เมแทบอไลท์ทุติยภูมิของราเอนโดไฟท์ไอโซเลต เออาร์อี1 จากใบน้อยโหน่ง

เรืออากาศเอกหญิง สุธีรา วัชระดิษย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชเวท ภาควิชาเภสัชเวท
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2545
ISBN 974-17-1414-9

SECONDARY METABOLITES OF ENDOPHYTIC FUNGUS ISOLATE ARE-1 FROM *ANNONA RETICULATA* LEAF

Flt. Lt. Sutheera Watcharadit

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy
Department of Pharmacognosy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2002

ISBN 974-17-1414-9

Thesis Title	SECONDARY METABOLITES OF ENDOPHYTIC FUNGUS ISOLATE ARE-1 FROM *ANNONA RETICULATA* LEAF
By	Flt. Lt. Sutheera Watcharadit
Field of Study	Pharmacognosy
Thesis Advisor	Assoc. Prof. Nijsiri Ruangrungsi, Ph.D.
Thesis Co-advisor	Assoc. Prof. Nongluksna Sriubolmas, Ph.D.
Thesis Co-advisor	Mr. Prasat Kittakoop, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree.

Boonyong Tantisira .....Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Boonyong Tantisira, Ph.D.)

THESIS COMMITTEE

Sumphan Wongseripipatana .....Chairman

(Associate Professor Sumphan Wongseripipatana, Ph.D.)

Nijsiri Ruangrungsi .....Thesis Advisor

(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

Nongluksna Sriubolmas .....Thesis Co-advisor

(Associate Professor Nongluksna Sriubolmas, Ph.D.)

.....Thesis Co-advisor

(Mr. Prasat Kittakoop, Ph.D.)

Suthep Wiyakrutta .....Member

(Assistant Professor Suthep Wiyakrutta, Ph.D.)

สุธีรา วัชระดิษย์ : เมแทบอไลท์ทุติยภูมิของราเอนโดไฟท์ไอโซเลต เออาร์อี1 จากใบน้อยโหน่ง (SECONDARY METABOLITES OF ENDOPHYTIC FUNGUS ISOLATE ARE-1 FROM *ANNONA RETICULATA* LEAF) อ. ที่ปรึกษา : รศ. ดร. นิจศิริ เรืองรังษี, อ. ที่ปรึกษาร่วม : รศ. ดร. นงลักษณ์ ศรีอุบลมาศ, ดร. ประสาท กิตตะคุปต์, 131 หน้า. ISBN 974-17-1414-9

ในการศึกษาเพื่อหาสารทุติยภูมิจากราเอนโดไฟท์ไอโซเลต เออาร์อี1ที่แยกได้จากใบน้อยโหน่ง พบว่าเมื่อทำการแยกสารบริสุทธิ์จากน้ำหมักเชื้อและเซลล์ของราเอนโดไฟท์ไอโซเลต เออาร์อี1 ด้วยวิธีทางโครมาโทกราฟี พบว่าได้สารบริสุทธิ์ 5 ชนิด คือ succinic acid monoethyl ester, phenylacetic acid, 2-(4'hydroxy) ethyl acetate, 4-hydroxyphenethyl alcohol และ ergosterol ซึ่งเป็นเมแทบอไลท์ปฐมภูมิที่เป็นองค์ประกอบสำคัญในเยื่อหุ้มเซลล์ของรา การพิสูจน์โครงสร้างทางเคมีของสารเหล่านี้ใช้วิธีการวิเคราะห์ข้อมูล UV IR MS และ NMR spectroscopy ร่วมกับการเปรียบเทียบข้อมูลที่มีรายงานมาแล้ว พบว่า ergosterol แสดงฤทธิ์ต้านเชื้อวัณโรคอย่างอ่อนด้วยค่า MIC 12.5 μg/ml การศึกษาทางสัณฐานวิทยาพบว่า ราเอนโดไฟท์ไอโซเลต เออาร์อี1ไม่สร้างสปอร์ จึงทำการจำแนกประเภทโดยการวิเคราะห์ลำดับนิวคลีโอไทด์ ในบริเวณ ITS1-5.8S-ITS2 ของ rDNAสามารถจำแนกประเภทราเอนโดไฟท์ไอโซเลตเออาร์อี1ไว้ในวงศ์ Valsaceae โดยมีความใกล้เคียงทางวิวัฒนาการกับ *Diaporthe caulivora*

ภาควิชา เภสัชเวท
สาขาวิชา เภสัชเวท
ปีการศึกษา 2545

ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4276604033 : MAJOR PHARMACOGNOSY
KEY WORD : ENDOPHYTIC FUNGUS/ SECONDARY METABOLITES/ VALSACEAE/ ITS/ ANTITUBERCULOSIS ACTIVITY
SUTHEERA WATCHARADIT: SECONDARY METABOLITE OF ENDOPHYTIC FUNGUS ISOLATE ARE-1 FROM *ANNONA RETICULATA* LEAF. THESIS ADVISOR: ASSOCIATE PROFESSOR NIJSIRI RUANGRUNGSI, Ph.D., THESIS CO-ADVISOR: ASSOCIATE PROFESSOR NONGLUKSNA SRIUBOLMAS, Ph.D., MR. PRASAT KITTAKOOP, Ph.D., 131 pp. ISBN 974-17-1414-9

In this study we investigated for secondary metabolites of the endophytic fungus isolate ARE-1 from *Annona reticulata* L. (Annonaceae) leaf. Chromatographic techniques were used to isolate compounds from the YES culture broth and mycelia of the endophytic fungus isolate ARE-1. Five known compounds were isolated and identified as succinic acid monoethyl ester, phenylacetic acid, 2-(4′hydroxy) ethyl acetate, 4-hydroxyphenethyl alcohol and ergosterol, a primary metabolite that is a major component of fungal cell membrane. The chemical structures of the isolated compounds were elucidated through extensive analyses of UV, IR, MS and NMR spectroscopic data and comparison with literatures. Ergosterol exhibited weak antituberculosis activity with MIC value of 12.5 μg/ml. Based on conventional method, the fungus isolate ARE-1 limited in spore formation. Nucleotide sequencing of ITS1-5.8S-ITS2 sequences of rDNA was applied to classify the endophytic fungus isolate ARE-1. It was found to be in the family Valsaceae with evolution closely related to *Diaporthe caulivora*.

Department Pharmacognosy
Field of study Pharmacognosy
Academic year 2002

Student's signature Sutheera Watcharadit
Advisor's signature Nijsiri Ruangrungsi
Co-advisor's signature Nongluksna Sriubolmas
Co-advisor's signature

ACKNOWLEDGEMENTS

The author wishes to express her deepest grateful appreciation to her advisor, Associate Professor Dr. Nijsiri Ruangrungsi for his guidance, suggestion, financial aid, encouragement and great kindness throughout the research study.

The author wishes to express her sincere appreciation to her co-advisor, Associate Professor Dr. Nongluksna Sriubolmas for her great valuable advice and guidance throughout the research study.

The author wishes to express her thanks to her co-advisor, Dr. Prasat Kittakoop of the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA) for his helpful suggestion and guidance throughout the research study.

The author wishes to thank the Bioassay Research Facility at the BIOTEC for biological activities test.

The author wishes to thank Mr. Wattana Punphut for his help in PCR experiment.

The author wishes to thank the committee for their constructive suggestion and critical review of this thesis.

The author wishes to thank the Graduate School of Chulalongkorn University, the research fund of Faculty of Pharmaceutical Sciences, Chulalongkorn University and the Biodiversity Research and Training Program (BRT) for granting partial financial support to conduct this study.

The author wishes to thank her friends and the officers in the Department of Pharmacognosy and Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University and the BIOTEC

Finally, the author wishes to express her infinite gratitude to her family for their love, support, understand and encouragement.

CONTENTS

Page

ABSTRACT (Thai).....iv

ABSTRACT (English).....v

ACKNOWLEDGEMENTS.....vi

CONTENTS.....vii

LIST OF TABLES.....x

LIST OF FIGURES.....xii

LIST OF SCHEMES.....xv

LIST OF ABBREVIATIONS.....xvi

CHAPTER

I INTRODUCTION.....1

II REVIEW OF LITERATURE.....3

1. Association of the endophytic fungi and plant.....3

2. Study of secondary metabolites from the endophytic fungi.....3

III EXPERIMENTAL.....5

1. Sample collection and isolation of the endophytic fungus ARE-1.....5

2. Classification of the endophytic fungus isolate ARE-1.....5

2.1 Conventional method basing on morphology.....5

2.1.1 Macroscopic morphology.....5

2.1.2 Microscopic morphology.....6

2.2 Molecular method basing on ribosomal RNA gene sequence.....6

2.2.1 DNA extraction.....6

2.2.2 Polymerase chain reaction.....7

2.2.3 DNA sequencing and phylogenetic analysis.....7

2.3 Preservation of the endophytic fungus isolate ARE-1.....9

3. Fermentation.....9

CONTENTS (Continued)

Page

4. Chromatographic Techniques.....9
    4.1 Analytical thin-layer chromatography.....9
    4.2 Column chromatography.....10
        4.2.1 Gel filtration chromatography.....10
        4.2.2 High performance liquid chromatography.....10
5. Spectroscopy.....11
    5.1 Ultraviolet (UV) spectroscopy.....11
    5.2 Infrared (IR) spectroscopy.....11
    5.3 Mass spectroscopy (MS).....11
    5.4 Proton (^{1}H) and Carbon (^{13}C) nuclear magnetic resonance (^{1}H and ^{13}C-NMR) spectroscopy.....11
6. Solvent.....12
7. Extraction.....12
8. Isolation of compounds.....14
    8.1 Isolation of compound AREB 3575 HP22.....14
    8.2 Isolation of compound AREB 485 HP4 and AREB 485 HP5.....18
    8.3 Isolation of compound AREB 485 HP2+3/4.....21
    8.4 Isolation of compound ARHM(H)76.....23
9. Physical properties of isolated compounds.....25
    9.1 AREB 3575 HP22.....25
    9.2 AREB 485 HP4.....25
    9.3 AREB 485 HP5.....26
    9.4 AREB 485 HP2+3/4.....26
    9.5 ARHM(H)76.....27
10. Determination of biological activities.....27
    10.1 Antimalarial activity.....27
    10.2 Antituberculosis activity.....28

CONTENTS (Continued)

Page

IV RESULTS AND DISCUSSION.....29
1. Isolation of the endophytic fungus ARE-1.....29
2. Classification of the endophytic fungus isolate ARE-1.....29
2.1 Conventional method.....29
2.2 Molecular method.....29
2.2.1 The PCR product of ITS1-5.8S-ITS2 regions of ribosomal DNA.....32
2.2.2 Nucleotide sequences of partial 18S sequence, complete ITS1-5.8S-ITS2 sequences and partial 28S sequence of the isolate ARE-1.....33
2.2.3 Phylogenetic analysis.....41
3. Structure elucidation of the isolated compounds.....43
3.1 Structure elucidation of compound AREB 3575 HP22.....43
3.2 Structure elucidation of compound AREB 485 HP4.....45
3.3 Structure elucidation of compound AREB 485 HP5.....48
3.4 Structure elucidation of compound AREB 485 HP2+3/4.....50
3.5 Structure elucidation of compound ARHM(H)76.....53
4. Biological activities.....56

V CONCLUSION.....57
REFERENCES.....58
APPENDICES.....66
APPENDIX A.....67
APPENDIX B.....101
APPENDIX C.....129
VITA.....131

LIST OF TABLES

Table		Page
1	Sources and biological activities of secondary metabolites of the endophytic fungi	67
2	Primer for amplification and sequencing of complete ITS1, 5.8S and ITS2 sequences of rRNA gene	8
3	Fractions obtained from AREB 3	14
3.1	Fractions obtained from AREB 35	15
3.2	Fractions obtained from AREB 357	15
3.3	Fractions obtained from AREB 3575	16
3.4	Fractions obtained from AREB 3575HP2	16
4	Fractions obtained from AREB 4	18
4.1	Fractions obtained from AREB 48	19
4.2	Fractions obtained from AREB 485	19
4.3	Fractions obtained from AREB 485HP2+3	21
5	Fractions obtained from ARHM(H)	23
5.1	Fractions obtained from ARHM(H)7	24
6	The ^{1}H, ^{13}C-NMR and HMBC spectral data of AREB 3575HP22 in $CDCl_3$	45
7	The ^{1}H, ^{13}C-NMR and HMBC spectral data of AREB 485HP4 in $CDCl_3$	47
8	^{1}H-NMR (500 MHz in $CDCl_3$) and ^{13}C-NMR (125 MHz in $CDCl_3$) spectral data of AREB 485HP5 and ^{1}H-NMR (300 MHz in $CDCl_3$) and ^{13}C-NMR (60 MHz in $CDCl_3$) spectral data of phenylacetic acid	47
9	The ^{1}H, ^{13}C-NMR and HMBC spectral data of AREB 485HP5 in $CDCl_3$	49
10	The ^{1}H, ^{13}C-NMR and HMBC spectral data of AREB 485HP2+3/4 in acetone *d*6 : $CDCl_3$ (9:1)	52

LIST OF TABLES (Continued)

Table **Page**

11 ^{1}H-NMR (500 MHz in acetone *d*6 : $CDCl_3$, 9:1) and ^{13}C-NMR (125 MHz in acetone *d*6 : $CDCl_3$, 9:1) spectral data of AREB 485 HP2+3/4 and ^{1}H-NMR (300 MHz in $CDCl_3$ and DMSO-*d*) and ^{13}C-NMR (60 MHz in $CDCl_3$ and DMSO-*d*) spectral data of 4-hydroxyphenethyl alcohol.....53

12 ^{13}C-NMR (125 MHz in $CDCl_3$) spectral data of ARHM(H)76 and ergosterol....55

LIST OF FIGURES

Figure		Page
1	Leaf of *Annona reticulata* L. (Annonaceae)	30
2	Colony morphology of the endophytic fungus isolate ARE-1 on five different mycological media	31
3	Agarose gel electrophoresis of PCR product	32
4	Nucleotide sequences of the partial 18S sequence, complete ITS1-5.8S-ITS2 sequences and partial 28S sequence of the isolate ARE-1	33
5	Alignment data of complete ITS1-5.8S-ITS2 sequences of isolate ARE-1 and 25 reference taxa	34
6	Maximum-parsimony tree generated from the ITS1-5.8S-ITS2 sequences of 26 taxa	42
7	IR spectrum of compound AREB 3575 HP22	101
8	UV spectrum of compound AREB 3575 HP22 (in methanol)	101
9	ESI-TOF mass spectrum of compound AREB 3575 HP22	102
10	500 MHz ^{1}H-NMR spectrum of compound AREB 3575 HP22	103
11	125 MHz ^{13}C-NMR spectrum of compound AREB 3575 HP22	103
12	DEPT 135 spectrum of compound AREB 3575 HP22	104
13	HMQC spectrum of compound AREB 3575 HP22	105
14	^{1}H-^{1}H COSY spectrum of compound AREB 3575 HP22	105
15	^{1}H-^{1}H correlations of compound AREB 3575 HP22	44
16	HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of compound AREB 3575 HP22	106
17	HMBC spectrum ($^{n}J_{HC}$ = 4 Hz) of compound AREB 3575 HP22	106
18	^{1}H-^{13}C long-range correlations of compound AREB 3575 HP22	44
19	IR spectrum of compound AREB 485 HP4	107
20	UV spectrum of compound AREB 485 HP4 (in methanol)	107
21	ESI-TOF mass spectrum of compound AREB 485 HP4	108
22	500 MHz ^{1}H-NMR spectrum of compound AREB 485 HP4	109
23	125 MHz ^{13}C-NMR spectrum of compound AREB 485 HP4	109

LIST OF FIGURES (Continued)

Figure		Page
24	DEPT 135 spectrum of compound AREB 485 HP4	110
25	HMQC spectrum of compound AREB 485 HP4	110
26	HMBC spectrum of compound AREB 485 HP4	111
27	^{1}H-^{13}C long-range correlations of compound AREB 485 HP4	46
28	NOESY spectrum of compound AREB 485 HP4	111
29	IR spectrum of compound AREB 485 HP5	112
30	UV spectrum of compound AREB 485 HP5 (in methanol)	112
31	ESI-TOF mass spectrum of compound AREB 485 HP5	113
32	500 MHz ^{1}H-NMR spectrum of compound AREB 485 HP5	114
33	125 MHz ^{13}C-NMR spectrum of compound AREB 485 HP5	114
34	DEPT 135 spectrum of compound AREB 485 HP5	115
35	HMQC spectrum of compound AREB 485 HP5	116
36	HMQC spectrum (partial expanded: δH 0.5-4.8 ppm, δC 8-73 ppm) of compound AREB 485 HP5	116
37	HMQC spectrum (partial expanded: δH 6.5-7.8 ppm, δC 60-140 ppm) of compound AREB 485 HP5	117
38	^{1}H-^{1}H COSY spectrum of compound AREB 485 HP5	117
39	^{1}H-^{1}H correlations of compound AREB 485 HP5	48
40	HMBC spectrum of compound AREB 485 HP5	118
41	HMBC spectrum (partial expanded: δH 0.5-5 ppm, δC 10-85 ppm) of compound AREB 485 HP5	118
42	HMBC spectrum (partial expanded: δH 0.5-5 ppm, δC 115-185 ppm) of compound AREB 485 HP5	119
43	^{1}H-^{13}C long-range correlations of compound AREB 485 HP5	50
44	IR spectrum of compound AREB 485 HP2+3/4	120
45	UV spectrum of compound AREB 485 HP2+3/4 (in methanol)	120
46	ESI-TOF mass spectrum of compound AREB 485 HP2+3/4	121
47	500 MHz ^{1}H-NMR spectrum of compound AREB 485 HP2+3/4	122

LIST OF FIGURES (Continued)

Figure		Page
48	125 MHz ^{13}C-NMR spectrum of compound AREB 485 HP2+3/4	122
49	DEPT 135 spectrum of compound AREB 485 HP2+3/4	123
50	HMQC spectrum of compound AREB 485 HP2+3/4	124
51	HMQC spectrum (partial expanded: δH 6.3-7.9 ppm, δC 95-150 ppm) of compound AREB 485 HP2+3/4	124
52	^{1}H-^{1}H COSY spectrum of compound AREB 485 HP2+3/4	125
53	^{1}H-^{1}H correlations of compound AREB 485 HP2+3/4	51
54	HMBC spectrum of compound AREB 485 HP2+3/4	126
55	HMBC spectrum (partial expanded: δH 6.4-7.5 ppm, δC 20-180 ppm) of compound AREB 485 HP2+3/4	126
56	^{1}H-^{13}C long-range correlations of compound AREB 485 HP2+3/4	52
57	IR spectrum of compound ARHM(H)76	127
58	UV spectrum of compound ARHM(H)76 (in methanol)	127
59	500 MHz ^{1}H-NMR spectrum of compound ARHM(H)76	128
60	125 MHz ^{13}C-NMR spectrum of compound ARHM(H)76	128
61	Chemical structure of compound ARHM(H)76	54

LIST OF SCHEMES

Scheme		Page
I	Extraction of the fermentation broth and mycelia of the endophytic fungus isolate ARE-1	13
II	Isolation of compound AREB 3575 HP22	17
III	Isolation of compound AREB 485 HP4 and AREB 485 HP5	20
IV	Isolation of compound AREB 485 HP2+3/4	22
V	Isolation of compound ARHM(H)76	24

LIST OF ABBREVIATIONS

acetone-*d*6	=	deuterated acetone
ARE-1	=	*Annona reticulata* endophyte-1
br	=	broad (for NMR spectral data)
°C	=	degree Celsius
^{13}C NMR	=	carbon-13 nuclear magnetic resonance
$CDCl_3$	=	deuterated chloroform
CD_3OD	=	deuterated methanol
cf	=	confer
$CHCl_3$	=	chloroform
CH_2Cl_2	=	methylene chloride
cm	=	centimeter
CzYA	=	Czapek yeast autolysate agar
δ	=	chemical shift
d	=	doublet (for NMR spectral data)
dd	=	doublet of doublets (for NMR spectral data)
DEPT	=	distortionless enhancement by polarization transfer
∈	=	molar absorptivity
EtOAc	=	ethyl acetate
ESI-TOF MS	=	Electronspray Ionization Time of Flight Mass
g	=	gram
μg	=	microgram
h	=	hour
^{1}H-^{1}H COSY	=	Homonuclear (proton-proton) correlation spectroscopy
^{1}H NMR	=	proton nuclear magnetic resonance
HMBC	=	^{1}H-detected heteronuclear multiple bond correlation
HMQC	=	^{1}H-detected heteronuclear multiple quantum coherence
HPLC	=	high performance liquid chromatography
Hz	=	Hertz

LIST OF ABBREVIATIONS (Continued)

IR	=	infrared
ITS	=	internally transcribed spacers
J	=	coupling constant
L	=	liter
µl	=	microliter
λ_{max}	=	wavelength at maximum absorption
$[M+Na]^+$	=	pseudomolecular ion
m	=	multiplet (for NMR spectral data)
MCzA	=	malt Czapek agar
MEA	=	malt extract agar
MeCN	=	acetronitrile
MeOH	=	methanol
mg	=	milligram
MIC	=	minimum inhibitory concentration
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
MHz	=	megahertz
MS	=	mass spectroscopy
m/z	=	mass to charge ratio
ν_{max}	=	wave number at maximum absorption
nm	=	nanometer
NMR	=	nuclear magnetic resonance
PCR	=	polymerase chain reaction
PDA	=	potato dextrose agar
ppm	=	part per million
q	=	quartet (for NMR spectral data)

LIST OF ABBREVIATIONS (Continued)

s	=	singlet (for NMR spectral data)
SDA	=	Sabouraud's dextrose agar
sp.	=	species
t	=	triplet (for NMR spectral data)
TAE	=	Tris-HCl, acetate and EDTA
TE	=	Tris-HCl and EDTA
TLC	=	thin layer chromatography
T*r*	=	retention time
UV	=	ultraviolet
YES	=	yeast extract sucrose