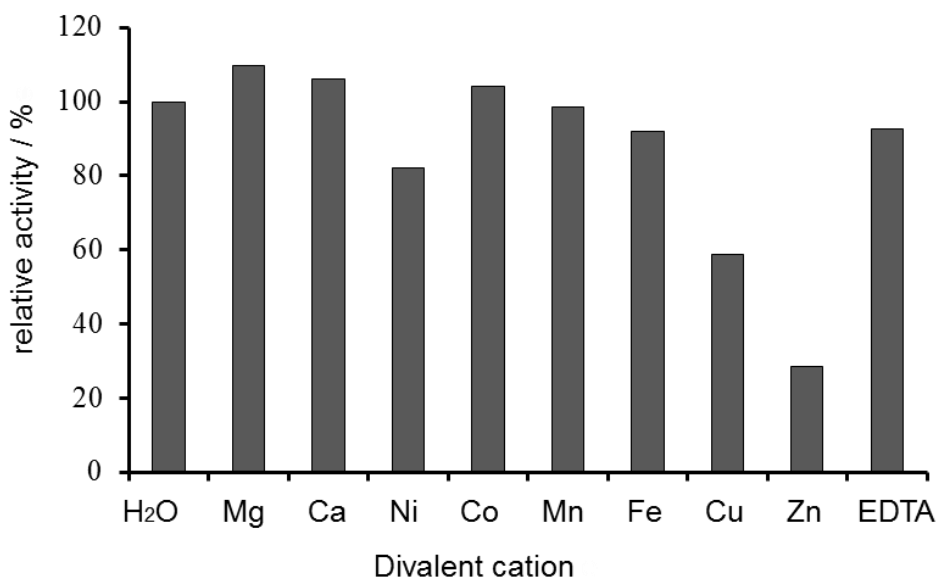


Figure 3.11 Effects of metal ions on AtmD activity. Each of divalent ions (5 mM), or 5 mM EDTA was added to the reaction mixture. The mixture was incubated at 30°C for 10 min.

3.3.2.5.4 Kinetic parameters of AtmD for Paxilline and DMAPP

The steady-state kinetic parameters of AtmD were also determined by fitting to the Michaelis-Menten equation. The enzyme reaction followed Michaelis-Menten kinetics. By using Hanes-Woolf plots, the K_m values were calculated as $13.8 \pm 0.9 \mu\text{M}$ for paxilline and $2.3 \pm 0.1 \mu\text{M}$ for DMAPP. The k_{cat} value was calculated as $0.38 \pm 0.01/\text{sec}$ (Fig 3.12).

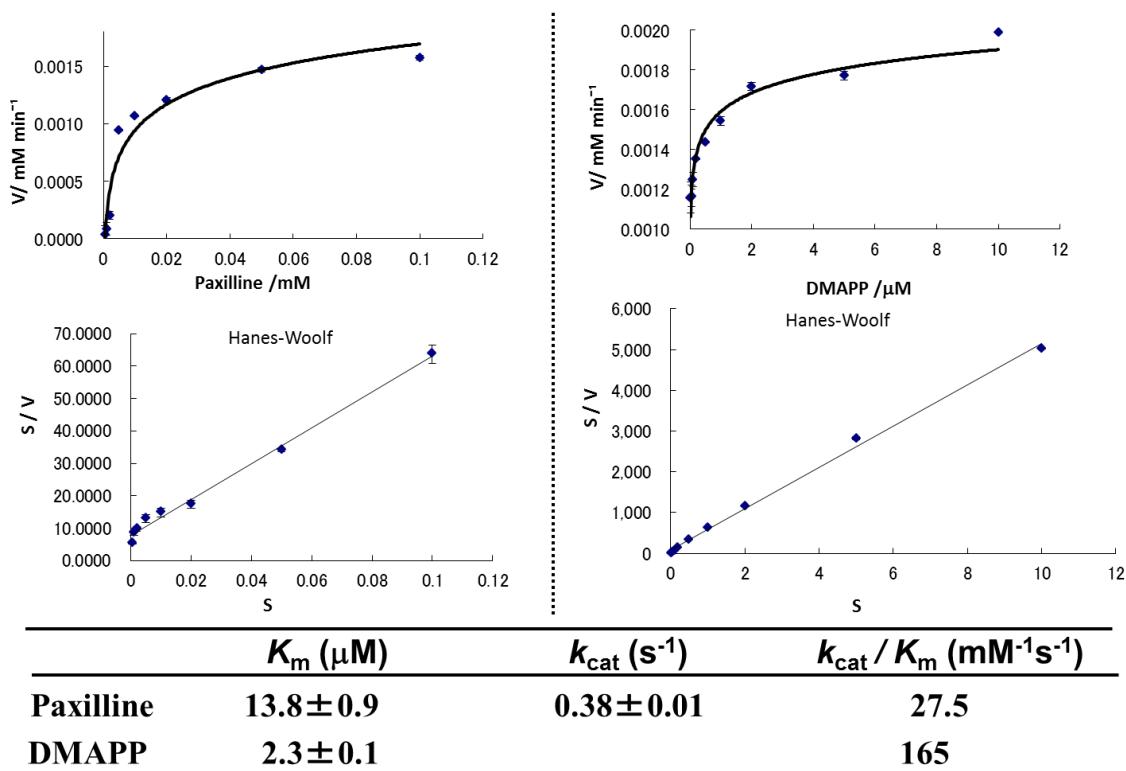


Figure 3.12 Hanes-Woolf plots for calculation of K_m and k_{cat} values of AtmD for paxilline and DMAPP.

3.3.3 Substrate specificity

The substrate specificity of AtmD was investigated. For the prenyl acceptor, compounds related to indole diterpene biosynthesis, such as tryptophan, indole, indole-3-glycerol phosphate, paspaline, farnesyl indole (FI), geranylgeranyl indole (GGI) were examined with DMAPP as a prenyl donor. Cyclo-dipeptides and hydroxynaphthalenes (see 2.3.3.7.1) were also tested because they were reported to be utilized by many fungal prenyltransferases (88).

Of these compounds, paspaline (Fig 3.13), FI (Fig 3.20) and GGI (Fig 3.23) were utilized as the substrate and all the products were suggested to be mono-prenylated ones by LC-ESI-MS analysis.

I also examined the substrate specificity of the prenyl donors. Aside from DMAPP, geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and geranylgeranyl diphosphate (GGDP) were examined. However, no products were formed with paxilline as the prenyl acceptor.

3.3.3.1 Analysis of reaction product formed from paspaline and DMAPP by AtmD

As mentioned above, AtmD accepted paspaline as the prenyl acceptor (Fig 3.13). I then try to determine the exact structure of the product by NMR analysis but the sample was a mixture of two closely related compounds.

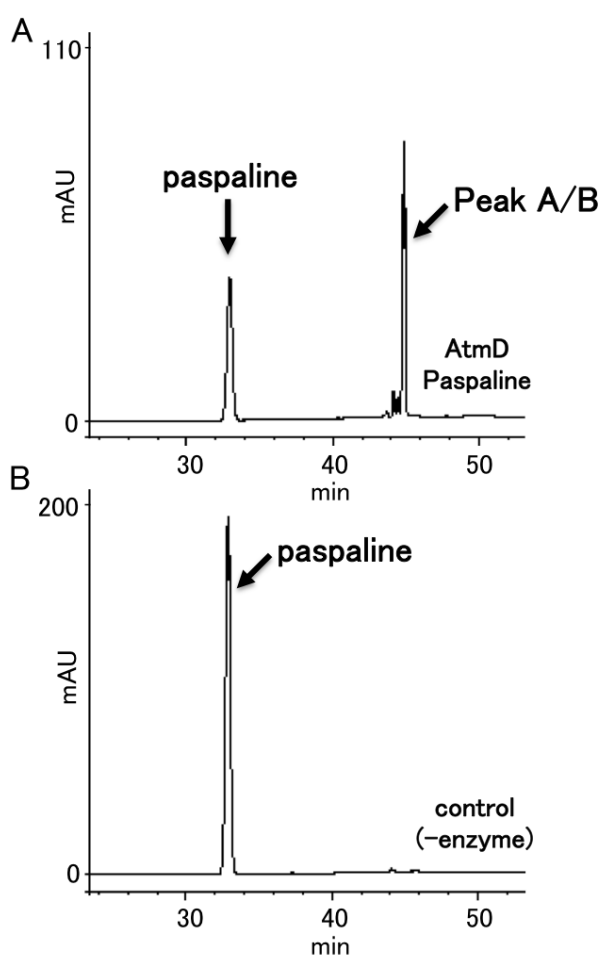


Figure 3.13 HPLC analysis of reaction products formed from paspaline and DMAPP with (A) and without (B) AtmD.

3.3.3.1.1 LC/ESI-MS analysis

Because the product was a mixture, I used Waters ACQUITY UPLC BEH Phenyl 1.7 μm column ($2.1 \times 50 \text{ mm}$) for LC/ESI-MS analysis. The LC/ESI-MS trace showed two specific peaks with molecular masses corresponding to mono-prenylated paspaline. Moreover, selected ion chromatograms and their mass spectra strongly suggested that both products were mono-prenylated paspaline (Fig 3.14).

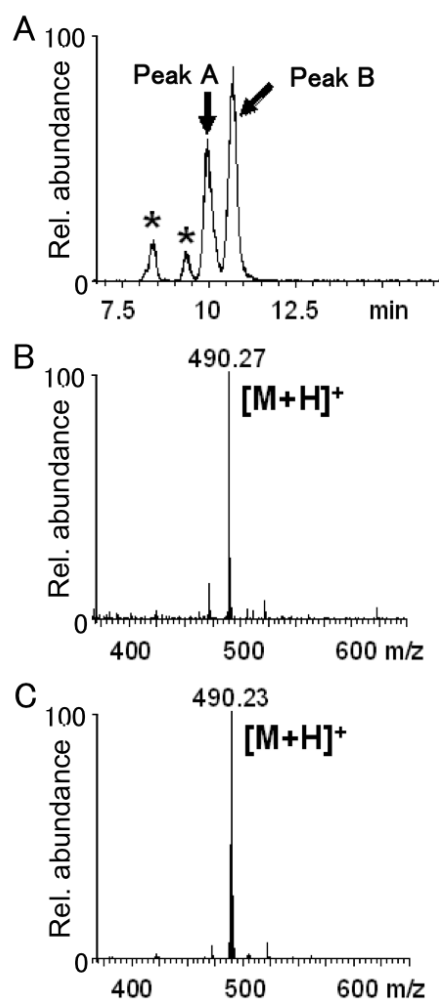


Figure 3.14 LC/ESI-MS analysis of the reaction products formed from paspaline and DMAPP by AtmD. (A), Selected ion chromatograms (m/z 490 $[\text{M}+\text{H}]^+$); and spectra of Peak A (B) and Peak B (C) are shown. Asterisks indicate unknown products.

3.3.3.1.2 NMR analyzed of products

After purification of the products, the exact structures of the two major products (Peak A/B) were analyzed. The ^1H -NMR spectra of Peak A showed new signals for a regularly prenylated moiety at $\delta = 3.40$ (d, 2H), $\delta = 5.38$ (m, 1H), $\delta = 1.73$ (s, 3H), $\delta = 1.75$ (s, 3H). Peak B also showed similar ^1H -NMR spectra with characteristic signals at $\delta = 3.41$ (d, 2H), $\delta = 5.37$ (m, 1H), $\delta = 1.73$ (s, 3H), $\delta = 1.73$ (s, 3H). Subsequently, extensive NMR data analysis, including COSY, HSQC, HMBC, and NOESY, proved the structure was regularly mono-prenylated paspaline at position 21 or 22. Key HMBC, H-H COSY, and NOESY correlations are shown below (Fig 3.15).

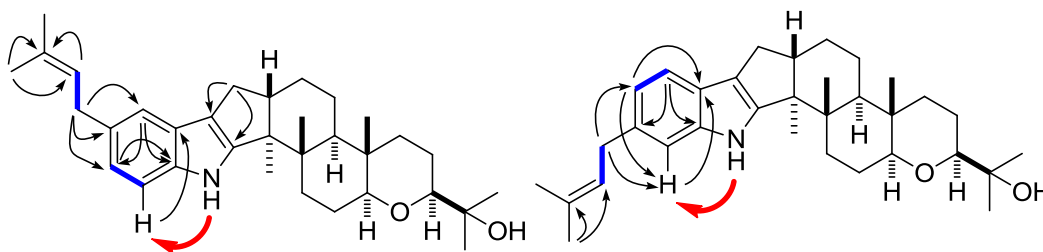


Figure 3.15 Key HMBC, H-H COSY, and NOESY correlations of 21 or 22 mono-prenylated paspaline.

Black arrow: long-range couplings obtained from HMBC, blue line: H-H coupling obtained from H-H COSY, and red arrow: NOE obtained from NOESY experiments.

3.3.3.1.3 Kinetic parameters

I investigated the kinetic parameters with paspaline as the substrate. The enzyme reaction followed Michaelis-Menten kinetics. By using Hanes-Woolf plots, The K_m values were calculated as $131 \pm 5 \mu\text{M}$ and $302 \pm 11 \mu\text{M}$ for paspaline and DMAPP, respectively (Fig 3.16). The k_{cat} value was $0.09 \pm 0.001/\text{sec}$ and the k_{cat}/K_m value was considerably lower than for paxilline. This low value was consistent with the fact that prenylated paspaline has not been reported as a natural product.

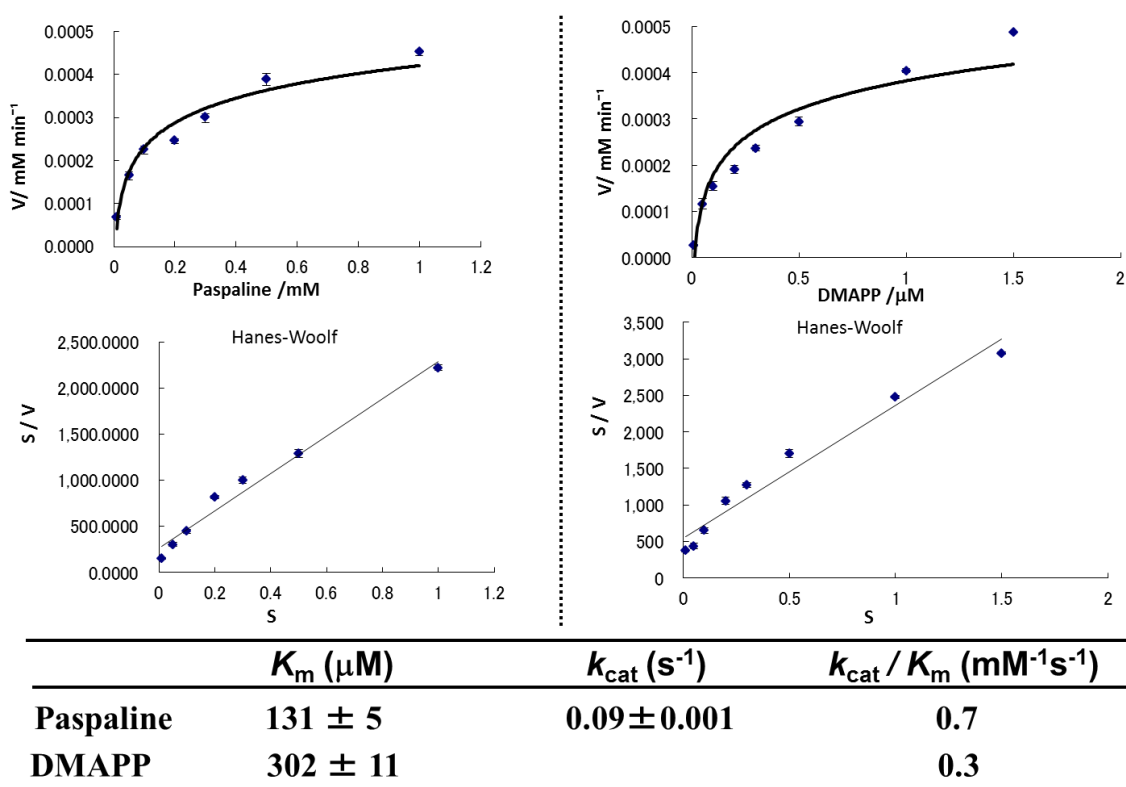


Figure 3.16 Hanes-Woolf plots of AtmD for paspaline and DMAPP.

3.3.3.2 Analysis of reaction product formed from paspaline and DMAPP by PaxD

Since AtmD accepted paspaline, I examined whether PaxD, which catalyzed the step-wise regular di-prenylation at the 21,22-positions of paxilline, was also able to utilize paspaline as the prenyl acceptor. By HPLC analysis, one specifically peak was detected (Fig 3.17). However, the peak was confirmed to be a mixture of two closely related compounds with Waters ACQUITY UPLC BEH Phenyl 1.7 μ m column, in the similar manner as AtmD products (Fig 3.18). After purification of the products, the exact structures of the both products were elucidated by NMR analysis and confirmed to be the same compounds formed by AtmD.

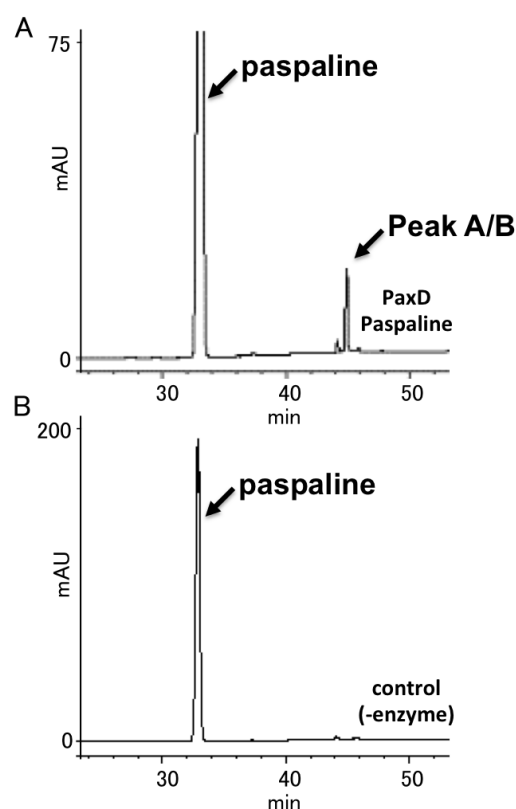


Figure 3.17 HPLC analysis of reaction products formed from paspaline and DMAPP with (A) and without (B) PaxD.

3.3.3.2.1 LC/ESI-MS analysis

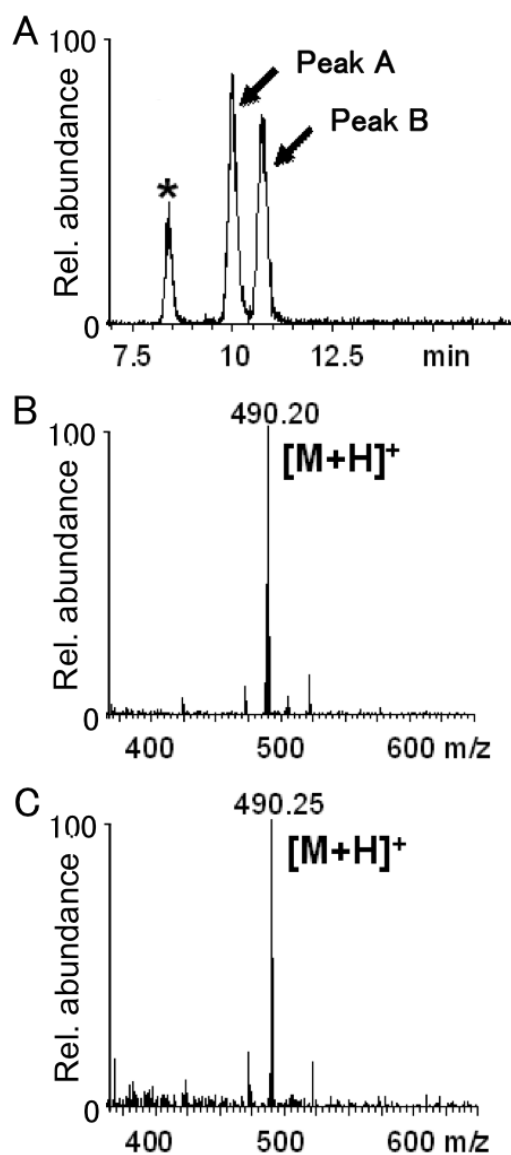


Figure 3.18 LC/ESI-MS analysis of reaction products formed from paspaline and DMAPP by PaxD. (A), Selected ion chromatograms (m/z 490 $[M+H]^+$); and spectra of Peak A (B) and Peak B (C) are shown. Asterisks indicate unknown products.

3.3.3.2.2 Kinetic parameters

I investigated the kinetic parameters of PaxD for paspaline. The enzyme reaction followed Michaelis-Menten kinetics. By using Hanes-Woolf plots, the kinetic parameters of PaxD were compared to those of AtmD. PaxD showed a similar K_m value for paspaline ($124 \pm 8 \mu\text{M}$) and a very low value for DMAPP ($7.9 \pm 0.4 \mu\text{M}$). The k_{cat} value (0.07/sec) was almost the same as that of AtmD (Fig 3.19).

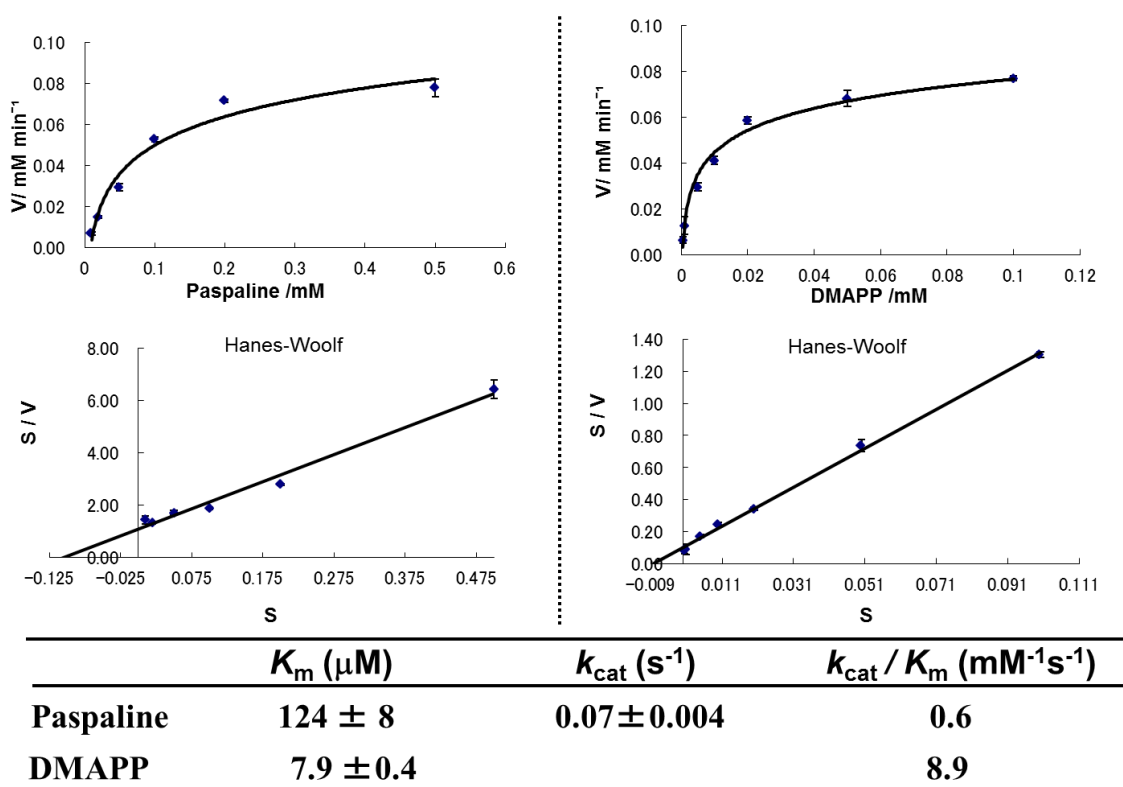
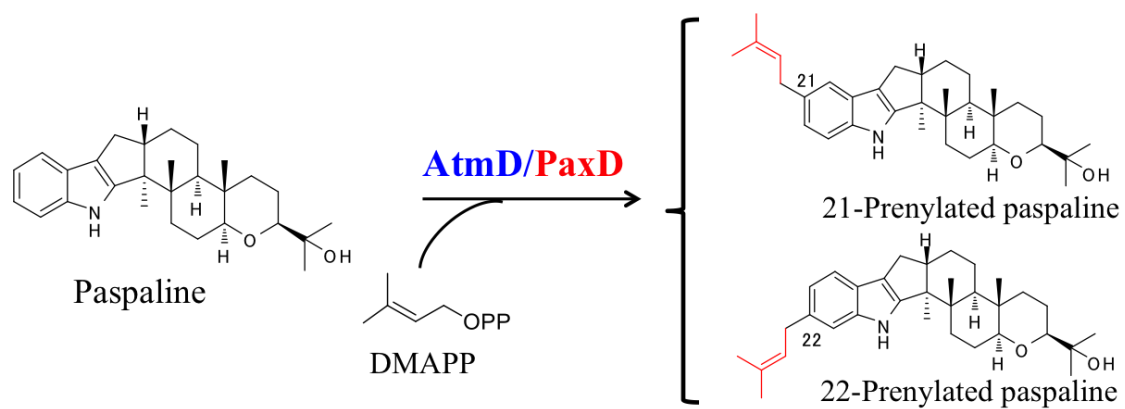


Figure 3.19 Hanes-Woolf plots of PaxD for paspaline and DMAPP.

In summary, AtmD and PaxD could accept paspaline and catalyzed regular mono-prenylation at either the 21- or 22-position (Scheme 3.3).



Scheme 3.3 Reaction products formed from paspaline and DMAPP by AtmD and PaxD.

3.3.3.3 Substrate specificities of AtmD and PaxD (1)

Next, I examined whether the other indole diterpene related compounds could be accepted as the substrates of AtmD and PaxD. For the prenyl acceptor, farnesyl indole (FI) was examined with DMAPP as a prenyl donor. By HPLC/LC-MS analysis, one specific peak was detected in both AtmD and PaxD reactions (Fig 3.20). Selected ion chromatograms (m/z 390 $[M+H]^+$) and their mass spectra strongly suggested that the product was mono-prenylated FI (Fig 3.21).

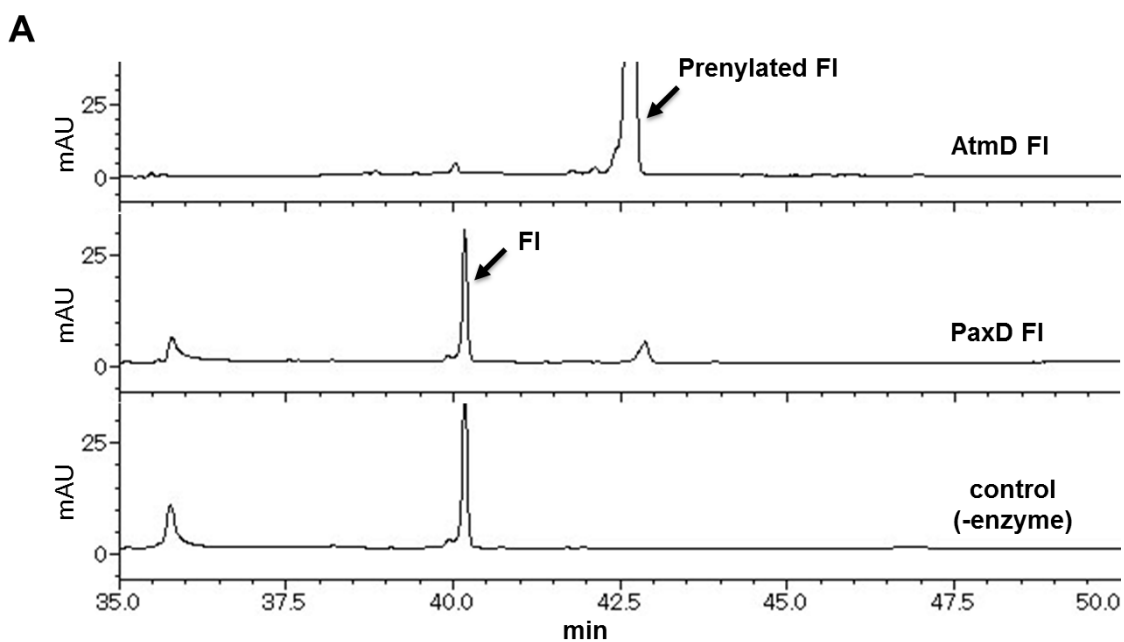


Figure 3.20 HPLC analysis of reaction products formed from FI and DMAPP by AtmD and PaxD.

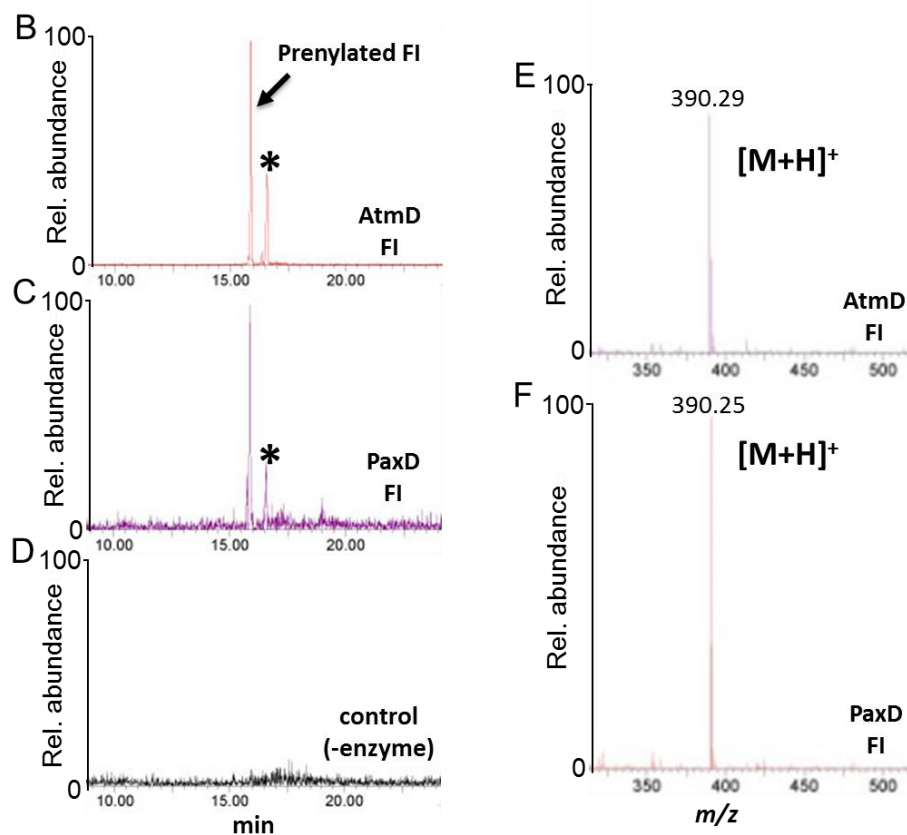
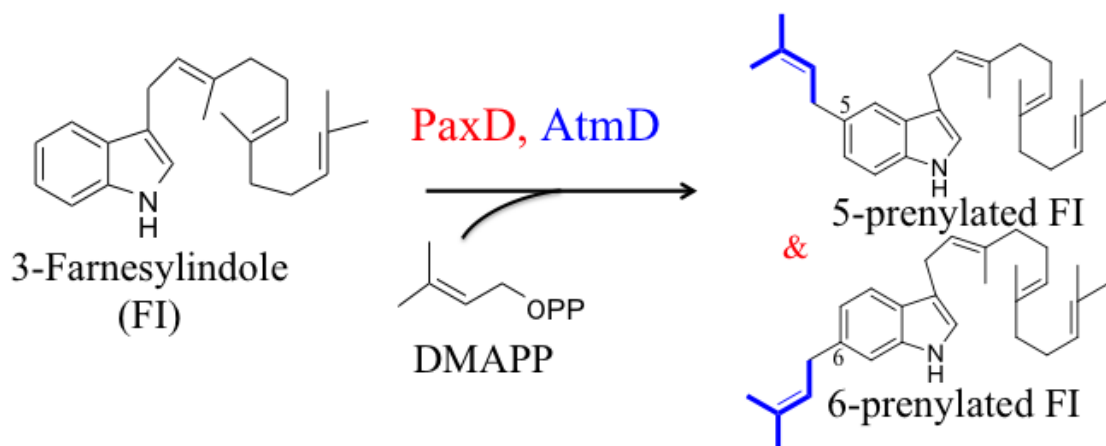


Figure 3.21 LC/ESI-MS (B-F) analysis of reaction products formed from FI and DMAPP by AtmD and PaxD. Selected ion chromatograms (m/z 390 $[M+H]^+$) (B, C) and control experiment (without enzyme, D). Mass spectra of the products formed by AtmD (E) and PaxD (F) are shown. Asterisks indicate impurities.



Scheme 3.4 Reaction products formed from FI and DMAPP by AtmD and PaxD.

3.3.3.3.1 NMR analyzed of mono-prenylated FI

To determine the exact structure of the mono-prenylated FI formed by AtmD, the compound was purified and subjected to NMR analysis. Consequently, the product was found to be a mixture of two related compounds. I conducted NMR analysis without separation of the two regioisomers (1:2.6 mixture) because of their low yields. Typical signals for a regular dimethylallyl moiety were found at $\delta = 3.44$ (m, 2H) and $\delta = 5.38$ (m, 1H). Key HMBC, H-H COSY, and NOESY correlations are similar to those of 21/22-prenylated paspaline. These results suggested that the prenylation takes place at 5- or 6-position on the indole moiety. Taken together, the products were determined as regularly mono-prenylated FI at 5- and 6-positions (Fig 3.22, Scheme 3.4).

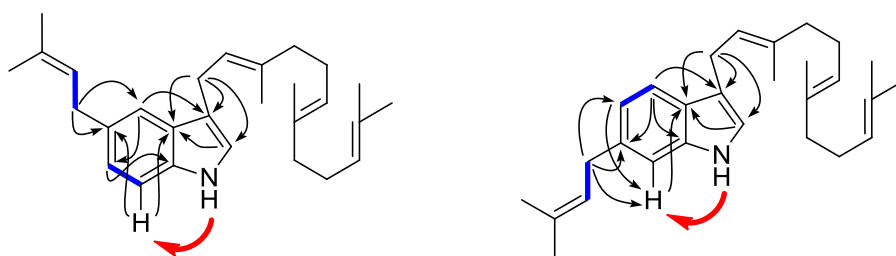


Figure 3.22 Key HMBC, H-H COSY, and NOESY correlations of 5- or 6- prenylated FI.

Black arrow: long-range couplings obtained from HMBC, blue line: H-H coupling obtained from H-H COSY, and red arrow: NOE obtained from NOESY experiments.

3.3.3.4 Substrate specificities of AtmD and PaxD (2)

Next, I used geranylgeranyl indole (GGI) as the prenyl acceptor. By HPLC analysis, one specific peak was detected in the AtmD reaction mixture, but not in the PaxD reaction mixture (Fig 3.23). I could not determine the structure of the compound because of the low yield. However, by LC/ESI-MS analysis, the product was suggested to be mono-prenylated GGI. Selected ion chromatograms (m/z 458 $[M+H]^+$) and its mass spectrum strongly suggested that the product was mono-prenylated GGI (Fig 3.24, Scheme 3.5).

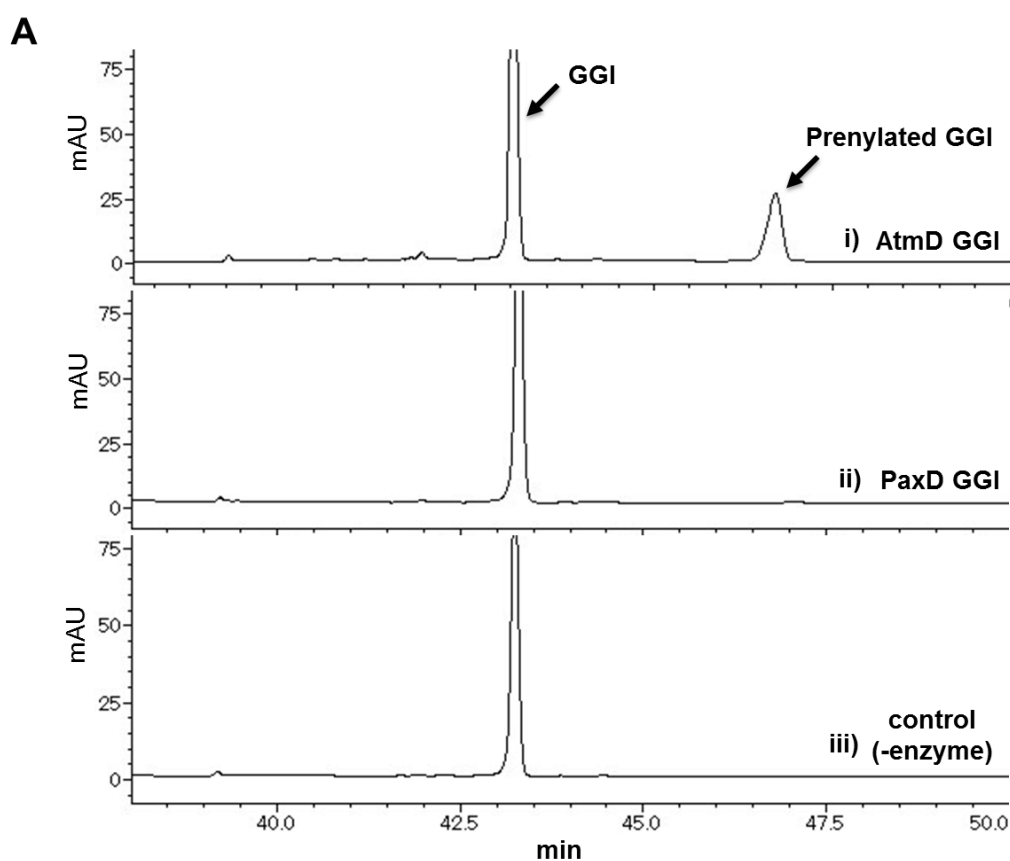


Figure 3.23 HPLC analysis of reaction products formed from GGI and DMAPP by AtmD and PaxD.

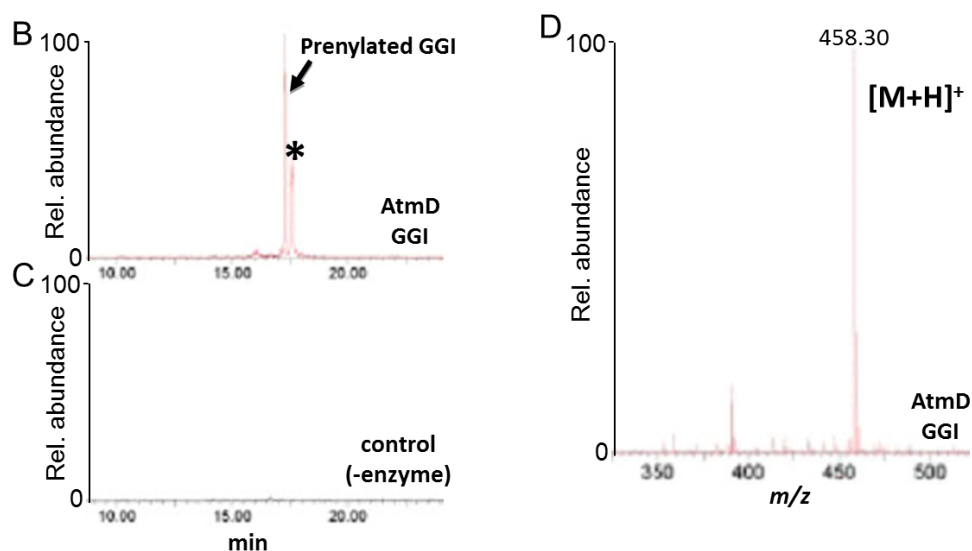
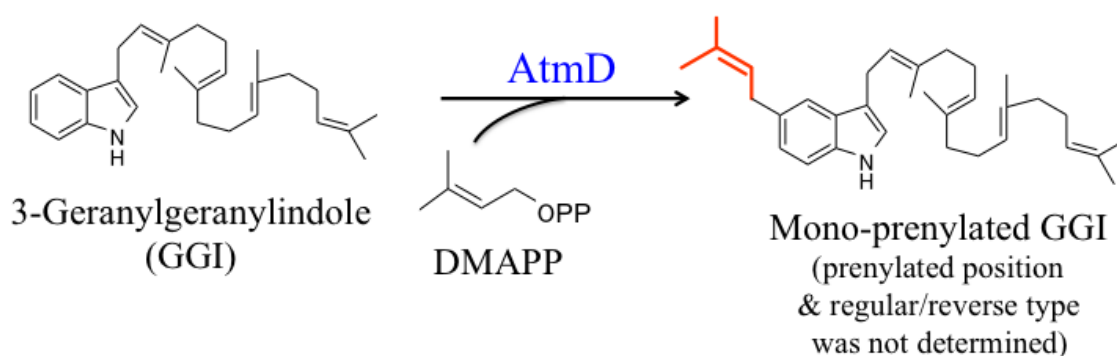


Figure 3.24 LC/ESI-MS (B-D) analysis of reaction products formed from GGI and DMAPP by AtmD.

The reaction products formed from GGI and DMAPP by AtmD (B) was analyzed by LC/ESI-MS, together with control experiment (without enzyme, C). Selected ion chromatograms (m/z 458 $[M+H]^+$) (B) and spectra of the products formed by AtmD (D) are shown. Asterisks indicate impurities.



Scheme 3.5 Reaction product formed from GGI and DMAPP by AtmD.

3.4 Discussion

In this chapter, I showed that AtmD and PaxD could accept the intermediate of paxilline biosynthesis. AtmD whose intrinsic substrate is paspaline (32) could utilize paxilline, paspaline, FI, and GGI. PaxD also accepted paspaline and FI besides its real substrate paxilline. More importantly and surprisingly, AtmD catalyzed prenylation of paxilline and paspaline at different positions and with regular/reverse specificities. AtmD catalyzed a reverse mono-prenylation either at position 20 or 21 with paxilline and DMAPP as substrates. In contrast, regular mono prenylation either at position 21 or 22 was observed with paspaline as a substrate. Moreover, PaxD, which was shown to produce a regularly di-prenylated product at the 21,22-positions from paxilline, catalyzed the same reactions as those of AtmD with paspaline (Scheme 3.6). I am unable to estimate the reaction mechanisms to explain why these enzymes altered their position specificity, the regular/reverse mode for prenylation, and the number of introduced DMAPP to structurally related compounds; additional experiments such as molecular evolution engineering and site-directed mutagenesis based on x-ray structures of the enzymes may give us an answer. In summary, the prenyltransferases responsible for indole diterpene biosynthesis possess the broad substrate specificities.

Chapter 4

Characterization of prenyltransferases in silent indole diterpene biosynthetic gene cluster in *Phomopsis amygdali*

4.1 Introduction

In the chapter 2 and 3, I studied the PaxC/D and AtmD responsible for indole-diterpene biosynthesis such as the prenylation of indole/indole-3-glycerol phosphates by PaxC, the step-wise regular-type di-prenylation at the 21,22-positions of paxilline by PaxD, and the reverse mono-prenylation of paxilline at either the 20- or 21-position by AtmD.

As part of the survey of a new fungal indole diterpene prenyltransferase, I identified a putative indole diterpene biosynthetic gene cluster composed of 8 genes in genome database of *P. amygdali* (Fig 4.1), a diterpene glucoside fusicoccin A producer (159). The six genes of them (*amyG*, *amyC*, *amyM*, *amyB*, *amyP*, and *amyQ*) have significant similarities to the corresponding the *pax* genes essential for paxilline biosynthesis (38). The other two genes are similar to prenyltransferases (*amyD*) and cytochrome P450s (*amyJ*), suggesting that the cluster might be responsible for biosynthesis of a prenylated- and oxidized-paxilline such as lolilline and lolitrem.

In this chapter, I investigated the substrate specificities of AmyD together with the PaxG and PaxC orthologs (AmyG and AmyC) with recombinant enzymes.

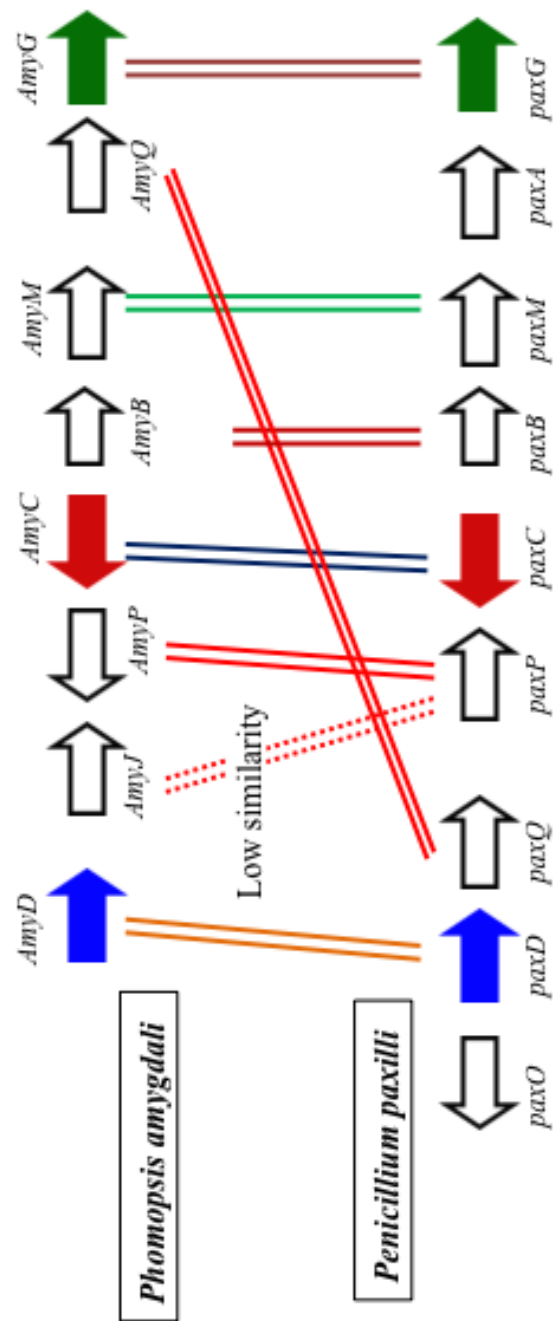


Figure 4.1 Comparison of *pax* and *amy* gene cluster.

4.2 Materials and Methods

4.2.1 Transformations

4.2.1.1 Transformation of *Aspergillus oryzae*

A spore suspension of *A. oryzae* M-2-3 was grown in DP (2% dextrin, 1% polypeptone, 0.5% KH_2PO_4 , 0.1% NaNO_3 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 mL) medium. After 3 days incubation at 30 °C, mycelia was collected by filtration and washed with water. Protoplasting was performed using Yatalase (Takara; 0.5 mg mL⁻¹) in Solution 1 (0.8 mM of NaCl, 10 mM of NaH_2PO_4 , pH 6.0) at 30 °C for 2 h. Protoplast formation was monitored microscopically. Protoplasts were recovered by filtration with Miracloth (Calbiochem), centrifuged at 1,800 X g and 4°C for 10 min, and washed twice with ice-cold 0.8 M NaCl. They were gently resuspended in 300 ml of STC buffer (1.2 M of Sorbitol, 10 mM of Tris-HCl (pH 7.5), and 10 mM of CaCl_2) and immediately used for transformation. Transformation was done with 10 mg of plasmid DNA in 10 ml of 10 mM of TE buffer to which a 100-ml suspension of 10^8 protoplasts was added. After the mixture was left for 10 min on ice, 1 ml of 60% (vol/vol) polyethylene glycol 4000 in a buffer containing Tris-HCl (10 mM, pH 7.5) and CaCl_2 (10 mM) was added and mixed gently by pipetting. The mixture was appropriately diluted with STC buffer and 100- μl samples were plated onto the Czapek-Dox agar plate supplemented with 0.8 M of NaCl and appropriate nutrients and then overlaid with the soft-top agar (1.2 M of sorbitol,

3.5% of Czapek-Dox, 0.6% of agar). The plates were incubated at 30 °C for 3-7 days.

4.2.1.2 Transformation of *Phomopsis. amygdali*

P. amygdali Niigata-2 was grown in 100 ml of medium containing 2.4% potato dextrose broth at 25°C for 4 days in a reciprocal shaker (125 strokes/min). Transformation procedure was the same as described above (4.2.1.1) and the transformants were selected on YPSA plate containing 100 µg hygromycin B per mL.

YPSA

Yeast extract	0.1%
Tryptone	0.1%
Sucrose	0.8M
Agar	1.5%

↓
Autoclave
↓
121°C, 15min

+

100 µg hygromycin B per mL

The recombinant protein was purified with amylose affinity column chromatography (New England Biolabs Japan Inc). After elution of the recombinant enzymes, purity was analyzed by an SDS-PAGE on 10% gels. Protein concentration was determined by a protein-dye standard assay (Bio-Rad) using bovine serum albumin as a standard.

4.2.4 Culture conditions of *P. amygdali* (17 different conditions)

#1. 26g rice bran, 13g wheat bran, 40ml DW, no shaking, room temperature, two weeks.

#2. 26g rice bran, 13g wheat bran, 1g sucrose, 40ml DW, no shaking, room temperature, two weeks.

#3. 26g rice bran, 13g wheat bran, 1g yeast extract, 40ml DW, no shaking, room temperature, two weeks.

#4. 26g rice bran, 13g wheat bran, 1g sucrose, 1g yeast extract, 40ml DW, no shaking, room temperature, two weeks.

#5. 20ml V8 juice, 75mg CaCO₃, 80ml DW, no shaking, room temperature, three weeks.

#6. 50ml V8 juice, 15g oatmeal, no shaking, room temperature, three weeks.

#7. 50ml V8 juice, 15g oatmeal, +TE, no shaking, room temperature, three weeks.

#8. 20ml V8 juice, 75mg CaCO₃, 80ml DW, 125rpm, 25°C, 4 days.

- #9. 50ml V8 juice, 15g oatmeal, 50ml DW, 125rpm, 25°C, 4 days.
- #10. 50ml V8 juice, 15g oatmeal, +TE, 50ml DW, 125rpm, 25°C, 4 days.
- #11. 2.4g potato dextrose broth, 100ml DW, 125rpm, 25°C, 4 days.
- #12. 3.5g Czapek-Dox, 100ml DW, 125rpm, 25°C, 4 days.
- #13. 3.5g Czapek-Dox, +TE, 100ml DW, 125rpm, 25°C, 4 days.
- #14. 3.5g Czapek-Dox, 0.5g yeast extract, 100ml DW, 125rpm, 25°C, 4 days.
- #15. 3.5g Czapek-Dox, 0.5g yeast extract, +TE, 100ml DW, 125rpm, 25°C, 4 days.
- #16. 0.5g pharma media, 3g sucrose, 0.5g KH₂PO₄, 0.1g MgSO₄·7H₂O, 100ml DW, 125rpm, 25°C, 4 days.
- #17. 0.1g yeast extract, 0.05g tryptone, 0.05g peptone, 1g glucose, 0.02g KH₂PO₄, 0.025g MgSO₄·7H₂O, 0.015 NaCl, 100ml DW, 125rpm, 25°C, 4 days.
- × 200 TE (trace element); 5g FeSO₄ · 7H₂O, 5g ZnSO₄ · 7H₂O, 1.43g ZnSO₄ · 5H₂O, 0.5g CuSO₄ · 5H₂O, 0.4g CoCl₂ · 6H₂O up to 100ml.

※ V8 juice (V8 vegetable juice, Campbell Soup Company), DW (distilled water), TE (trace element)

All the medium were autoclaved at 121°C for 15min.

4.2.5 Culture conditions with 5-Azacytidine and SAHA

Component

PDB(potato dextrose broth) 2.4g

DW 100ml

↓
Autoclave



121°C, 15min

#1. Control

#2. +100 µm 5-Azacytidine

#3. +100 µm SAHA (Suberoylanilide Hydroxamic acid)

#4. +100 µm 5-Azacytidine and 100 µm SAHA

The *p. amygdali* wild type strain was cultivated in the above 4 different culture medium at 25°C for 4 days with a reciprocal shaker (125 rpm/min). The products were extracted and analyzed by HPLC/LC-MS.

4.2.6 In vitro assay of AmyG

The assay mixture of AmyG contained, in a final volume of 100 µL, 0.5 mM of allylic substrates, 0.5 mM of IPP, 50 mM Tris-HCl (pH 8.0), 5mM Mg²⁺, and a suitable amount of AmyG. This mixture was incubated at 30°C for overnight. After centrifugation at 15,000 rpm for 30 min, the precipitated products were solubilized with

100 μ L water and analyzed by HPLC. The analytical conditions were follow as Merck Mightisil RP-18GP Aqua column (250 mm $\times$ 4.6 mm); mobile phase of acetonitrile in 10 mM potassium phosphate (pH 7.4) and 0.5% ion-pair reagents (0.5 mol/L tetrabutylammonium dihydrogenphosphate solution; Wako, Osaka, Japan) (0 to 15 min, 40% acetonitrile; 15 to 30 min, 40 to 100%; 30 to 45 min, 100%); flow rate, 1.0 mL/min; detection, 205 nm.

4.2.7 In vitro assay of AmyC/D

The assay mixture and analytical conditions were same as PaxC/D and described in the materials and methods 2.2.

4.2.7.1 Kinetic analysis of AmyD

The assay was linear with respect to protein concentration up to 1 μ g for 30 min incubation and no substrate inhibition was observed with paxilline and DMAPP up to 1.0 mM of each of the substrate. The assays for determination of the kinetic parameters of paxilline contained, in a final volume of 100 μ L, 50 mM Tris-HCl (pH 8.0), 0.5 mM DMAPP, 0.5 μ g of the AmyD, and 1 μ M to 0.5 mM paxilline. When the concentration of paxilline was fixed at 0.25 mM, the concentration of DMAPP was varied from 5 μ M to 0.5 mM. This mixture was incubated at 30°C for 20 min.

4.2.8 Structural analysis of the reaction product

The reaction product formed from paxilline and DMAPP by AmyD was fractionated with a preparative HPLC for purification. The ^1H - and ^{13}C -NMR spectra were recorded on a Bruker AMX-500 spectrometer: NMR δH (CDCl_3 , 500 MHz) (Fig. S37) 1.04 (s, 3H), 1.28 (s, 3H), 1.30 (s, 3H), 1.32 (s, 3H), 1.46 (m, 1H), 1.64 (m, 1H), 1.68 (s, 3H), 1.70 (s, 3H), 1.72 (s, 3H), 1.78 (m, 1H), 1.80 (s, 3H), 1.90 (m, 1H), 2.04 (m, 1H), 2.07 (m, 1H), 2.32 (m, 1H), 2.57 (m, 1H), 2.79 (m, 1H), 2.85 (m, 1H), 3.36 (d, $J = 6.8$ Hz, 2H), 3.61 (m, 2H), 3.73 (d, $J = 2.0$ Hz, 1H), 4.86 (brt, $J = 9.7$ Hz, 1H), 5.18 (m, 1H), 5.27 (m, 1H), 5.89 (d, $J = 1.7$ Hz 1H), 6.91 (d, $J = 8.3$ Hz 1H), 7.09 (d, $J = 8.3$ Hz 1H), 7.65 (s, 1H). NMR δC (CDCl_3 , 125 MHz) 16.2, 17.9, 18.2, 19.7, 20.9, 24.2, 25.7, 25.8, 26.6, 28.0, 28.5, 29.1 (x2), 31.1, 34.4, 43.2, 49.5, 50.4, 72.5, 72.6, 77.6, 83.3, 109.4, 116.9, 119.6, 122.9, 124.4, 124.7, 125.3, 130.4 (x2), 130.6, 130.9, 138.4, 151.1, 168.2, 199.3. High resolution (HR)-ESIMS: calcd. for $\text{C}_{37}\text{H}_{50}\text{NO}_4$ $[\text{M}+\text{H}]^+$: 572.3734, observed: 572.3754.

4.2.9 Accession numbers

The nucleotide sequences of cDNAs of amyG, amyC, and amyD were deposited to the DDBJ/GenBankTM/EBI Data Bank with accession numbers, AB839408, AB839409, and AB839410, respectively.

4.3 Results

4.3.1 Identification of putative indole diterpene gene cluster

By searching for *P. amygdali* draft genome sequence by tBLAST with PaxG as a query, I found an ortholog of geranylgeranyl diphosphate (GGDP) synthases (AmyG). Moreover, the gene, *amyG*, was suggested to be clustered with additional seven genes responsible for indole diterpene biosynthesis. The six genes of them (*amyG*, *amyC*, *amyM*, *amyB*, *amyP*, and *amyQ*) have significant similarities to the corresponding the *pax* genes essential for paxilline biosynthesis (38). Moreover, *amyD* and *amyJ* are similar to *ltmE*, a prenyltransferase, and *ltmJ*, a cytochrome P450 (Table 4.2), both of which were previously identified in the lolitrems biosynthetic gene cluster in *Epichloë festucae* (42).

Table 4.2 Predicted functions of *amy* genes identified in *P. amygdali*.

<i>amy</i> genes	Homologous protein (accession No.)	e-value	Function
<i>amyG</i>	<i>P. paxilli</i> PaxG (AAK11531)	5e-34	GGDP synthase
<i>amyQ</i>	<i>P. paxilli</i> PaxQ (AAK11527)	4e-32	Cytochrome P450 monooxygenase
<i>amyM</i>	<i>P. paxilli</i> PaxM (AAK11530)	2e-79	FAD-dependent monooxygenase
<i>amyB</i>	<i>P. paxilli</i> PaxB (ADO29934)	3e-38	Cyclase
<i>amyC</i>	<i>P. paxilli</i> PaxC (AAK11529)	2e-65	Prenyltransferase
<i>amyP</i>	<i>P. paxilli</i> PaxP (AAK11528)	5e-97	Cytochrome P450 monooxygenase
<i>amyJ</i>	<i>E. festucae</i> LtmJ (AFO85405)	e-167	Cytochrome P450 monooxygenase
<i>amyE</i>	<i>E. festucae</i> LtmE (AFO85404)	5e-83	Prenyltransferase

4.3.2 Attempt to identify a compound governed by the cluster

I first searched for an indole diterpene-related compound in culture broth of *P. amygdali*. However, no production was suggested by LC-MS analysis. Since the *amy* genes were silent, I then tried activating the genes by the several methods.

4.3.2.1 Optimization of culture medium and conditions

I first tried activating the genes by varying the culture medium and conditions (160). After *P. amygdali* was cultivated with 17 different media (see method), I tried preparing cDNA. However, no cDNAs were obtained, suggesting that all the *amy* genes were not expressed under the conditions employed. I also analyzed the culture broth to investigate the production of indole diterpene-related compounds. However, no products were detected by HPLC and LC-ESI-MS analysis (data not shown).

4.3.2.2 Utilization of histone deacetylase-inhibitor or DNA methylation-inhibitor

To wake up fungal silent secondary metabolic pathways, small-molecule epigenetic modifiers such as DNA methyltransferase and histone deacetylase inhibitors were reported (48, 161-3) to be effective tools as described in General Introduction. Therefore, I applied this strategy to *P. amygdali*. However, no indole diterpene-related compounds were again detected (data not shown).

4.3.2.3 Activation of transcriptional regulators

Some gene clusters for secondary metabolites in fungi were reported to be control by transcription factors (51). In the *amy* gene cluster, a candidate gene, Zn2Cys6 gene, was located at the upper stream region. Then, I tried overexpressing the gene to activate the *amy* cluster. The Zn2Cys6 gene was amplified by PCR and inserted into the pSH75, in which the Zn2Cys6 could be expressed under the control of the *trpC* promoter and terminator of *Aspergillus. nidulans* (164). Then, compound produced by the transformants were examined by HPLC/LC-MS.

However, no indole diterpene related compounds were detected (data not shown).

4.3.2.4 Brief summary

I tried activating the silent gene cluster of *P. amygdali* by optimization of culture medium and conditions, adding histone deacetylase-inhibitors and DNA methylation-inhibitors into culture broth, and overexpression of the transcriptional regulator (Zn2Cys6). However, no indole diterpene-related compounds were detected by HPLC and LC-ESI-MS analysis.

4.3.3 cDNA preparation by heterologous expression in *Aspergillus oryzae*

Since I could not express the *amy* gene cluster, I then tried to prepare cDNAs by its heterologous expression in *A. oryzae*. The coding regions including intron sequences of *amyC*, *amyD*, *amyG* and *amyJ* genes were amplified by PCR with *P. amygdali* Niigata-2 (165) genomic DNA and the primer set as shown in Table 4.1. The amplified DNA fragments were inserted into the corresponding sites of the expression vector pTAex3 (166), to create pTAex3-*amyG*, pTAex3-*amyC*, and pTAex3-*amyD*, pTAex3-*amyJ*. Each of the plasmids was introduced into *A. oryzae* (64). Transformation and cDNA preparation were described in the materials and methods.

cDNA of all the 4 genes were successfully obtained. The predicted gene products of AmyG, AmyC, AmyD, and AmyJ were consisted of 339, 347, 457, and 530 amino acids, respectively. Most homologous enzymes to AmyG and AmyC were PaxG (32% amino acid identity) and PaxC (40%), respectively. Moreover, AmyJ and AmyD had high similarities to LtmJ (64% identity), a cytochrome P450 monooxygenase, and LtmE (58%), both of which were reported to be responsible for the lolitremanes formation (42). suggesting that the *amy* gene cluster would be responsible for indole diterpene biosynthesis.

4.3.4 Functional analysis of AmyG

The sequence analysis of *amyG* gene predicted the presence of seven introns (Fig 4.2), cDNA of *amyG* was composed of 1020 base sequence and encoding 339 amino acids. By clustalW analysis, AmyG was 31% identical to PaxG, 34% to both AtmG and LtmG (Table 4.3).

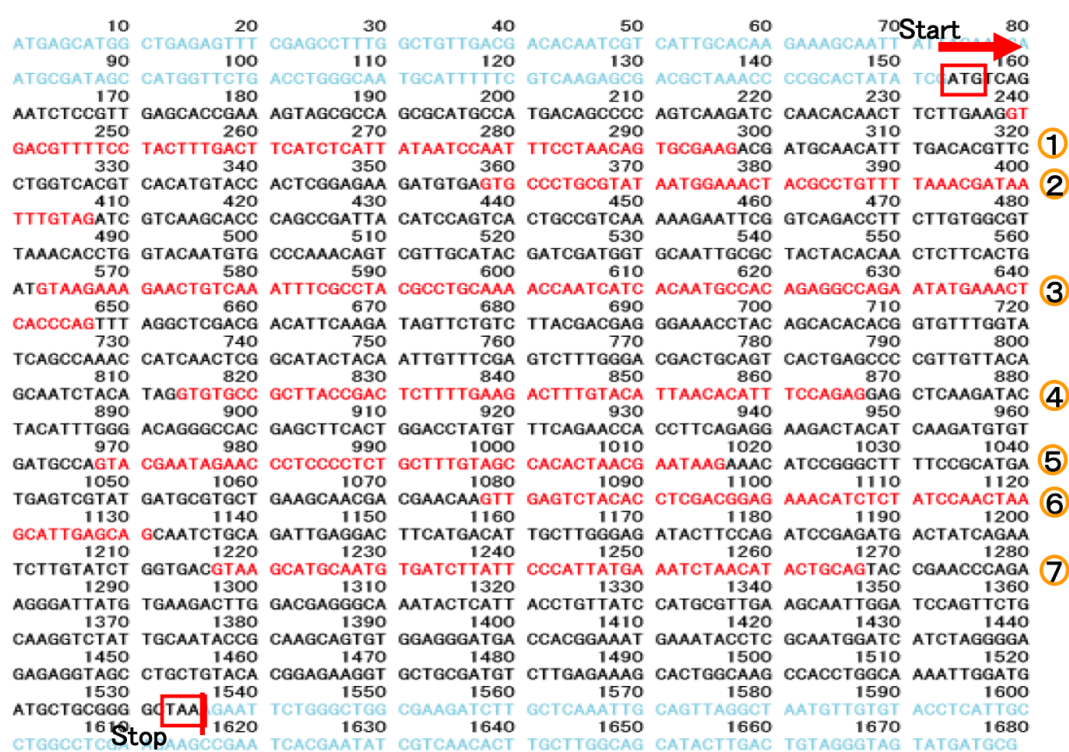


Figure 4.2 *amyG* nucleotide sequence including intron regions

Sequence identities / %

Enzyme	LtmG	AtmG	PaxG	AmyG
AmyG	34	34	31	100
PaxG	46	51	100	
AtmG	52	100		
LtmG	100			

Table 4.3 Sequence identities among AmyG/PaxG/AtmG/LtmG.

PaxG, *Penicillium paxilli* (GenBank: AAK11531); AtmG, *Aspergillus flavus* (AAT65717); LtmG, *Epichloe festucae* (AFO85411).

Alignment of amino acid sequences of AmyG, PaxG, AtmG and LtmG showed that all four enzymes possess the two aspartate-rich motifs, DDXXD and DDXXN/D (Fig 4.3), which are known to function as substrate binding with Mg^{2+} in GPP synthase (45).

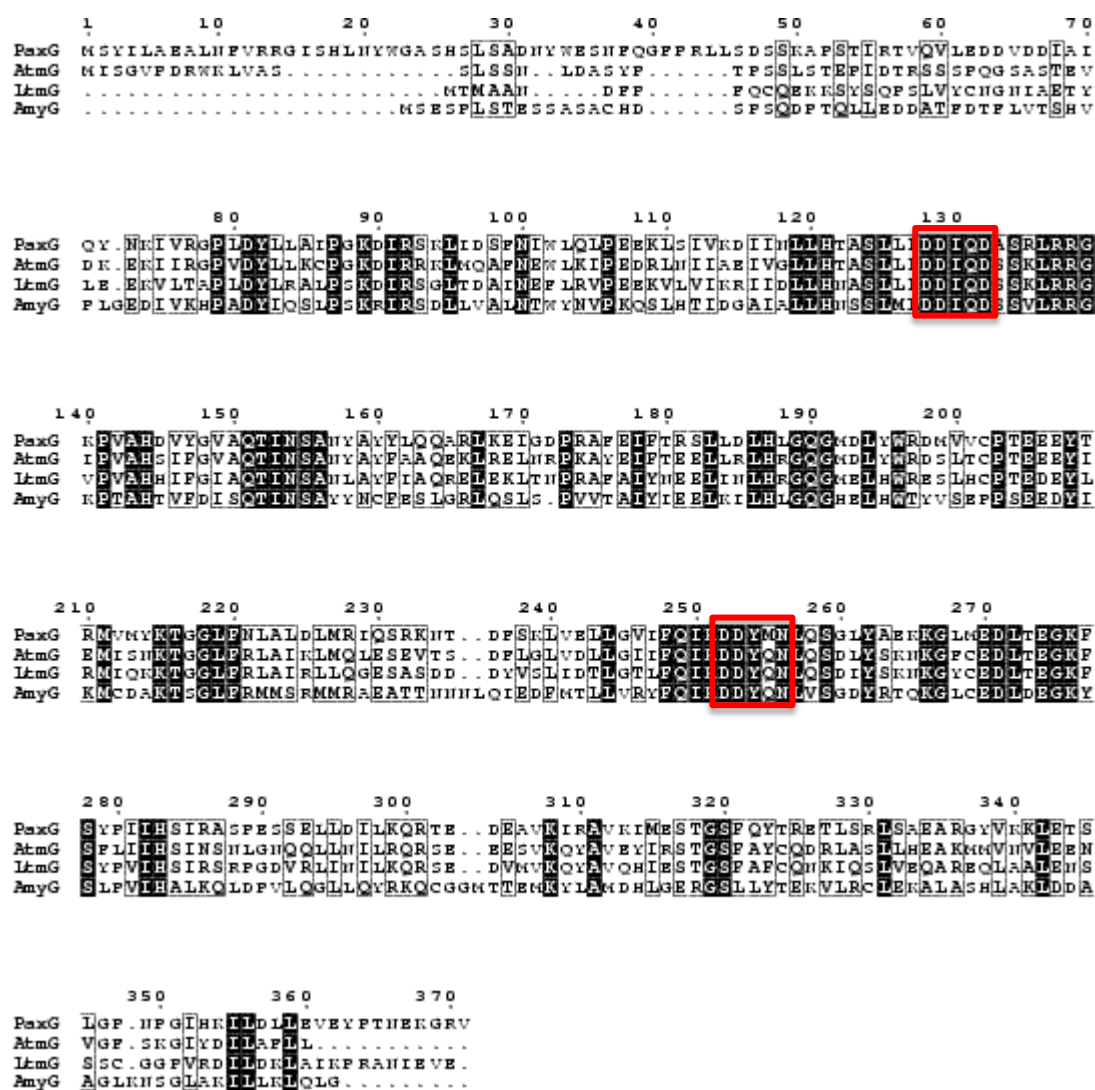


Figure 4.3 Alignment of amino acid sequences of AmyG, PaxG, AtmG and LtmG.

4.3.4.1 Overexpression and purification of AmyG

The cDNA carrying amyG gene was amplified by PCR using gene specific primers (Table 4.1) and introduced into pMAL-c2X. The recombinant protein was expressed as an *N*-terminal maltose binding protein (MBP)-fused protein in *E. coli* TB1 (NEB). The cell culture condition and protein purification were described in the materials and methods.

MBP-fused AmyG recombinant enzyme was analyzed by an SDS-PAGE on 10% gels. The recombinant enzyme was successfully expressed in a soluble form (Fig 4.4).

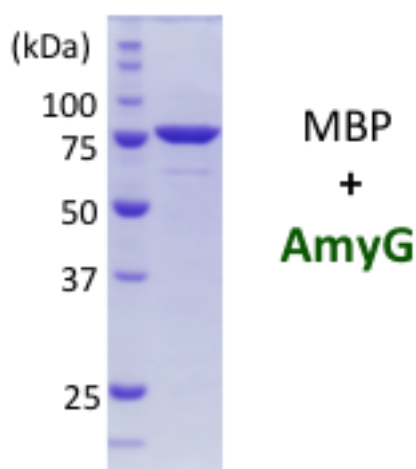


Figure 4.4 SDS-PAGE analysis of purified AmyG

4.3.4.2 In vitro assay of AmyG

After the recombinant AmyG was incubated with IPP and DMAPP, GDP, or FDP, the reaction product was analyzed by HPLC. When FDP and GDP were used, a peak, which eluted at the same retention time with that of standard GGDP was detected together with another peak, which was suggested to be farnesylgeranyl diphosphate (C25) by taking account of the retention times of FDP (C15) and GGDP as shown in Fig 4.5 A and B. However, DMAPP was not utilized (data not shown). Considering that amyG clustered with many orthologs of the paxilline biosynthetic genes (Fig 4.1 and Table 4.2) and that the chain length of the product generated by in vitro assay with GGDP synthase was known to vary depends on the reaction conditions (167), AmyG was suggested to be a GGDP synthase (Scheme 4.1).

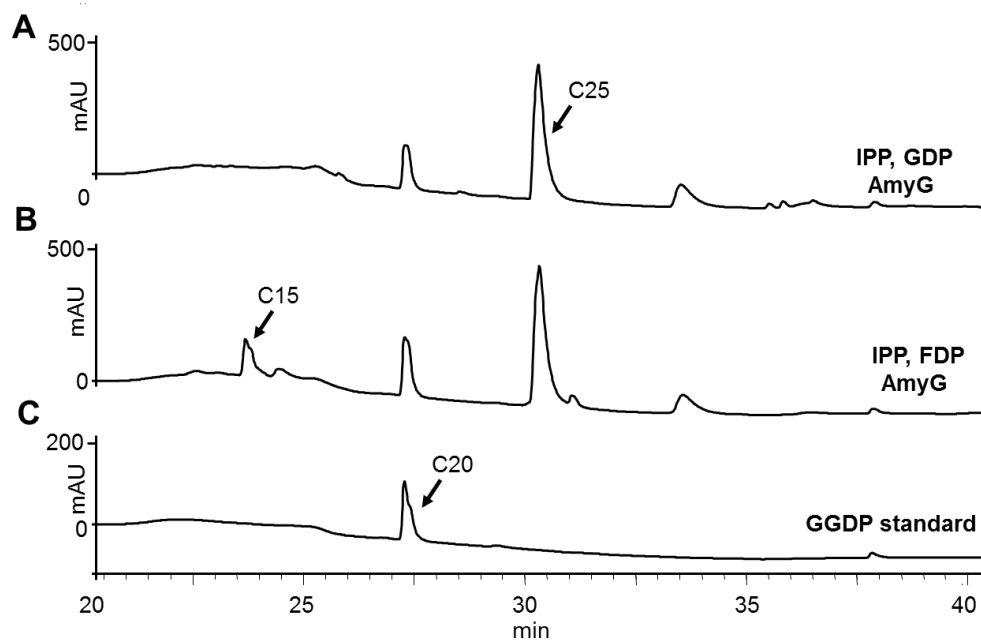
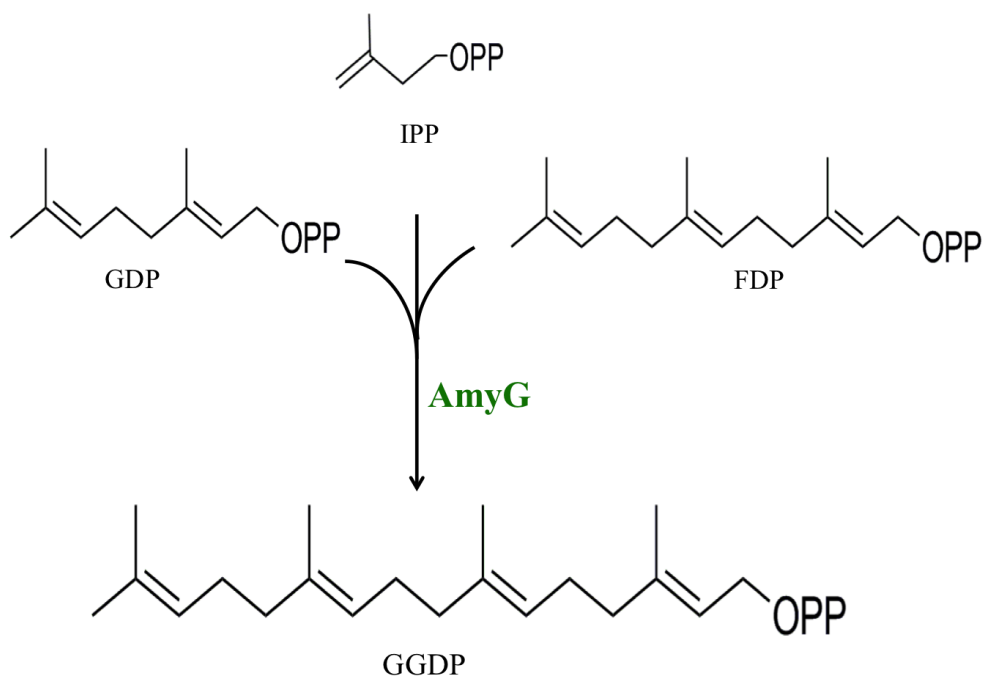


Figure 4.5 HPLC profiles of the AmyG reaction products;
A) with IPP and FDP; B) with IPP and GDP; C) GGDP standard.



Scheme 4.1 AmyG-catalyzed reaction.

4.3.5 Functional analysis of AmyC

The sequence analysis of AmyC predicted the presence of one intron (Fig 4.6), cDNA of *amyC* was composed of 1044 base sequence encoding 347 amino acids. By clustalW analysis, AmyC was 40% identical to PaxC, 42% to AtmC, and 49% to LtmC (Table



Figure 4.6 *amyC* nucleotide sequence including intron regions.

Sequence identities / %

	LtmC	AtmC	PaxC	AmyC
AmyC	49	42	40	100
PaxC	37	56	100	
AtmC	40	100		
LtmC	100			

Table 4.4 Sequence identities among AmyC/PaxC/AtmC/LtmC.

PaxC, *Penicillium paxilli* (GenBank: AAK11529); AtmC, *Aspergillus flavus* (AAT65718); LtmC, *Epichloe festucae* (AFO85409).

Since AmyC had a significant similarity to PaxC (Fig 4.7), which was confirmed to catalyze the transfer of GGD_P to indole-3-glycerol phosphate (IGP) in the chapter 1, I examined whether AmyC also would catalyze the same reaction.

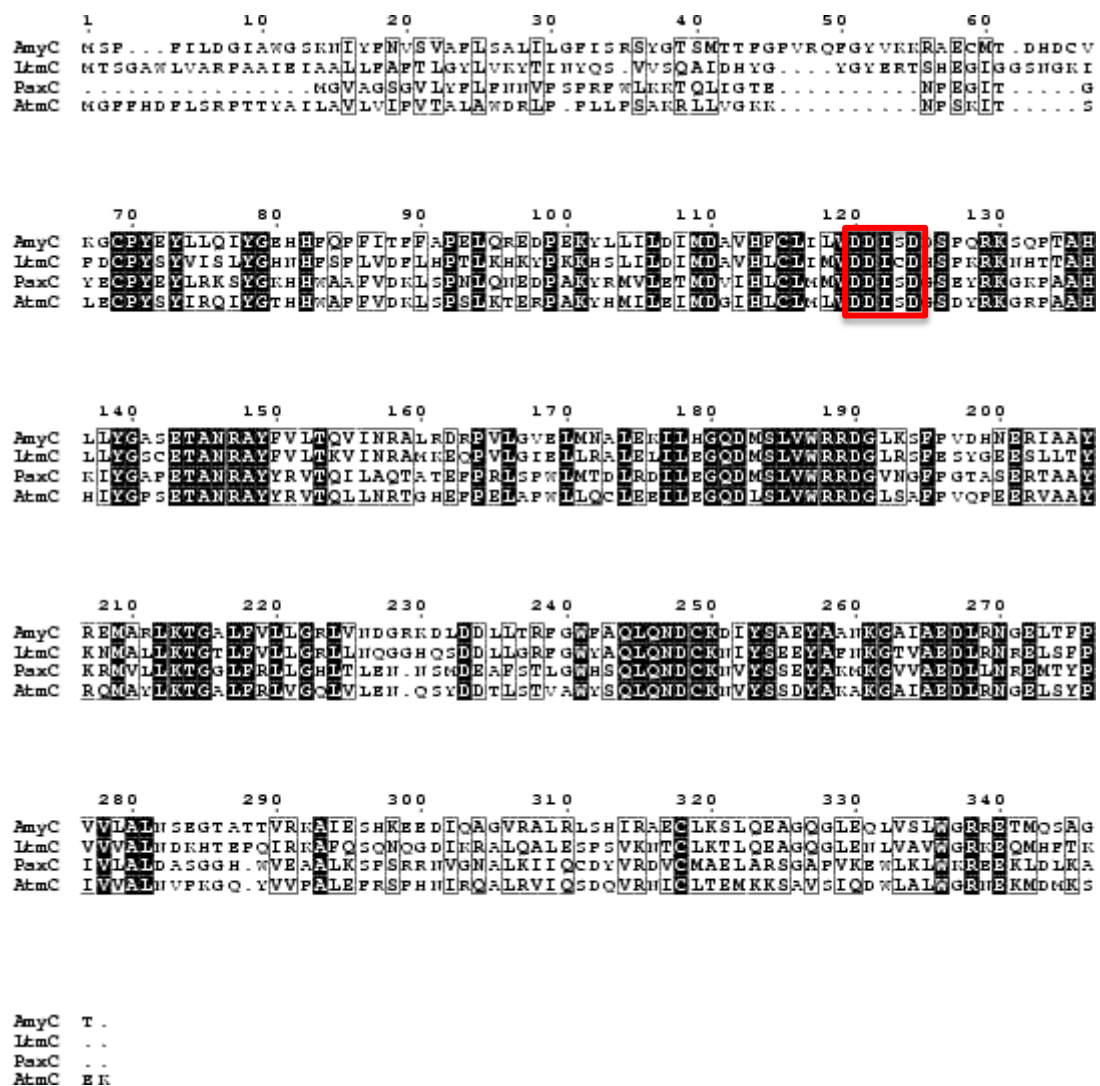


Figure 4.7 Alignment of amino acid sequences of AmyC PaxC, AtmC and LtmC.

4.3.5.1 Overexpression and purification of AmyC

The cDNA carrying amyC gene was amplified by PCR using gene-specific primers (Table 4.1) and introduced into pMAL-c2X, the recombinant protein was expressed in *E. coli* TB1. The recombinant AmyC enzyme was checked by SDS-PAGE (Fig 4.8).

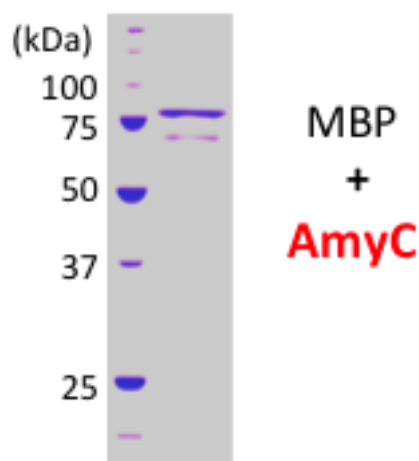


Figure 4.8 SDS-PAGE analysis of purified AmyC.

4.3.5.2 In vitro assay of AmyC

The AmyC was incubated with GGDP and indole, IGP or tryptophan. By HPLC analysis, one major product was specifically detected, which eluted at the same retention time with that of standard GGI when IGP and GGDP were used as the substrates. Moreover, a minor peak was also detected at the same retention time with indole and GGDP (Fig 4.9). In contrast, no product was formed with tryptophan (data not show). These results clearly showed that AmyC was a GGI synthase (Scheme 4.2) like PaxC. As mentioned in Chapter 1, PaxC catalyzed the transfer of FDP into IGP to yield farnesyl indole, but AmyC did not accept FDP.

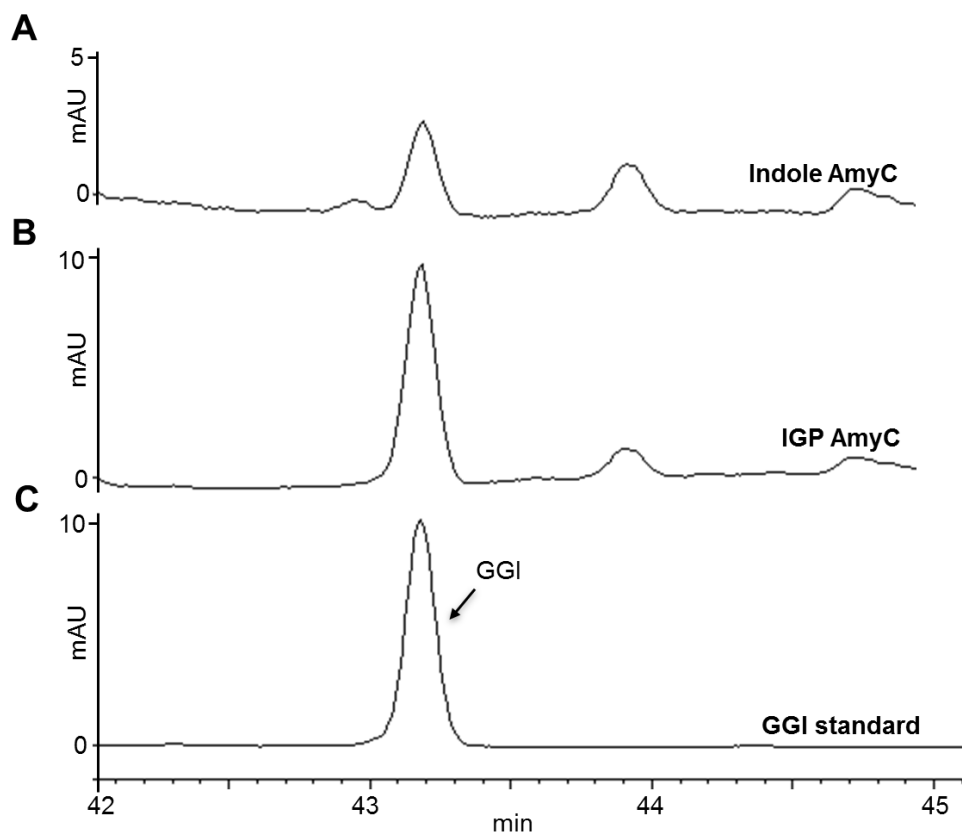
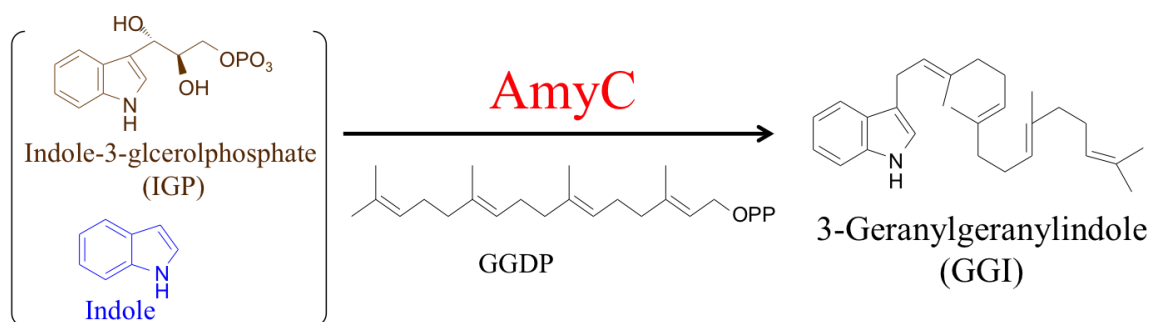


Figure 4.9 HPLC profiles of the AmyC reaction product;
A) with indole and GGDP; B) with IGP and GGDP; C) GGI standard.



Scheme 4.2 AmyC-catalyzed reaction.

4.3.6 Functional analysis of AmyD

The sequence analysis of AmyD predicted the presence of two introns (Fig 4.10). The cDNA was composed of 1374 base sequence encoding 457 amino acids. By clustalW analysis, AmyD was 13% identical to PaxD, 17% to AtmD, and 58% to LtmE (Table



4.3.6.1 Overexpression and purification of AmyD

The cDNA carrying amyD gene was amplified by PCR using gene-specific primers (Table 4.1) and introduced into pMAL-c5X, the recombinant protein was expressed in *E. coli* TB1. After elution of the recombinant enzymes, purity was analyzed by an SDS-PAGE on 10% gels. The recombinant enzyme was expressed in a soluble form (Fig 4.11)

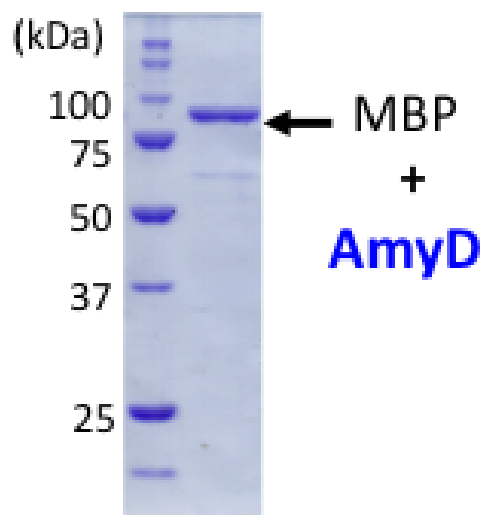


Figure 4.11 SDS-PAGE analysis of purified AmyD.

For the molecular mass and subunit structure determination of the enzyme was analysis by gel filtration chromatography. The result is shown in Fig 4.12, a peak with a calculated molecular mass of 104 kDa was detected, suggesting that AmyD forms a

monomer structure different from the recombinant PaxD and AtmD, both of which showed homo-dimer structures.

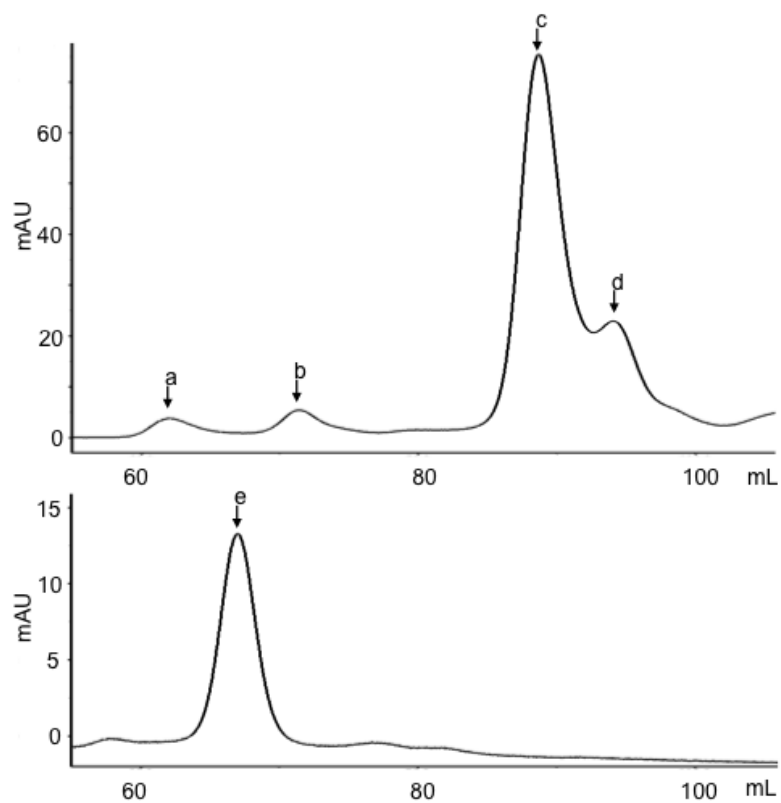
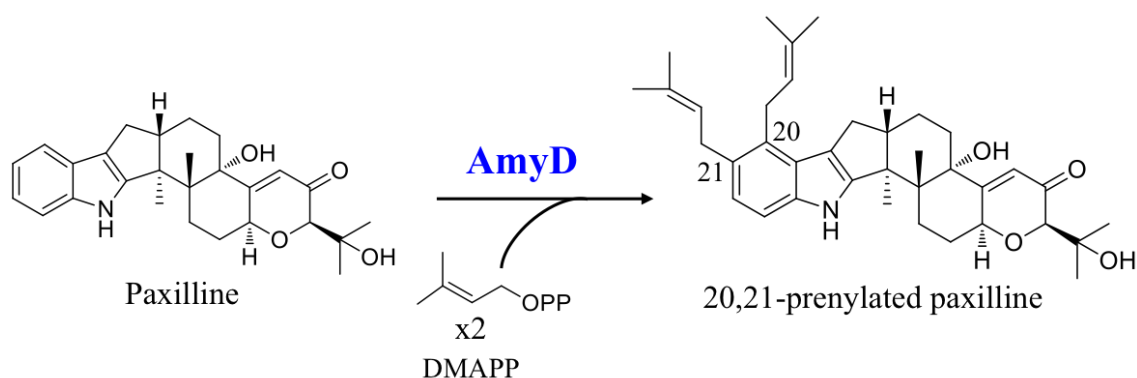


Figure 4.12 Gel filtration chromatography of AmyD. Lane 1 molecular mass markers: elution profiles of the standard proteins [aldolase (a, 158 kDa), albumin (b, 67 kDa), chymotrypsinogen A (c, 25 kDa), and ribonuclease A (d, 13.7kDa); top] Lane 2: purified AmyD (bottom, e)

4.3.6.2 In vitro assay of AmyD

Since *amy* gene cluster is highly similar to paxilline biosynthesis gene cluster, I thought that AmyD might catalyze a prenylation of paxilline. The purified recombinant AmyD was therefore incubated with paxilline and DMAPP. By HPLC analysis, a major product was specifically detected (Fig 4.13A). Selected ion chromatograms obtained by LC/ESI-MS analysis showed the specific peak with molecular masses corresponding to di-prenylated paxilline (Fig 4.13 C, D) (Scheme 4.3).



Scheme 4.3 AmyD-catalyzed reaction with paxilline and DMAPP.

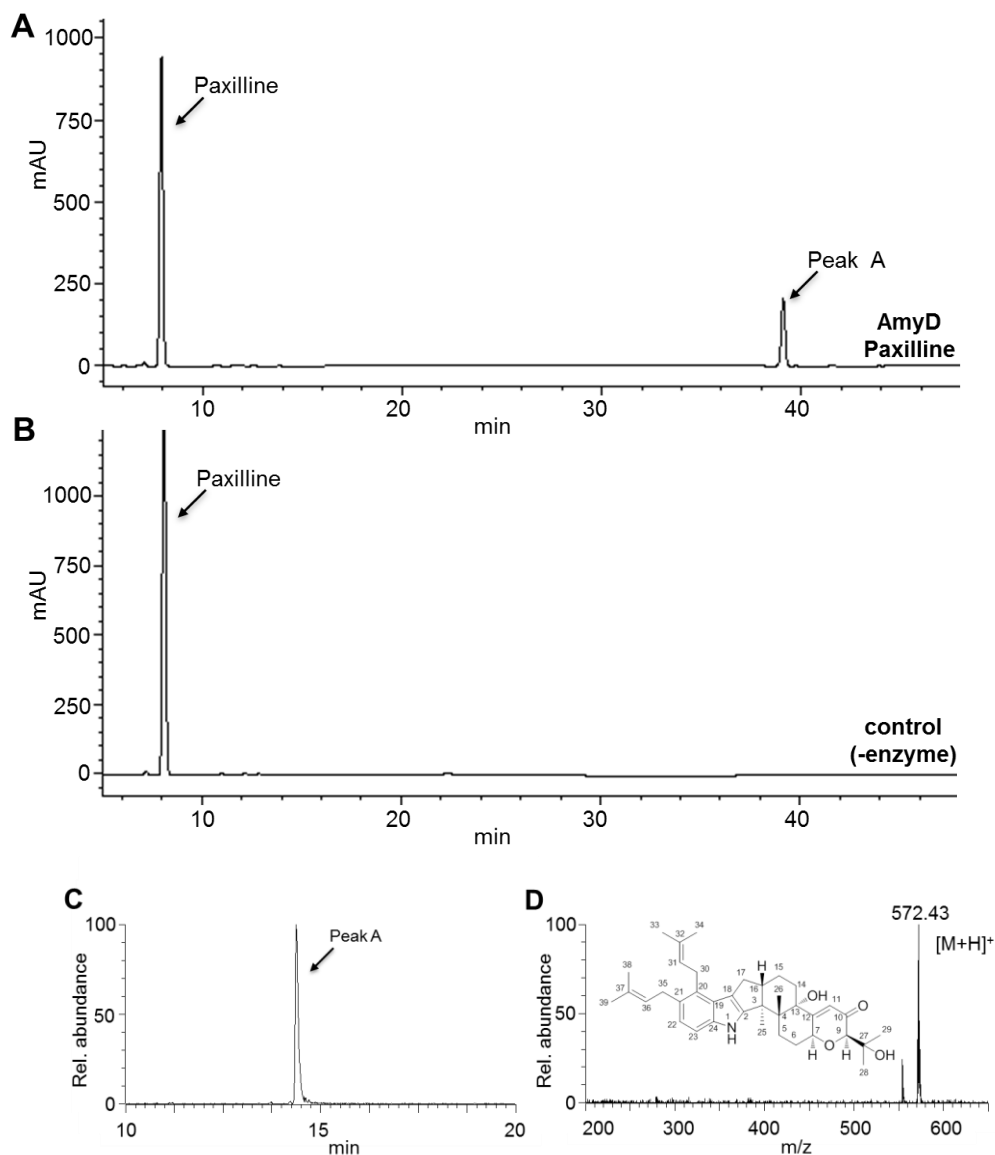


Figure 4.13 HPLC and LC/ESI-MS analysis of reaction products formed from paxilline and DMAPP by AmyD. The reaction products formed with (A, C) and without (B) AmyD were analyzed by HPLC (A, B) and LC/ESI-MS (C, D). Selected ion chromatograms (C) and spectra of the peak (D) are shown.

4.3.6.3 NMR analysis of reaction product formed from paxilline and DMAPP by AmyD

The exact structure of the major product (Peak A) was analyzed. The ^1H -NMR spectra showed the characteristic signals for the regular prenyl moieties [two olefinic protons: 5.18 (m, 1H), 5.27 ppm (m, 1H); two allylic methylene protons; 3.61 (m, 2H), 3.36 (d, $J = 6.8$ Hz, 2H)] and for the 4,5-disubstituted indole signals [aromatic protons: 6.91 (d, $J = 8.3$ Hz, 1H), 7.09 ppm (d, $J = 8.3$ Hz, 1H)], indicating the substitution of the prenyl moieties at the C-20 and C-21 positions on the indole moiety. Finally, extensive NMR data analysis, including hetero-nuclear single quantum coherence (HSQC), hetero-nuclear multiple quantum coherence (HMBC), correlation spectroscopy (COSY), and nuclear overhauser effect spectroscopy (NOESY) proved that the structure was 20,21-di-prenylated paxilline (Fig 4.14).

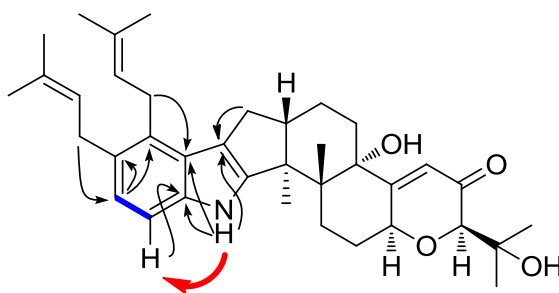


Figure 4.14 Key HMBC, H-H COSY, and NOESY correlations of 20,21-prenylated paxilline.

Black arrow: long-range couplings obtained from HMBC, blue line: H-H coupling obtained from H-H COSY, and red arrow: NOE obtained from NOESY experiments.

4.3.6.4 Biochemical characterization of AmyD

4.3.6.4.1 Temperature effect

The biochemical properties of the enzyme were investigated using paxilline and DMAPP as the substrates. The assay was conducted at a temperature range from 20 to 60°C.

The optimal temperature was 55°C (Fig 4.15)

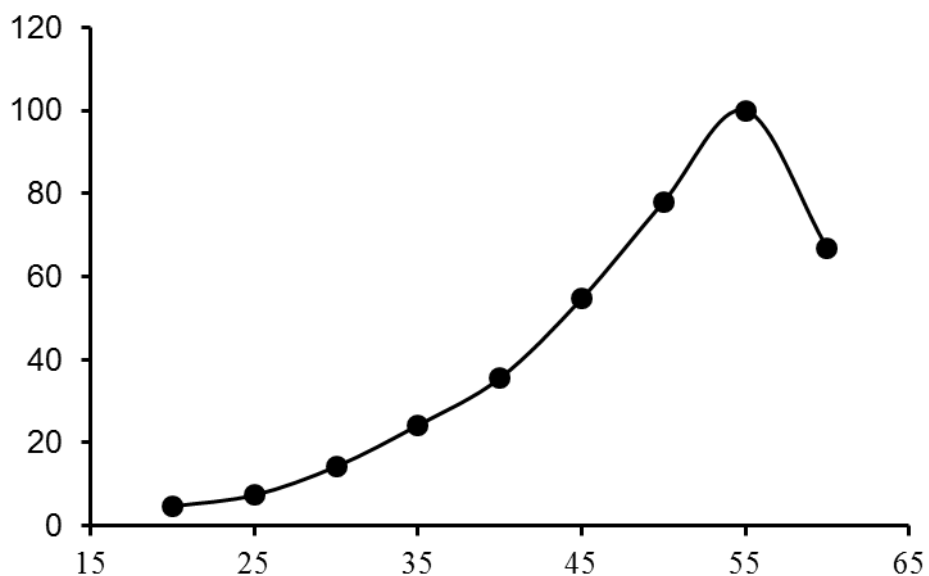


Figure 4.15 Effects of temperature on AmyD activity. The assay mixture contained, in a final volume of 100 μ L, 0.25 mM of paxilline, 0.5 mM of DMAPP, 50 mM Tris-HCl (pH 8.0), and 0.2 μ g of AmyD. This mixture was incubated at each temperature for 20 min.

4.3.6.4.2 pH effect

The pH effect of the enzyme was investigated using paxilline and DMAPP as the substrates at different pH range from 5.0 to 10, employing a mixed buffer system with citrate buffer (pH 5.0~6.0), MES buffer (pH 6.0~7.0), Tris-HCl buffer (pH 7.0~9.0) and borate-NaOH buffer (pH 9.0~10.0).

The optimal pH was 7.0 (Fig 4.16)

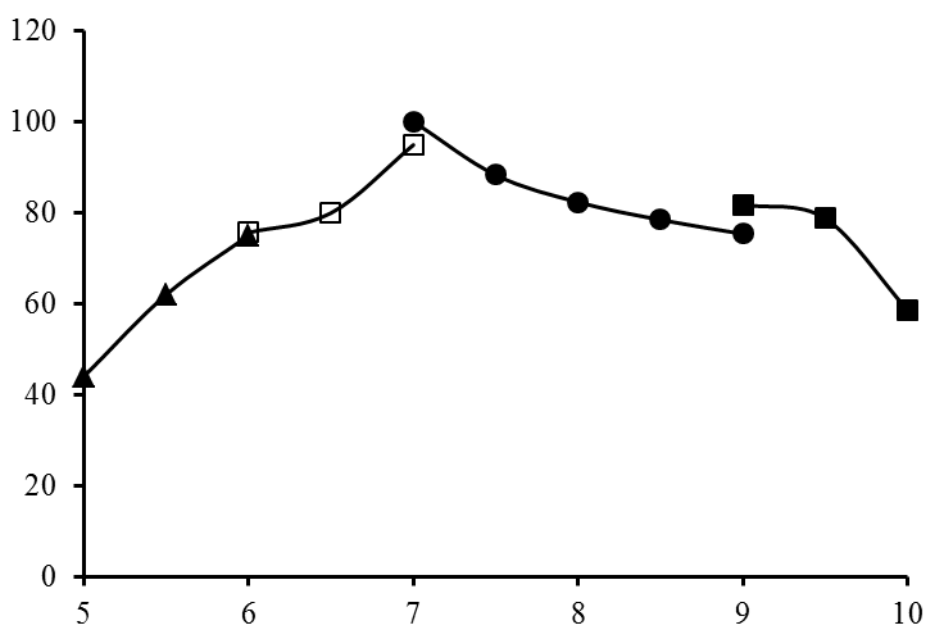


Figure 4.16 Effects of pH on AmyD activity. The assay was conducted at a pH range from 5.0 to 10, with 50 mM citrate (solid triangles), 50 mM MES (open squares), 50 mM Tris-HCl (solid circles), and 50 mM borate-NaOH buffer (solid squares). The mixture was incubated at 30°C for 20 min.

4.3.6.4.3 Metal dependency of AmyD

Since some prokaryotic prenyltransferases require divalent cations for the activity, the effect and dependence of divalent metal ions on AmyD activity were tested. The addition of Mg^{2+} and Ca^{2+} enhanced the enzymatic activity in contrast to EDTA, which decreased the activity to less than 50% of the control experiment. The activity was lost when Cu^{2+} and Zn^{2+} were added (Fig 4.17).

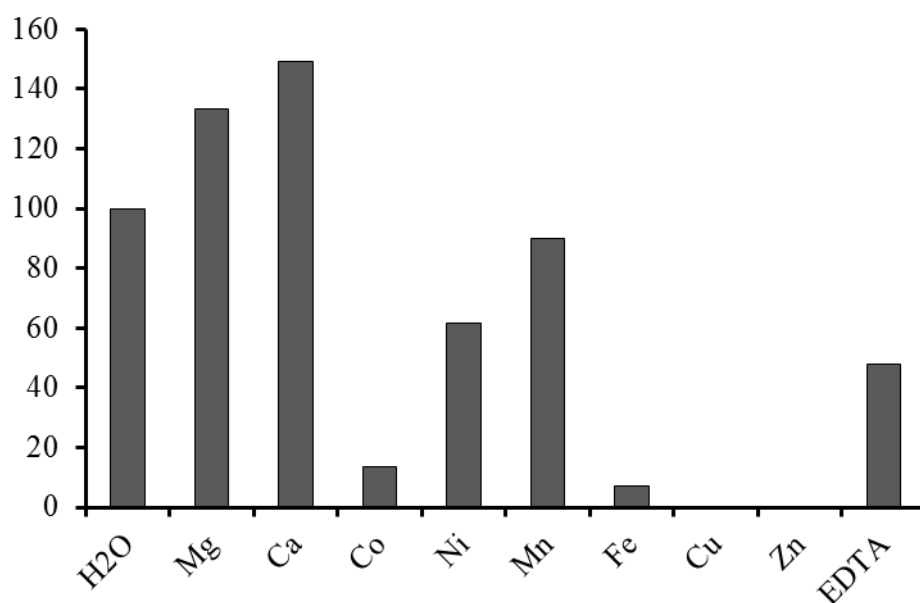


Figure 4.17 Effects of divalent metal ions on AmyD activity. Each of divalent cation (5 mM), or 5 mM EDTA was added to the reaction mixture. The mixture was incubated at 30°C for 20 min.

4.3.6.4.4 Kinetic parameters

The steady-state kinetic parameters of AmyD were determined by fitting to the Michaelis-Menten equation. The enzyme reaction followed Michaelis-Menten kinetics. By using Hanes-Woolf plots, the K_m values were calculated as $7.6 \pm 0.5 \mu\text{M}$ for paxilline and $17.9 \pm 1.7 \mu\text{M}$ for DMAPP. The k_{cat} value was calculated as $0.12 \pm 0.003/\text{sec}$ (Fig 4.18).

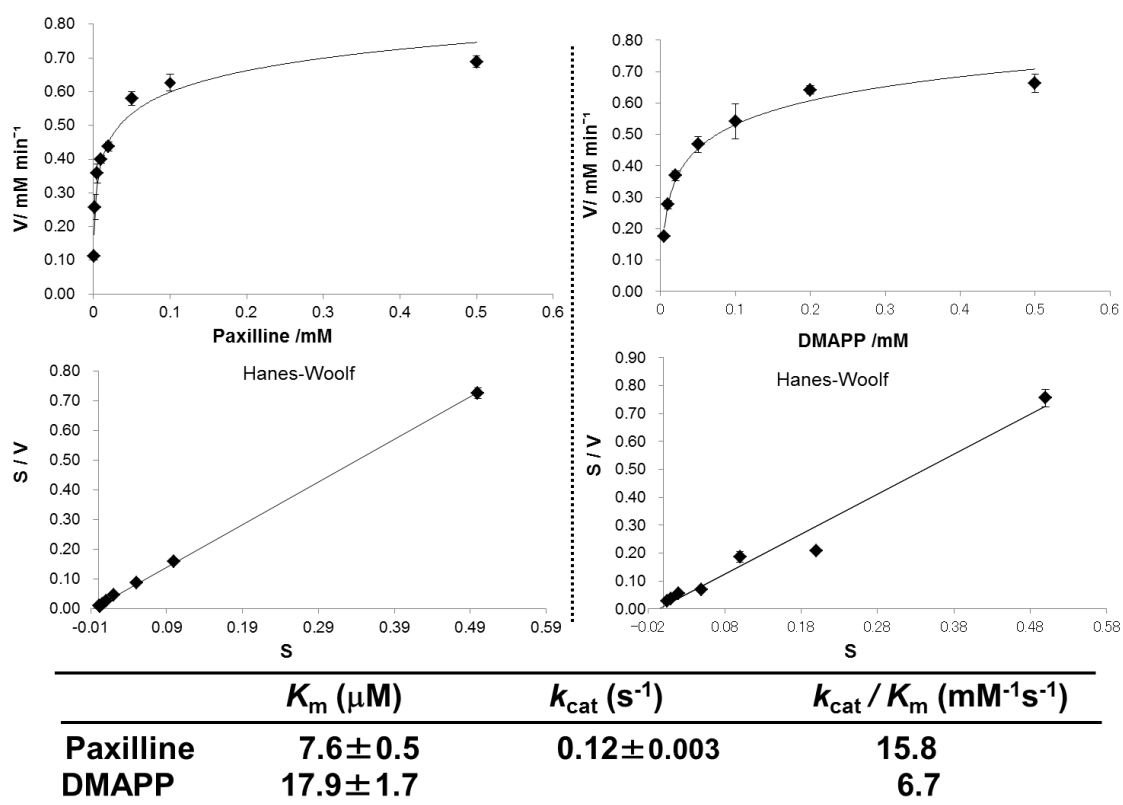


Figure 4.18 Hanes-Woolf plots of AmyD for paxilline and DMAPP.

4.3.6.5 Substrate specificity of AmyD

The substrate specificity of AmyD was investigated with the same compounds used for AtmD analysis (see 3.2.3). Of the compounds, paspaline (Fig 4.19), FI (Fig. 4.22) and GGI (Fig 4.23) were suggested to be prenylated by LC-ESI-MS analysis.

4.3.6.5.1 Analysis of the reaction product formed from paspaline and DMAPP by AmyD.

Reaction product formed from paspaline and DMAPP by AmyD was analyzed by HPLC and three small peaks were detected (Fig 4.19).

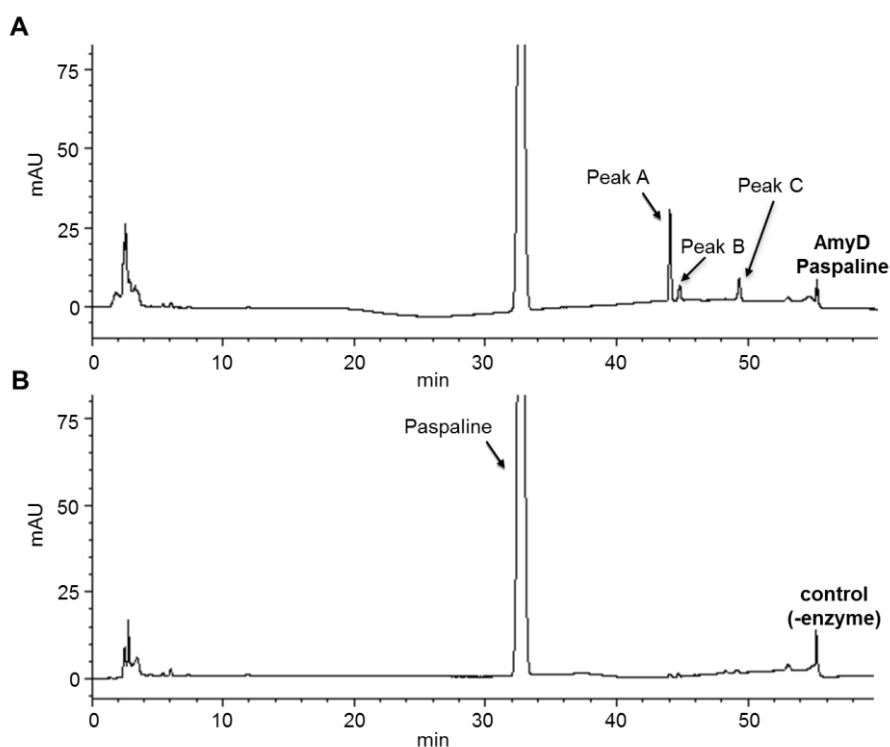


Figure 4.19 HPLC analysis of reaction products formed from paspaline and DMAPP with (A) and without (B) AmyD.

To estimate the structure of the prenylated paspaline by AmyD, I analyzed the compounds by LC/ESI-MS with Waters ACQUITY UPLC BEH C18 1.7 μm column. Three peaks were detected and one was suggested to be a di-prenylated paspaline (Fig 4.20 Peak C) and the other two peaks were mono-prenylated paspalines (Fig 4.20 Peak A and B). However, I could not determine the structures of these compounds because of their low yields.

Although I could not determine the structure formed from paspaline and DMAPP by AymD reaction, the retention time of one of the mono-prenylated paspaline (peak B in Fig 4.19A) was the same as that of the 21- or 22- mono-prenylated paspaline (both compounds were eluted at the same retention time with a conventional reverse-phase column), which were formed from the same substrate by both AtmD and PaxD as described in Chapter 3. Interestingly, the product formed by AmyD was also found to be a mixture of the two compounds with the XBridgeTM Phenyl Column analysis (Fig 4.21). Since I did not access the standard compound of the 20-mono-prenylated paspaline, I could not examine the retention time of the compound with the XBridgeTM Phenyl Column analysis. Considering that AmyD catalyzed the prenylation at the 20 and 21-positions rather than the 21 and 22-positions, the prenylation at the 20-position was also undeniable. Taking these results together, AmyD was suggested to catalyze the prenylation at the 20, 21, or 22-position of paspaline (Scheme 4.4).

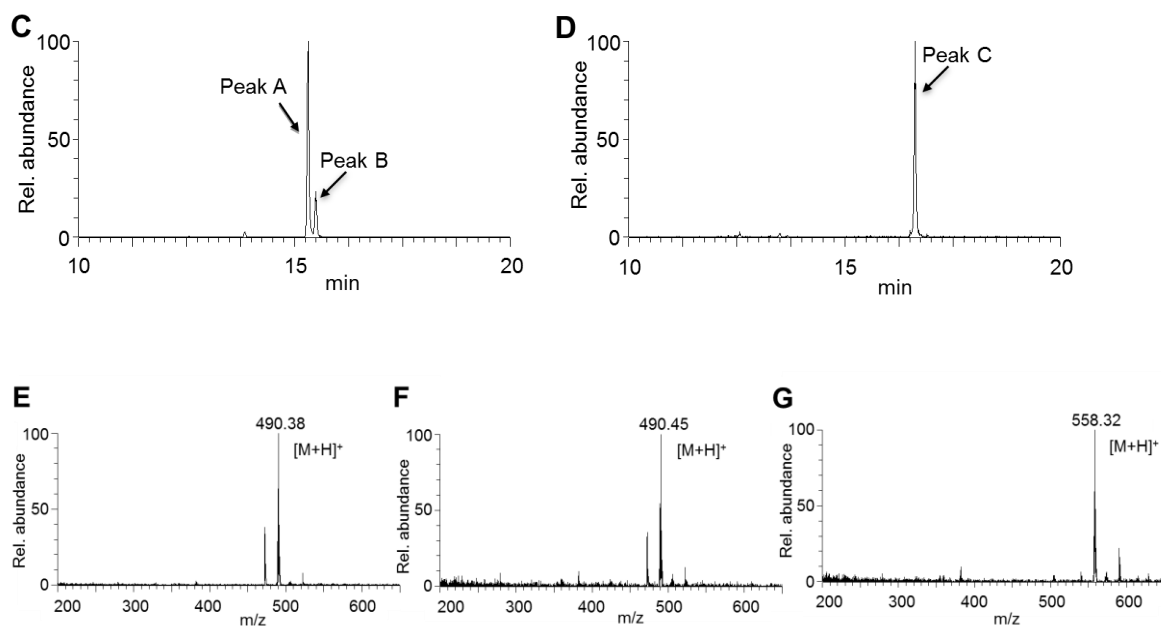


Figure 4.20 LC/ESI-MS analysis of reaction products formed from paspaline and DMAP by AmyD.

Selected ion chromatograms: C, m/z 490 $[M+H]^+$ corresponding to mono-prenylated paspaline; D, m/z 558 $[M+H]^+$ corresponding to di-prenylated paspaline; and mass spectra of peak A (E), peak B (F), and peak C (G) are shown.

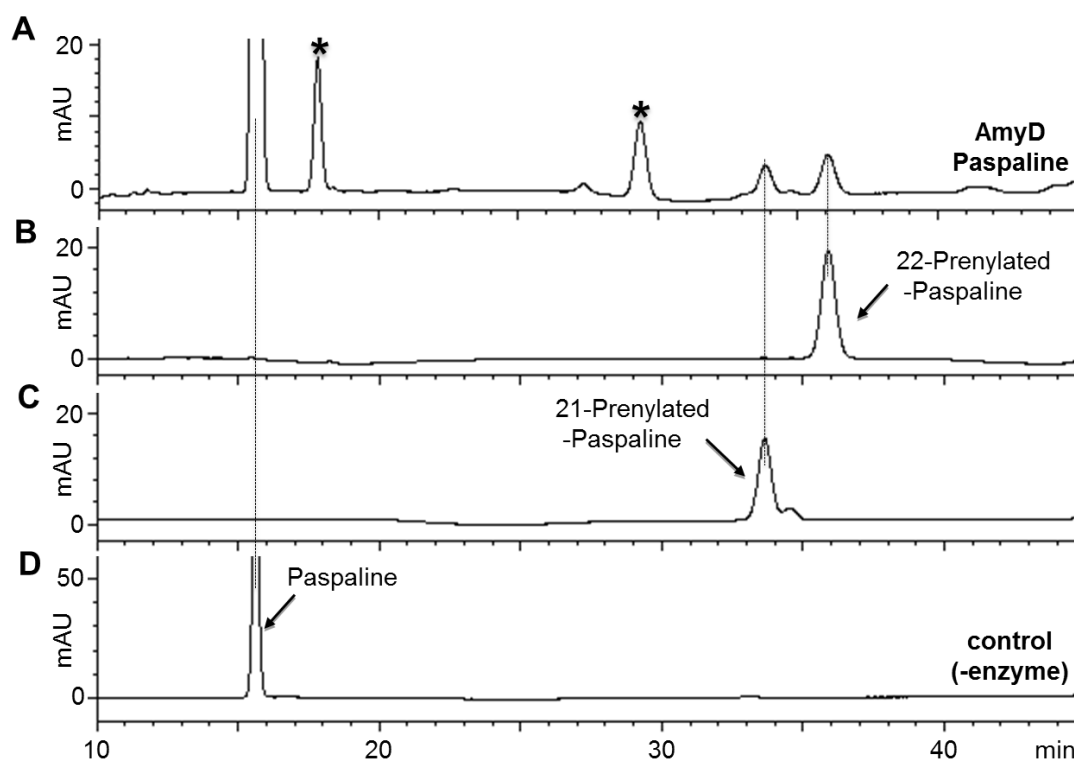
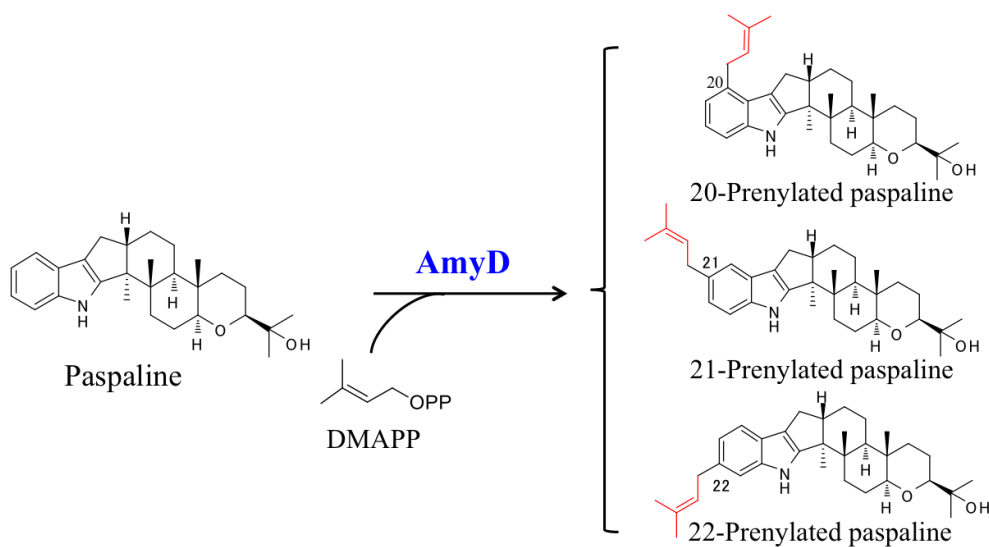


Figure 4.21 HPLC analysis of the closely related products formed from paspaline and DMAPP by AmyD with XBridgeTM Phenyl Column.

The reaction products formed with (A) and without (D) AmyD were analyzed by HPLC. Two standard compounds, 22-prenylated paspaline (B) and 21-prenylated paspaline (C), were also analyzed. Asterisks indicate unknown products.

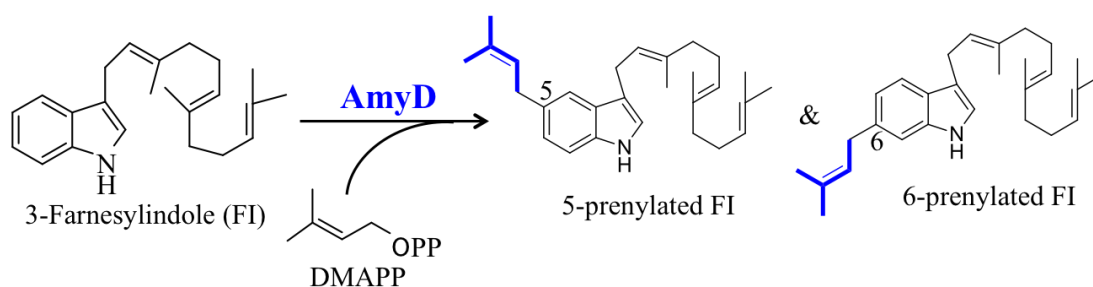


Scheme 4.4 Predicted reactions catalyzed by AmyD with paspaline and DMAPP.

4.3.6.5.2 Analysis of the reaction product formed from FI and DMAPP by AmyD.

Because PaxD and AtmD accepted farnesy indole (FI) to give 5 or 6-prenylated FI, I examined whether AmyD also catalyzed the same reaction.

By HPLC analysis, one specific peak was detected and its retention time was the same as that of the 5- or 6- mono-prenylated FI (Fig 4.22A), which were shown to be formed from the same substrate by both AtmD and PaxD. Moreover, by LC/ESI-MS analysis (Fig 4.22B), it was suggested to be 5- or 6- mono-prenylated FI (Scheme 4.5).



Scheme 4.5 Predicted reactions catalyzed by AmyD with FI and DMAPP.

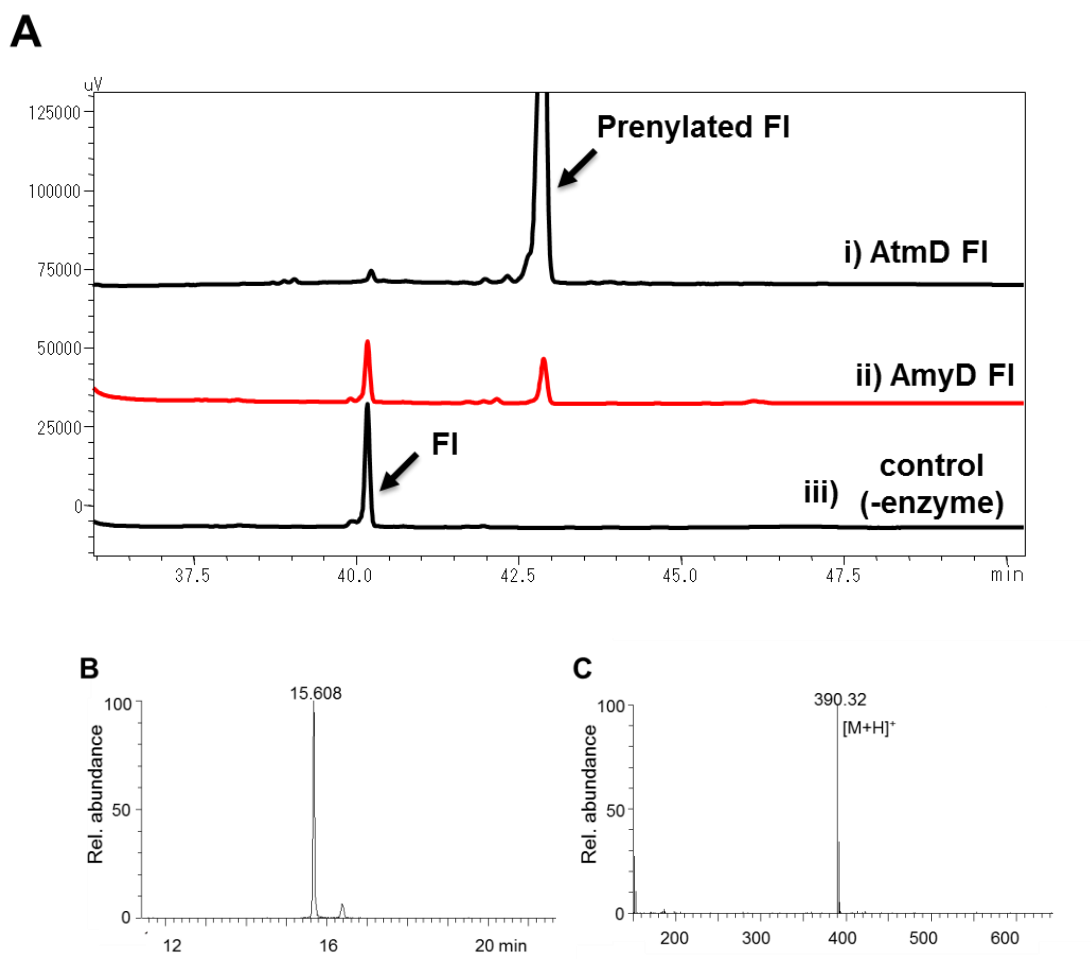


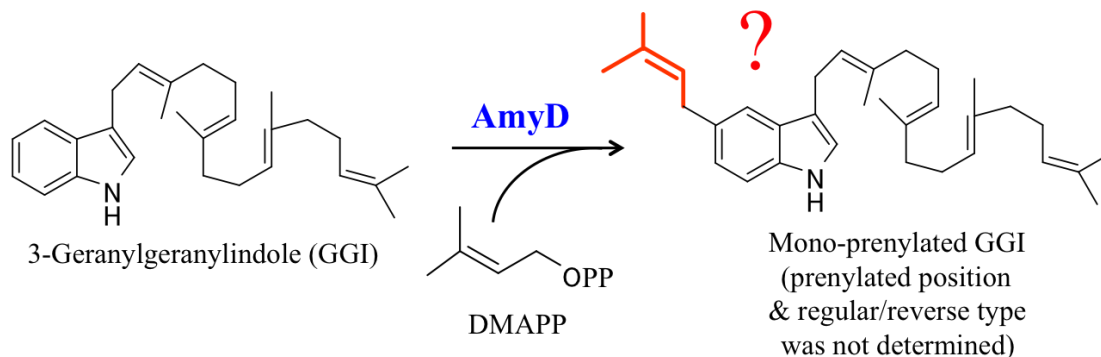
Figure 4.22 HPLC and LC/ESI-MS analysis of reaction products formed from FI and DMAPP by AtmD and AmyD.

(A), HPLC analysis of the reaction products formed from FI and DMAPP with AtmD (i), with AmyD (ii), and without enzyme (iii).

(B, C), LC/ESI-MS analysis by selected ion chromatograms (m/z 390 $[M+H]^+$) (B) and mass spectra of the products formed by AmyD (C).

4.3.6.5.3 Analysis of the reaction GGI and DMAPP by AmyD.

Because AtmD accepted GGI as the substrate to give mono-prenylated GGI, I used the same substrate for AmyD assay. The reaction and analytical conditions were the same as those of AtmD. By HPLC analysis (Fig 4.23A), one small specific peak was detected and the retention time was the same as that of AtmD product. I could not determine the structure of the compound because of the very low yield. However, by LC/ESI-MS analysis, it was suggested to be mono-prenylated GGI (Fig 4.23B, Scheme 4.6).



Scheme 4.6 predicted reaction catalyzed by AmyD with GGI and DMAPP.

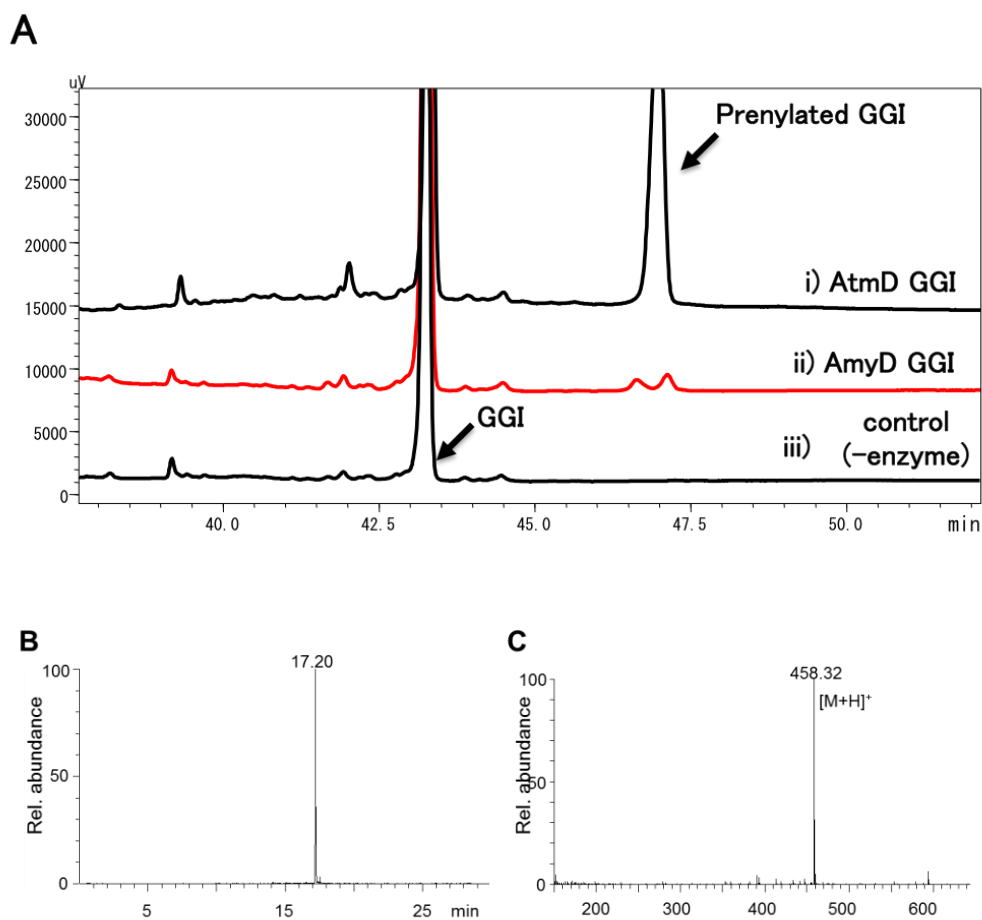


Figure 4.23 HPLC and LC/ESI-MS analysis of reaction products formed from GGI and DMAPP by AtmD and AmyD.

(A), HPLC analysis of the reaction products formed from GGI and DMAPP with AtmD (i), with AmyD (ii), and without enzyme (iii).

(B, C), LC/ESI-MS analysis by selected ion chromatograms (m/z 458 [M+H]⁺) (B) and mass spectra of the products formed by AmyD (C).

4.3.7 Functional analysis of AmyJ

The sequence analysis of *amyJ* predicted the presence of six introns (Fig 4.24). cDNA of *amyJ* consisted of 1593 base sequence encoding 530 amino acids. The most homologous enzyme to AmyJ was LtmJ (64% amino acid identity) (Fig 4.25), a cytochrome P450 monooxygenase, which is located in the lolitrem biosynthetic gene cluster in the grass endosymbiont *Epichloë festucae*. Scott et al examined the function of LtmJ gene by constructing a gene-deletion mutant and suggested that LtmJ catalyzed di-prenylation of A-ring of terpendole I, a paxilline-related compound (42).

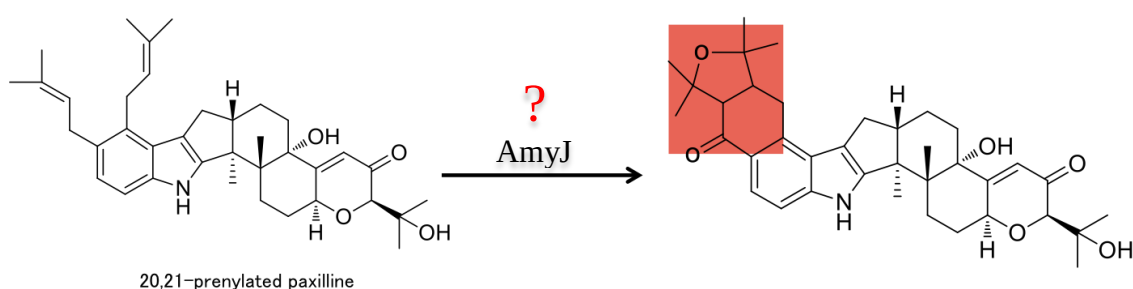
10	20	30	40	50	60	70	80	90	100
ATGGCTTTGG	ATCAACTTCT	ACTTCGGAAC	TGGGCGCTCG	AGCTGGATCA	TCTCGACCAG	CTTCATTGGG	CTCATGTCCT	CCTGGCGCTC	TTTGTATCT
110	120	130	140	150	160	170	180	190	200
GGACGATGTT	GTCCTGGATG	TCAGGGCCCA	CCGGCCCCAA	GGCATCTTTC	GTCGGTITTC	GTTTCCCGCT	TGAGCCCAAG	TTTTTGGTTC	GCATGCGTTT
210	220	230	240	250	260	270	280	290	300
CGTGAATCAA	GGCTTGAAGA	TGATGACTGA	GGGATACGAC	ACGGTAAGTC	TTGAAACCCA	TTTCTTTTGC	TTTCTCCATT	TTTAAAGAA	AGAAAAGTGA
310	320	330	340	350	360	370	380	390	400
AAAGAGAAGA	CGAAGTTTGG	AAACCCCTTG	TGACGATTCT	CGATACTACG	TATGCATCAG	TGCAAGAAGT	CCATGTTCAA	AATCACAACC	CACGATGCCG
410	420	430	440	450	460	470	480	490	500
ACTGGCTGGT	GGTCTCACAA	CGCTATCTTG	GGGATTGCA	GTCTCTCCCA	GCGGAGAGAT	TGAGCCACAC	AGATGCTTTA	GTTACTGTAG	GTAAGACTA
510	520	530	540	550	560	570	580	590	600
AATAACAAC	AACAATAGCA	ACCTTAAGAG	TCTGCGCTCG	GCTTACATAC	AGGCTCTGGC	TTTTCAAAA	TATAGATGTG	GGGCAGCAGC	CACGGACCGT
610	620	630	640	650	660	670	680	690	700
TTGCCCTGCT	TAACAAAAGC	GATCTCGGCT	CACGATCTCT	CGGTGTAAGC	GGGGAACCTT	GTCTTACATC	CAAGTGTGTG	TGAATACGGG	CCTAATGGAT
710	720	730	740	750	760	770	780	790	800
ATTTCAGGA	TGTGCTCGCC	CCAACTACG	TTAAGGACCT	GAATGGACTT	GCCGAAGAGC	TTCAGTATTC	GTTAGACCAC	GATATCCCTG	ATTACAACGG
810	820	830	840	850	860	870	880	890	900
TATGTCCTTA	TTGATGGGAT	TCACCTTTCA	GGGCGCTCTG	CGCATGTGCG	TCATTTTGAA	GGAAGAAGCTT	ACATGATATC	TGCAGATTGG	TCGACTGTTG
910	920	930	940	950	960	970	980	990	1000
ATGTGCTGGC	GCTGAGCTTC	AAACTGGTGC	TGCGTATCTC	ACAGCGGATC	CTCATCGGTG	AGCCAATGAG	TCGTGACGAA	GGGCTCCTCG	ACTGCGCACA
1010	1020	1030	1040	1050	1060	1070	1080	1090	1100
AGGCTACGCA	GATGCTGTA	AGAGCCAGAC	ACTCCTCCTC	CCTCCTCATG	AGAAAAGTAT	TCCTTTACCG	CCGTGCTAAG	CATTTCGCAC	ATCTCTGGGG
1110	1120	1130	1140	1150	1160	1170	1180	1190	1200
AGTAGCAACG	GTGGTGCACT	TCACCCTCAA	GCCGCTCCCA	CGTCTGCTCC	GCCCTCTTAT	ATACCATCTG	CTCCAGCCT	CAAGGCACGT	CAAGGCATTG
1210	1220	1230	1240	1250	1260	1270	1280	1290	1300
ATCCGCAAGA	CCAATAAGAT	TCTGGCCAAG	GAGCTCCAGC	GCCGCCGCCA	CCTGGAACAG	ACCGACCCAA	GCTACAAGAA	GCCCAAGGAC	CTGCTGCAGG
1310	1320	1330	1340	1350	1360	1370	1380	1390	1400
GCATGGTGGG	GCTGGATCCG	TGCCGATCCG	ACGACCAGTT	CGGTCTGATG	TTCTCTGATC	AGGCGCTAAT	CTCCCGCATG	GCCCGGTGG	TAACCATGGC
1410	1420	1430	1440	1450	1460	1470	1480	1490	1500
ACAAGCCCTG	ATAGACCTTT	CTCTTCAACC	GGAAGCTATC	GATGAGTTAC	GTAAGGAGAT	TGTGCAGGTG	ATGGGACAGA	ACGGCGAAGG	GCTGTGAGAT
1510	1520	1530	1540	1550	1560	1570	1580	1590	1600
TTACGGAAGT	CTCTGGCGAA	GCTCGATAAG	ATGGATAGCT	TCATCAGGGA	GTCGGCGAGG	ATGACTCCTC	TGTCCATGAG	TAAGTGTGTTG	ACAAATCTCT
1610	1620	1630	1640	1650	1660	1670	1680	1690	1700
ATCTGTGTCT	CTCCCATTCG	CGTTTTCCCT	TCTTTGTTTT	CTTTTTTTTG	AATCTAATCA	AGACTCCCTT	TTCTTCCGAG	TGACAATGCA	CCGCATTGTC
1710	1720	1730	1740	1750	1760	1770	1780	1790	1800
CAAGACGATG	CTGGCATCAC	TCTCCACGAC	GGCGTTTACC	TGCCCCGCGG	CACCCACCTA	GCGCTTCCCG	CCTACAACAT	TGGCCGAGAT	CCCGCTATGA
1810	1820	1830	1840	1850	1860	1870	1880	1890	1900
CACCCAACGC	GGAGGTCTTC	GACGGCTTGA	GGTGGTACCG	CCAGCGACAG	CAGAACCATC	AGTCGTCGGA	CGAAGATAGC	GGCGGCAAGT	GGCAGTTCTG
1910	1920	1930	1940	1950	1960	1970	1980	1990	2000
CACGCCCGAC	CCGAACATATC	TGACGTTCCG	CTCCGGCAAA	TACGTGTGCC	CAGGACGCTT	CATCGCCGCC	CACATGGTCA	AGCTGATGAT	GGTCGCGGTG
2010	2020	2030	2040	2050	2060	2070	2080	2090	2100
CTGTCCAGAT	ACGACGTGAA	GCGGGCTCCC	GACGCGCCCA	AACCGACGCA	GAAGTACCTG	CATGTCTTTT	CATTCCGAGA	TCAACGGACG	CTGTTGGTGA
2110	2120	2130	2140	2150	2160	2170	2180	2190	2200
AGCGACGTGT	AGGCGTGGAT	CAGGTCCAGA	TCATCTGA						

Figure 4.24 *amyJ* nucleotide sequence including intron regions.

4.3.7.1 Heterologous expression of AmyJ

Because AmyJ and LtmJ had significant identities, I predicted AmyJ catalyzed the same reaction as that by LtmJ (Scheme 4.7). To examine the possibility, *amyJ* gene was introduced into *A. oryzae* and the transformant was used for catalyst for bioconversion experiment with 20,21-diprenylated paxilline and DMAPP as the substrates. However, I did not detect any product by LC-ESI-MS analysis.

I also used *Saccharomyces cerevisiae* YPH500 as a host for a heterologous expression of *amyJ* gene together with a cytochrome P450 reductase (*PaP450Red*) of *p. amygdali* (168). Two genes were simultaneously introduced into the strain YPH500, in which both *AmyJ* and *AbP450Red* genes were expressed under the control of the galactose promoter. Microsomal fractions were prepared and incubated with 20, 21-diprenylated paxilline. However, no products were detected by LC-ESI-MS analysis.

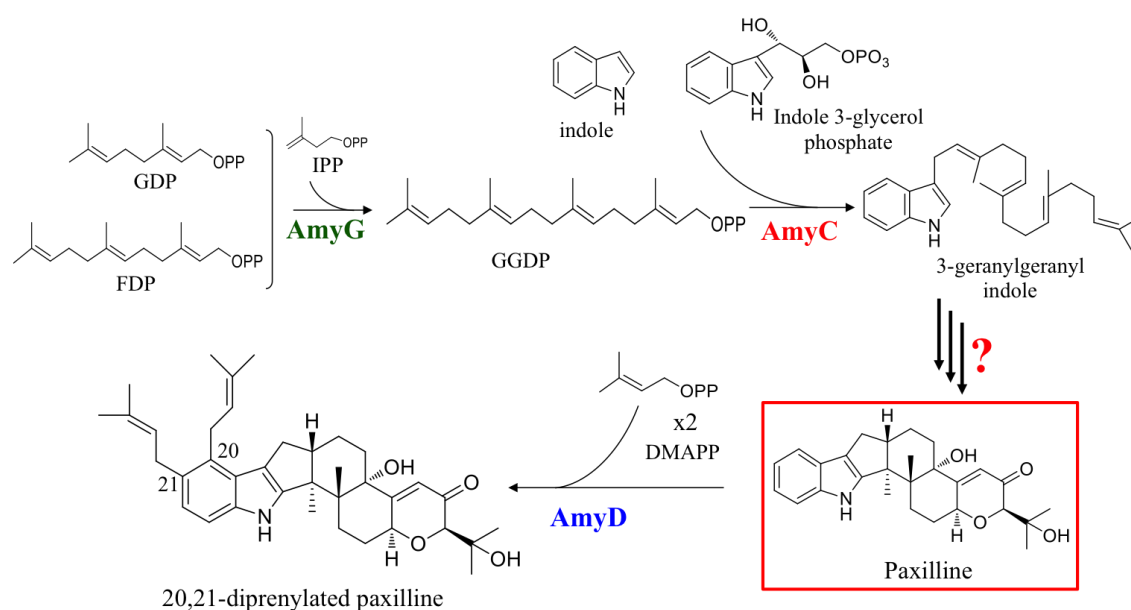


Scheme 4.7 Predicted AmyJ-catalyzed reaction.

4.4 Discussion

I identified the putative indole diterpene biosynthetic gene cluster composed of 8 genes in the genome database of *P. amygdali*, from which biosynthetic genes of fusicoccin A had been identified (169, 170). I tried to identify a compound governed by the cluster but all the attempts failed. The following results, however, suggested that the cluster might be responsible for the biosynthesis of a lolilline/lolitrem type of compound. AmyG and AmyC were suggested to be GGDP synthase and geranylgeranyl indole synthase (Scheme 4.8), respectively, both of which are the essential enzymes for biosynthesis of indole diterpene-related compounds. Moreover, AmyD has 58% amino acid identities to the product of *ltmE*, which was previously identified in the lolitrems biosynthetic gene cluster in *Epichloë festucae*, in contrast to the low similarities to AtmD and PaxD (17% and 13%). Scott et al examined the function of *ltmE* gene by constructing a gene-deletion mutant, introduction of the mutant into its host plant, and the structural analysis of a lolitrem-related compound produced by the endophyte. They suggested that LtmE catalyzed the di-prenylation of A-ring of terpendole I, a paxilline-related compound, at the 20,21-positions (42). Therefore, AmyD and LtmE would catalyze the regular di-prenylation at the same positions of the similar substrates. Furthermore, three P450 monooxygenase genes exist in the *amy* gene cluster. By searching for their homologous enzymes, two of them, AmyP and AmyQ, were found to

have similarities to PaxP/LtmP (28%/34% identity) and PaxQ/LtmQ (22%/27%), which catalyzed the successive reactions to convert paspaline to paxilline. The other one has high identity to LtmJ (64% identity), a cytochrome P450 monooxygenase, which was reported to be responsible for the lolitremanes formation (42). Taken these results together, the cluster identified in this study was suggested to participate in a lolilline/lolitreman type of compound. To confirm the probability, introduction of the *amy* genes into *A. oryzae* and analysis of the compound produced by the transformant might be also useful in this case.



Scheme 4.8 Summary of reactions catalyzed by AmyG, AmyC and AmyD.

Chapter 5

Conclusion

A number of indole diterpenes with various structural features and important biologically activities have been identified in different sources. Investigation on their biosynthesis with molecular biological and biochemical research began approximately ten years ago. Nowadays, the availability of more and more genome sequences provides us opportunities for the study of these metabolites.

In this study, I investigated the prenyltransferases involved in the indole diterpene biosynthesis pathway. Geranylgeranyl indole (GGI) was a putative common intermediate in indole diterpene biosynthesis, but its biosynthetic mechanism remains unclear. Therefore, I carried out in vitro analysis using recombinant PaxC and demonstrated that indole 3-glycerol phosphate (IGP) was the real substrate to yield GGI. I also confirmed that *paxD* encoded the prenyltransferase catalyzing a successive transfer of two molecules of DMAPP into paxilline to yield 21,22-diprenylated paxilline. This is the first example of the fungal prenyltransferase catalyzing di-prenylation reaction. Moreover, trace amounts of the product were detected in culture broth of *P. paxilli* by LC/ESI-MS analysis, showing that the final fermentation product governed by the *pax* gene cluster in *P. paxilli* is the 21,22-diprenylated paxillines rather than paxilline.

I found that AtmD accepted paxilline, paspaline, FI, and GGI as the substrate. More importantly and surprisingly, AtmD catalyzed prenylation of paxilline and paspaline at different positions and with different regular/reverse specificities. These results

suggested that prenyltransferases responsible for indole diterpene biosynthesis possess broad substrate specificities.

Genome projects have revealed that fungi have many secondary metabolite biosynthetic gene clusters. However, many of them are not expressed. A putative indole diterpene biosynthetic gene cluster composed of 8 genes was identified in a genome database of *Phomopsis amygdali*. Although I could not determine the product governed by this cluster, I showed that AmyG was a GGDP synthase, and that AmyC and AmyD were prenyltransferases catalyzing a transfer of GGDP to IGP and a regular di-prenylation at the 20,21-positions of paxilline, respectively. AmyD is the first example of the enzyme with this function.

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Acknowledgements

I would first like to thank my advisor, Professor Tohru Dairi for all his advice and invaluable guidance to my research. Thank you giving me this opportunity to work on this project, I am very grateful. With his innovative and creative research strategies and his powerful networking ability, he has shown me the basic steps to be a stronghold in the scientific field.

I would like to acknowledge Dr. Motoyoshi Noike, who has kindness encouragements and scrupulous assistance during my research. I would also like to thank Professor Hideaki Oikawa at Hokkaido University and Professor Hiroaki Toshima at Ibaraki University for their kindness and cooperation for support compounds paspaline and Farnesyl indole to use this research. I would also like to thank Dr. Eri Fukushi and assistant professor Atsushi Minami at Hokkaido University for their technical support in NMR measurements and structural analysis.

I would like to express my gratitude to assistant professor Yasuharu Satoh and all members of Dairi laboratory for their support to experiment, discussion and encouragement and enjoyable moments.

Finally, I also gratefully acknowledge my family for all their support and love.