ELICITORS AND PHYTOTOXINS FROM THE BLACKLEG FUNGUS: STRUCTURE, BIOACTIVITY AND BIOSYNTHESIS

A thesis submitted to the
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in the
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University of Saskatchewan
Saskatoon

By

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ABSTRACT

The phytopathogenic fungus *Leptosphaeria maculans* can cause blackleg disease on crucifers, which results in significant yield losses. Fungal diseases involve interactions between pathogenic fungi and host plants. One aspect of these interactions is mediated by secondary metabolites produced by both fungi and host plants. Phytotoxins and elicitors as well as phytoanticipins and phytoalexins are metabolites produced by fungi and plants, respectively. This thesis describes and discusses the isolation, structure, biological activity and biosynthesis of the secondary metabolites produced by *L. maculans*.

The elicitor-toxin activity bioassay guided isolation of elicitors and phytotoxins produced by *L. maculans* in a chemically defined medium lead to the isolation of general elicitors, sirodesmin PL (**165**) and deacetylsirodesmin PL (**166**), and specific elicitors, cerebrosides C (**14**) and D (**31**) from minimum medium (MM) culture under standard conditions. The known phytotoxins sirodesmin PL (**165**) and deacetylsirodesmin PL (**166**) induced the production of phytoalexin spirobrassinin (**122**) in both resistant plant species (brown mustard, *Brassica juncea* cv. Cutlass) and susceptible plant species (canola, *B. napus* cv. Westar). A mixture of cerebrosides C (**14**) and D (**31**) induced the production of the phytoalexin rutalexin (**127**) in resistant plant species (brown mustard, *B. juncea* cv. Cutlass) but not in susceptible plant species (canola, *B. napus* cv. Westar). New metabolites leptomaculins A-E (**267-269**, **272** and **274)** and deacetylleptomaculins C-E (**270**, **273** and **275**) were isolated from elicitor-phytotoxin active fractions but did not display detectable elicitor activity or phytotoxicity after purification.

New metabolites maculansins A (**299**) and B (**300**), which were not detected in cultures of *L. maculans* incubated in MM, were isolated from cultures of *L. maculans* incubated in potato dextrose broth (PDB). Maculansins A (**299**) and B (**300**) displayed higher phytotoxicity on brown mustard than on canola and white mustard (*Sinapis alba* cv. Ochre) but did not elicit detectable production of phytoalexins in either brown mustard or canola. Metabolite 2,4-dihydroxy-3,6-dimethylbenzaldehyde **(212)** was produced in higher amount in cultures of *L. maculans* incubated in PDB than in MM and displayed strong inhibition effect on the root growth of brown mustard and canola.

L. maculans incubated in MM amended with high concentration of NaCl produced a new metabolite, 8-hydroxynaphthalene-1-sulfate (**293**), and a known metabolite, bulgarein (**294**), which are likely involved in the self-protection.

The potential intermediates involved in the biosynthesis of sirodesmin PL (**165**) were investigated using deuterium labeled precursors: [3,3-$^2H_2$]-L-tyrosine (**251a**), [3,3-$^2H_2$]*O*-prenyl-L-tyrosine (**312a**), *E*-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-L-tyrosine (**312b**), [5,5-$^2H_2$]phomamide (**171a**), [2,3,3-$^2H_3$]-L-serine (**233d**) and [5,5-$^2H_2$]cyclo-L-tyr-L-ser (**252a**). Intact incorporation of [5,5-$^2H_2$]phomamide (**171a**) into sirodesmin PL (**165**) suggested that leptomaculin D (**272**) and E (**274**), and deacetylleptomaculin D (**273**) and E (**275**) are not intermediates in the biosynthesis of sirodesmin PL (**165**). They are more likely the catabolic metabolites of sirodesmin PL (**165**). Phomamide (**171**), the intermediate in the biosynthetic pathway of sirodesmin PL (**165**), is likely biosynthesized by coupling of prenyl tyrosine (**312**) with serine (**233**) rather than prenylation of cyclo-L-tyr-L-ser (**252**). When [3,3-$^2H_2$]-L-tyrosine (**251a**), [3,3-$^2H_2$]*O*-prenyl-L-tyrosine (**312a**), and *E*-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-L-tyrosine (**312b**) were fed into cultures of *L. maculans*, a β proton exchange was detected by ^{1}H NMR through intrinsic steric isotope effect, which occurs before the formation of phomamide (**171**). The biosynthesis and catabolism of sirodesmin PL (**165**) were proposed based on the results obtained in this work.

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DEDICATION

To my mother,

Zhen Liu
刘真

And to my father,

Zhi Jie Yu
喻子杰

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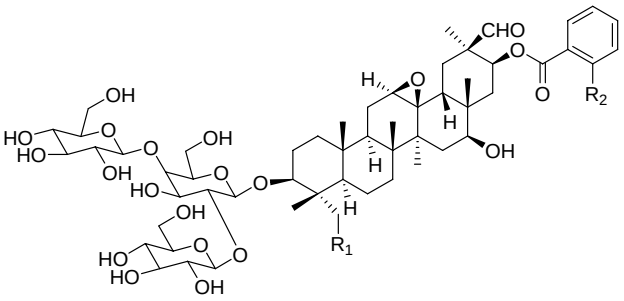
^{1}H NMR	proton nuclear magnetic resonance
^{13}C NMR	carbon-13 nuclear magnetic resonance
A. alternata	*Alternaria alternata*
ABC	ATP-binding cassette
ABq	AB quartet
Ala	alanine
aq.	aqueous
A. brassicae	*Alternaria brassicae*
A. infectoria	*Alternaria infectoria*
A. radicina	*Alternaria radicina*
A. fumigatus	*Aspergillus fumigatus*
A. terreus	*Aspergillus terreus*
A. hypochondriacus	*Amaranthus hypochondriacus*
A. thaliana	*Arabidopsis thaliana*
A. rabiei	*Ascochyta rabiei*
A. sativa	*Avena sativa*
Avr	avirulence
B. cinerea	*Botrytis cinerea*
B. campestris	*Brassica campestris*
B. juncea	*Brassica juncea*
B. napus	*Brassica napus*
BJ-125	virulent isolate of *L. maculans*
Boc	*tert*-butoxycarbonyl
br	broad
calcd.	calculated
COSY	correlation spectroscopy
cv.	cultivar
C. arvense	*Cirsium arvense*
C. carbonum	*Cochliobolus carbonum*
C. heterostrophus	*Cochliobolus heterostrophus*
C. victoriae	*Cochliobolus victoriae*
DMA	N,N-dimethylaniline
DMSO	dimethyl sulfoxide
EDCI	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EI	electron impact
EPTs	epipolythiodioxopiperazines
ESI-MS/MS	electron spray ionization-mass spectrum/mass spectrum
Et2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
F. oxysporum	*Fusarium oxysporum*
F. verticillioides	*Fusarium verticillioides*
FCC	flash column chromatography

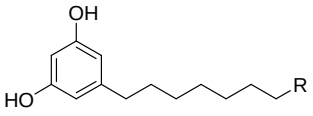
FTIR	fourier transformed infrared
G. graminis var. *tritici*	*Gaeumannomyces graminis* var. *tritici*
G. graminis var. *avenae*	*Gaeumannomyces graminis* var. *avenae*
G. viride	*Gliocladium viride*
G. deliquescens	*Gliocladium deliquescens*
GC-MS	gas chromatography-mass spectrometry
h	hours
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum correlation
HPLC	high performance liquid chromatography
HSTs	host selective toxins
HR	hypersensitive response
HRMS	high resolution mass spectrum
Hz	Hertz
IBCN	international blackleg crucifer network
ISDI	intrinsic steric deuterium isotope
J	coupling constant
LAH	lithium aluminum hydride
L. biglobosa	*Leptosphaeria biglobosa*
LC-MS/DAD	liquid chromatography-mass spectroscopy/diode array detector
L. maculans	*Leptosphaeria maculans*
M. grisea	*Magnaporthe grisea*
m/z	mass/charge ratio
MAMPs	microbial associated molecular patterns
MeOH	methanol
MHz	megahertz
min	minutes
MM	minimal medium
mmol	millimole
m.p.	melting point
MS	mass spectrum
Nep1	necrosis-eliciting protein 1
NLPs	Nep1-like proteins
NOE	nuclear Overhauser enhancement
NOESY	nuclear Overhauser effect spectroscopy
NPP1	necrosis-inducing *phytophthora* protein 1
P. lingam	*Phoma lingam* (blackleg fungus)
PDA	potato dextrose agar
PDB	potato dextrose broth
Phe	phenylalanine
P. oryzae	*Pyricularia oryzae*
ppm	parts per million
prep. TLC	preparative thin layer chromatography
P. wasabi	*Phoma wasabi*
P. sojae	*Phytophthora sojae*
P. infestans	*Phytophthora infestans*

P. cichorri	*Pseudomonas cichorri*
P. syringae	*Pseudomonas syringae*
ROS	reactive oxygen species
RP-FCC	reverse phase flash column chromatography
rpm	revolutions per minute
R. solani	*Rhizoctonia solani*
rt	room temperature
R_t	retention time
s	seconds
S. alba	*Sinapis alba*
SGF	spore germination fluids
Ser	serine
T. aestivum	*Triticum aestivum*
TEA	triethylamine
THF	tetrahydrofuran
TLC	thin layer chromatography
T. viride	*Trichoderma viride*
Tyr	tyrosine
UV	ultraviolet
UV/Vis	ultraviolet/visible
v	volume

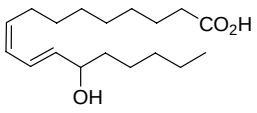
CHEMICAL STRUCTURES MENTIONED IN THIS THESIS



1 R_1=OH, R_2=NHMe
2 R_1=OH, R_2=H
3 R_1=H, R_2=NHMe
4 R_1=H, R_2=H



5 $AR_{17:1}$ R=CH=CH$(CH_2)_7CH_3$
6 $AR_{13:0}$ R=$(CH_2)_5CH_3$
7 $AR_{15:1}$ R=CH=CH$(CH_2)_5CH_3$
8 $AR_{15:0}$ R=$(CH_2)_7CH_3$
9 $AR_{17:0}$ R=$(CH_2)_9CH_3$



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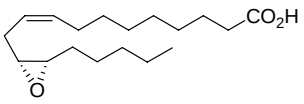

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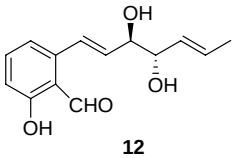

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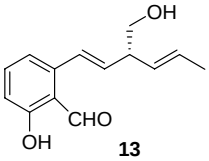

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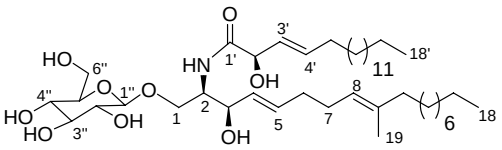

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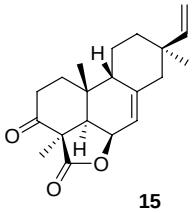

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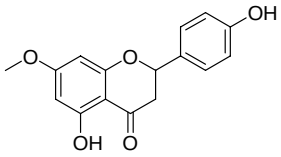

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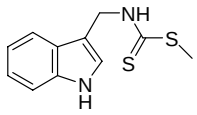

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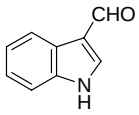

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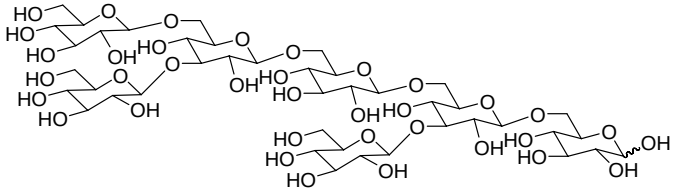

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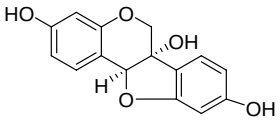

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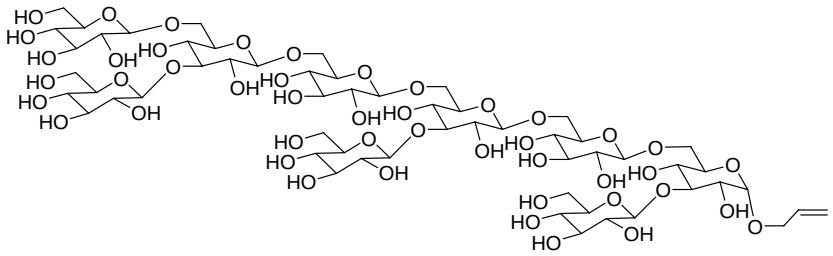

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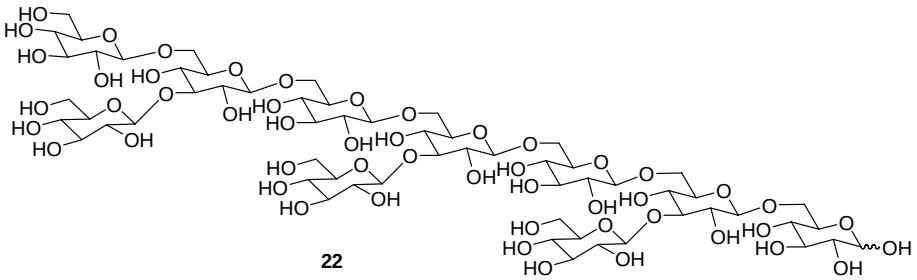

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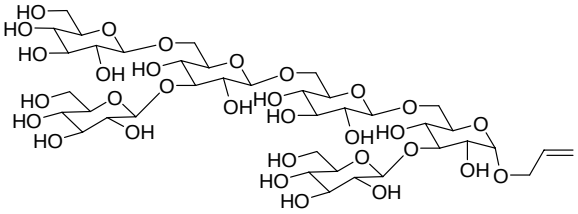

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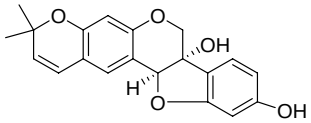

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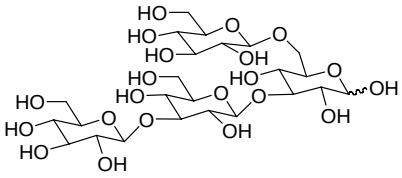

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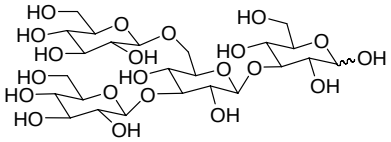

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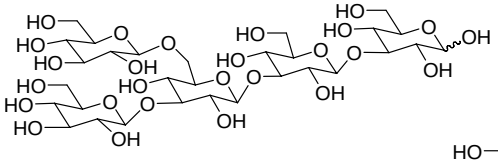

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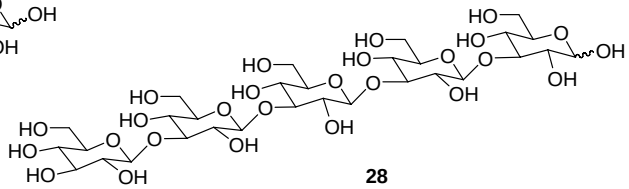

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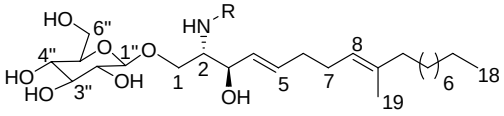

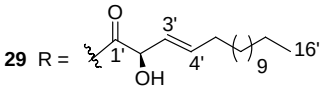

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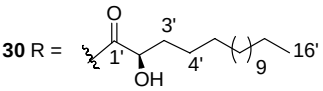

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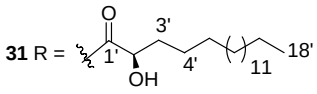

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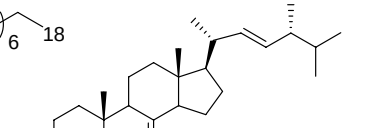

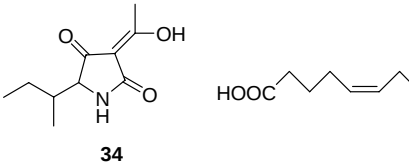

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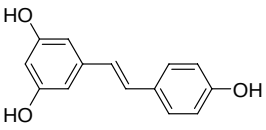

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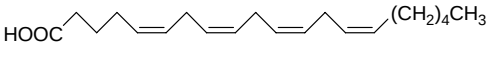

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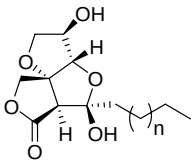

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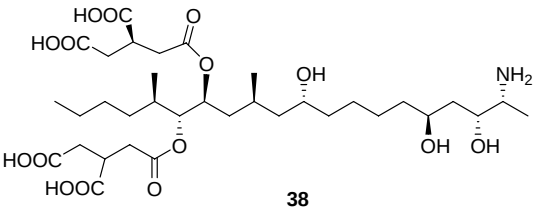

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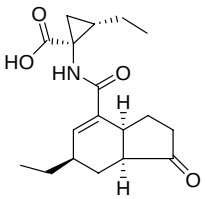

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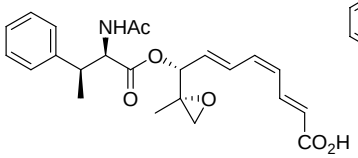

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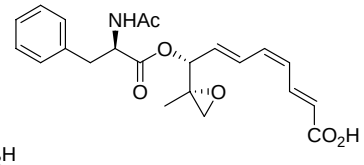

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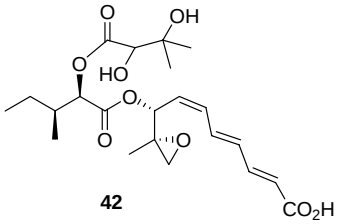

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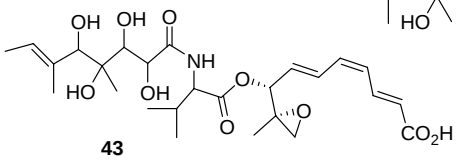

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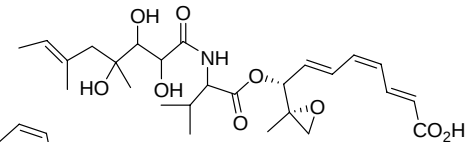

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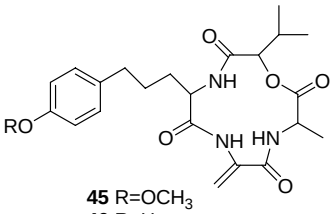
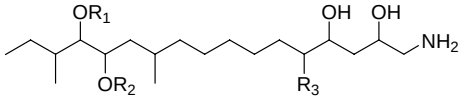
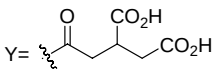
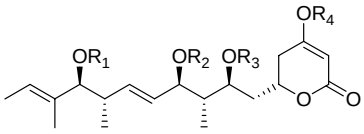


45 R=OCH_3
46 R=H
47 R=OH

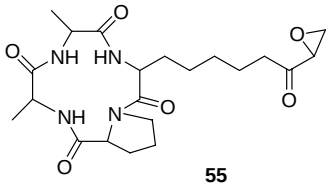


48 R_1=H, R_2=Y, R_3=OH
49 R_1=Y, R_2=H, R_3=OH
50 R_1=R_2=R_3=H
51 R_1=R_2=Y, R_3=H





52 R_1=R_2=R_3=Ac, R_4=Me
53 R_1=R_2=R_3=H, R_4=Me
54 R_1=R_2=R_3=R_4=H



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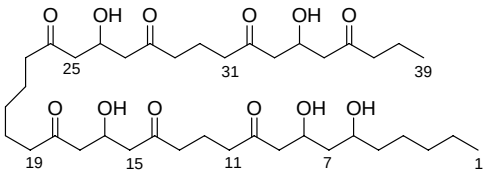

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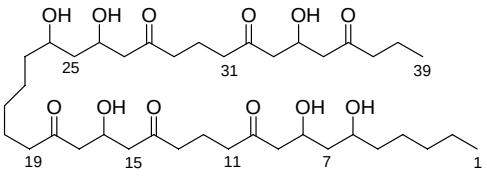

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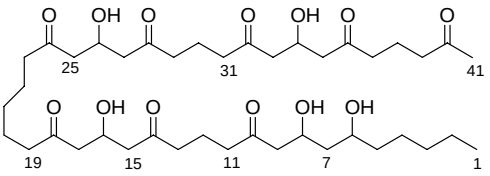

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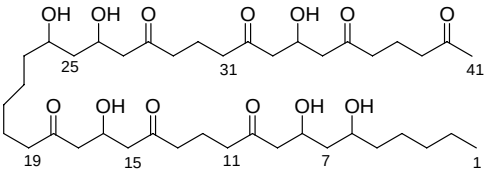

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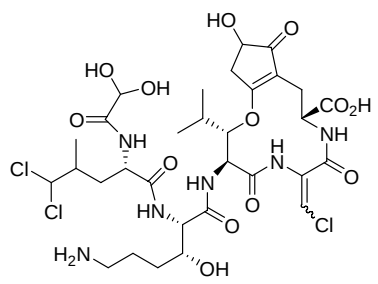

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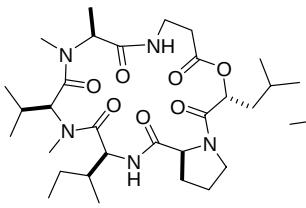

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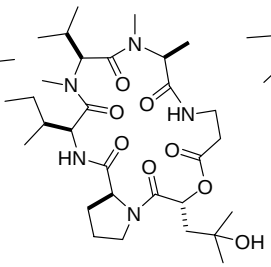

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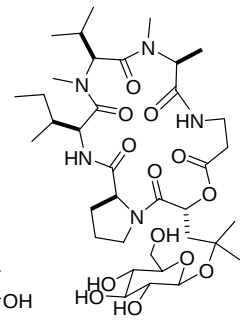

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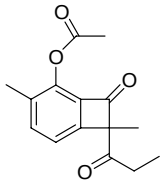

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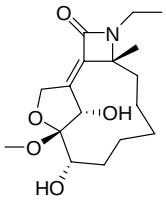

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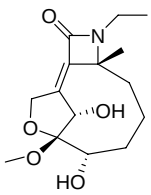

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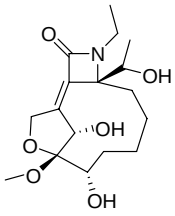

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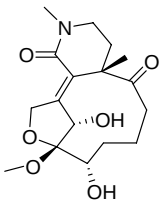

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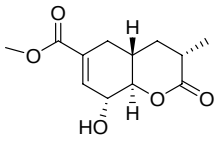

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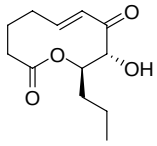

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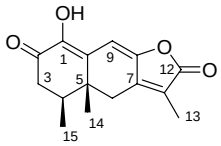

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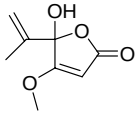

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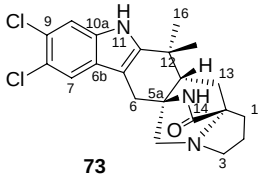

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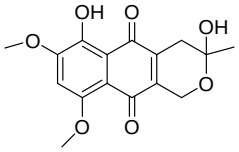

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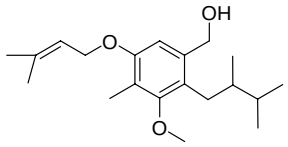

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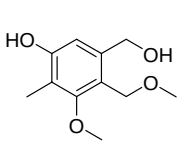

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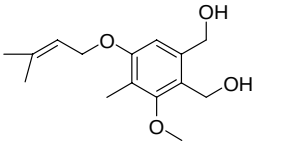

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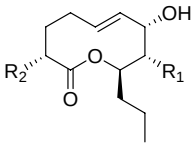
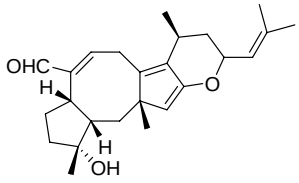


78 R_1=OH, R_2=H
79 R_1=R_2=OH
80 R_1=R_2=H



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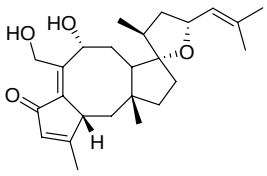

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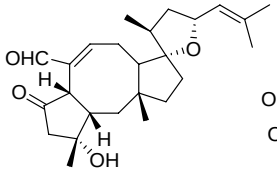

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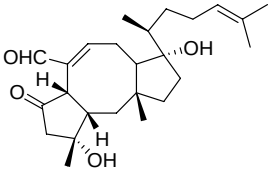

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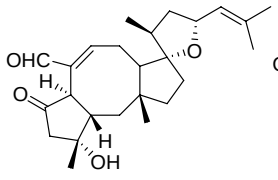

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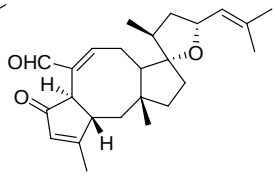

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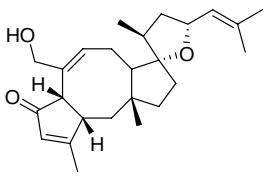

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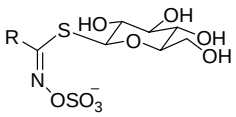
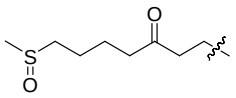
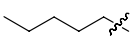


88

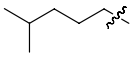
88A R=



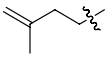
88B R=



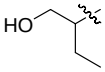
88C R=



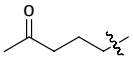
88D R=



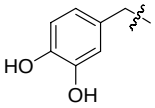
88E R=



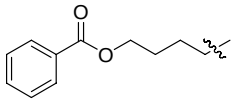
88F R=



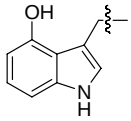
88G R=



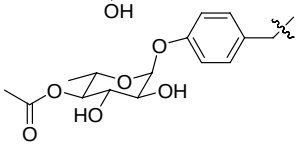
88H R=

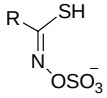


88I R=



88J R=





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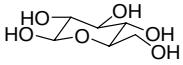


90

R−N=C=S

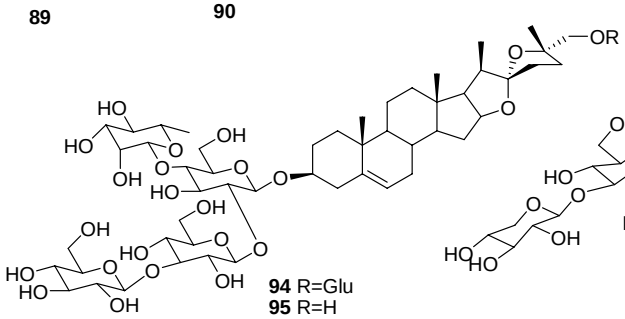
91

R−C≡N

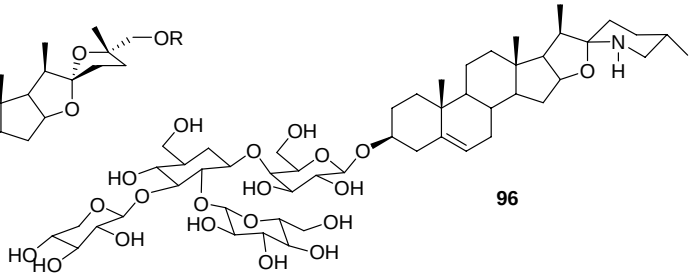
92

R−S−C≡N

93



94 R=Glu
95 R=H



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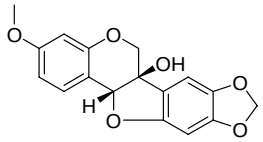

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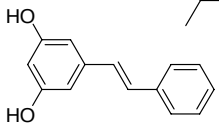

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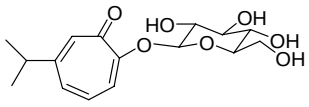

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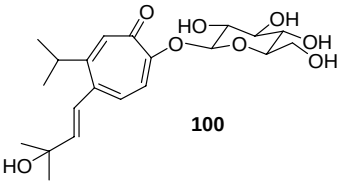
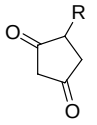
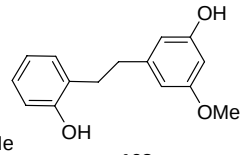


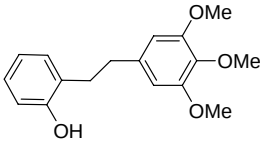
100



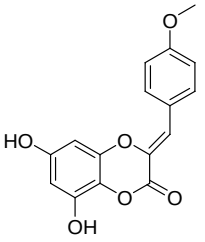
101 R= $(CH_2)_7Me$
102 R= $(CH_2)_8Me$



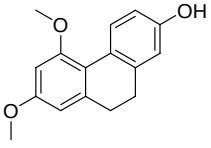
103



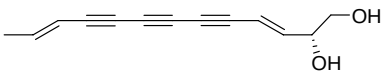
104



105



106



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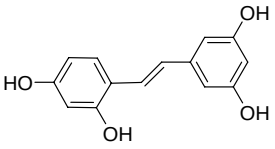

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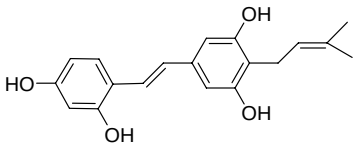

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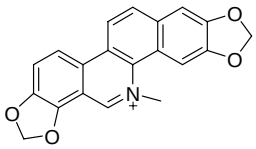
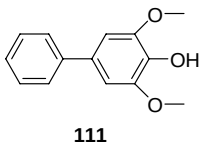
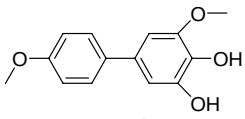


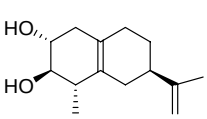
110



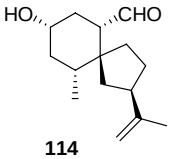
111



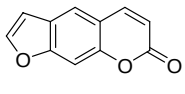
112



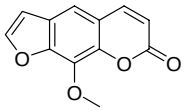
113



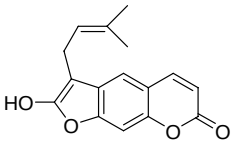
114



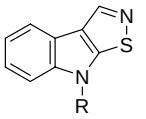
115



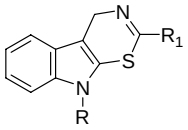
116



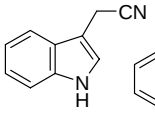
117



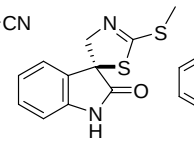
118 R=H
130 R=OMe



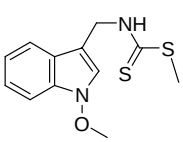
119 R=H; R1=SMe
120 R=H; R1=S(O)Me
128 R=OMe; R1=S(O)Me
129 R=OMe; R1=SMe



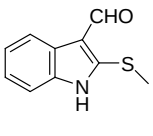
121



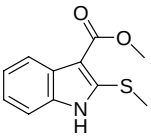
122



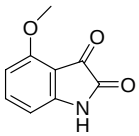
123



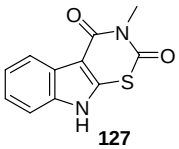
124



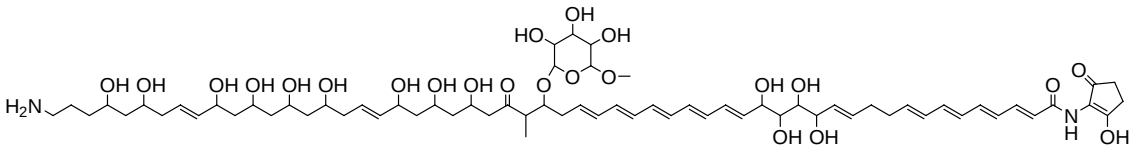
125



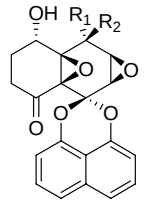
126



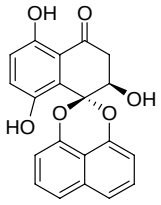
127



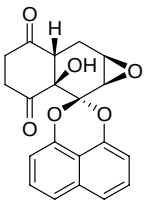
131



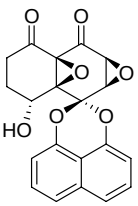
132 R_1=H, R_2=OH
133 R_1, R_2=O



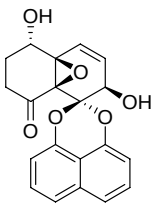
134



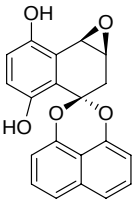
135



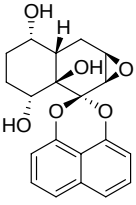
136



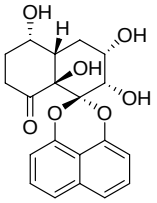
137



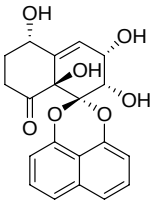
138



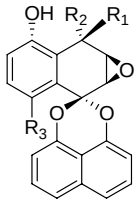
139



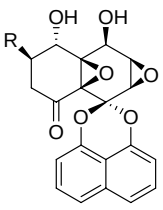
140



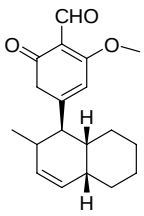
141



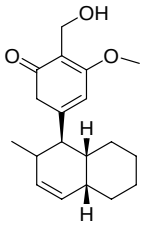
142 R_1,R_2=O, R_3=H
143 R_1, R_2=O, R_3=OH
144 R_1=OH, R_2=H, R_3=OH



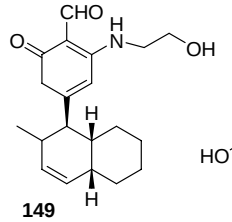
145 R=OH
146 R=H



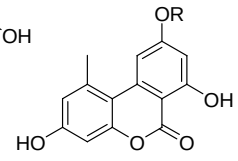
147



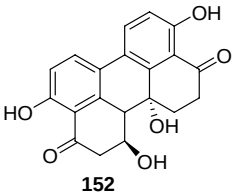
148



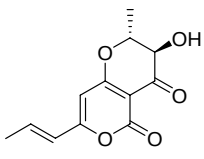
149



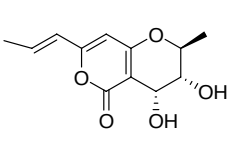
150 R=H
151 R=Me



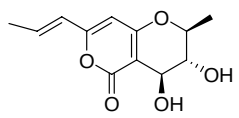
152



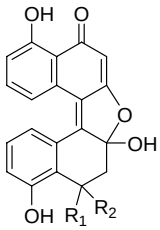
153



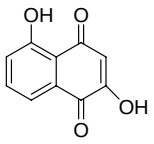
154



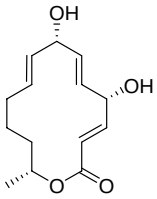
155



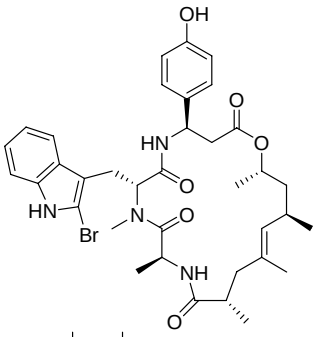
156 R_1,R_2=O
157 R_1=H, R_2=OH



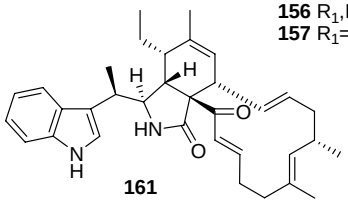
158



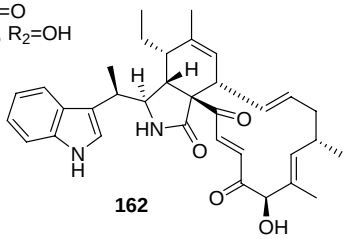
159



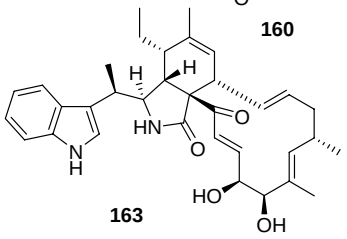
160



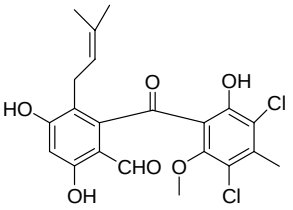
161



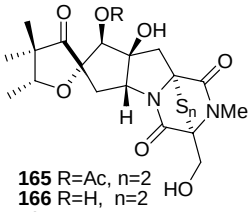
162



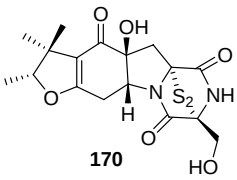
163



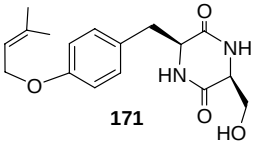
164



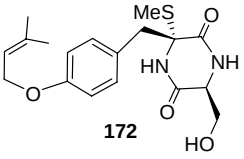
165 R=Ac, n=2
166 R=H, n=2
167 R=Ac, n=1
168 R=Ac, n=3
169 R=Ac, n=4



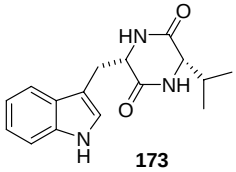
170



171



172



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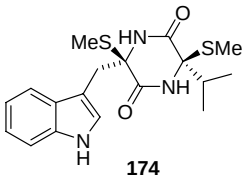

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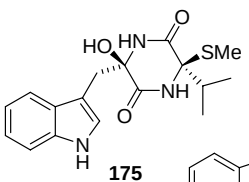

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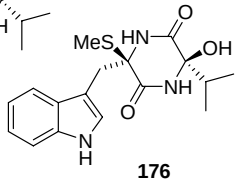

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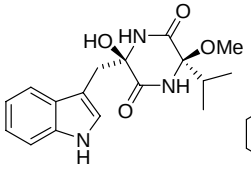

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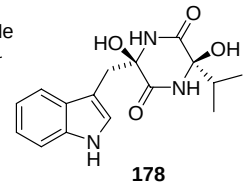

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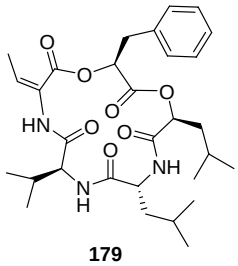

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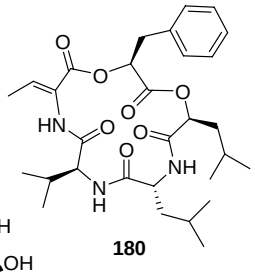

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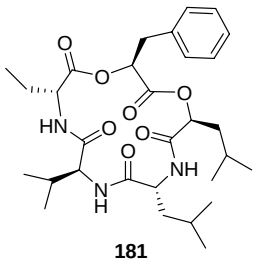

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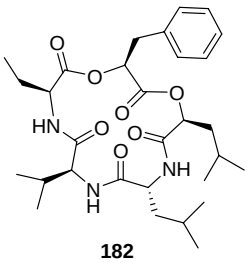

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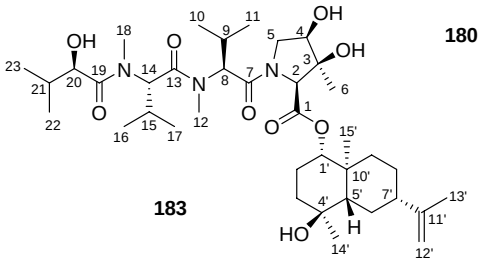

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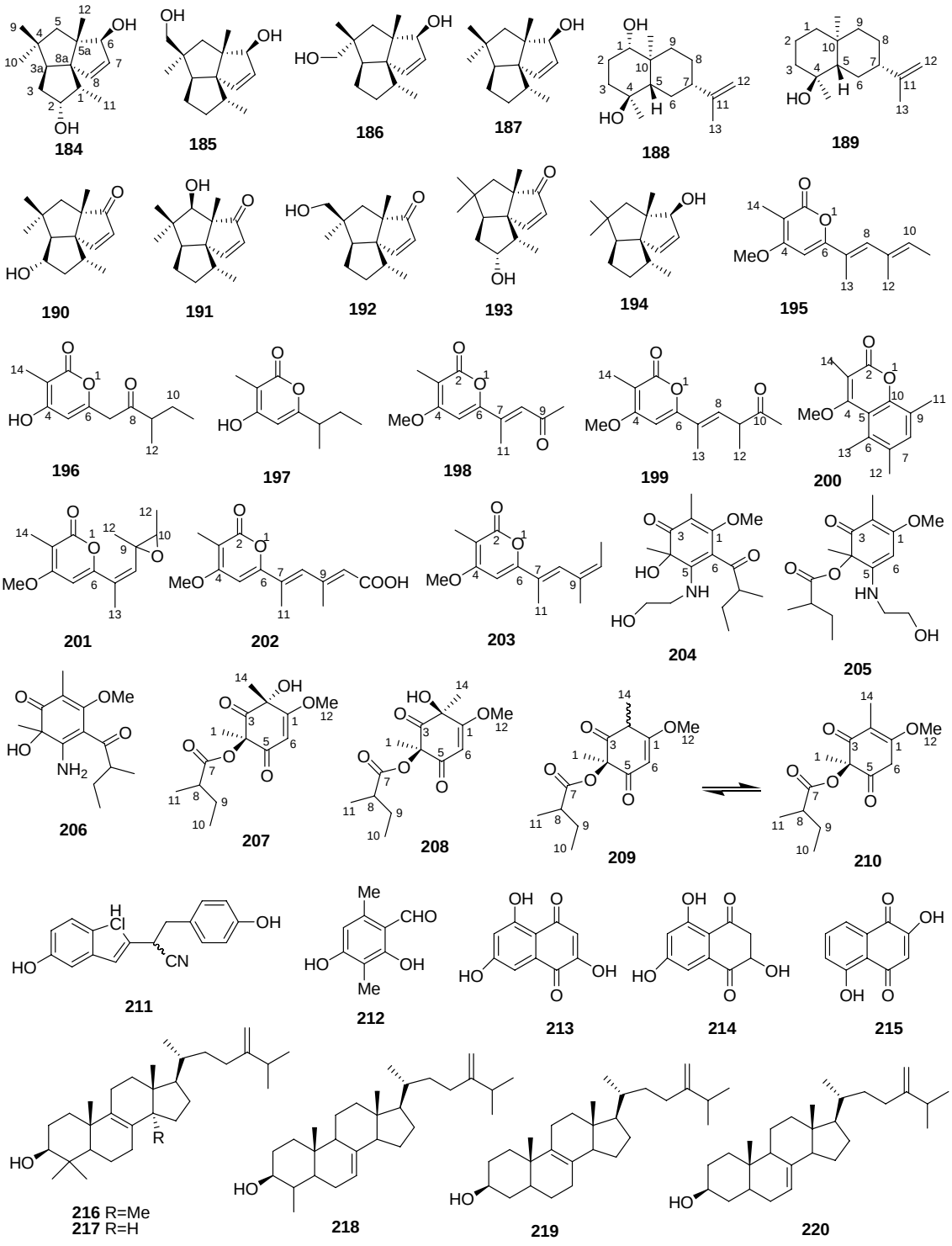
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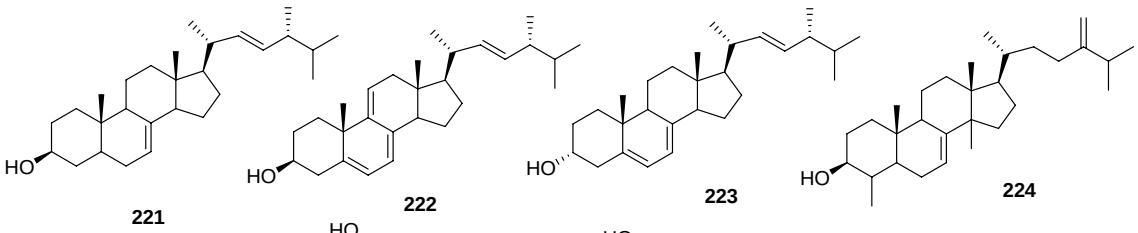
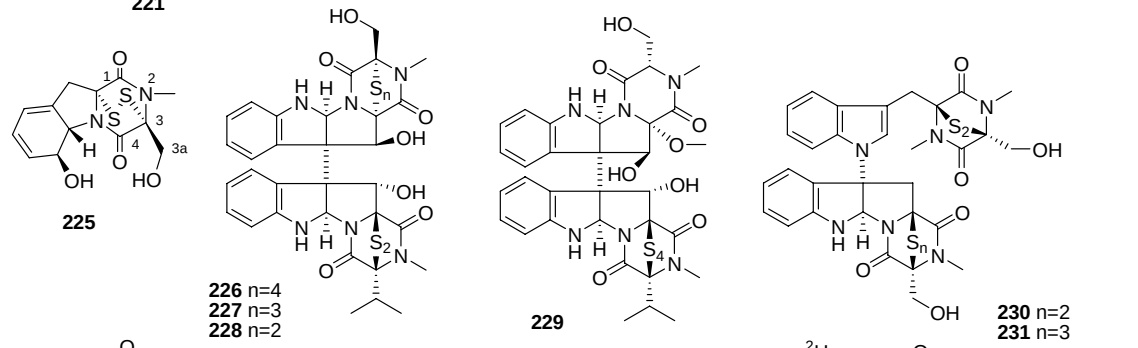
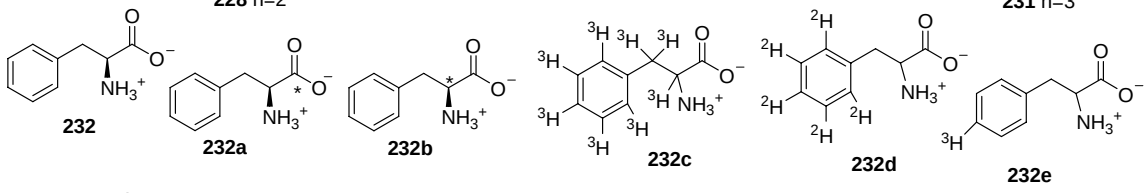


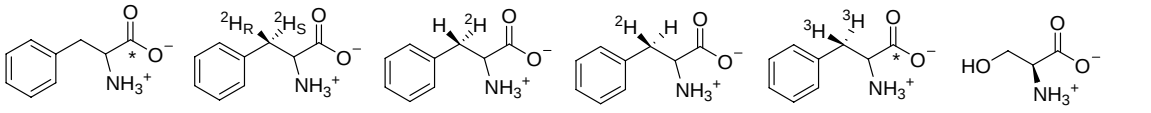
221 **222** **223** **224**



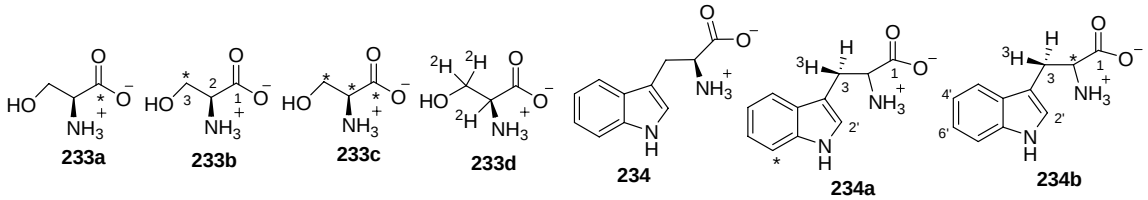
225 **226** n=4 **227** n=3 **228** n=2 **229** **230** n=2 **231** n=3



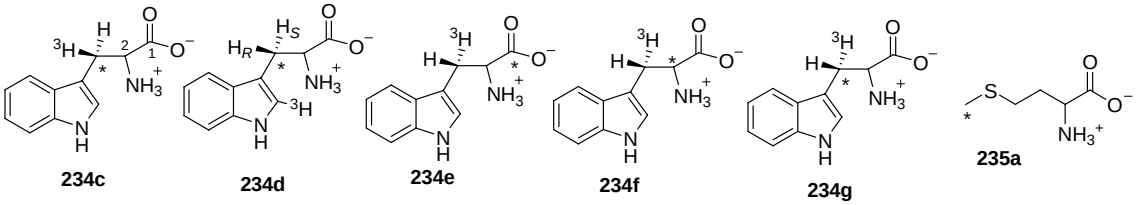
232 **232a** **232b** **232c** **232d** **232e**



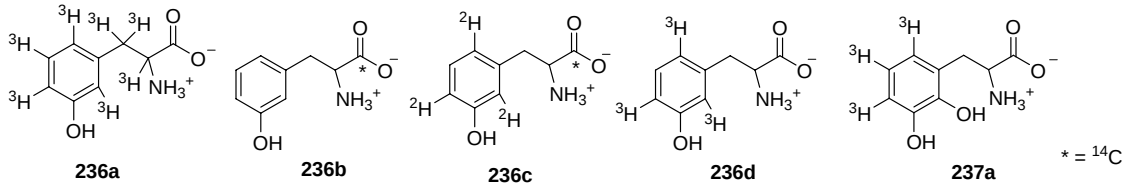
232f **232g** **232h** **232i** **232j** **233**



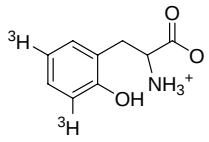
233a **233b** **233c** **233d** **234** **234a** **234b**



234c **234d** **234e** **234f** **234g** **235a**



236a **236b** **236c** **236d** **237a** * = ^{14}C



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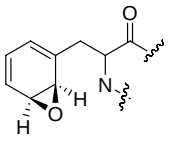

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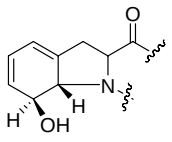

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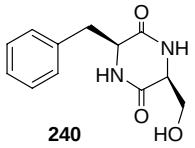

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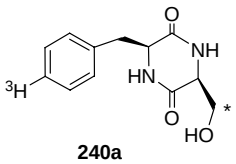

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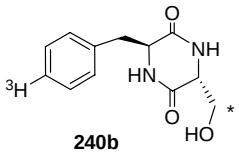

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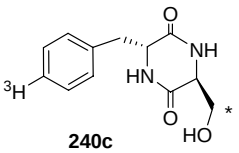

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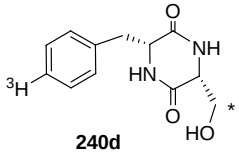

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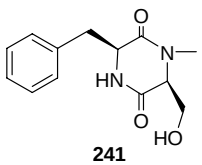

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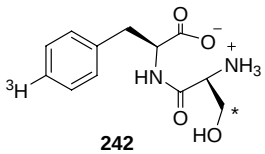

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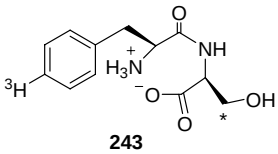

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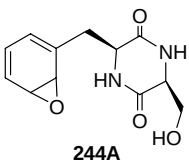

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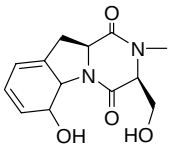

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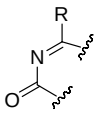

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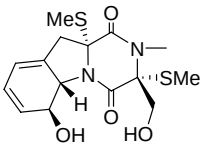

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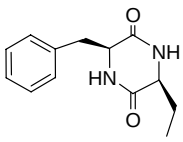

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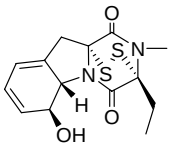

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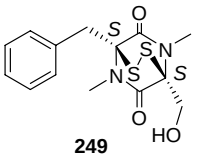

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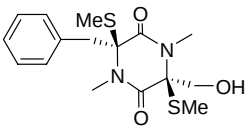

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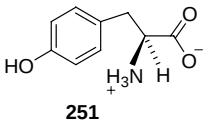

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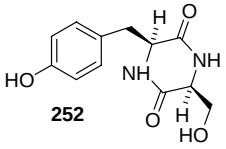

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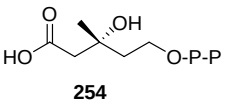

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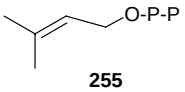

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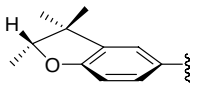

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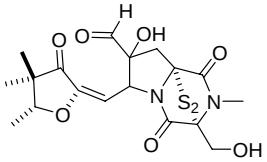

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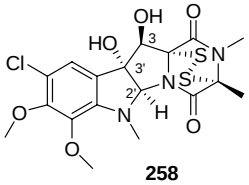

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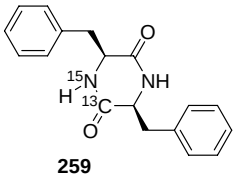

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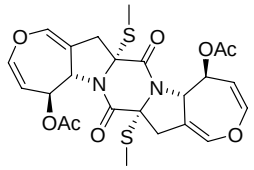

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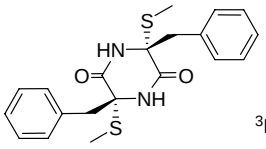

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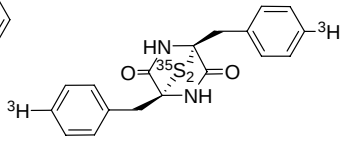

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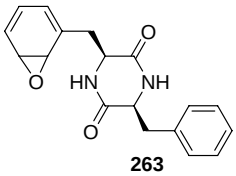

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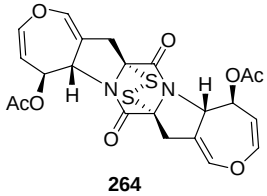

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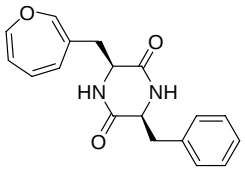

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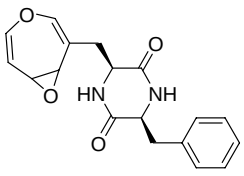

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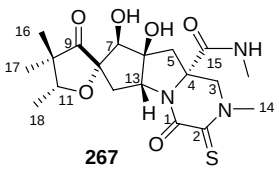

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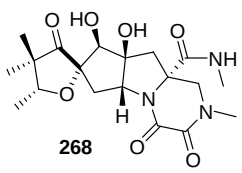

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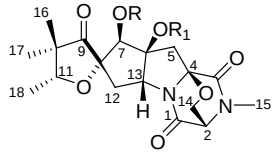
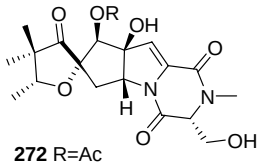
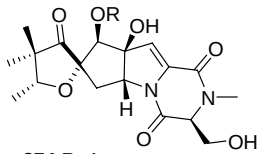


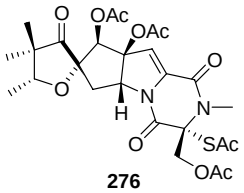
269 R=Ac, R_1=H
270 R=R_1=H
271 R=R_1=Ac



272 R=Ac
273 R=H



274 R=Ac
275 R=H



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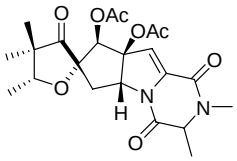

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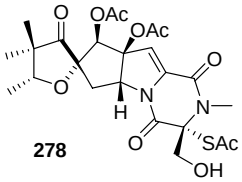

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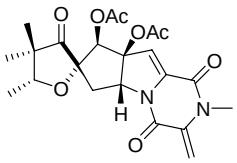

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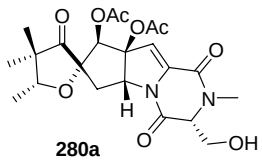

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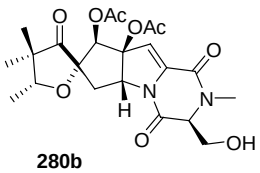

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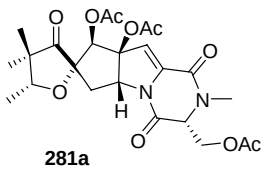

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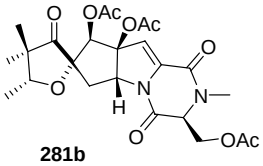

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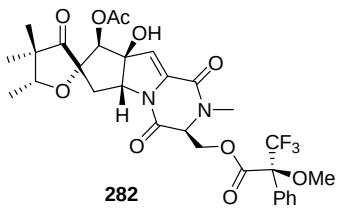

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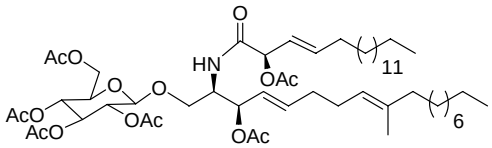

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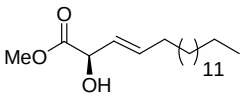

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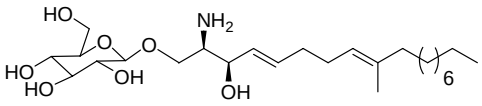

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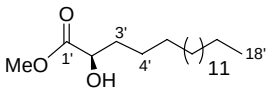

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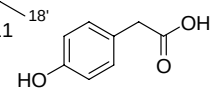

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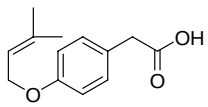

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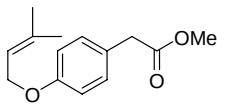

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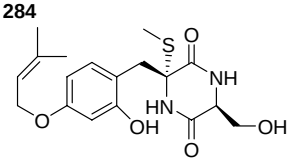

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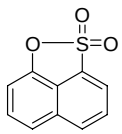

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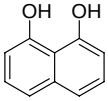

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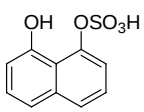

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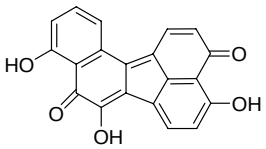

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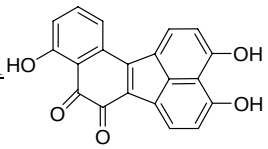

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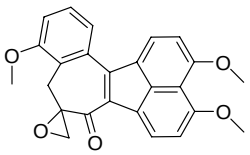

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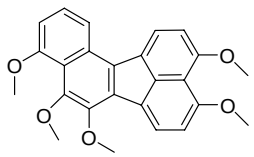

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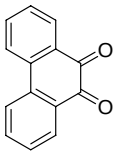

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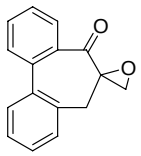

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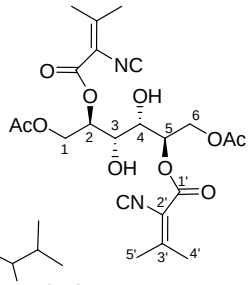

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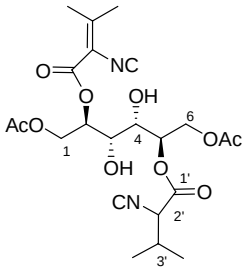

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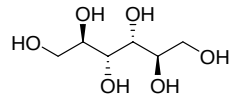

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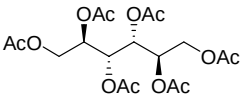

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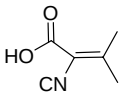

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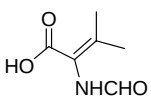

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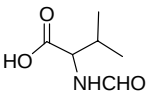

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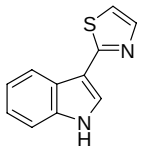

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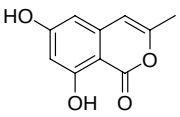

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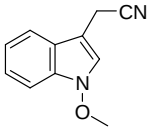

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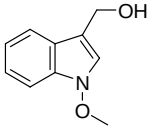

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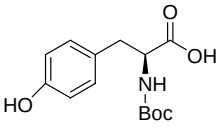

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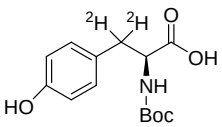

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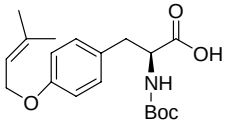

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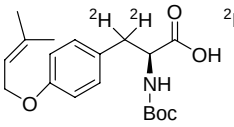

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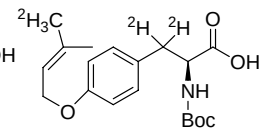

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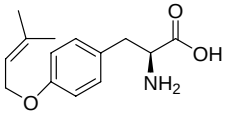

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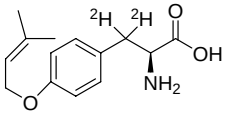

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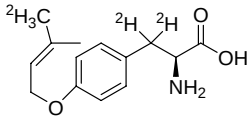

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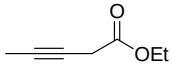

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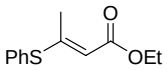

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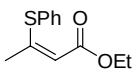

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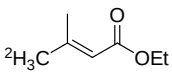

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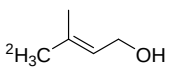

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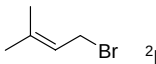

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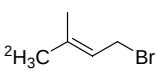

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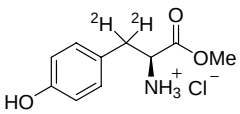

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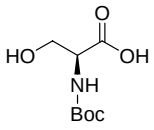

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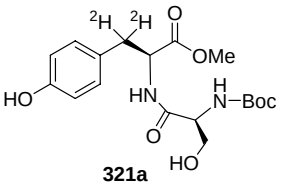

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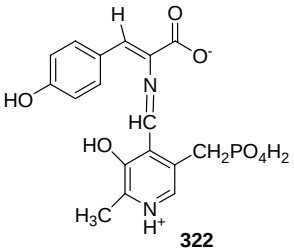

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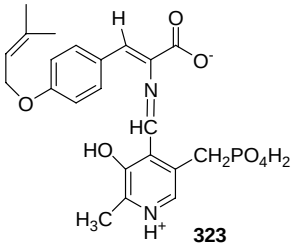

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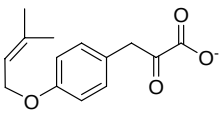

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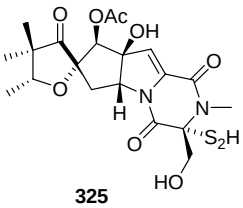

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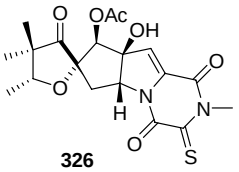

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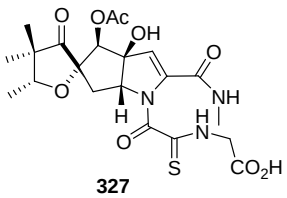

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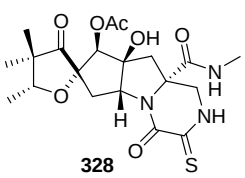

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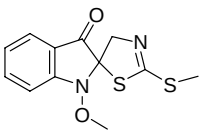

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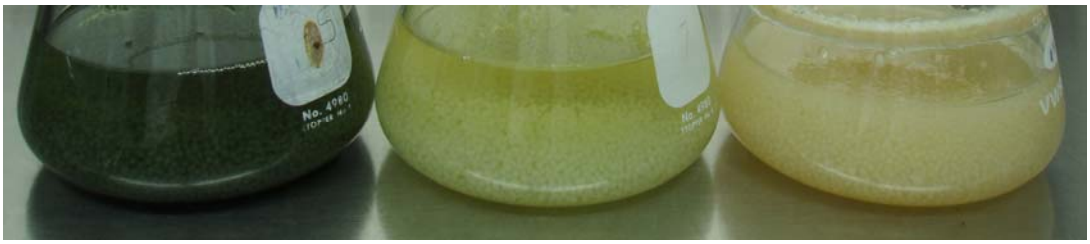
COLOR FIGURES



MM + NaCl
7 days incubation

MM + NaCl
6 days incubation

MM Control
7 days incubation

Figure I. 1. Cultures of *Leptosphaeria maculans* in minimal medium (MM) and MM containing high NaCl concentration (0.7 M).

Maculansin A (**299**)

Sirodesmin PL (**165**)

Figure I. 2. A leaf of *Brassica juncea* cv. Cutlass treated with maculansin A (**299**) and sirodesmin PL (**165**).

Control Maculansin A (**299**)

Figure I. 3. A leaf of *Brassica juncea* cv. Cutlass treated with maculansin A (**299**) and control.

Figure I. 4. Plants (rows: A, *Brassica juncea* cv. Cutlass; B, *B. napus* cv. Westar; C, *Sinapis alba* cv. Ochre) treated with maculansin A (**299**) (from left to right: 1.0 mM (sirodesmin PL (**165**)), 1.0 mM, 0.2 mM, 0.1 mM, and control).

Treated with SGF Control

Figure I. 5. A leaf of *Brassica juncea* cv. Cutlass treated with spore germination fluids (SGF) produced by *Leptosphaeria maculans* inoculated on the leaves of *B. napus* cv. Westar and control.

CHAPTER 1: INTRODUCTION

1.1 GENERAL OBJECTIVES

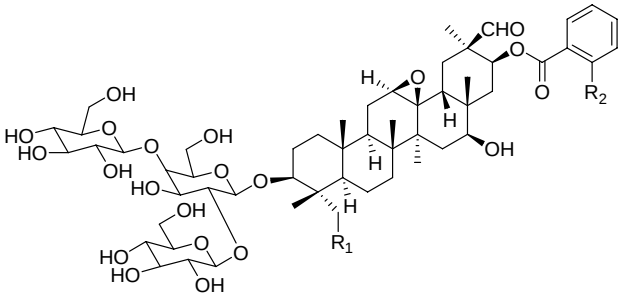
The phytopathogenic fungus *Leptosphaeria maculans* can cause blackleg disease on crucifers, which results in significant yield losses. Fungal diseases involve interactions between pathogenic fungi and host plants. One aspect of these interactions is mediated by secondary metabolites produced by both fungi and host plants. Phytotoxins and elicitors as well as phytoanticipins and phytoalexins are secondary metabolites produced by fungi and plants, respectively. The study of phytotoxins and elicitors produced by *L. maculans* will help to understand the chemical means utilized by this pathogenic fungus to infect its host plants. This thesis describes and discusses the isolation, structure, biological activity and biosynthesis of the secondary metabolites produced by *L. maculans*. This research includes:

- Isolation of secondary metabolites with phytotoxin and/or elicitor activity
- Structural elucidation of metabolites isolated from *L. maculans*
- Biological assays of metabolites isolated from *L. maculans*
- Study of sirodesmin PL biosynthetic precursors (a phytotoxin produced by *L. maculans*)

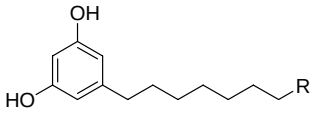
1.2 CHEMICAL INTERACTIONS BETWEEN FUNGI AND THEIR HOST PLANTS

Metabolites synthesized and utilized by all living organisms are called primary metabolites, i.e. metabolites essential for life: carbohydrates, amino acids, common fatty acids, and nucleotides, (Mann, 1994). On the other hand, the metabolites that are synthesized and utilized by a specific group of living organisms are called secondary metabolites and have very diverse chemical structures. Based on the biosynthetic origin, secondary metabolites include polyketides, isoprenoids, shikimates, alkaloids and metabolites of mixed biosynthetic origin (Mann, 1994). Secondary metabolites are produced by living organisms during a particular stage of growth and development or during periods of stress caused by nutritional limitation or microbial attack. Secondary metabolites are not essential for life but are important in fitness and survival and play important roles in the plant-microbe interaction. After hundreds of millions of years of co-evolution, plants evolved sophisticated defense mechanisms to protect them from the attack of pathogens. One of these defense mechanisms involves preformed and induced metabolites. One group of these preformed metabolites is called phytoanticipins, which are low molecular weight, antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from preexisting constituents (Grayer and Kokubun, 2001; Osbourn, 1996; Vanetten et al., 1994). The presence of phytoanticipins, acting as the first layer of chemical barriers, can inhibit the growth of fungi. For example, the preformed saponins avenacins (**1** to **4**) are the first layer of chemical barriers in oats (*Avena sativa*). Oats are resistant to the wheat (*Triticum aestivum*) root pathogen *Gaeumannomyces graminis* var. *tritici* due to the presence of avenacins in healthy oat plants. Mutants of oats that do not produce avenacins are susceptible to *G. graminis* var. *tritici* (Papadopoulou et al., 1999). Therefore, the phytoanticipins avenacins effectively inhibit the growth of the pathogen *G. graminis* var. *tritici*. However, successful pathogens can detoxify phytoanticipins, and further infect host plants (Vanetten et al., 2001). The oat root pathogen *G. graminis* var. *avenae* produces a glycosidase that removes the sugar residue from avenacins. The resulting metabolites are not antifungal, therefore, oat plants are susceptible to *G. graminis* var. *avenae* (Thordal-Christensen, 2003; Vanetten et al., 1995). In another

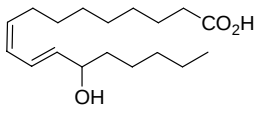
example, phytoanticipins produced by rice act as the first chemical barriers to inhibit the colonization by rice blast fungus *Pyricularia oryzae*. 5-(8'Z-Heptadecenyl)resorcinol (**5)** and a mixture of its homologues (**6**-**8** and **9**) were isolated from etiolated rice seedlings and showed antifungal activity against rice blast fungi (Suzuki et al., 1996). These 5-alkylresorcinols were newly produced after germination and the concentration reached to 50 μg/g fresh weight on day 6 (Suzuki et al., 1996). From older rice plants hydroxy and epoxy fatty acids (such as **10** and **11**) showing antifungal activity were isolated (Kato et al., 1993b). However, it seems that *P. oryzue* can avoid these phytoanticipins using a specific ATP-binding cassette (ABC) transporter, which can take away these antimicrobial metabolites produced by rice (Urban et al., 1999).



1 R_1=OH, R_2=NHMe
2 R_1=OH, R_2=H
3 R_1=H, R_2=NHMe
4 R_1=H, R_2=H



5 $AR_{17:1}$ R=CH=CH$(CH_2)_7CH_3$
6 $AR_{13:0}$ R=$(CH_2)_5CH_3$
7 $AR_{15:1}$ R=CH=CH$(CH_2)_5CH_3$
8 $AR_{15:0}$ R=$(CH_2)_7CH_3$
9 $AR_{17:0}$ R=$(CH_2)_9CH_3$



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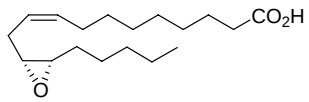

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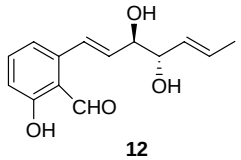

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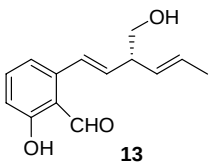

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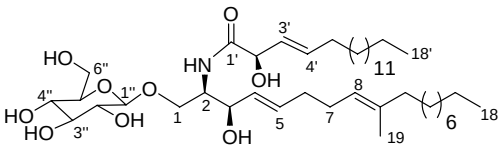
Fungi produce a variety of toxic metabolites, phytotoxins, which cause damage on plant tissues and usually play a role in the establishment of infection on plants

(Walton, 1996; Walton and Panaccione, 1993; Wolpert et al., 2002). Pyriculol (**12**) was the first phytotoxin isolated from the rice blast fungus (Iwasaki et al., 1969; Suzuki et al., 1987). Pyriculol (**12**) was shown to cause dark necrotic spots on rice leaves, similar to the rice blast fungus. Pyricuol (**13**), a pyriculol related metabolite, was isolated from the same fungus, and caused more serious damage on rice leaves than pyriculol (**12**) (Kim et al., 1998; Nakamura et al., 2005).



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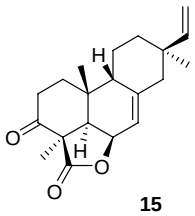
Once fungi overcome preformed barriers to initiate colonization of plants, the recognition of elicitors by plants becomes the key step determining the outcome of the interaction. Elicitors are signal molecules produced by pathogens (exogenous) or plants (endogenous) (Montesano et al., 2003). If elicitors are recognized by plants, defense mechanisms are induced, for example, the production of phytoalexins and reactive oxygen species (ROS), as well as the hypersensitive response (HR, a programmed cell death) at the site of infection. The timely recognition of elicitors and subsequent induction of defense mechanisms most of the time can stop further infection by fungi. For example, the sphingolipid cerebroside C (**14**), which was isolated from diverse pathogenic fungi (Koga et al., 1998; Umemura et al., 2004), induced the production of the rice phytoalexin momilactone A (**15**), and further conferred disease resistance of rice plants to the rice blast fungus (Umemura et al., 2004).



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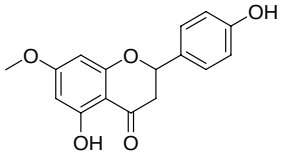

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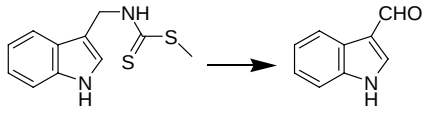
Phytoalexins, acting as the second chemical barriers of plant defense mechanisms, are low molecular weight, antimicrobial compounds that are both synthesized de novo by and accumulated in plants after exposure to microorganisms (Bailey and Mansfield, 1982). For example, the flavanone phytoalexin sakuranetin (**16**) was detected in blast-infected rice leaves and the content in a resistant cultivar after infection with *P. oryzue* was much higher than in a susceptible cultivar, which indicated that sakuranetin (**16**) contributed to defense in the resistant cultivar (Kodama et al., 1992). Many successful fungi have a way to circumvent phytoalexins. For example, brassinin oxidase, which is produced by *L. maculans*, oxidizes brassinin (**17**), a phytoalexin produced by crucifers, to indole-3-carboxaldehyde (**18**). Aldehyde **18** showed no antifungal activity against *L. maculans* (Scheme 1.1, Pedras and Ahiahonu, 2005).



17 **18**

Scheme 1.1. Detoxification of brassinin (**17**) by *Leptosphaeria maculans*.

The recognition, production, utilization, and detoxification of elicitors, phytotoxins, phytoanticipins and phytoalexins represent aspects of the chemical interactions between fungi and their host plants. The recent progress on the isolation, structure, and biosynthesis of elicitors, phytotoxins, phytoanticipins and phytoalexins as well as their roles in the chemical interactions of fungi and plants are presented.

1.2.1 Metabolites produced by fungi

The secondary metabolites produced by fungi have very diverse structures and different functions, many of which are still not clear. However, two types of metabolites produced by fungi are important and better understood in the plant-microbe interactions: elicitors and phytotoxins.

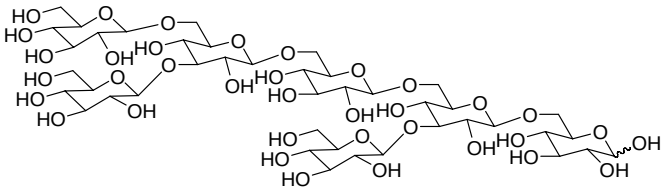
1.2.1.1 Elicitors

Elicitors induce various plant defense responses. Detection of plant defense responses can be used as an elicitor activity bioassay. The most widely used bioassay method is detection of phytoalexins. This method actually gave the original definition of elicitors, i.e. metabolites capable of inducing the biosynthesis of phytoalexins are called elicitors (Keen, 1975). This definition was extended to include all compounds that can induce any plant defense responses (Montesano et al., 2003). Elicitor bioassay methods include detection of: glycosylhydrolases, reactive oxygen species, callose, hydroxyproline-rich glycoproteins, lignin, and measurement of pH of cell cultures (Hahn, 1996; Montesano et al., 2003). Elicitors display a wide range of chemical structures with no particular motifs, but based on the mode of elicitation, two types of elicitors can be considered: general elicitors and race specific elicitors (Ferreira et al., 2006). General elicitors are able to trigger defenses both in host and non-host plants, whereas race specific elicitors induce defense responses only in specific hosts.

General elicitors

General elicitors are also called microbial associated molecular patterns (MAMPs), i.e. indispensable and unique constituents of microbes such as ergosterol, chitin, β-glucan and fungal-specific glycosylated proteins (Jones and Takemoto, 2004; Nurnberger et al., 2004). MAMPs are detected by plant transmembrane pattern recognition receptors and lead to non-host resistance (Jones and Dangl, 2006; Jones and Takemoto, 2004). The presence of MAMPs and their transmembrane pattern recognition receptors explain the observation that not all pathogens can infect all plant species. The attempt of infection from non-host pathogens is most of the time inhibited by plant defense mechanisms induced by MAMPs. For example, the β-glucan fragment **19**, a classic exogenous general elicitor, from the rice blast disease fungus *P. oryzae*, elicits the biosynthesis of the phytoalexins momilactones A (**15**) in rice cell suspension cultures (Yamaguchi et al., 2000). Examples of endogenous general elicitors are plant-cell-wall-derived oligogalacturonides, which are products of cell-wall-degrading enzymes produced by fungi. These oligogalacturonides are known to induce the biosynthesis of

the phytoalexin glycinol (**20**) in cotyledons of soybean (Nothnagel et al., 1983; Shibuya and Minami, 2001; Weinstein et al., 1981).



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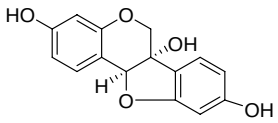

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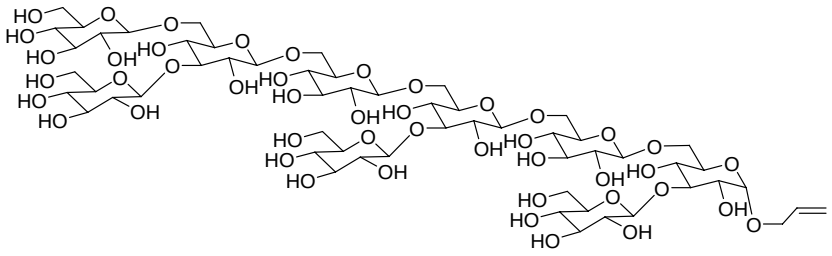
Unique to microbes, general elicitors are not produced by (potential) host plants, and appear to be indispensable for microbial fitness (Nurnberger et al., 2004). Although termed "general elicitors", the detection and consequent induction of defense response of general elicitors is relative, since some of them are recognized by only a restricted number of plants (Ferreira et al., 2006). For example, soybean and rice cells responded selectively to the glucan oligosaccharide elicitors prepared from *Phytophthora sojae* and *Magnaporthe grisea* that carry different structural features (Shibuya and Minami, 2001). Pep-13, a peptide elicitor isolated from *P. sojae*, was not recognized by host soybean but triggered a defense response in non-host plants such as parsley and potato (Brunner et al., 2002; Jabs et al., 1997; Shibuya and Minami, 2001).

(i). β-Glucan oligosaccharides

Oligosaccharide elicitors are among the first characterized general elicitors derived from the β-glucans by partial acidic hydrolysis or heating cell walls of fungi. A doubly-branched hepta-β-glucoside (**19**) that was generated from *P. sojae* glucan was shown to be a very active elicitor for glyceollin II (**24**) biosynthesis in soybean cotyledon cells (Sharp et al., 1984a; Sharp et al., 1984b; Sharp et al., 1984c). Hepta-β-glucoside elicitors of *P. sojae* showed elicitor activity not only on soybean but also on various plants of plant family Fabaceae (Cosio et al., 1996). To study structure-activity relationships more than 15 oligosaccharides were synthesized and tested for elicitor activity on soybean (Cheong et al., 1991). Synthetic (**21**-**22**) and naturally obtained (**19**)

oligosaccharides were found to have the highest elicitor activity. All three non-reducing terminal glucosyl residues and two β(1→3) branch linkages (compound **23**) are the minimum structural requirement for elicitor activity on soybean cotyledons (Cheong et al., 1991).



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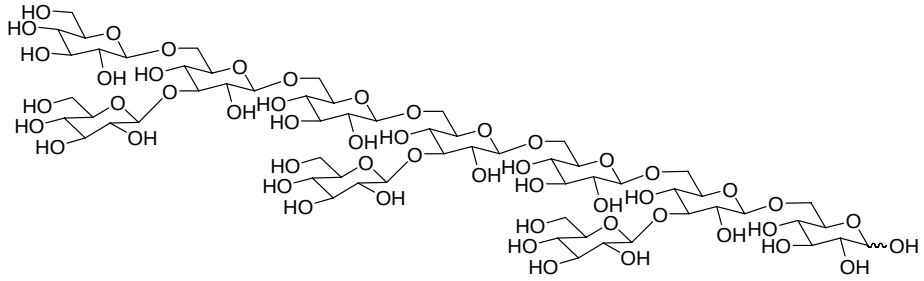

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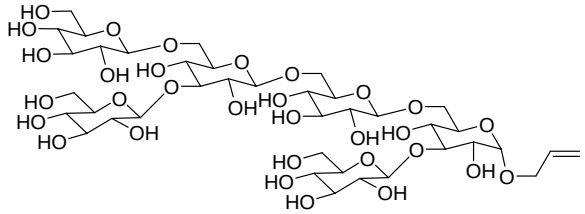

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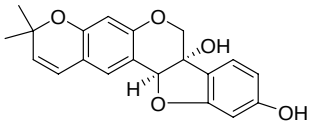

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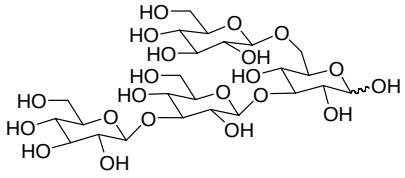
Tetraglucosyl glucitols (**25** - **27**) purified from an enzymatic digest of the β-glucan from the rice blast fungus induced phytoalexin biosynthesis in rice cell suspension cultures (Yamaguchi et al., 2000). The backbone of the elicitor-active glucopentaose has to be 1,3-linked residue and branched at the 6-position. Whileas in the hepta-β-glucoside elicitor from *P. sojae*, the backbone is a 1,6-linked β-glucooligosaccharide with branches at the 3-position (Yamaguchi et al., 2000).

Moreover, linear β-1,3-linked glucooligosaccharides but not hepta-β-glucosides showed elicitor activity on tobacco cells. Among tested linear β-1,3-linked glucooligosaccharides, laminaripentaose **(28**) is the smallest polymer found to have elicitor activity (Klarzynski et al., 2000). From these results it seems that recognition of β-glucan oligosaccharide elicitors by different plants is selective, although not race-specific.



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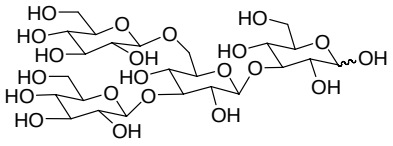

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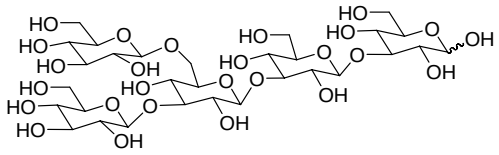

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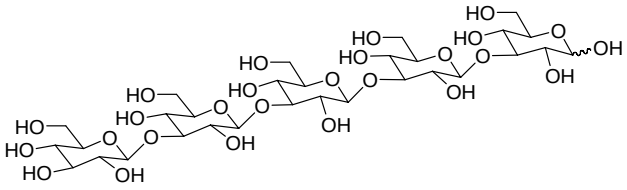

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(ii) Chitins

Chitins, β-1,4-linked polymers of *N*-acetylglucosamine, are indispensible components of fungal cell walls and not present in plants. For example, in powdery mildews cell wall chitin and β-1,3 glucans are detectable at all stages of haustorial development (Mims et al., 2004; Ramonell et al., 2005) The fragments of chitins (monomer to hexamer) were tested for elicitor activity in induction of lignification in wheat (Barber et al., 1989). Tetramer, pentamer and hexamer all possessed significant elicitor activity, but not other smaller fragments. It was also found that a pre-wounding was necessary to detect elicitor activity of these fragments (Barber et al., 1989). The

fragments of chitins have been shown to have strong elicitor activity in several other plant systems, such as induction of ion flux and protein phosphorylation in cultured tomato cells (Felix et al., 1993), and chitinase activity in melon (Roby et al., 1987). In suspension-cultures of rice cells, chitin fragments were also able to induce the biosynthesis of diterpene phytoalexins (Ren and West, 1992; Yamada et al., 1993) and generated reactive oxygen species (Kuchitsu et al., 1995) and some other defense responses (Shibuya and Minami, 2001).

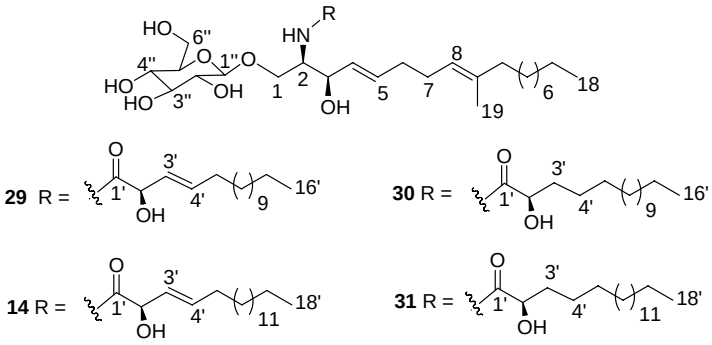
(iii) Chitosan

Chitosan, a collective term of deacetylated products of cell wall chitins, has also been shown to elicit the biosynthesis of phytoalexin momilactone A (**15**) and sakuranetin (**16**) (Agrawal et al., 2002). Chitosan or its fragments can induce plant defense responses including callose formation in soybean and parsley cells (Conrath et al., 1989), and phytoalexin biosynthesis in pea (Hadwiger and Beckman, 1980). However, the concentration required for these responses is usually much higher than those necessary for activity of chitin fragments. This may be related to the mode of action in which chitosan seemed to interact with negatively charged phospholipids rather than with receptor-like molecules (Kauss et al., 1989).

(iv) Cerebrosides

Cerebrosides, which belong to sphingolipids, are the essential components of fungal cell membranes (Dickson and Lester, 1999, 2002). A recent report indicated that conversion of sphingolipids to glycosphingolipids is essential for spore germination, hyphal growth and cell cycle in *Aspergillus* species (Levery et al., 2002). Cerebrosides A (**29**), B (**30**), C (**14**) and D (**31**) have been isolated as novel elicitors from *Fusarium oxysporum*, a common soilborne agent of wilt disease affecting a wide range of plant species. Later on, Cerebrosides A (**29**), B (**30**), C (**14**) and D (**31**) were found in a wide range of soilborne phytopathogens such as *Pythium graminicola*, *Botrytis allii*, *Glomerella cingulata*, and *Sclerotium cepivorum* (Koga et al., 1998; Umemura et al., 2004). Cerebroside elicitors can induce the production of phytoalexins and pathogenesis-

related proteins and also confer disease resistance in rice plants to the rice blast fungus in paddy fields (Umemura et al., 2004).



(v) Fungal proteins

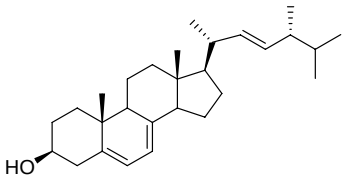
Fungal proteineous elicitors mainly include xylanases and Nep1-like proteins (NLPs). Xylanases are fungal endo-β-1,4-xylanases which aid the invasion of fungi by hydrolyzing plant cell wall cellulose, pectin, and xylan. However, these xylanases induce the plant defense response mechanisms. Application of xylanases on tobacco or tomato leaves induced biosynthesis of ethylene, phytoalexins and pathogenesis-related proteins as well as caused necrosis and hypersensitive cell death (Bailey et al., 1990; Fluhr et al., 1991; Avni et al., 1994). Interestingly, the enzyme activity of xylanases is not essential for their elicitor activity (Enkerli et al., 1999).

Nep1-like proteins (NLPs) were identified as protein elicitors from oomycetes, fungi and bacteria (Gijzen and Nurnberger, 2006). NLPs were able to elicit plant defense responses, but without clear function for pathogens except that maybe related with accelerating disease and pathogen growth in host plants (Gijzen and Nurnberger, 2006). Nep1 (necrosis-eliciting protein 1), a 24-KDa elicitor protein was first isolated from *F. oxysporum* as a necrosis and ethylene-inducing protein (Bailey, 1995). It caused the necrosis and ethylene production only on dicotyledonous plant species tested, but not in monocotyledons (Bailey, 1995). Later on, NPP1 (necrosis-inducing *phytophthora* protein 1) was characterized from cell walls of *Phytophthora* spp. and elicited the HP in parsley and *Arabidopsis* spp. (Fellbrich et al., 2002). The NPP1 domain was found to be present in all NLPs (Fellbrich et al., 2002). Recently, BcNEP1 and BcNEP2, two NPPs

from necrotrophic phytopathogenic fungus *B. cinerea*, were characterized and displayed elicitor activity only on dicotyledonous plant species tested (Schouten et al., 2008).

(vi) Others

Ergosterol (**32**) is another indispensible component in fungal cell walls. It triggers a defense reaction in tobacco and tomato cells such as production of reactive oxygen species (Granado et al., 1995), biosynthesis of the phytoalexin capsidiol in tobacco (Kasparovsky et al., 2003) and biosynthesis of the phytoalexin resveratrol (**33**) in grapevine (Laquitaine et al., 2006).



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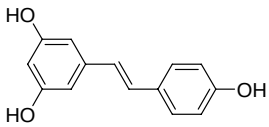

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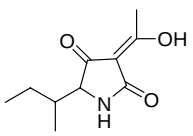
Tenuazonic acid (**34**), a toxin produced by rice blast fungus *P. oryzae*, can cause small brown necrotic spots on leaves of rice. This effect leads to local disease resistance by most likely inducing the generation of reactive oxygen species in rice leaves (Aver'yanov et al., 2007). Arachidonic acid (**35**) was isolated from the pathogenic fungus *Phytophthora infestans* (Bostock et al., 1981). It elicits phytoalexin production in potato (Bostock et al., 1981) and pepper (Bloch et al., 1984; Hoshino et al., 1994) but not in tobacco, although these three different species belong to the family Solanaceae (Garcia-Pineda and Lozoya-Gloria, 1999).



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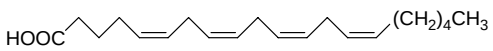

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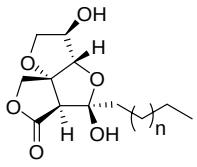
Specific elicitors

The majority of race-specific elicitors so far identified are proteins. The occurrence of specific elicitors is the result of the co-evolution between plants and microbes. It is assumed that individual phytopathogenic races or strains of a given pathogen species were able to overcome plant non-host resistance through the acquisition of virulence factors, which enable them to either evade or suppress plant immune systems (Nurnberger et al., 2004). Consequently, plants became hosts to these microbes. On the other hand, as a result of co-evolution, individual cultivars of a host plant species have evolved specific mechanisms to recognize virulence factors and initiate defense mechanisms. In such cases, virulence factors became specific signals of pathogens, i.e. race specific elicitors, which are encoded by avirulence (Avr) genes (Nurnberger et al., 2004).

Race-specific elicitors selectively trigger defense response in host plants carrying specific receptors, a nucleotide binding and leucine rich repeat protein encoded by most resistance genes (Jones and Dangl, 2006). The outcome of interaction is determined by complementary pairs of pathogen-encoded avirulence genes and plant resistance genes. Lack of either gene will result in infection on host plants (Flor, 1955; Keen, 1975). Nucleotide binding and leucine rich repeat protein-mediated disease resistance is effective against biotrophic and hemibiotrophic pathogens, but not against necrotrophic pathogens (Glazebrook, 2005).



36 n = 2
37 n = 4

Although almost all the specific elicitors identified so far are proteins or polypeptides, syringolides (**36** and **37**) are the only exceptions to date (Ji et al., 1998; Midland et al., 1993; Collmer and Gold, 2007). Syringolides (**36** and **37**) are a group of non-proteineous secondary metabolites isolated from bacterial *Pseudomonas* spp.

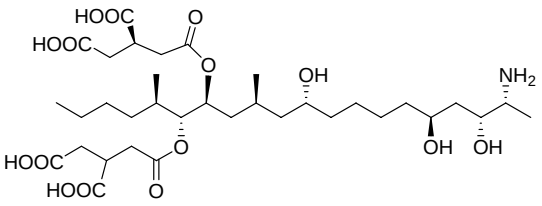
Syringolides (**36** and **37**) triggered defense responses, such as hypersensitive cell death and a hydrogen peroxide burst in cultivars of soybean carrying the *Rpg4* disease-resistance gene (Ji et al., 1998; Midland et al., 1993; Collmer and Gold, 2007). To date, syringolides A and B (**36** and **37**) are also the only nonproteinaceous specific elicitors of which the avirulence gene (*avrD*) and resistance gene (*Rpg4*) are identified (Ji et al., 1998; Midland et al., 1993; Collmer and Gold, 2007). Syringolides (**36** and **37**) have not been isolated from fungi.

1.2.1.2 Phytotoxins

Phytotoxins are secondary metabolites produced by fungi that damage the plant tissues and contribute to fungal colonization of plants (Walton, 1996; Wheeler and Luke, 1963). The phytotoxicity of metabolites is determined by phytotoxicity bioassays. The most frequently used bioassays include leaf puncture assays, electrolyte leakage assays, germination assays and root (radicle) growth assays (reviewed in Hoagland and Williams, 2004). By measuring sizes of necrotic or chlorotic lesions caused by test metabolites, the phytotoxicity can be directly compared with controls. This is the most employed method in determination of phytotoxicity of a metabolite. Electrolyte leakage assays measure the conductivity of cellular electrolytes which are caused by disruption of membranes by tested metabolites. Electrolyte leakage assays can be carried out using tissue slices, leaf disks or cell culture suspensions. Germination assays are more frequently used in allelopathy research. Test metabolites are dissolved in various concentrations and seeds are placed in solutions on supporters such as filter or germination blotter paper, or the test solution is solidified by mixing with PDA or other agents. Sometimes the seed germination is not sensitive to the effect of test metabolites. In this case, root (radicle) growth can be further used as a measurement to determine the phytotoxicity of test metabolites.

The dividing line between phytotoxins and elicitors sometimes is not clear. Depending on the type of interaction, some compounds can act as elicitors or

phytotoxins. For example, fumonisin B1 (**38**), isolated from *Fusarium verticillioides* (Stone et al., 2000), can act as an elicitor inducing hypersensitive responses (HR) in the plant *Arabidopsis thaliana*. It also acts as a phytotoxin in the interaction of the necrotrophic pathogen *F. verticillioides* with its host, maize (Desjardins et al., 2000). Coronatine (**39**) is a phytotoxin produced by certain races of the pathogen *Pseudomonas syringae* (Ichihara et al., 1977). It mimics jasmonic acid, acting as a plant hormone in *A. thaliana* (Feys et al., 1994; Kloek et al., 2001). Coronatine (**39**) also elicited the expression of defense genes in *A. thaliana* (Bohlmann et al., 1998), and biosynthesis of phytoalexins in rice leaves in the same manner as jasmonic acid (Tamogami and Kodama, 2000). Based on the spectrum of toxicity on different plants, phytotoxins are divided in host-selective phytotoxins and nonhost-selective phytotoxins. Host-selective phytotoxins induce the cell death only on host plants, whileas nonhost-selective phytotoxins causes damages or necrosis on both host and non-host plants (Markham and Hille, 2001; Pringle and Scheffer, 1964; Walton, 1996). Reviews about phytotoxins and their applications can be found in recent publications (Hoagland, 2001; Hoagland et al., 2007; Kimura et al., 2001; Strange, 2007; Singh et al., 2003).



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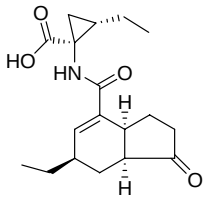

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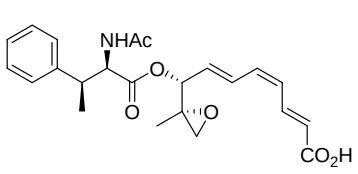
Host-selective phytotoxins

HSTs producing fungi are virulent to the plants that are sensitive to HSTs (host plants), but not virulent to plants that are not sensitive to HSTs (non-host plants). Disruption of gene(s) encoding the production of HSTs removes the virulence of these mutants to host plants (Walton, 1996; Wolpert et al., 2002). Therefore, HSTs are necessary for fungi to be virulent on host plants. It seems that only necrotrophic fungi produce HSTs which cause the lysis of plants cells. The non-living plant cells provide

the desired conditions for the colonization of necrotrophic pathogens (Friesen et al., 2008; Markham and Hille, 2001).

A few proteinaceous HSTs were isolated and identified in recent years. For example SnTox1, the first identified HST produced by *Stagonospora nodorum*, was shown to be a 10–30 kDa proteinaceous HST (Friesen et al., 2008). However, the proteinaceous HSTs will not be covered in this introduction. Most HSTs are secondary metabolites from different biosynthetic pathways; for example, polyketides, terpenoids, cyclic peptides and mixed pathways. The isolation, structure, mode of action and role of virulence in the plant-microbe interaction of HSTs has been reviewed in several articles (Walton, 1996, 2006; Wolpert et al., 2002). The HSTs produced by genera *Alternaria* and *Cochliobolus* (Table 1.1) have been extensively studied and provide excellent examples of chemical diversity and host selective phytotoxicity.



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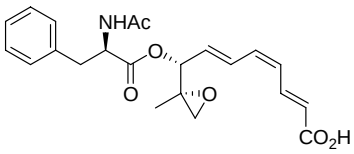

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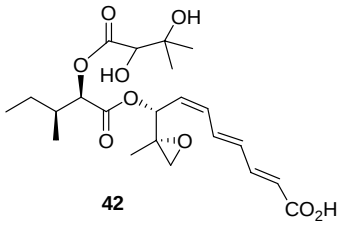

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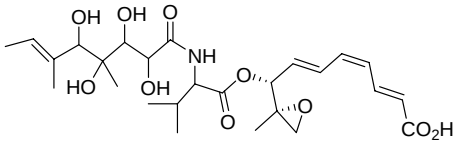

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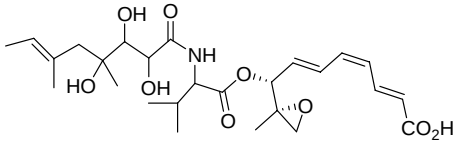

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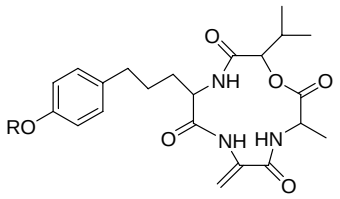
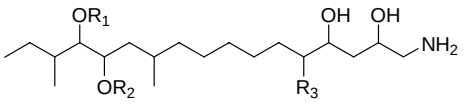


45 R=OCH_3
46 R=H
47 R=OH



48 R_1=H, R_2=Y, R_3=OH
49 R_1=Y, R_2=H, R_3=OH
50 R_1=R_2=R_3=H
51 R_1=R_2=Y, R_3=H

Y=

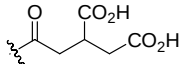
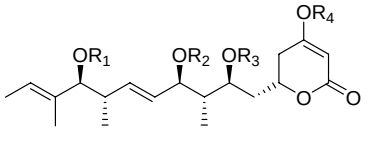
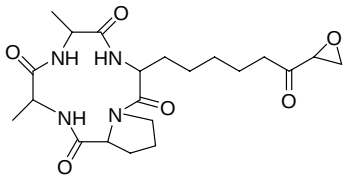


Table 1.1. HSTs produced by genera *Alternaria* and *Cochliobolus* (Pedras et al., 2002; Walton, 1996, 2006; Wolpert et al., 2002).

Species	Host plants	HSTs	Chemical types
A. alternata spp.	Japanese pear	AK-toxin I (**40**) and II (**41**)	Epoxy-decatrienoic
A. alternata spp.	Strawberry	AF-toxin I (**42**)	Epoxy-decatrienoic
A. alternata spp.	Tangerine	ACT-toxin Ib (**43**) and Ic (**44**)	Epoxy-decatrienoic
A. alternata spp.	Apple	AM-toxin I (**45**), II (**46**) and III (**47**)	Cyclic tetrapeptide
A. alternata spp.	Tomato	AAL-toxins (**48** – **51**)	Aminopentol
A. alternata spp.	Rough lemon	ACR(L)-toxin (**52**-**54**)	Terpenoid
A. brassicae	Brassica spp.	Destruxin B (**61**)	Cyclodepsipeptide
C. carbonum	Corn	HC-toxin (**55**)	Cyclic tetrapeptide
C. heterostrophus	Corn	T-toxins (**56** – **59**)	Polyketides
C. victoriae	Oats	Victorin C (**60**)	Chlorinated depsipeptide



52 $R_1=R_2=R_3=Ac$, $R_4=Me$
53 $R_1=R_2=R_3=H$, $R_4=Me$
54 $R_1=R_2=R_3=R_4=H$



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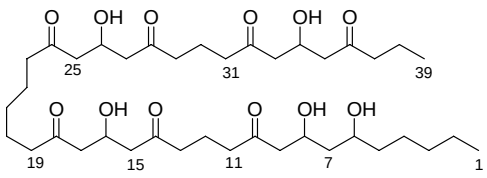

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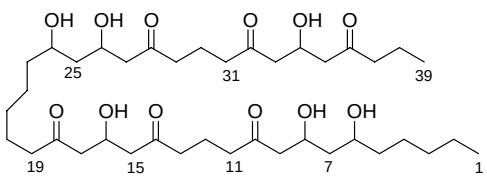

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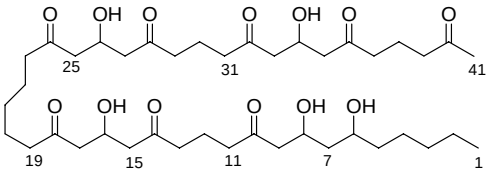

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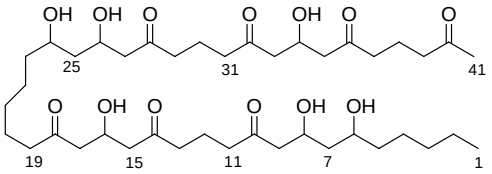

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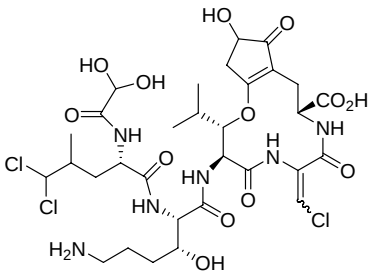

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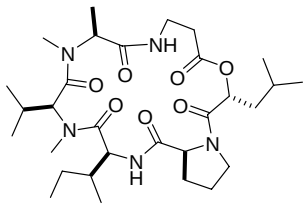
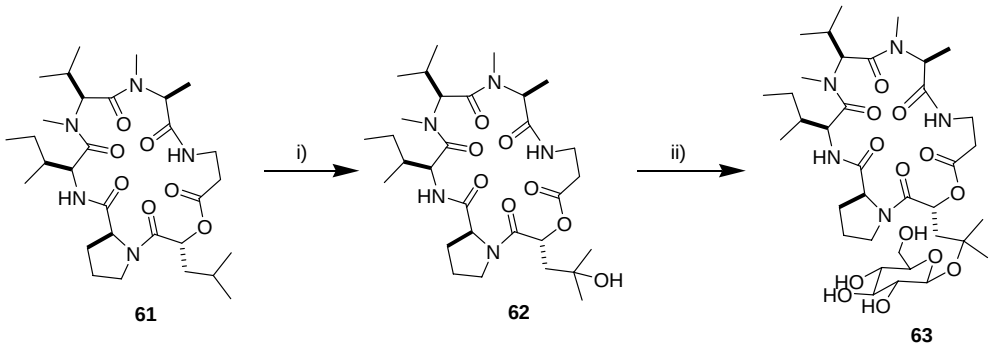


61

Each pathotype of *A. alternata* spp. produces HSTs that cause disease symptoms on its host plant species. Japanese pear pathotype produced epoxydecatrienoides AK-toxins I (**40**) and II (**41**), which caused black spot on Japanese pear at very low concentration (10^{-8}M). AF toxins for example AF-toxin I (**42**), produced by germinating spores of strawberry pathotype of *A. alternata* spp., caused black spot specifically on strawberry. The other epoxy decatrienoic acid type HSTs ACT-toxin Ib (**43**) and Ic (**44**) produced by tangerine pathotype of *A. alternata* spp displayed high toxicity (< 10^{-6}M) and selectivity (same host range as producing fungal isolates), suggesting the essential role of ACT-toxin Ib (**43**) and Ic (**44**) in disease development in susceptible tangerines. AM-toxins I (**45**), II (**46**) and III (**47**) were isolated from Alternaria blotch of apple causing pathotype *A. alternaria* f. sp. *mali* and displayed potent toxicity (10^{-8}M), causing electrolyte loss and necrosis in susceptible apple tissue. AM toxins possess a four-membered cyclic depsipeptide consisting of a molecule of L-α-hydroxyisovaleric acid, L-alanine, α-amino acrylic acid and derivatives of L-α-amino-δ-phenylvaleric acid. AAL-toxins (**48** – **51**), produced by Alternaria stem canker causing pathotype *A. alternaria* f. sp.*lycopesici*, caused necrotic lesion on tomato leaves similar to that caused by fungal isolate. AAL-toxins, structurally related to fumonisin B1 (**38**), containing an aminopolyol backbone that is esterified to one (**48** – **50**) or two tricarboxylic acids (**51**). ACR(L)-toxins (**52** – **54**) caused serious damage on cultivars of rough lemon susceptible

to pathotype of *A. alternaria* f. sp.*citri*, the ACR(L) toxin producing pathogen (reviewed in Walton, 1996, 2006; Wolpert et al., 2002).

Destruxin B (**61**), a cyclodepsipeptide produced by *A. brassicae*, caused necrotic and chlorotic symptoms in different plant species, of which *Brassica* species was the most sensitive (reviewed in Pedras et al., 2001). Further, the sensitivity to destruxin B (**61**) within the *Brassica* species decreased as the sensitivity of plant species to the fungus *A. brassicae* decreased, suggesting that destruxin B (**61**) is a HSTs. Destruxin B (**61**) was detected in infected *B. napus* and identified in germinating conidia of *A. brassica* at early stages of the infection process, suggesting the important role of destruxin B (**61**) in the plant-pathogen interaction. Further important evidence came from the detoxification of destruxin B (**61**) in susceptible plant *B. napus* and resistant plant *B. juncea*. Destruxin B (**61**) was detoxified through sequential hydroxylation and glucosylation in both susceptible and resistant plants. However, hydroxylation was the rate limiting step in susceptible plant species but not in resistant plant species. Hydroxydestruxin B (**62**) acted as an elicitor to induce the biosynthesis of phytoalexins in resistant plant species but not in the susceptible plant species (Pedras et al., 2001, 2002).



Scheme 1.2. Detoxification of destruxin B (**61**) in *Brassica napus* and *B. juncea* i) hydroxylation, ii) glucosylation.

HC-toxin (**55**), a tricyclic peptide, is produced by *C. carbonum* race 1, causal agent of Northern leaf spot of maize. It causes necrosis and inhibited growth of seedlings

of susceptible maize whereas it has no effect on maize resistant to *C. carbonum* race 1. Application of HC-toxin on *C. carbonum* race 1 susceptible maize resulted on colonization by *C. carbonum* which was unable to infect *C. carbonum* race 1 susceptible maize. These results suggested the host selectivity of this toxin and its determinant factor of host-selectivity and pathogenicity of *C. carbonum* race 1 (Pedras et al., 2002; Walton, 1996, 2006; Wolpert et al., 2002).

T-toxins (**56** – **59**) are a group of C_{39} and C_{41} polyketo-polyhydroxy metabolites produced by *C. heterostrophus* race T, the causal agent of Southern corn blight disease of maize. T-toxins (**56** – **59**) displayed high toxicity and selectivity toward Texas male sterile maize (Walton, 1996, 2006; Wolpert et al., 2002).

Victorin C (**60**) is the major component of HSTs isolated from cultures of *C. victoriae*, the causal agent of Victoria blight of oats. The structure of victorin C (**60**) was established (Macko et al., 1985) more than 20 years after its isolation (Scheffer and Pringle, 1963). Victorin C (**60**) is a cyclic peptide consisting a glyoxylic acid and a cyclic combination of five unusual amino acids: 5,5-dichloroleucine, threo-β-hydroxylysine, erythro-β-hydroxyleucine, α-amino-β-chloroacrylic acid, and 2-alanyl-3,5-dihydroxycyclopentenone. Victorin C (**60**) inhibited the root growth of susceptible oat (0.1 ng/mL), suppressed dark CO_2 fixation of oat leaf slices and application of victorin C (**60**) on susceptible oat leaf reproduced disease symptom. Further, mutants that did not produce victorin C (**60**) were not able to infect *C. victoriae* susceptible oat (Walton, 1996, 2006; Wolpert et al., 2002).

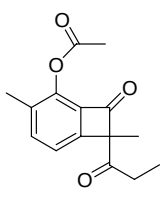
Nonhost-selective phytotoxins

This part covers the nonhost-selective phytotoxins identified from 2003-2008. The phytotoxins isolated from *L. maculans* will be discussed separately in section 1.3.2.

(i) Polyketides

The majority of new phytotoxins identified in this period were polyketides. Four phytotoxins, phyllostoxin (**64**), and phyllostictines A (**65**), B (**66**), and D (**68**) were isolated from *Phyllosticta cirsii*, a fungal pathogen of the perennial weed *Cirsium*

arvense (Evidente et al., 2008a; Evidente et al., 2008b). Phyllostoxin (**64**) and phyllostictine A (**65**) were highly phytotoxic to *C. arvense*, causing rapid necrosis on punctured leaves. The phytotoxicity decreased in phyllostictines B (**66**) and D (**68**). No phytotoxicity was detected in phyllostin (**69**) and phyllostictine C (**67**). Phyllostoxin (**64**) was elucidated as a new pentasubstituted bicyclo-octatrienyl acetic acid ester, whileas phyllostictines A-D were macrocyclic oxazatricycloalkenones. These metabolites could lead to the discovery of herbicides against *C. arvense* (Evidente et al., 2008a; Evidente et al., 2008b).



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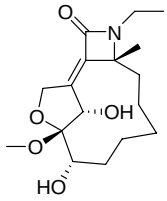

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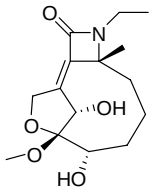

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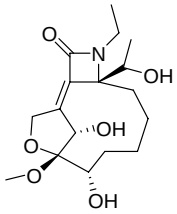

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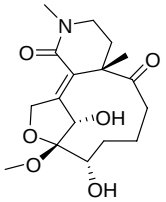

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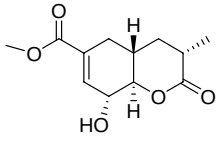

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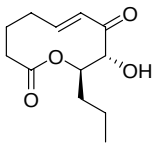
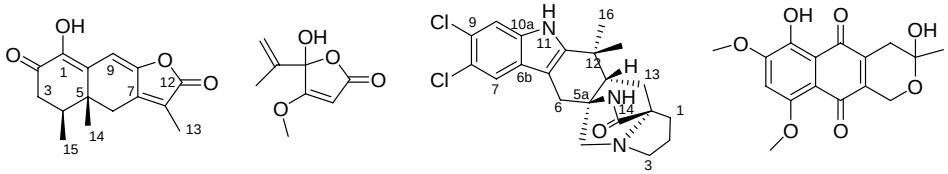
Stagonolide (**70**) was isolated from fungus *Stagonospora cirsii*, pathogenic to the weed *C. arvense*, and the structure was determined to be (8*R*,9*R*)-8-hydroxy-7-oxo-9-propyl-5-nonen-9-olide, which is similar to herbarumins (Rivero-Cruz et al., 2003). Stagonolide (**70**) caused large necrotic lesions not only on host plant leaves of *C. arvense* but also on leaves of hollyhock, sunflower, lettuce, sow-thistle, radish, and peppermint. However, leaves of tomato and pepper (both Solanaceae) were less sensitive to stagonolide (**70**) at similar concentration (5×10^{-3} M). Stagonolide (**70**) also displayed selective inhibitory effect on root growth in seedlings of *C. arvense* and some other Asteraceae species but not on cucumber, and weak effect on wheat and radish (Yuzikhin et al., 2007).



70

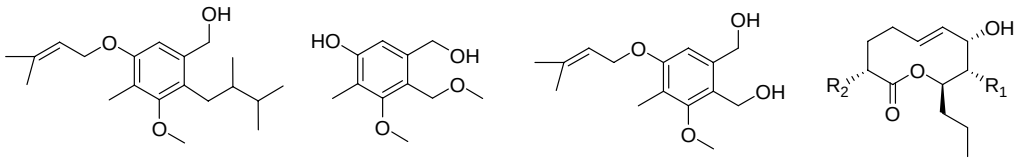
From fungal culture of *Malbranchea aurantiaca*, 1-hydroxy-2-oxoeremophil-1(10),7(11),8(9)-trien-12(8)-olide (**71**) was isolated together with penicillic acid (**72**). Both **71** (IC_{50} = 6.57 µM) and **72** (IC_{50} = 3.86 µM) displayed weaker inhibition of radicle growth of *Amaranthus hypochondriacus* than malbrancheamide (**73**) (Martinez-Luis et al., 2006).



71 **72** **73** **74**

8-*O*-Methylfusarubin (**74**) was isolated from *Fusarium acutatum*, the causal pathogen of chickpea plants. The purified 8-*O*-methylfusarubin (**74**) caused permanent wilting of chickpea cuttings and the LD_{50} value in a cell bioassay was 327 ng/ml (Gopalakrishnan et al., 2005).

Two new phytotoxins 2-(2'',3''-dimethyl-but-1-enyl)-zinniol (**75**), 8-zinniol methyl ether (**76**) were isolated from *Alternaria solani*. These two toxins, structurally related to known phytotoxin zinniol (**77**), caused necrosis on potato leaves (Moreno-Escobar et al., 2005)

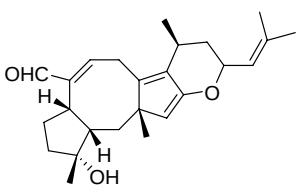


75 **76** **77**

78 R_1=OH, R_2=H
79 R_1=R_2=OH
80 R_1=R_2=H

From the combined extracts of the fermentation broth and mycelium of the fungus *Phoma herbarum* three nonenolide phytotoxins herbarumins I (**78**), II (**79**) and III (**80**) were isolated. These phytotoxins displayed potent inhibitory effect on radicle growth of seedlings of *Amaranthus hypochondriacus* (Rivero-Cruz et al., 2000). It seemed that the phytotoxicity decreased as the number of hydroxyl groups increased. Herbarumins III (**80**) (IC_{50} = 2 × 10^{-5} M) was ten fold more toxic than the positive control commercial herbicide 2,2-dichlorophenoxyacetic acid [2,4-D] (IC_{50} = 2 × 10^{-4} M) (Rivero-Cruz et al., 2003). Herbarumin I (**78**) (IC_{50} = 5 × 10^{-5} M) was more toxic than II (**79**) (IC_{50} = 1 × 10^{-4} M). Metabolites **78** - **80** also displayed inhibitory effect on the calmodulin-dependent enzyme cAMP phosphodiesterase (Rivero-Cruz et al., 2003).

(ii) Terpenoids



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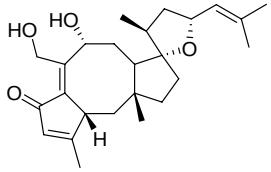

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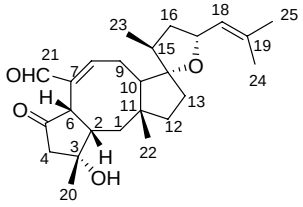

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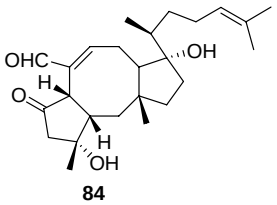

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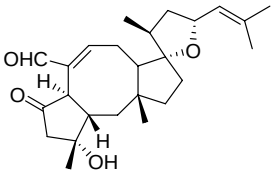

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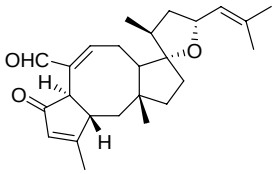

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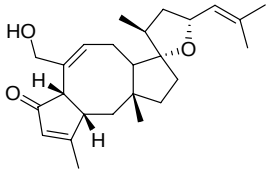

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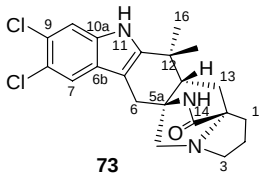
Two new ophiobolins, namely ophiobolin E (**81**) and 8-epi-ophiobolin J (**82**), were isolated from both liquid and solid cultures of *Drechslera gigantea*, a fungal pathogen of large crabgrass (*Digitaria sanguinalis*), together with five known metabolites, ophiobolin A (**83**) and B (**84**), 6-epi-ophiobolin A (**85**), 3-anhydro-6-epi-ophiobolin A (**86**) as well as ophiobolin I (**87**) (Evidente et al., 2006a; Evidente et al., 2006b). Among these metabolites, ophiobolin A (**83**) was more phytotoxic, which may

be due to the hydroxy group at C-3, the stereochemistry at C-6, and the aldehyde group at C-7. Dicotyledoneae appeared to be less sensitive than monocotyledoneae when these metabolites (**81** to **87**) were applied on punctured detached leaves (Evidente et al., 2006a; Evidente et al., 2006b).

(iii) Alkaloids

A novel phytotoxic alkaloid malbrancheamide (**73**) was isolated from the fungus *Malbranchea aurantiaca*. Malbrancheamide (**73**) is a member of the brevianamide type of alkaloids (containing bicyclo [2.2.2] diazaoctane ring system). Malbrancheamide (**73**) caused moderate inhibition of radicle growth of *A. hypochondriacus* (IC_{50}=0.37 μM) (Martinez-Luis et al., 2006).



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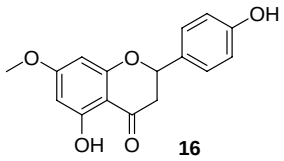
1.2.2 Metabolites produced by plants

Plants also produce a large variety of secondary metabolites with different biological activities. Phytoanticipins and phytoalexins are two important antifungal metabolites produced by both Monocotyledoneae and Dicotyledoneae and involved in the plant-microbe interaction (Bailey and Mansfield, 1982; Grayer and Harborne, 1994; Harborne, 1999; Kuc, 1992, 1995; Osbourn, 1996). Phytoanticipins are preformed antifungal secondary metabolites present in the healthy plants. For example, glucosinolates are stored in vacuoles. Upon the break down of plant cell wall by fungal hyphae, glucosinolates come into contact with a glycosidase, which is separated from glucosinolates in the intact cell. The products of hydrolysis of glucosinolates are toxic and antifungal (Grubb and Abel, 2006). Although these newly produced metabolites by hydrolysis are not detected in healthy plants and induced by the fungal attack, they are still considered as phytoanticipins, because the glycosidase is present in the healthy plants and stored in other compartments of the cells. Sometimes, an antifungal

metabolite may be a phytoanticipin in one organ but a phytoalexin in another organ within the same plant. For example, momilactone A (**15**) was induced in rice leaves, but detected as a constitutive metabolite in rice husks and rice stems. In another case, an antifungal metabolite may be a phytoalexin in one plant species but a phytoanticipin in another. For example, the flavanone sakuranetin (**16**) was isolated from ultraviolet irradiated rice leaves as a phytoalexin, but was released as a phytoanticipin on the surface of leaves of blackcurrant (*Ribes nigra*, Grossulariaceae) from the glands storing it early in the season (Atkinson and Blakeman, 1982).



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Both phytoanticipins and phytoalexins are antifungal metabolites. The detection and determination of antifungal activity were carried out using several methods, such as bioautography on thin-layer plates, radial growth, disk diffusion and microdilution bioassays (Engelmeier and Hadacek, 2006). Bioautography on thin-layer plates (TLC) is useful in detection of antifungal metabolites from crude extracts. The crude extracts are separated by TLC and plates are sprayed with fungal spore suspensions followed by incubation; the bands with antifungal metabolites show no fungal growth. Radial growth is used to determine the antifungal activity after a metabolite is purified. The metabolite is mixed with agar to make a solid medium plate, and a fungal mycelia plug is placed on the surface of the agar medium. The mycelial growth will be inhibited if a metabolite has antifungal activity. Microdilution is a highly efficient method to screen the antifungal activity of pure metabolites or crude extracts. Media with the test metabolites prepared by serial dilution are added to microwells. A fixed amount of conidia suspension is added to the test solution and incubated. The morphological deformations such as curling caused by the antifungal activity of test metabolites can be observed using a microscope. Those antifungal bioassays were reviewed recently (Engelmeier and Hadacek, 2006).

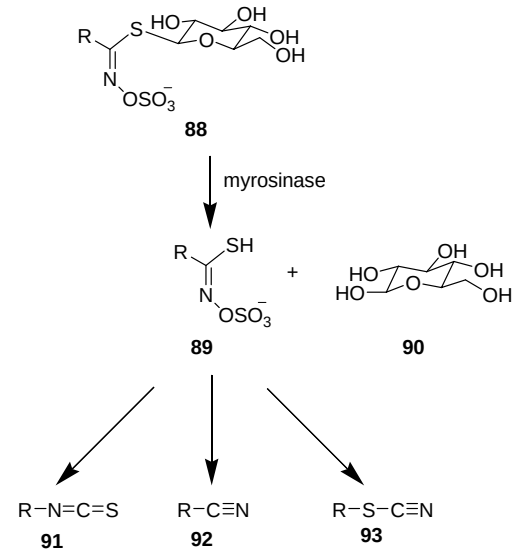
1.2.2.1 Phytoanticipins

Phytoanticipins (coined by Mansfield) are defined as "low molecular weight, antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from preexisting constituents" (Vanetten et al., 1994). Phytoanticipins are commonly sequestered in vacuoles or organelles in healthy plants but some are concentrated in the outer cell layer of plant organs or secreted to the surface of leaves from trichomes (Grayer and Kokubun, 2001; Kelsey et al., 1984; Osbourn, 1996; Shepherd and Wagner, 2007).

A large number of constitutive plant compounds have been reported to have antifungal activity. Well-known examples include phenols and phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenic glycosides, and glucosinolates (reviewed in Ingham, 1973; Schonbeck and Schlosser, 1976; Fry and Myers, 1981; Mansfield, 1983; Bennett and Wallsgrove, 1994; Osbourn, 1996; Grayer and Harborne, 1994; Grayer and Kokubun, 2001; Shepherd and Wagner 2007). The representative glucosinolate and saponin phytoanticipins are described below.

Glucosinolates

Glucosinolates are produced mainly in cruciferous plants, although some 500 other plant species produce one or a few glucosinolates (Grubb and Abel, 2006; Morant et al., 2008). Glucosinolates (also known as (*Z*)-(or *cis*)-*N*-hydroximinosulfate esters) are sulfur-containing glucosides with a sulfur-linked β-D-glucopyranose moiety and a side chain (R). Most glucosinolates are weakly toxic or non-toxic, however, they can be hydrolyzed to toxic metabolites by myrosinase, a hydrolyase stored in all compartments separated from glucosinolates. Once plant tissues are damaged by fungi, myrosinase is released from vacuoles and becomes in contact with glucosinolates, which are hydrolyzed to yield glucose and an unstable aglycone (Scheme 1.3). This aglycone, the thiohydroxamate-O-sulfonate, undergoes spontaneous rearrangement to give different products, of which, nitriles and isothiocyanates are toxic to fungi (Grubb and Abel, 2006; Morant et al., 2008).



Scheme 1.3. Hydrolysis of glucosinolates (**88**).

Glucosinolates are divided into three groups, i.e. aliphatic, indolyl and aryl amino acid glucosinolates, based on the different side chains (R) (Osbourn, 1996). One hundred and twenty glucosinolates were grouped by Fahey and co-workers into A-J groups, based on the similarity of the side chains (Fahey et al., 2001). Table 1.2 lists representative glucosinolates of each group.

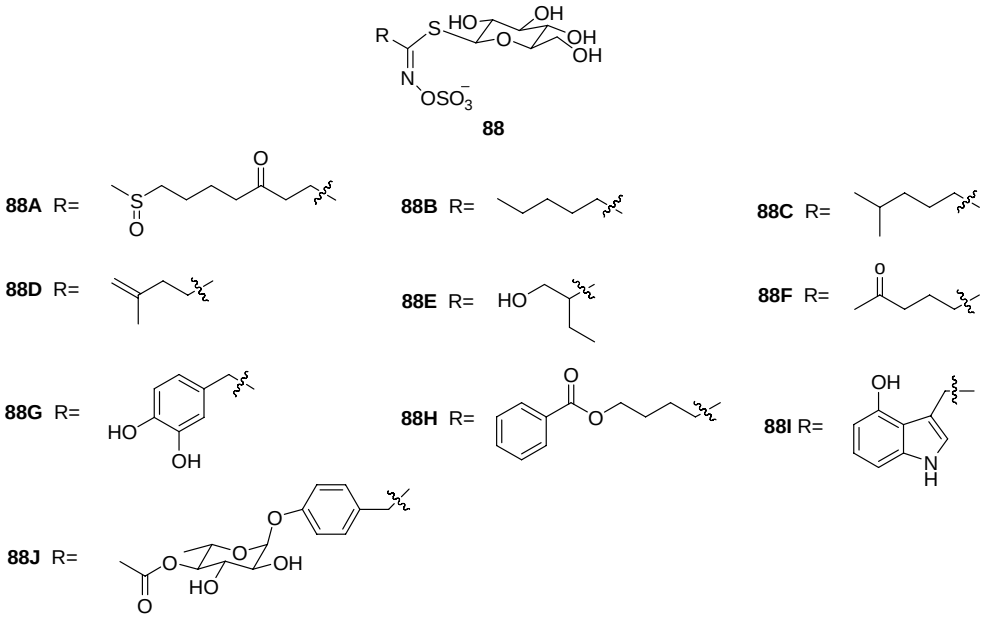


Table 1.2. Examples of glucosinolates based on the types of side chains (Fahey et al., 2001).

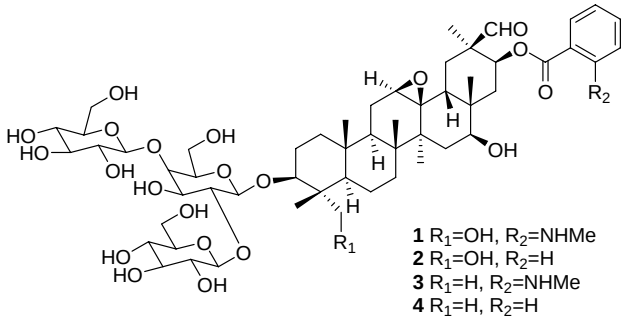
Types of side chains	Chemical name (-glucosinolate)	Common name
Sulfur-containing	7-Methylsulfinyl-3-oxoheptyl (**88A**)	
Straight aliphatic chain	*n*-Pentyl (**88B**)	
Branched aliphatic chain	4-Methylpentyl (**88C**)	
olefins	3-Methyl-3-butenyl (**88D**)	
Aliphatic alcohols	1-Ethyl-2-hydroxylethyl (**88E**)	Glucosisautricin
Aliphatic ketones	4-Oxopentyl or 3-(methylcaronyl)propyl (**88F**)	
aromatic	3,4-Dihysroxybenzyl (**88G**)	Glucomatronalin
ω-hydroxyalkyl (Benzoates)	4-(Benzoyloxy)butyl (**88H**)	
indoles	4-Hydroxyindol-3-ylmethyl (**88I**)	4-Hydroxyglucobrassicin
Others	4-(4'-*O*-Acetyl-α-L-rhamnopyranosyloxy)benzyl (**88J**)	

Saponins

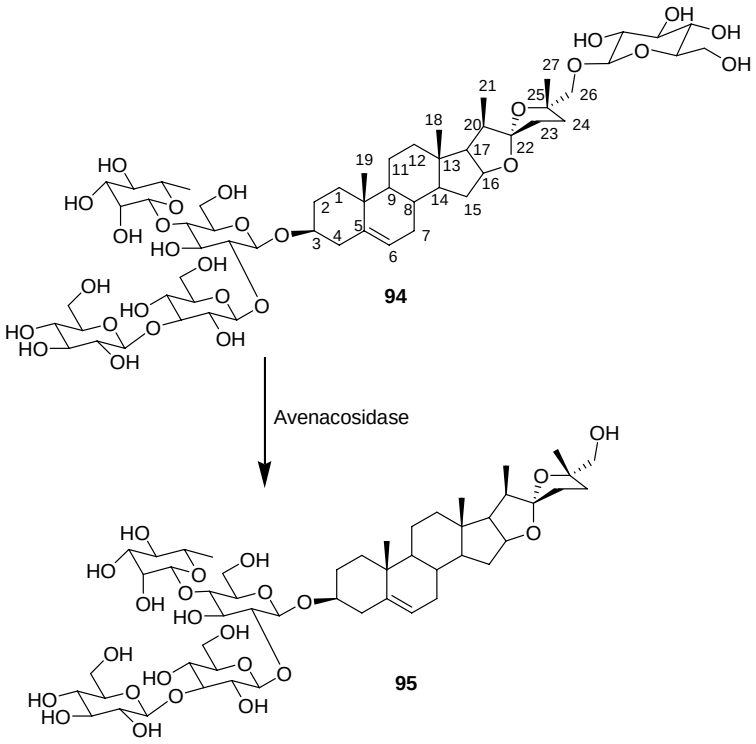
Saponins are glycosylated metabolites that include three major groups, triterpenoid, steroid and steroidal glycoalkaloids. Triterpenoid saponins and steroid saponins were found in both monocots and dicots, while steroidal glycoalkaloid saponins

are found primarily in members of the family Solanaceae (i.e. potato and tomato) and Liliaceae (Osbourn, 1996).

The representative triterpenoid saponins are avenacins in which the sugar unit is a branched trisaccharide, and the aglycones are β-amyrin-derived pentacyclic triterpenoids esterified with either N-methylanthranilic acid (avenacin A-1 (**1**) and avenacin B-1(**3**)) or benzoic acid (avenacin A-2 (**2**) and avenacin B-2 (**4**)). The fungal soil pathogen *G. graminis* var. *tritici* causes the "take-all" disease on wheat and barley but not on oat seedlings. It has been demonstrated that avenacins are directly related to the disease resistance of oat seedlings (Crombie and Crombie, 1986). Avenacins appear to be found exclusively in oat roots (Trojanowska et al., 2000).

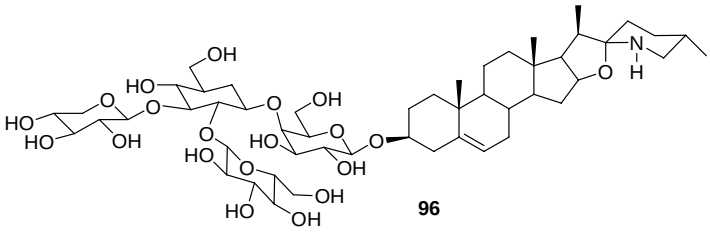


The representative steroidal saponins are avenacosides, which were also isolated from oats (Morant et al., 2008; Tschesch.R et al., 1969). Avenacoside B (**94**), which has an additional *O*-glucose attached to C-26 of their steroidal aglycones, is activated to 26-desglucoavenacoside B (**95**) by a specific plant glucosyl hydrolase upon fungal attack (Scheme 1.3, reviewed in Morant et al., 2008; Osbourn, 1996).



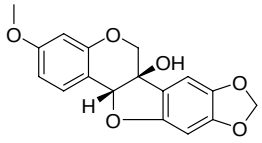
Scheme 1.4. Activation of avenacoside B (**94**) to 26-desglucoavenacoside B (**95**) by a specific plant glucosyl hydrolyase.

α-Tomatine (**96**) is a steroidal glycoalkaloid in which the C-3 of steroid aglycone is attached to two D-glucose residues and then branched at the second D-glucose unit (β-1→4) which is bonded to D-galactose (β-1→2) and D-xylose (β-1→3). α-Tomatine is the active form and is present in particularly high levels in leaves, flowers and green fruits of tomato (Roddick, 1974; Osbourn, 1996).



1.2.2.2 Phytoalexins

Phytoalexins, the plant defensive metabolites against the infection attempts from potential pathogens, are produced by plants *de novo* (Bailey and Mansfield, 1982; Grayer and Harborne, 1994). Phytoalexins are often detected a few hours after elicitation and reach a maximum within a certain period of time. The hypothesis that phytoalexins are induced metabolites produced as a plant self-defense mechanism was proposed by Műller and Boger (Műller and Boger, 1940). They demonstrated that application of an incompatible race of *P. infestans* on potato tuber tissue induced resistance of the tissue to a subsequent infection attempt of a compatible race of *P. infestans*. They hypothesized that potato tubers produced nonspecific metabolites (phytoalexins) under elicitation by the incompatible race of *P. infestans*. These metabolites inhibited the infection attempt by the compatible race of *P. infestans*. The first phytoalexin, pisatin (**97**), was isolated from *Pisum sativum* more than ten years after the proposal of the phytoalexin concept (Perrin and Bottomley, 1961; Hammerschmidt, 1999). Phytoalexins were originally defined as chemical compounds produced as a result of invasion of living cells by a parasite (Műller and Boger, 1940, Grayer and Kokubun, 2001). However, abiotic challenges such as UV and heavy metal ions can also induce the production of antifungal metabolites. Therefore, phytoalexins were defined in many ways. For example, "antibiotics formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors" was the definition of phytoalexins by Ingham (1973). After more than 60 years of phytoalexin research, these metabolites are an indisputable component of plant-microbe interactions (Bailey and Mansfield, 1982; Hammerschmidt, 1999).



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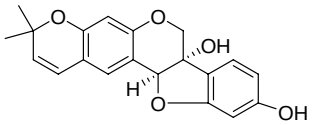
The majority of the plant species studied showed ability to produce phytoalexins upon different elicitation treatments. A few plant families, such as Cucurbitaceae and Rosaceae, did not respond with sufficient production of phytoalexins upon elicitation, which may be due to the presence of high concentration of phytoanticipins or other defense mechanisms (Harborne, 1999). Table 1.3 lists the representative phytoalexins isolated from ten plant families including Gymnospermae and Angiospermae.



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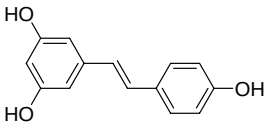

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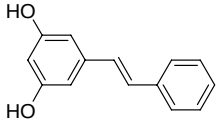
Phytoalexins have diverse structures which derive from the major secondary metabolite biosynthetic pathways, such as polyketides, terpenoids and alkaloids. Tables 1.3 and 1.4 are adapted from reviews (Grayer and Harborne, 1994; Harborne, 1999) and list some representative phytoalexins isolated from ten plant families. The structures of phytoalexins isolated from the same plant family often share structural motifs. For example, isoflavonoids are major phytoalexins isolated from the family Leguminosae. The phytoalexins isolated from Cruciferae contain an indole or related moiety and one or two sulfur atoms. There are also overlaps of phytoalexins between taxonomically non-related plant families. For example, the phytoalexin resveratrol (**33**) was found in the dicots peanut (Leguminosae) and vine (Vitaceae) as well as in gymnosperms in *Pinus* sapwoods (Harborne, 1999). The legume phytoalexin glyceollin II (**24**) was isolated from the dicot soyabean (*Glycine max*) and monocot *Costus speciosus* (Costaceae) (Harborne, 1999; Lyne et al., 1976).

Table 1.3. Selected phytoalexins produced by plants of Gymnospermae (Grayer and Harborne, 1994; Harborne, 1999).

	Family	Chemical type	Examples
Gymnosperms	Pinaceae	Stilbenes	pinosylvin (Schultz et al., 1992) (**98**) and resveratrol (**33**)
	Cupressaceae	Terpenoids	cupressotropolone A (**99**) and B (**100**), (Madar et al., 1995)



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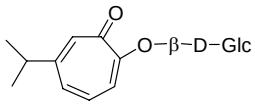

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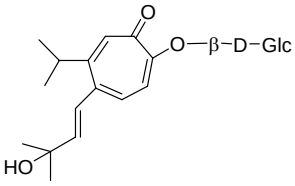

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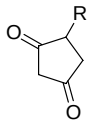
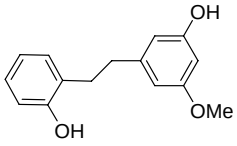


101 R= $(CH_2)_7Me$
102 R= $(CH_2)_8Me$



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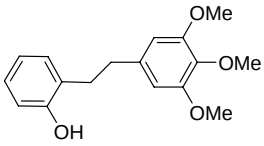

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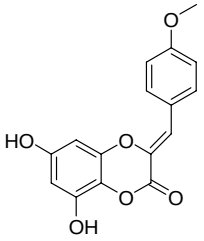

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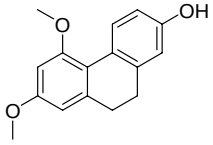

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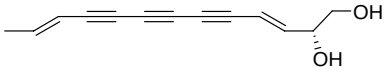

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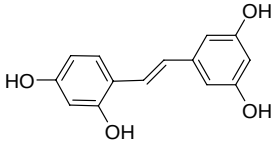

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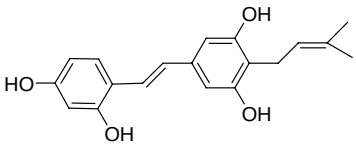

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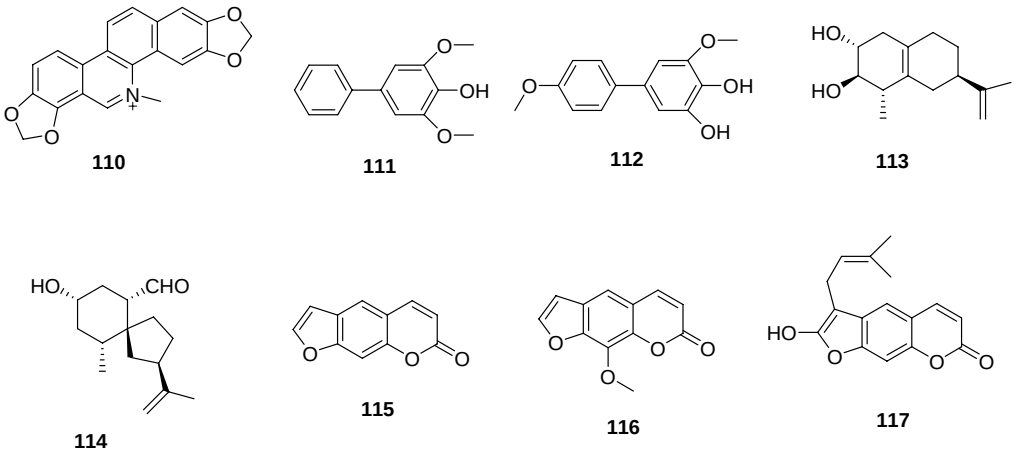
Table 1.4. Selected phytoalexins produced by plants of Angiospermae (Grayer and Harborne, 1994; Harborne, 1999).

	Family	Chemical type	Examples
Angiospermae (Monocotyledons)	Alliaceae	Cyclic dione	5-alkyl-cyclopenta-1,3-diones (**101**) and B (**102**), (Tverskoy et al., 1991)
	Dioscoreaceae	Bibenzyl	Batatasin IV (**103**) and Batatasin V (**104**), (Hashimoto and Tajima, 1978)
	Liliaceae	Benzodioxin-2-one	Yurinelide (**105**), (Monde et al., 1992)
	Orchidaceae	Phenanthrene	Orchinol (**106**), (Fisch et al., 1973)
Angiospermae (Dicotyledons)	Compositae	Acetylenic	Safynol (**107**), (Allen and Thomas, 1971)
	Leguminosae	Isoflavonoid	Pisatin (**97**) (Perrin and Bottomley, 1961)
	Moraceae	Stilbenes	Oxyresveratrol (**108**) and 4'-prenyloxyresveratrol (**109**), (Takasugi et al., 1978)
	Papaveraceae	Alkaloid	Sanguinarine (**110**), (Furuya et al., 1972)
	Rosaceae	Biphenyl	Aucuparin (**111**), Rhaphiolepsin (**112**) (Kokubun and Harborne, 1995)
	Solanaceae	Sesquiterpene	Rishitin (**113**) and lubimin (**114**), (Brindle et al., 1988)
	Umbelliferae	Furanocoumarin	Psoralen (**115**), Xanthotoxin (**116**) and demethylsuberosin (**117**), (Masuda et al., 1998)



So far indole-sulfur containing phytoalexins are exclusively found in Brassicaceae (Cruciferae). Over 40 cruciferous phytoalexins have been isolated from crucifers (Pedras et al., 2003; Pedras et al., 2007b), starting from brassinin (**17**), the first phytoalexin isolated from Chinese cabbage (*B. campestris* L. ssp. *pekinensis*) after infection with the bacterium *P. cichorri* (Takasugi et al., 1986). Table 1.5 lists phytoalexins produced by four representative cruciferous plant species and elicitation agents.

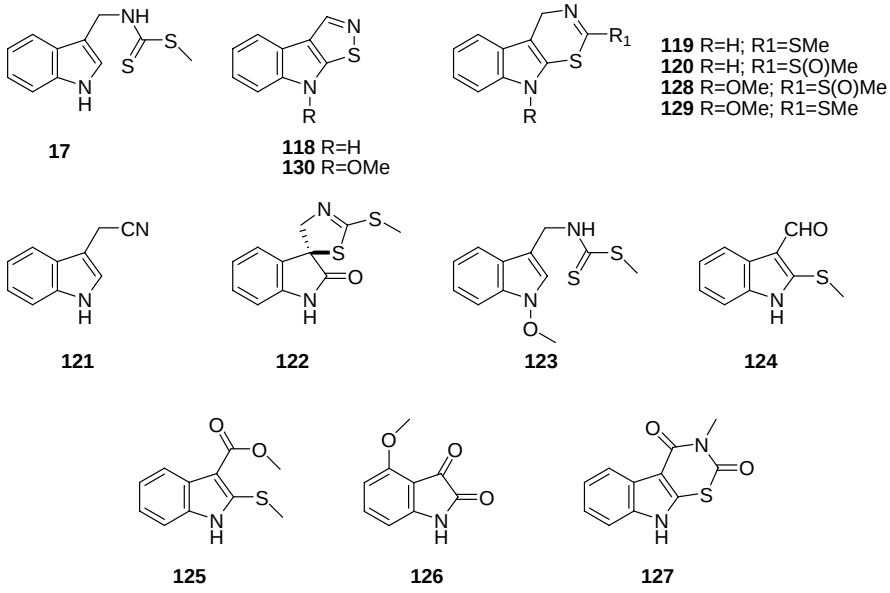


Table 1.5. Selected phytoalexins produced by cruciferous plants (adapted from Pedras et al., 2007b; Pedras et al., 2003).

Species (common name)	Elicitors	Phytoalexins
Brassica. juncea (brown mustard)	$CuCl_2$, $AgNO_3$, *L. maculans*, *A. brassicae*	Brassilexin (**118**), cyclobrassinin (**119**), cyclobrassinin sulfoxide (**120**), indole-3-acetonitrile (**121**), spirobrassinin (**122**)
B. napus (rapeseed)	$CuCl_2$, *L. maculans*	Brassilexin (**118**), cyclobrassinin (**119**), cyclobrassinin sulfoxide (**120**), 1-methoxybrassinin (**123**), spirobrassinin (**122**)
B. napus ssp. rapifera (rutabaga)	UV, *R. solani*	Brassicanal A (**124**), brassicanate A (**125**), brassilexin (**118**), brassinin (**17**), isalexin (**126**), 1-methoxybrassinin (**123**), rutalexin(**127**), spirobrassinin (**122**)
Sinapis. alba (white mustard)	Destruxin B, $CuCl_2$, *A. brassicae*, *L. maculans*	Sinalbin A (**128**), sinalbin B (**129**), sinalexin (**130**)

1.2.3 Diversity of secondary metabolites produced by one fungal species

It is well known that very diverse secondary metabolites are produced by a wide range of organisms, notably microorganisms (Firn and Jones, 2003). However, the diversity of secondary metabolites produced by one fungal species did not receive sufficient attention until work published recently (Bode et al., 2002; Bode and Műller, 2005; Van Lanen and Shen, 2006). Because of the rapid development of genomic projects, a large number of orphan pathways (discussed in section 1.2.3.1) have been discovered in fungal species, indicating that diverse and novel secondary metabolites can be produced by a single fungal species. Diverse secondary metabolites can be obtained from manipulation of known culturable microorganisms (Bode et al., 2002; Bode and

Műller, 2005; Gross, 2007; Van Lanen and Shen, 2006). This manipulation can be done genetically, which is beyond the scope of this thesis, or by variation of culture conditions, which will be discussed in section 1.2.3.2.

1.2.3.1 Orphan pathways

It has been hypothesized that most of the investigated microorganisms have a broader genetic capacity to produce natural products than those isolated from a particular strain or species (Gross, 2007). Many "new" gene clusters were accidentally found during research to characterize specific gene clusters encoding known metabolites. The function and the encoded products of these "new" gene clusters are not known. These gene clusters were described as "pathways for previously undetected metabolites", "yet-to-be isolated compounds" or "cryptic gene clusters encoding putative natural products" (Gross, 2007). However, to avoid confusion and the fact that a notation is necessary for communication, the term "orphan pathways" was proposed to refer to gene clusters (biosynthetic loci) for which the corresponding metabolites are unknown (Gross, 2007).

According to the concept of orphan pathways, the potential to discover diverse bioactive natural products from microorganisms is much higher than previously expected. Recently, natural product research efforts in pharmaceutical areas are diminishing despite that more than 75% of all antibacterial and approximately 50% of all anticancer compounds currently in clinical use are either natural products themselves or derivatives thereof (Newman et al., 2003). Instead, synthetic chemicals have been emphasized because of certain advantages, for example, isolation and characterization of synthetic chemicals are easier, analogues can be chemically synthesized, and there is no limitation of source (Bode and Műller, 2005). However, as a result of the rapid progress of genome projects worldwide, a large number of gene clusters are identified as orphan pathways by bio-informatics. Recent progress suggests that the structures of new natural products can be predicted, many "unnatural" natural products can be produced from cultures of microorganisms, and the biological source will not be limited by genetic manipulation of culturable microorganisms (Gross, 2007). Overall there is still tremendous potential to discover natural-product diversity (Gross, 2007).

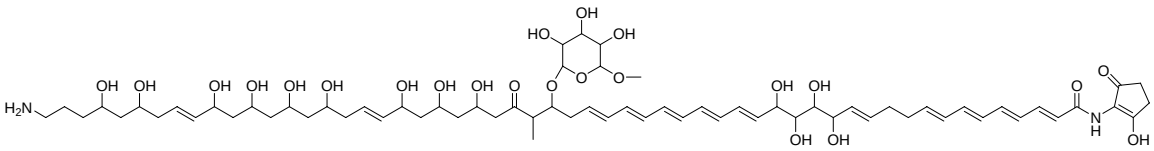
Identification of orphan pathways can be the first step to isolate novel natural products encoded by such pathways. The most important method is bio-informatic search for the orphan gene clusters, which is usually termed 'genome mining', 'data mining' or 'metabolic pathway mining'. Identification of orphan pathways is closely related to molecular biology which is beyond the scope of this thesis (reviewed by Gross, 2007).

1.2.3.2 Culture conditions

After orphan pathways have been identified, several strategies can be used to characterize the metabolites encoded by the orphan pathways and elucidate the function of the unknown gene clusters. These strategies include, for example, bio-informatic guided screening, heterologous expression and gene inactivation (Gross, 2007). Variation of culture conditions may not be necessary when strategies such as heterologous expression and gene inactivation are employed. However, variation of culture conditions is crucial for the strategy of bio-informatic guided screening. This is done by varying cultural medium components or addition of inhibitors or stimulators (Gross, 2007; Strange, 2007). Recent examples are discussed to illustrate the success of obtaining diverse bioactive metabolites by variation of culture conditions:

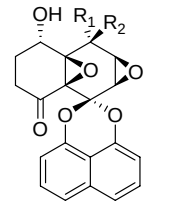
Variation of medium components

Based on bio-informatic analysis of a type I PKS gene cluster in *Streptomyces aizunensis*, a polyketide of 1,257 Da with characteristic UV absorbance at around 300 nm was predicted (McAlpine et al., 2005). After culture conditions were optimized (about 50 different medium components), metabolite ECO-02301 (**131**) was detected using LC-MS/DAD. The optimized medium contained soluble starch, glucose, Pharmamedia (Sigma), corn steep liquor (Sigma), and Proflo oil (pH of 7.2, Traders Protein). This metabolite showed anti-fungal activity (4 μg/ml MIC against *Candida albicans*) (McAlpine et al., 2005).

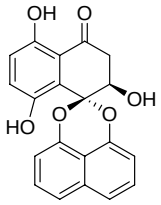


131

The strain F-24'707 of the fungal species *Sphaeropsidales* was described to produce mainly the antifungal spirobisnaphthalene cladospirone bisepoxide (**132**) in medium A (containing oat meal 2%, degreased soy meal 2%, and glucose 2%) (Thiergardt et al., 1995). However, using a combination of different media and cultivation vessels, eight new spirobisnaphthalenes (cladospirones B to I (**134** to **141**) and six known members of this class of compounds (palmarumycins C_2 (**142**), C_3 (**143**), and C_{12} (**144**), diepoxins σ (**133**), η (**145**), and δ (**146**) were isolated in yields of up to 2.6 g/L (Bode et al., 2000b). The medium was thought to be closer to natural living conditions (Bode et al., 2002).



132 R_1=H, R_2=OH
133 R_1, R_2=O



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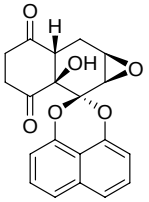

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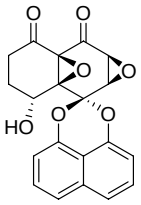

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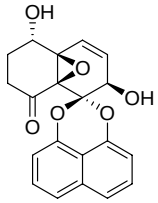

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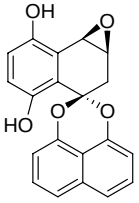

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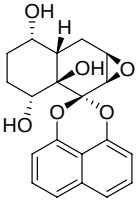

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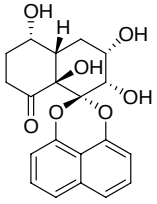

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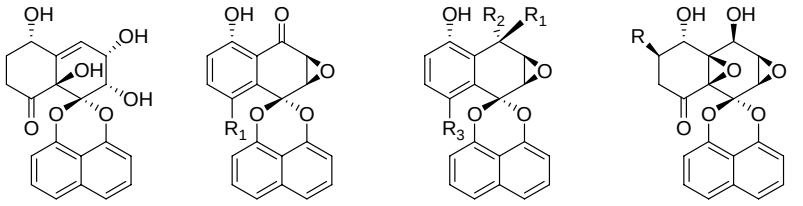
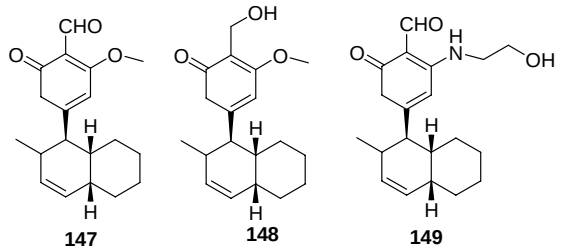


141 **142** R_1=H **143** R_1=OH **144** R_1=OH, R_2=H, R_3=OH **145** R=OH **146** R=H

Addition of chickpea extract or a mixture of inorganic salts, amino acids and vitamins into Czapek-Dox medium induced the production of solanapyrone toxins **147**, **148**, and **149** by *A. rabiei*. After systematic removal of each component of this mixture, divalent cations such as Zn^{2+}, Mn^{2+}, Cu^{2+} or Ca^{2+} were found to be essential for the production of solanapyrone toxins **147**, **148**, and **149** by *A. rabiei* (Chen and Strange, 1991).

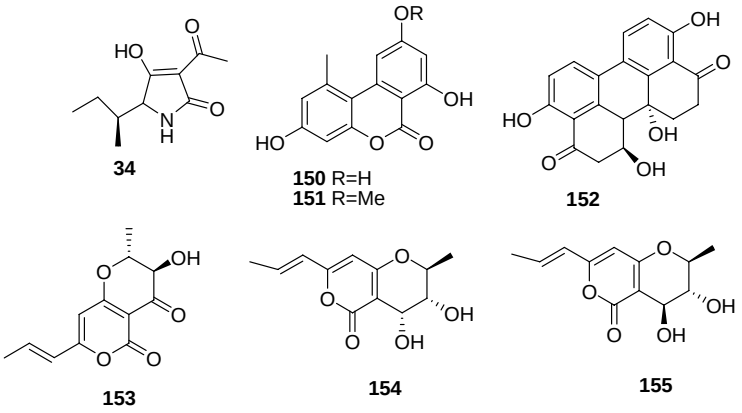


147 **148** **149**

Mycotoxins and phytotoxins produced by isolates of the fungi *Alternaria alternata* and *A. radicina* on rice and carrot discs are different (Table 1.6). Most of the isolates of *A. alternata* produced tenuazonic acid (**34**), alternariol (**150**), alternariol methyl ether (**151**) and altertoxin-I (**152**) on rice, but only alternariol (**150**) and alternariol methyl ether (**151**) on carrot discs. *A. radicina* produced radicinin (**153**), epi-radicinol (**154**) and radicinol (**155**) on carrot discs, but only radicinin and radicinol on rice.

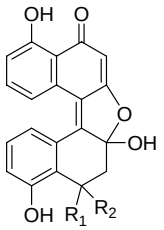
Table 1.6. Production of phytotoxins by *Alternaria alternata* and *A. radicina* on rice and carrot discs.

Fungi\Media	Rice	Carrot discs
A. alternata	**34**, **150**, **151**, **152**	**150**, **151**
A. radicina	**153**, **155**	**153**, **154**, **155**

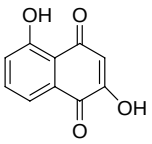


Addition of inhibitors or stimulators

The metabolites produced by the strain F-24'707 of *Sphaeropsidales sp*. in liquid culture medium were analyzed after addition of tricyclazole, a commercially available antifungal agrochemical that inhibits 1,3,8-trihydroxynaphthalene reductase. As expected, when tricyclazole was added, 1,3,8-trihydroxynaphthalene accumulated in culture due to inhibition of the breakdown of natural dihydroxynaphthalene melanin and spirobisnaphthalene biosynthesis cladospirone bisepoxide (**132**). In addition, two new bisnaphthalenes, named sphaerolone (**156**) and dihydrosphaerolone (**157**), 2-hydroxyjuglone (**158**) were produced (Bode and Zeeck, 2000). More interesting mutolide (**159**) was produced in medium when tricyclazole was added. That is the addition of an inhibitor stopped the known dihydroxynaphthalene biosynthesis pathway, but induced a new polyketide biosynthetic pathway leading to the production of mutolide (**159**) (Bode et al., 2000a).



156 R_1,R_2=O
157 R_1=H, R_2=OH



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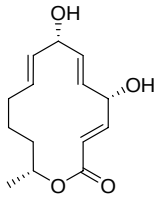
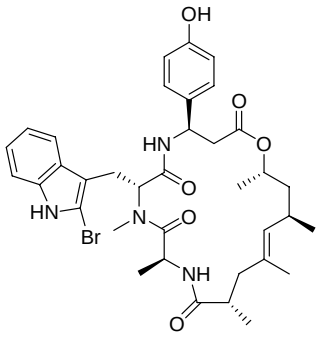


159

When cultures of the marine-derived fungus *Phomopsis asparagi* were challenged with the known F-actin inhibitor jasplakinolide (**160**), three new secondary metabolites, chaetoglobosin-510 (**161**), -540 (**162**), and -542 (**163**) were isolated. Chaetoglobosin-542 displayed antimicrofilament activity and was cytotoxic toward murine colon and leukemia cancer cell lines (Christian et al., 2005).



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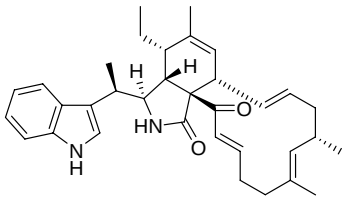

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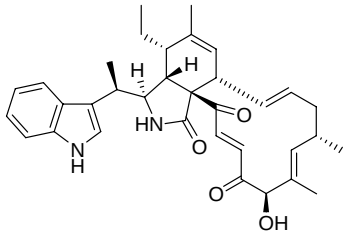

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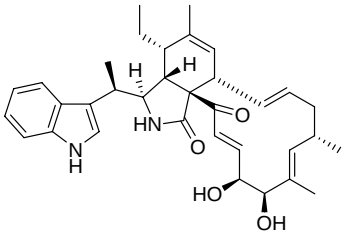

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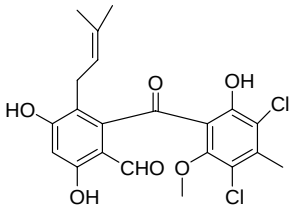


164

Co-incubation of the marine fungus *Pestalotia* sp. with an unidentified gram-negative bacterium led to the isolation of pestalone (**164**), a new and potent benzophenone antibiotic (Cueto et al., 2001). Therefore, addition of another microorganism could induce the production of new biological active metabolites.

AB-toxin, a HST, is produced by germinating spores of *Alternaria brassicicola* only on host leaves. The specific production of AB-toxin was found to be the elicitation effect of an 1.3 kDa oligosaccharide derived from host plants (Oka et al., 2005). This report presented evidence that production of HSTs can be induced upon elicitation of host derived molecules (Oka et al., 2005).

1.3 METABOLITES FROM *LEPTOSPHAERIA MACULANS*

The fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm can cause blackleg disease of crucifers, which leads to large yield losses (Gugel and Petrie, 1992; Kutcher et al., 2007). *L. maculans* comprises pathotype groups and subgroups. Initially, those fungal isolates that caused blackleg disease on canola were called aggressive, highly virulent, or "A" group (McGee and Petrie, 1978, Williams and Fitt, 1999). The fungal isolates that did not cause disease symptoms on canola were called non-aggressive, weakly virulent, or "B" group (McGee and Petrie, 1978). Further study, based on the pathogenicity of isolates on cotyledons of *B. napus* cultivars Westar, Quinta and Glacier, regrouped isolates into four categories: PG1, PG2, PG3 and PG4. PG1 did not cause disease symptoms on cotyledons of *B. napus* cultivars Westar, Quinta and Glacier. PG2 was virulent on Westar, PG3 was virulent on Westar and Glacier and PG4 was virulent on all three cultivars tested (Koch et al., 1991; Mengistu et al., 1991). All these isolates were under the name *L. maculans*, until a reclassification into *L. biglobosa* was introduced to enclose the isolates traditionally known as avirulent, weakly virulent, "B" group or PG1 (Chen and Fernando, 2006; Howlett et al., 2001). However, reversed pathogenicity was observed in two isolates Laird 2 and Mayfair 2, which were not virulent on *B. napus* but virulent on *B. juncea*, a plant species resistant to the blackleg fungus (Pedras et al., 1999a). These two isolates were not considered in the reclassification of *L. maculans*. The metabolites produced by different isolates of *L. maculans* and *L. biglobosa* are unique to some groups and thus have taxonomic value. These metabolites are discussed according to their structural types in section 1.3.2.

1.3.1 Elicitors

A gene-for-gene genetic model appears to explain the interactions between brassicas and *L. maculans* (Howlett, 2004). Nine avirulence genes (*AvrLm1–9*) and complementary resistance genes (*Rlm1–9*) from *Brassica spp*. and *L. maculans*, respectively, have been identified and mapped (Gout et al., 2006). Recently, a protein (sp1) secreted during fungal infection has been identified by sequencing random genes of *L. maculans* (Wilson et al., 2003). The protein fractions containing sp1 induced autofluorescent defense response in leaves of canola. However, when a mutant unable to secret this protein was applied to leaves of canola it caused lesions similar to those caused by the wildtype fungus, suggesting that this protein does not play a significant role in disease development (Wilson et al., 2003).

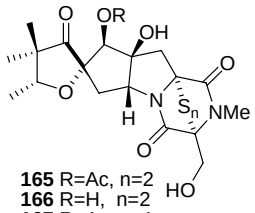
To date, neither proteins/enzymes encoded by avirulence genes or corresponding metabolites , nor elicitors from *L. maculans* been reported. Therefore, studies to discover elicitors produced by *L. maculans* are necessary.

1.3.2 Phytotoxins and other metabolites

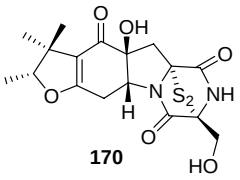
1.3.2.1 Dioxopiperazines

Sirodesmins are epipolythiodioxopiperazines, which are derived from the condensation of two amino acids and contain a sulfur bridge (Curtis et al., 1977; Ferezou et al., 1980; Ferezou et al., 1977). Sirodesmin PL (**165**) and deacetylsirodesmin PL **(166)** are the first two non-selective phytotoxins isolated from *L. maculans* (Férézou et al., 1977). Later on, sirodesmins with a various number of sulfur atoms, i.e. sirodesmin H (**167**) (Pedras et al., 1988), sirodesmin J and K (Pedras et al., 1990) were isolated from a chemically defined medium. Sirodesmin J and K are not stable, decomposing to **165**, **169** and **168** on standing in MeOH solution within 24 hours (Pedras et al., 1990). The toxicity of EPTs comes from its disulfur bridge, which may conjugate with cysteine residue in proteins or generate reactive oxygen species through redox cycling (Hurne et al., 2002; Hurne et al., 2000). Sirodesmins cause yellowish necrosis on leaves of both

resistant and susceptible plants. By investigating the toxicity of acetylated derivatives of sirodesmin PL (Pedras et al., 1990), it was suggested that the –OH group at C-14 may be involved in the mechanism of toxicity.



165 R=Ac, n=2
166 R=H, n=2
167 R=Ac, n=1
168 R=Ac, n=3
169 R=Ac, n=4



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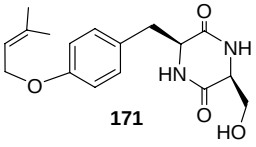

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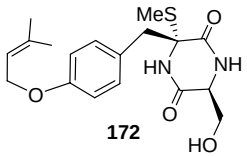
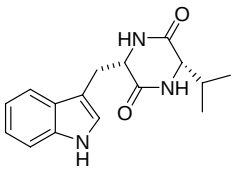


172

Phomalirazine (**170**), another non-selective phytotoxin, was isolated from minimal medium still culture incubated for 21 days (Pedras et al., 1989). It showed phytotoxicity (10^{-5} M) towards both resistant and susceptible plants (Pedras et al., 1989). Phomalirazine (**170**) possesses a disulfur bridge similar to sirodesmins but no methyl group attached to the nitrogen atom and no spiro-fused tetrahydrofuranone ring are present. These structural differences suggested that it is a possible intermediate in the biosynthesis of sirodesmins (discussed in section 1.4).

Six dioxopiperazines polanrazines A (**173**), B (**174**), C (**175**), D (**176**), E (**177**) and F (**178**), derived from tryptophan and valine, were isolated from Polish isolates of *L. maculans* (Pedras et al., 1998; Pedras and Biesenthal, 2001). Polanrazines B (**174**) and C (**175**) were also isolated from Laird 2 isolate (Pedras et al., 2005a). Polanrazines A (**173**), C (**175**) and E (**177**) showed moderate toxicity to leaves of brown mustard but not to canola (Pedras et al., 1998; Pedras, 2001; Pedras and Biesenthal, 2001).



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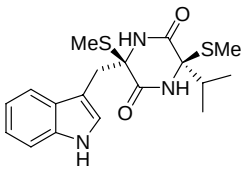

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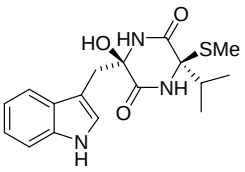

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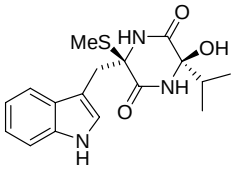

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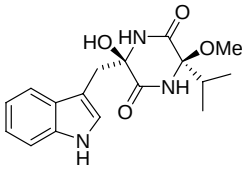

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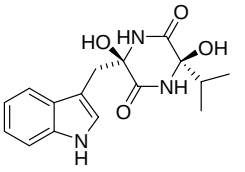


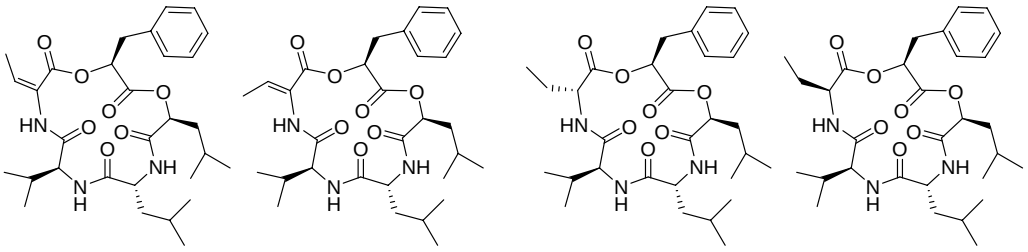
178

1.3.2.2 Depsipeptides

Phomalide (**179**), the first host-selective phytotoxin isolated from virulent isolates of *L. maculans*, represents a rare cyclic peptide with three α-amino acids and two α-hydroxy acids (Pedras, 2001; Pedras et al., 1993b). Phomalide (**179**) caused disease symptoms (necrotic, chlorotic, and reddish lesions) on canola (susceptible to *L. maculans*) but not on brown mustard or white mustard (resistant to *L. maculans*) at concentrations ranging from 10^{-5} M to 10^{-4} M (Pedras et al., 1993b). The production of phomalide was detected in 30 to 60-hour-old cultures. Once the cultures started to produce sirodesmins, the production of phomalide stopped (Pedras and Biesenthal, 1998; Pedras et al., 1993a; Pedras et al., 1993b). Spores of virulent isolates co-incubated with sirodesmin PL (**165**) in MM did not produce phomalide (**179**). Therefore, the above results suggested that the production of phomalide (**179**) is inhibited by sirodesmins. Phomalide (**179**) was also detected in the infected leaves of canola, suggesting a biological role. The detection of phomalide (**179**) but not sirodesmin PL (**165**) in infected leaves of canola is consistent with the inhibition effect of sirodesmin PL (**165**) on the production of phomalide (**179**) in the culture media. However, traces of sirodesmin PL (**165**) were detected using LC-MS in the leaves of canola infected with *L. maculans* (Elliott et al., 2007).

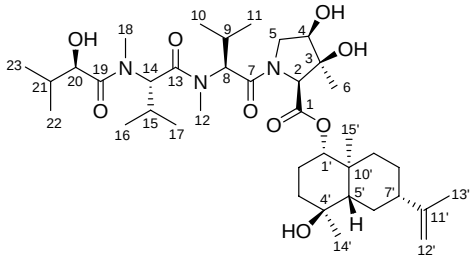
Phomalide (**179**) together with three analogues **180**, **181** and **182** were synthesized and the phytotoxicity of these compounds was tested (Ward et al., 1999). The *Z*-isomer (**180**, 5×10^{-4} M) did not have phytotoxicity while compounds **181** and

182 (5×10^{-5} M) caused necrosis on brown mustard but not on canola or white mustard (Ward et al., 1999).



179 **180** **181** **182**

Depsilairdin (**183**), another host-selective phytotoxin produced by the isolate Laird 2, possesses a tripeptide coupled with a sesquiterpene moiety. The tripeptide moiety contains (2*S*,3*S*,4*S*)-3,4-dihydroxy-3-methylproline, a novel amino acid motif. Depsilairdin (**183**) causes the same diseases symptom as the pathogen and mimics the host selectivity. Plant leaves of brown mustard treated with depsilairdin (**183**) showed strong necrotic and chlorotic lesions but no such symptoms were observed in canola in a wide range from μM to mM (Pedras et al., 2004). The total synthesis of depsilairdin is underway, and the sesquiterpene moiety (lairdinol A (**188**)) was synthesized recently (Pardeshi and Ward, 2008).



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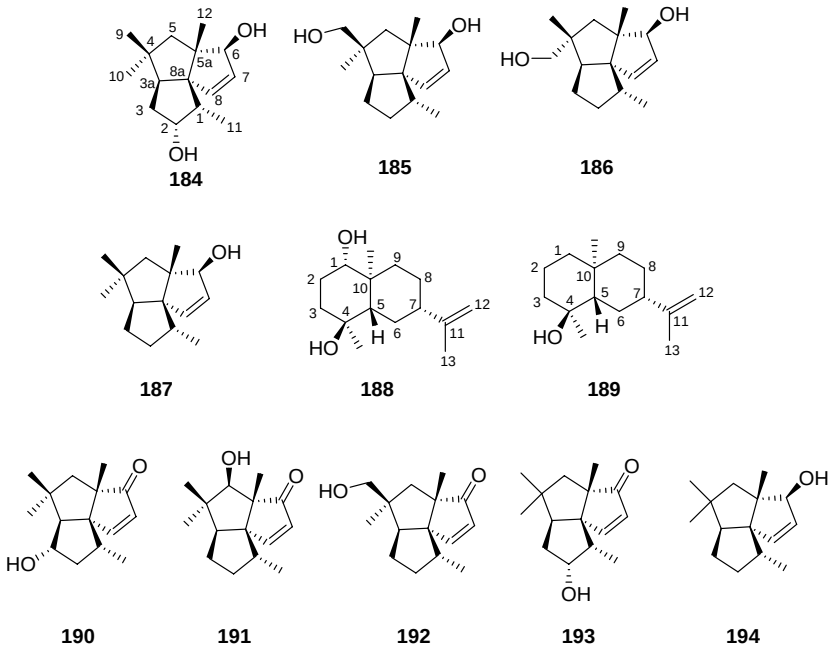
1.3.2.3 Sesquiterpenes

Ten sesquiterpenes have been isolated from extracts of cultures of *L. maculans* (Pedras et al., 1999; Pedras et al., 2005b). Phomalairdenones A (**193**), B (**190**) and C (**191**) were isolated from a fungal isolate IBCN 18 (the International Blackleg of Crucifers Network), which is virulent to canola. Phomalairdenones A (**193**) and D (**192**), phomalairdenols A (**184**), B (**185**), C (**186**) and D (**187**), lairdinol A (**188**) as well as **189** and **194** were isolated from Laird 2 and Mayfair 2 which are virulent to brown mustard but not to canola. Among these, metabolites **189** and **194** were isolated before from *Dugaldia hoopesii* and *Podocarus dacrydioides*, respectively. The tricyclic ring system of phomalairdenone A (**193**), was assigned based on analysis of X-ray crystallographic data of a single crystal (Pedras et al., 1999).



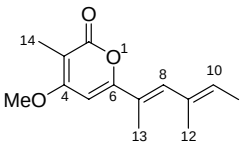
Phomalairdenone A (**193**) (5×10^{-4} M) causes necrotic, chlorotic, and reddish lesions on brown mustard but not on canola (Pedras et al., 1999). Phomalairdenones A (**193**) and D (**192**), phomalairdenol A (**184**) as well as lairdinol A (**188**) also caused lesions (3–4 mm diameter) on brown mustard but not on canola (Pedras et al., 2005b).

The selective phytotoxicity is consistent with the virulence range of isolates Laird 2 and Mayfair 2.

Only phomalairdenol A (**184**) displayed phytotoxicity among all the tested phomalairdenols B (**185**), C (**186**) and D (**187**). Structure-activity analysis of all phomalairdenols suggested that the hydroxyl group at C-2 is important for the phytotoxicity in phomalairdenols. It is also interesting to note that lairdinol A (**188**), a eudesmene type sesquiterpene, is a structural moiety of selective-phytotoxin depsilairdin (**183**). Lairdinol A (**188**) also showed selective activity on brown mustard but less toxicity than depsilairdin (**183**) (Pedras et al., 2004).

1.3.2.4 Polyketides

Nine 2-pyrone type metabolites, phomapyrones A-C (**195**–**197**) and D-G (**198**–**201**), as well as phomenin B (**203**) and infectopyrone (**202**) were isolated from cultures of isolates Laird 2 (Pedras et al., 1999; 2005a) and FAN10B5 (Pedras et al., 1994) incubated in MM. Phomenin B (**203**) and infectopyrone (**202**), were isolated from *Phoma tracheiphila* (Tringali et al., 1993) and from *A. infectoria*, respectively (Larsen et al., 2003). The biosyntheses of these phomapyrones were demonstrated to follow a polyketide pathway using ^{13}C-labeled acetate and malonate as well as deuterated methionine as precursors. Because of the limited amount of phomapyrones isolated, only phomenin B (**203**), and phomapyrones A (**195**) and D (**198**) were tested for the phytotoxicity. None of these pyrones caused any necrosis even at high concentration (10^{-3} M); however, 2-pyrones isolated from some pathogenic fungi have displayed phytotoxicity, cytotoxicity, or antibiotic activity (Dickinson, 1993).



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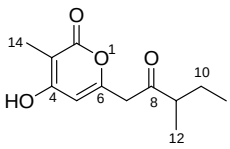

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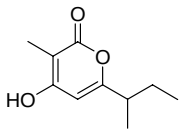

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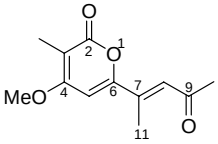

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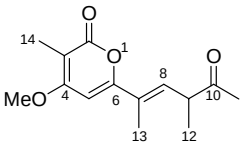

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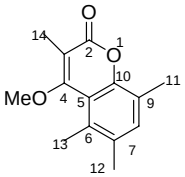

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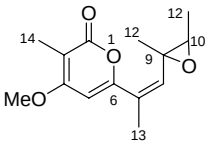

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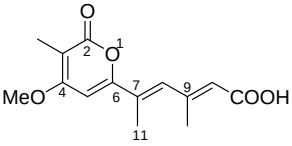

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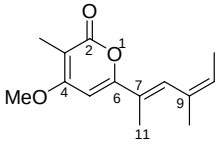
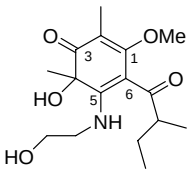


203

In work to find the yellow pigments produced by weakly virulent isolates of *L. maculans* three yellow metabolites, phomaligin A (**204**) and wasabidienones B (**205**) and E (**206**), were isolated (Pedras, 1996; Pedras et al., 1995). However, these three metabolites displayed the lowest toxicity among all the phytotoxins isolated from *L. maculans* and *L. biglobosa*.



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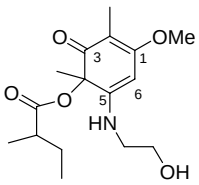

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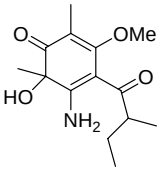
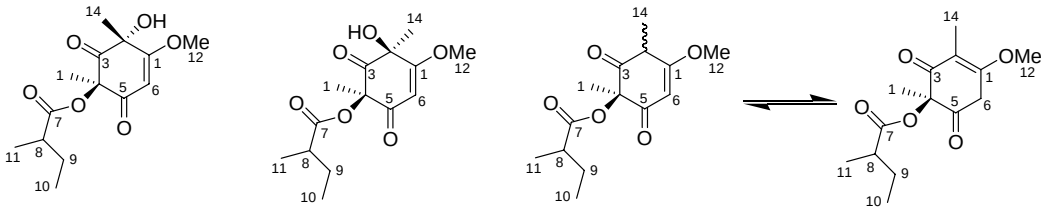


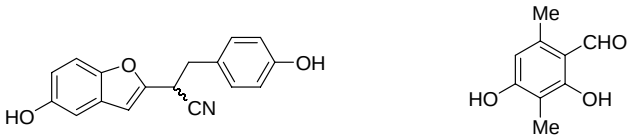
206

Phomaligol A (**207**) and A_1 (**208**), phomaligadiones A (**209**) and B (**210**) as well as wasabidienone B (**206**) and E (**205**) were isolated from a weakly virulent isolate of *L. maculans*. These metabolites have also been found in *P. wasabi*, suggesting a similarity between these two pathogens.



207 **208** **209** **210**

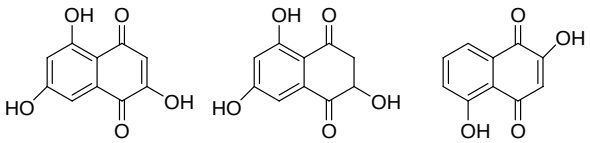
2-[2-(5-Hydroxybenzofuranyl)]-3-(4-hydroxyphenyl)-propanenitrile (**211**) was isolated from Laird 2 isolate of *L. maculans* grown in PDB. The structure of this metabolite was elucidated by analysis of spectroscopic data. However, the configuration of stereocenter C-8' was not determined since racemization at C-8' could happen as H-8' is acidic (Pedras et al., 2007a).



211 **212**

2,4-dihydroxy-3,6-dimethylbenzaldehyde (**212**) was isolated from Canadian V isolates grown in PDB (Pedras, et al., 2005). It was reported to have root and hypocotyl growth inhibition effects on lettuce seedlings (Jiao et al., 1994).

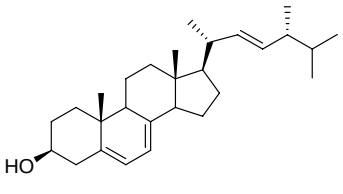
Flaviolin (**213**), 4-hydroxyscytalone (**214**) and 2-hydroxyjuglone (**215**) were isolated from a solid culture of *L. maculans* when tricyclazole, a systemic fungicide and a melanin synthesis inhibitor, was supplied in the PDA solid medium. These metabolites were suggested to be the shunt metabolites of melanin (Dahiya and Rimmer, 1988).



213 **214** **215**

1.3.2.5 Sterols

The composition of sterols in mycelia of *L. maculans* has been studied using GC-MS spectrometry. Ergosterol (**32**) comprised 95% of the total sterols. The remaining sterols were 24-methylene dihydrolanosterol (**216**), 4,4-dimethyl fecosterol (**217**), 4-methyl fecosterol (**218**), fecosterol (**219**), episterol (**220**), ergosta-7,22-dienol (**221**), ergosta-5,7,9(11),22-tetraenol (**222**) and epiergosterol (**223**) and obtusifoliol (**224**) (Griffiths et al., 2003).



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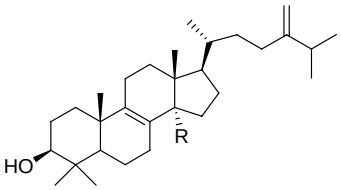
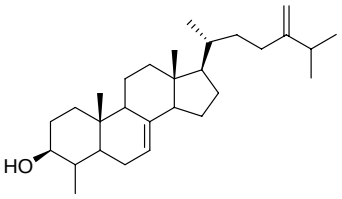


216 R=Me
217 R=H



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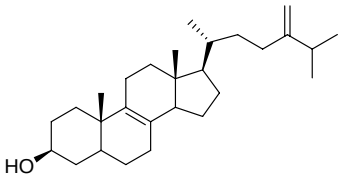

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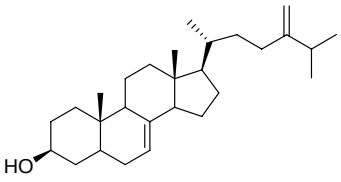

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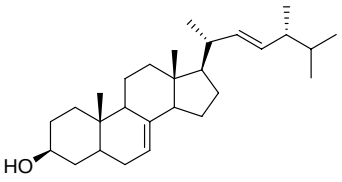

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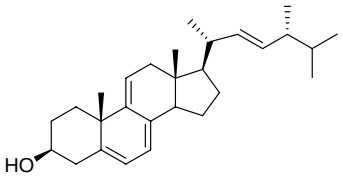

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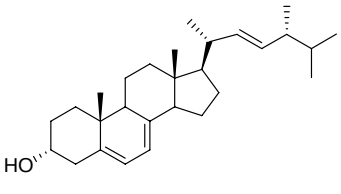

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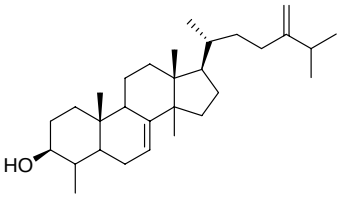


224

The metabolites produced by both *L. maculans* and *L. biglobosa* are summarized in Table 1.7.

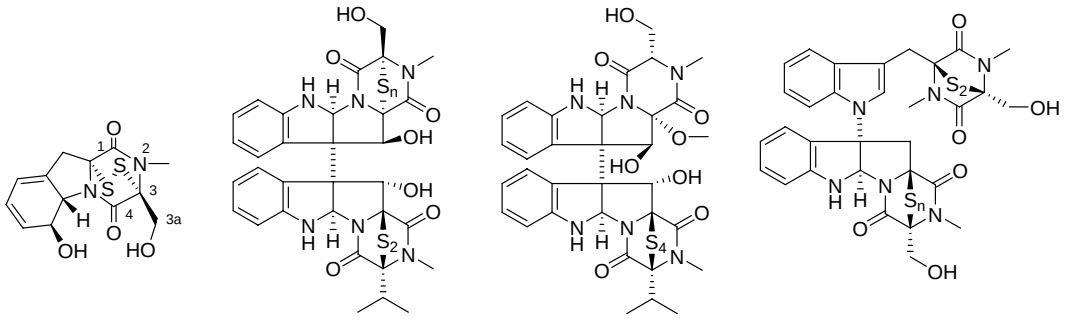
Table 1.7. Secondary metabolites produced by different isolates of *Leptosphaeria maculans* and *L. biglobosa*.(Pedras, 2001; Pedras et al., 2007a; Pedras et al., 2005a; Pedras et al., 2005b).

Isolates / Types	*L. maculans* (virulent isolates)[a]	*L. maculans* (Mayfair 2/ Laird 2)[b]	*L. biglobosa*[c]	*L. biglobosa* (Polish isolates)[d]
Dioxopiperazines	Sirodesmins (**169** to **166**) Phomalirazine (**170**)	Polanrazines B (**174**), C (**175**)	-	Polanrazines A (**173**), B (**174**), C (**175**), D (**176**), E (**177**), F (**178**)
Depsipeptides	Phomalide (**179**)	Depsilairdin (**183**)	-	-
Sesquiterpenes	Phomalairdenones A (**193**), B (**190**), C (**191**)	Phomalairdenones A (**193**), D (**192**); Phomalairdenols A (**184**), B (**185**), C (**186**), D (**187**); Lairdinol A (**188**); **189** and **194**	-	-
Polyketides	**212**, **213**, **214**, **215**	Phomapyrones A-C (**195**–**197**), D-G (**198**–**201**); Phomenin B (**203**); Infectopyrone (**202**)	**204**, **205**, **206**	**211**
Sterols	**216** to **224**	-	-	-

[a]isolates virulent on canola; [b]isolates virulent on brown mustard; [c]isolates avirulent on canola; [d]isolates avirulent on canola.

1.4 BIOSYNTHESES OF EPIPOLYTHIODIOXOPIPERAZINES

A large number of epipolythiodioxopiperazines have been isolated from more than 20 fungal genera. These metabolites display diverse biological activities (Gardiner et al., 2005; Rezanka et al., 2006). For example, gliotoxin (**225**) has antibacterial and antiviral properties and selective toxicity to cells of the hematopoietic system (Rezanka et al., 2006), leptosins A (**226**), B (**227**), C (**228**) and M (**229**) displayed antitumor and cytotoxicity (Takahashi et al., 1994; Yamada et al., 2002), and chetomin (**230**) and chetoseminudin A (**231**) displayed immunonodulatory activity (Fujimoto et al., 2004). In recent years the study of genes responsible for the biosynthesis of EPTs has been carried out. For example, 18 genes have been cloned and identified in a sirodesmin biosynthetic gene cluster (Gardiner et al. 2004). The function of each of the 18 genes has been proposed and denoted with a prefix *sir* followed by a capital letter indicating the function, for example gene *sirP* corresponds to a non-ribosomal peptide synthetase, *sirD* (prenyl transferase) and *sirT* (thioredoxin reductase). Mutants with specific sirodesmin biosynthetic genes disrupted were also generated and tested for the ability to produce sirodesmin PL (**165**) (Gardiner et al. 2004). After the first gene of sirodesmin PL biosynthetic gene cluster from *L. maculans* was cloned, homologues of the gene in the cluster responsible for the biosynthesis of gliotoxin were identified in expressed sequence tags of the EPT producing fungus *Chaetomium globosum* (Gardiner et al. 2004). A putative gliotoxin biosynthesis gene cluster has been indentified in *Aspergillus fumigatus* by a bioinformatic and expression analysis (Gardiner and Howlett 2005). The identification of a gene cluster responsible for the biosynthesis of EPTs may facilitate the discovery of new strategies to cope with EPT related diseases (reviewed in Fox and Howlett, 2008).



225

226 n=4
227 n=3
228 n=2

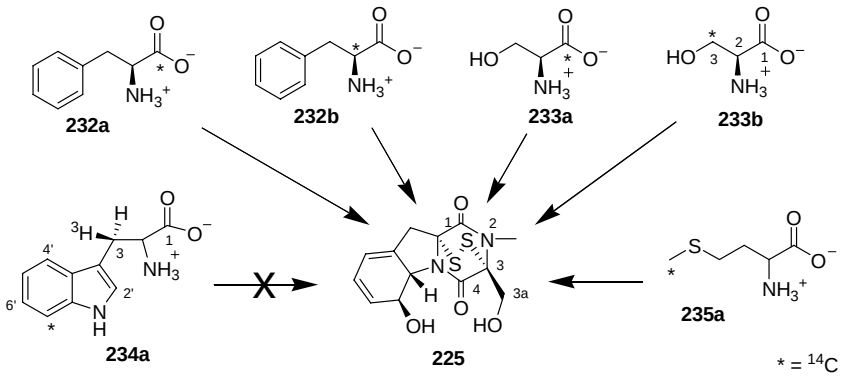
229

230 n=2
231 n=3

The biosynthesis of EPTs has been studied using isotopically labeled potential precursors. The biosynthesis of gliotoxin and sirodesmin PL has been extensively studied and the basic biosynthetic route has been established based on the incorporation of isotopically labeled potential precursors.

1.4.1 Biosynthesis of gliotoxin (225)

The first reported EPTs was gliotoxin (**225**), a toxin produced by *Gliocladium fimbriatum*. Gliotoxin was also isolated from *Aspergillus* spp. and *Penicillium* spp. (reviewed in Rezanka et al., 2006). Studies of the biosynthesis of gliotoxin started in 1958 with isotopically labeled potential precursors (Suhadolnik and Chenoweth, 1958). DL-[7a-^{14}C]tryptophan (**234a**), [2-^{14}C]sodium acetate, DL-[methyl-^{14}C]methionine (**235a**), DL-[1-^{14}C]Phenylalanine (**232a**), DL-[2-^{14}C]Phenylalanine (**232b**) were fed to cultures of *Trichoderma viride* (syn. *Gliocladium viride*) incubated in a chemically defined medium (Scheme 1.5). DL-[7a-^{14}C]tryptophan was not incorporated into gliotoxin, instead, DL-[1-^{14}C]Phenylalanine (**232a**) and DL-[2-^{14}C]Phenylalanine (**232b**) were incorporated into gliotoxin (**225**) efficiently (4-12%). These results suggested that, although gliotoxin (**225**) has an indole related moiety, this moiety derived from phenylalanine not tryptophan (Suhadolnik and Chenoweth, 1958).

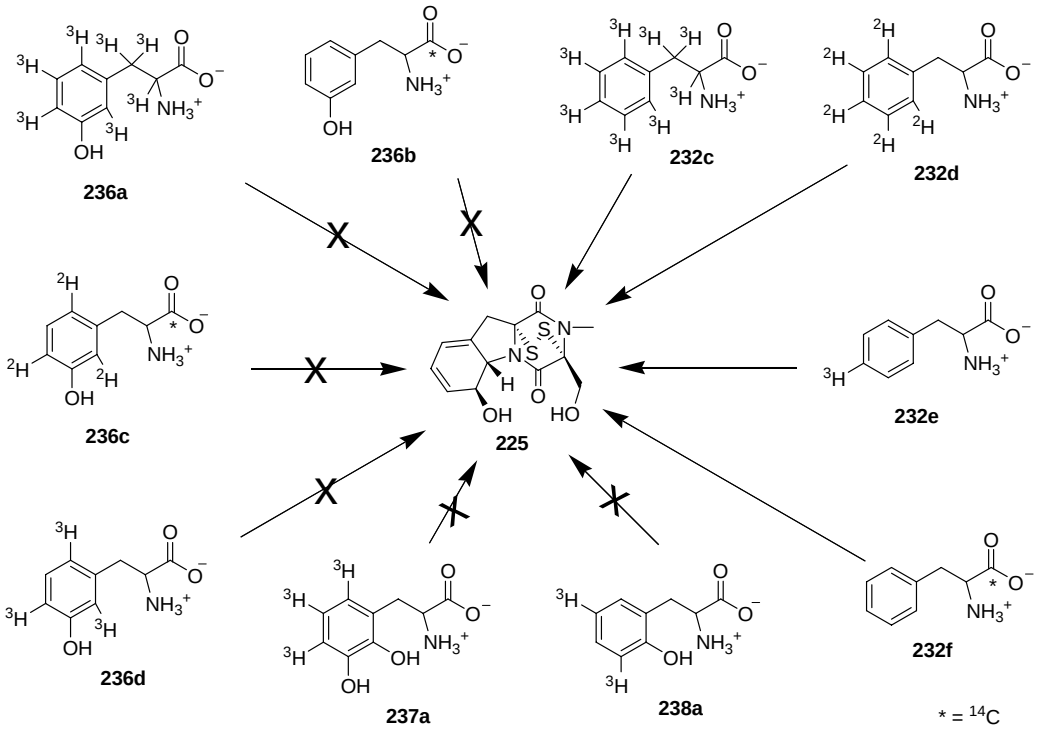


Scheme 1.5. Incorporation of potential isotopically labeled precursors into gliotoxin (**225**). (X denotes no incorporation of potential precursors into gliotoxin (**225**)).

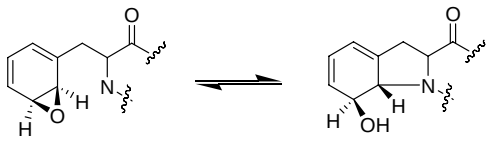
[1-^{14}C]Serine (**233a**) was incorporated into C-3,3a or 4 of gliotoxin more efficiently than [3-^{14}C]serine (**233b**) (Winstead and Suhadolnik, 1960). The result suggested that serine is another potential biosynthetic precursor to gliotoxin. The incorporation ratio of DL-[methyl-^{14}C]methionine (**235a**), [3-^{14}C]serine (**233b**) and [1-^{14}C]serine (**233a**) into the N-methyl group of gliotoxin were about 72, 25 and 0% in total radioactivity in gliotoxin obtained from each of the feeding experiments respectively (determined after hydrolysis of gliotoxin). It seemed methionine is the potential N-methyl donor of the N-methyl group of gliotoxin and serine is also utilized as a methyl group donor (Suhadolnik and Chenoweth, 1958).

Winstead and Suhadolnik (1960) also found that m-[2,3,3,5,7,8,9-$^{3}H_7$]tyrosine (**236a**) was incorporated into gliotoxin (**225**) in a higher ratio than that of [2,3,3,5,6,7,8,9-$^{3}H_8$]phenylalanine (**232c**), which strongly indicated that hydroxylation of the phenyl ring can happen before the coupling or cyclization of both amino acids. However, this was proved not to be the case. Ten years later, Bűlock and Ryles found that neither m-tyrosine nor o-tyrosine is the intermediate in the biosynthetic pathway of gliotoxin. Feeding of isotopically labeled m-tyrosine (**236b**, **236c** and **236d**), o-tyrosine (**238a**) and 2,3-dihydroxyl-tyrosine (**237a**) led to about zero incorporation (Scheme 1.6). More important evidence was the intact incorporation of [2,3,4,5,6-$^{2}H_5$]phenylalanine (**232d**) into gliotoxin, which eliminated the possibility of formation of m-tyrosine as an intermediate in the biosynthesis of gliotoxin (Bűlock and Ryles, 1970). An epoxide

pathway (Scheme 1.7) was proposed similar to the formation of aranotin (**264**) (see Section 1.4.3, scheme 1.17) (Neuss et al., 1968). In another independent experiment carried out by Johns and Kirby, [3-^{3}H]phenylalanine (**232e**) was incorporated into gliotoxin (**225**) without losing or migration of ^{3}H at the C-3 position of phenylalanine (Johns and Kirby, 1971). Only DL-[1-^{14}C]phenylalanine (**232e**) was incorporated into gliotoxin (**225**) when a mixture of DL-[2',4',6'-$^{3}H_3$]-m-tyrosine (**236d**) and DL-[1-^{14}C]phenylalanine (**232e**) were fed into the cultures of *G. deliquescens* (previously called *T. viride*). Again these results demonstrated that hydroxybenzene derivatives are not intermediates in the biosynthetic pathway of gliotoxin (Johns and Kirby, 1985).

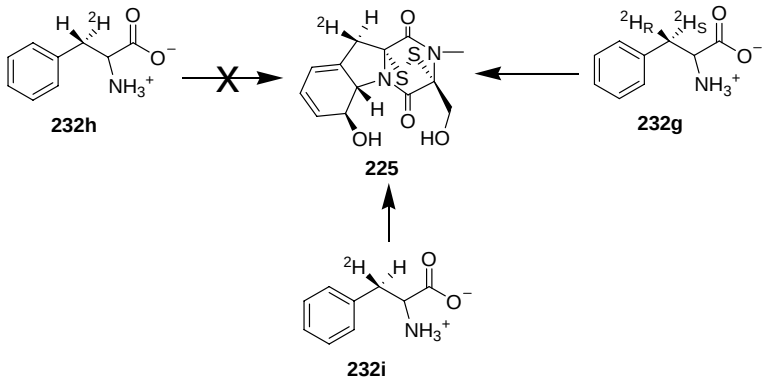


Scheme 1.6. Incorporation of potential isotopically labeled precursors into gliotoxin (**225**). (X denotes no incorporation of potential precursors into gliotoxin (**225**)).



Scheme 1.7. Plausible intermediates **239a** and **239b** involved in the proposed epoxide pathway of formation of gliotoxin (**225**) (Bűlock and Ryles, 1970).

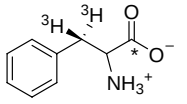
When [^{15}N]glycine was added into the culture of *T. viride*, both nitrogen atoms in gliotoxin were labeled although not to the same extent. Further, when a mixture of [1-^{14}C]phenylalanine and [^{15}N]phenylalanine was fed to the culture of *T. viride*, the nitrogen atom showed very large isotope dilution (11 times) compared with the carbon atom (4 times). These results indicated that an extensive deamination and reamination must occur when these labeled precursors were fed to the culture. Therefore, it is difficult to determine whether phenylalanine is incorporated intact into gliotoxin (Bose et al., 1968).



Scheme 1.8. Incorporation results of **232g**, **232h** and **232i** into gliotoxin in culture of *Trichoderma viride.* (X denotes no incorporation of potential precursors into gliotoxin (**225**)).

When DL-[3',3'-$^{2}H_2$]phenylalanine (**232g**) was fed to the culture of *T. viride*, a monodeuterated gliotoxin (**225a**) was observed, in which ^{2}H at the pro-S position in

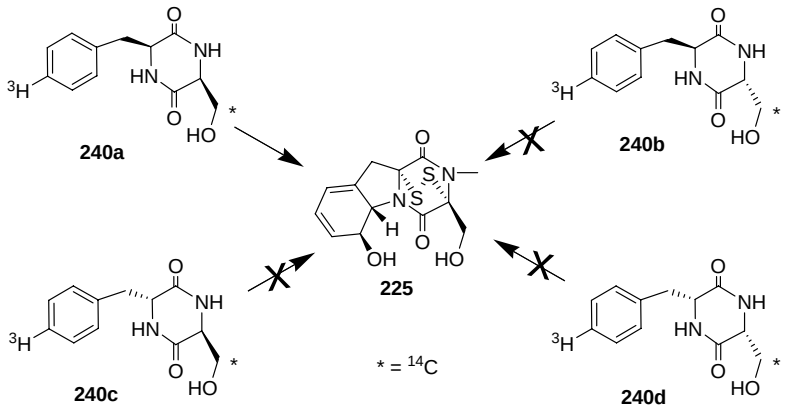
phenylalanine was not incorporated into gliotoxin (**225**) (Bűlock et al., 1972). No incorporation of deuterated phenylalanine was observed when DL-3'*S*-[3'-^{2}H] -phenylalanine (**232h**) was fed to the culture of *T. viride*. Whileas L-3'*R*-[3'-^{2}H]-phenylalanine (**232i**) was determined to be incorporated into gliotoxin, efficiently (scheme 1.8). It seemed that when phenylalanine was fed to the culture of *T. viride*, a process independent of the biosynthesis of gliotoxin happened that caused the loss of H_S at the C-3 position of phenylalanine. To further confirm this conclusion, DL-[1-^{14}C-3-$^{3}H_2$]-phenylalanine (**232j**) was fed to the culture of *T. viride* and harvested after a short incubation time (22 hours). Examination of the ^{14}C: ^{3}H ratio of DL-[1-^{14}C-3-$^{3}H_2$]-phenylalanine (**232j**) fed and recovered from the culture of *T. viride* indicated that up to a 35% loss of ^{3}H happened in this short incubation time during which the biosynthesis of gliotoxin had not started yet. Therefore, a β methylene proton exchange happened when phenylalanine was fed to the culture of *T. viride*, which is independent of the biosynthesis of gliotoxin. The authors proposed that this β methylene proton exchange involved an intermediate of α-keto-acid (Bűlock et al., 1972).



232j * = ^{14}C

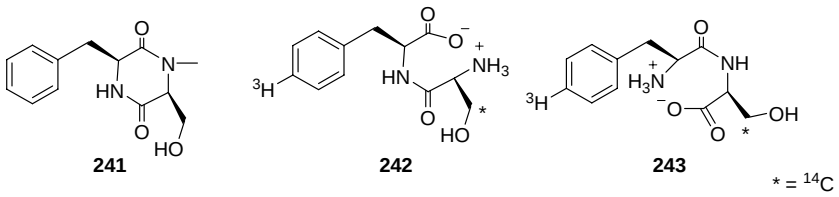
Although it is generally accepted that dioxopiperazines are the intermediates corresponding to their final EPTs, in early biosynthetic studies of gliotoxin, two groups had different results and explanations. MacDonald and Slater reported that *cyclo*-(L-phe-L-ser) (**240**) did not incorporate into gliotoxin, and proposed that the true intermediate is enzyme-bound (Macdonald and Slater, 1975). However, Bűlock and Leigh (1975) reported an intact incorporation of *cyclo*-(L-phe-L-ser) into gliotoxin with a high incorporation (21±3%) in the culture of *T. viride*. Therefore, Bűlock and Leigh concluded that *cyclo*-(L-phe-L-ser) indeed was the precursor of gliotoxin and that no incorporation of *cyclo*-(L-phe-L-ser) into gliotoxin was due to two possible reasons (Bűlock and Leigh, 1975). The first one, which was less likely, was that a different

fungus (*Penicillium terlikowksi*) was used by MacDonald and Slater. The second reason was that the excess amount of *cyclo*-(L-phe-L-ser) fed to the culture by MacDonald and Slater was liable to generate the misleading results. However, the results from Bűlock and co-workers also need some consideration due to the high dilution of ^{14}C (×59±7) (Bűlock and Leigh, 1975).

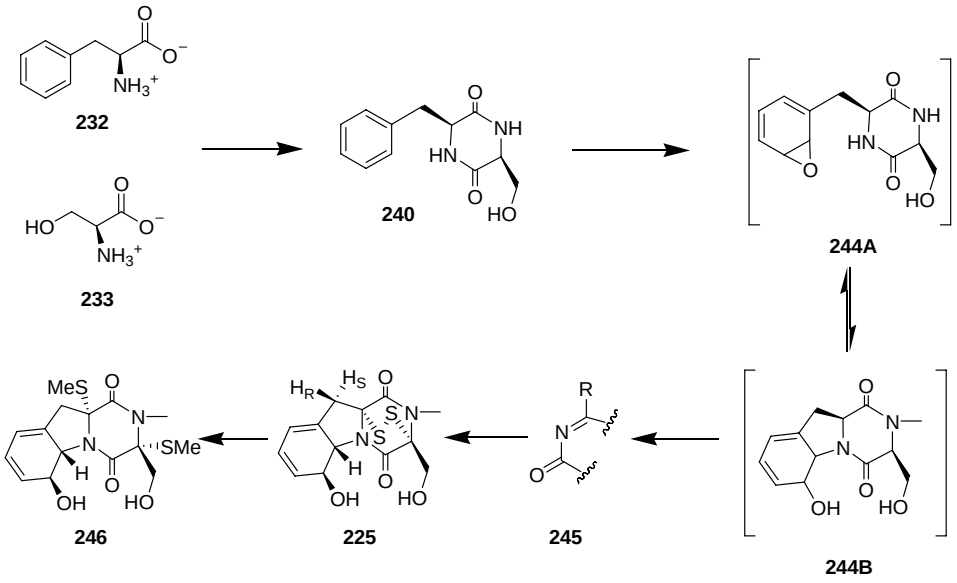


Scheme 1.9. Incorporation results of **240a** – **240d** into gliotoxin (**225**) in cultures of *Trichoderma viride.* (X denotes no incorporation of potential precursors into gliotoxin (**225**)).

Four stereoisomers of *cyclo*-([4'-^{3}H]-phe-[3-^{14}C]-ser) (**240a** – **240d**) were fed to the culture of *T. viride*, only *cyclo*-([4'-^{3}H]-L-phe-[3-^{14}C]-L-ser) (**240a**) gave an efficient incorporation (48%) and constant ^{3}H: ^{14}C ratio between precursor (**240**) and gliotoxin (**225)**, (Scheme 1.9, Kirby et al., 1978). The other three isomers had only trace amounts of incorporation. These results support the results obtained by Bűlock and Leigh (1975). Further, radioactive *cyclo*-(L-phe-L-ser) (1.3% radioactivity) was recovered from cultures of *T. viride* fed successively with non-radioactive *cyclo*-(L-phe-L-ser) (**240**) and L-[U-^{14}C] phenylalanine. These results suggest that dioxopiperazine *cyclo*-(L-phe-L-ser) (**240**) is "either an intermediate, or is interconvertible with an intermediate, on the biosynthetic pathway from phenylalanine to gliotoxin" (Kirby et al., 1978).



No incorporation of compound **241** into gliotoxin suggested that N-methylation is not a step immediately after the formation of dioxopiperazine. Linear dipeptides **242** and **243** were not incorporated into gliotoxin intact, but were incorporated into gliotoxin after they first were hydrolyzed to amino acids. Once again these results support the claim that cyclo-(L-Phe-L-Ser) (**240**) is resistant to enzymatic hydrolysis and is incorporated into gliotoxin intact (Boente et al., 1991).

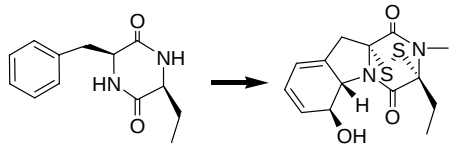


Scheme 1.10. Biosynthesis and catabolism of gliotoxin (**225**). (Adapted from references: Bűlock and Leigh, 1975; Kirby et al., 1980; Boente et al., 1991; Johns and Kirby, 1985).

A new metabolite related to gliotoxin, bisdethiobis-(methylthio)gliotoxin (**246**), was isolated and its biosynthetic pathway was studied (Kirby et al., 1980). ^{14}C-gliotoxin was incorporated into **246** (8.6% incorporation), whileas ^{14}C-**246** was not converted into

gliotoxin by *T. viride*. Therefore, **246** was biosynthesized irreversibly from gliotoxin through reduction and methylation by *T. viride* (Kirby et al., 1980) (Scheme 1.10).

Interestingly, *cyclo*-(L-phe-L-ala) (**247**), an unnatural precursor of gliotoxin, was converted to an analogue of gliotoxin (**248**) by *T. viride* (Scheme 1.11). The conversion was about 20% without appreciable dilution, despite a significant structural difference between serine and alanine, i.e. –OH to –H. Gliotoxin was not detected when *cyclo*-(L-phe-L-ala) was fed to the culture. These results suggested that enzymes responsible for N-methylation and sulfur insertion in *T. viride* had broader ability to catalyze different substrates (Kirby and Robins, 1976).

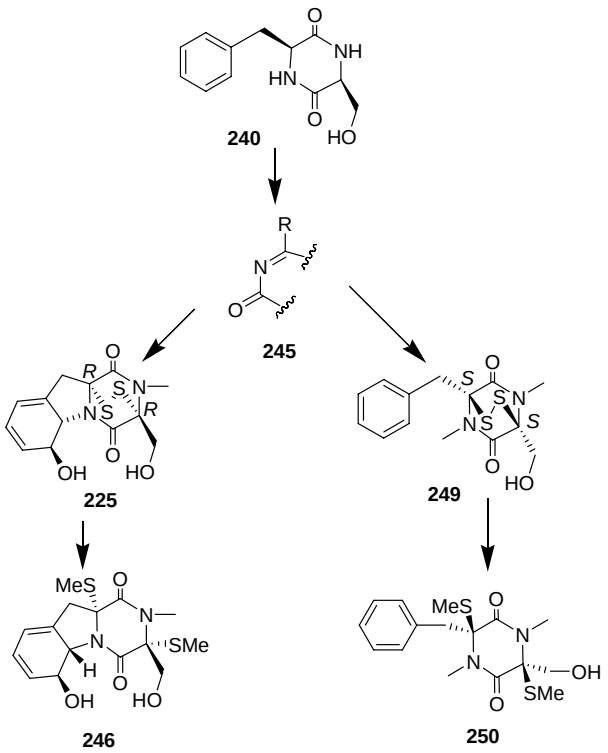


247 **248**

Scheme 1.11. Incorporation of *cyclo*-(L-phe-L-ala) (**247**) into analogue of gliotoxin (**248**) by *Trichoderma viride*.

While the majority of EPTs have 3*R*,6*R*-configurations, hyalodendrin (**249**) produced by *Hyalodendron* sp. represented a rare EPT of 3*S*,6*S*- configurations (Ahmed and Przybylska, 1977). 3*S*,6*S*-Didethiobis(methylthio)hyalodendrin (**250**), the co-metabolite of hyalodendrin (**249**), was predominant under high incubation temperature and long incubation time. The biosynthesis of **250**, which was easily purified by crystallization, was studied using isotopically labeled compounds. Only cyclo-(L-[U-^{14}C]-phe-L-ser), the same stereoisomer precursor of gliotoxin, was incorporated into 3*S*,6*S*-didethiobis(methylthio) hyalodendrin (**250**) by *Hyalodendron* sp. (Boente et al., 1991). The other three stereoisomers (**240b, 240c** and **240d**) were not incorporated into **250**, which indicated that cyclo-(L-Phe-L-Ser) (**240**) is the common precursor for EPTs containing phenylalanine and serine moieties in the skeleton. Further, the result also indicated that the introduction of sulfur does not proceed with stereospecific retention of

configuration. The authors proposed an imino compound (**245**) as an intermediate which could be attacked from either side of the imino bond (Scheme 1.12, Boente et al., 1991).

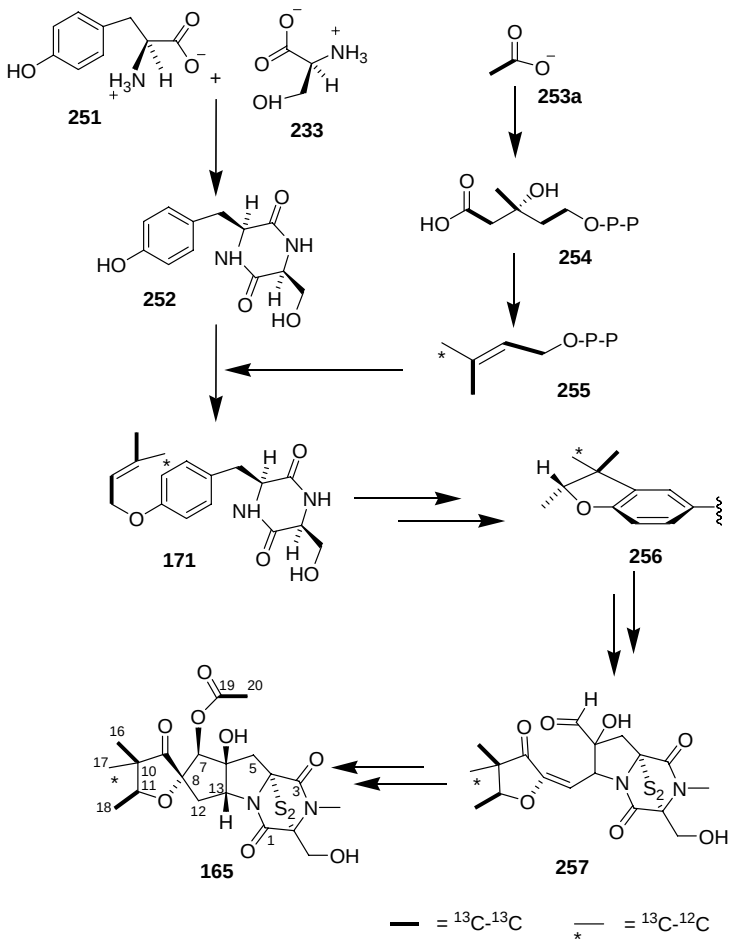


Scheme 1. 12. Proposed biosynthesis of metabolites of **246** and **250**.

1.4.2 Biosynthesis of sirodesmin PL (165)

The biosynthetic pathway of sirodesmin PL has been studied for more than two decades (Bűlock and Clough, 1992; Ferezou et al., 1980). [1-^{14}C]-, [1-^{13}C]-, and [1,2-$^{13}C_2$]-acetates were incorporated into sirodesmin PL. Analysis of the ^{13}C NMR incorporation pattern from [1,2-$^{13}C_2$]-acetate (**253a**) indicated that C-10, C-16, C-11 and C-18 were derived from the intact incorporation of [1,2-$^{13}C_2$]-acetate (**253a**) and C-17 was derived from decarboxylated acetate moiety of mevalonic acid (Scheme 1.13). These results suggested a mevalonic origin of the tetrahydrofuranone ring of sirodesmin PL (Férézou et al., 1980b). L-[U-^{14}C]Serine (**233c**), L-[U-^{14}C]tyrosine (**251**), *cyclo*-(L-[U-^{14}C]-tyr-L-ser) (**252**) and cyclo-*O*-(3',3'-dimethylally)-L-[U-^{14}C]try-L-ser

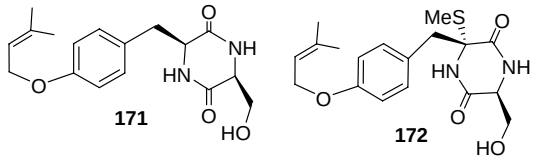
([^{14}C]phomamide) (**171**) were incorporated into sirodesmin PL (**165**). [^{14}C]Phomamide (**171**) was incorporated into sirodesmin PL (**165**) with the highest ratio (25.5%), followed by *cyclo*-(L-[U-^{14}C]-tyr-L-ser) (**252**) (12.5%). L-[U-^{14}C]Serine (**233c**) and L-[U-^{14}C]tyrosine (**251**) were very slightly incorporated into sirodesmin PL, 0.29% and 0.49% respectively. Based on these results a biosynthetic pathway (Scheme 1.13) was proposed (Férézou et al., 1980b).



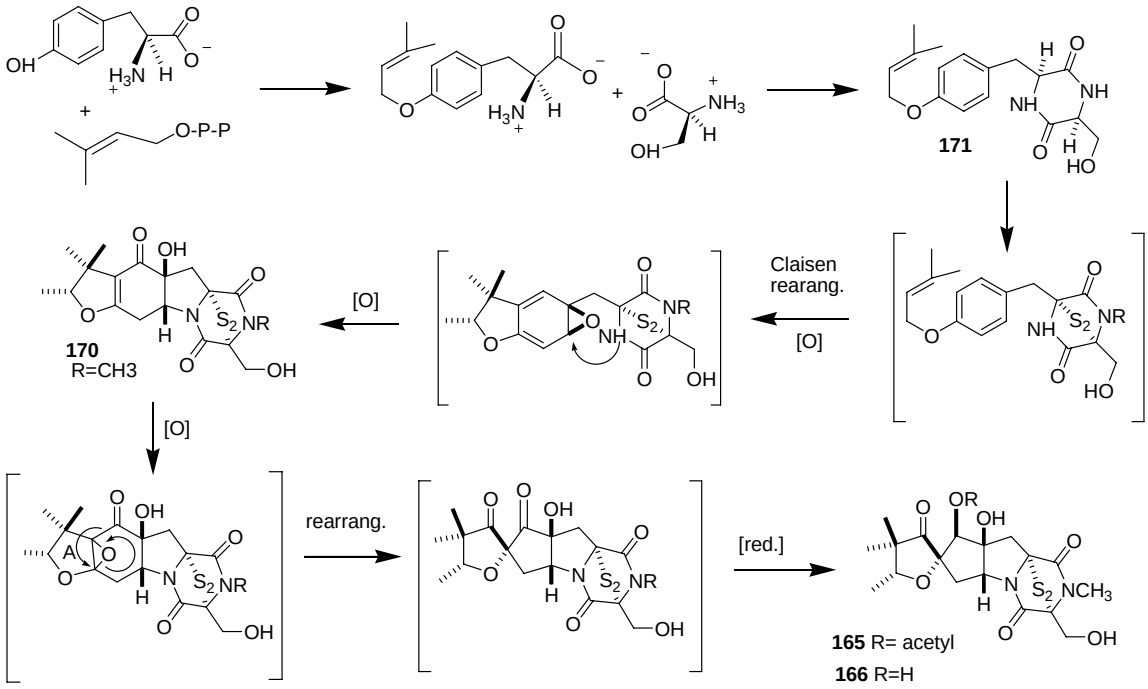
Scheme 1.13. Proposed biosynthetic pathway of sirodesmin PL (**165**) (adapted from Férézou et al., 1980).

Bűlock and co-workers also demonstrated incorporation of [1,2-$^{13}C_2$]-acetate (**253a**) into sirodesmin PL and proposed a more detailed steric rearrangement from the

dimethylallyl moiety to the tetrahydrofuranone ring (Bűlock and Clough, 1992). Doubly labeled L-[3,5-$^{3}H_2$, U-^{14}C]tyrosine (**251**) was incorporated as expected with retention of one ^{3}H and nine ^{14}C atoms into sirodesmin A, an epimer of sirodesmin PL at C-8, suggesting that a precursor of sirodesmin PL is L-tyrosine (**251**) (Bűlock and Clough, 1992).



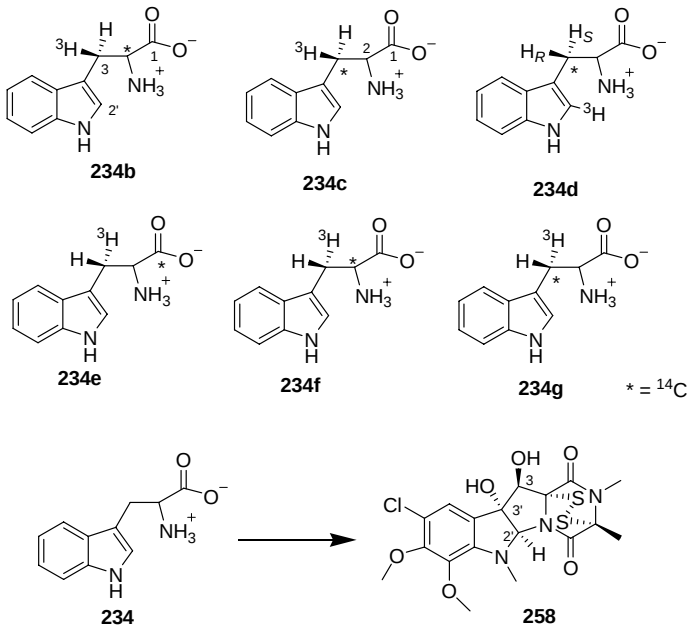
In addition to phomamide (**171**), phomalirazine (**170**) was also isolated from cultures of *L. maculans*, and was proposed to be an intermediate in the biosynthetic pathway of sirodesmin PL (Pedras et al., 1989). The isolation of 3-(methylthio)phomamide (**172**) also gave a hint of when sulfur is introduced into sirodesmin PL (**165**) (Pedras et al., 1990). The biosynthesis of sirodesmin PL was proposed to follow the scheme 1.14.



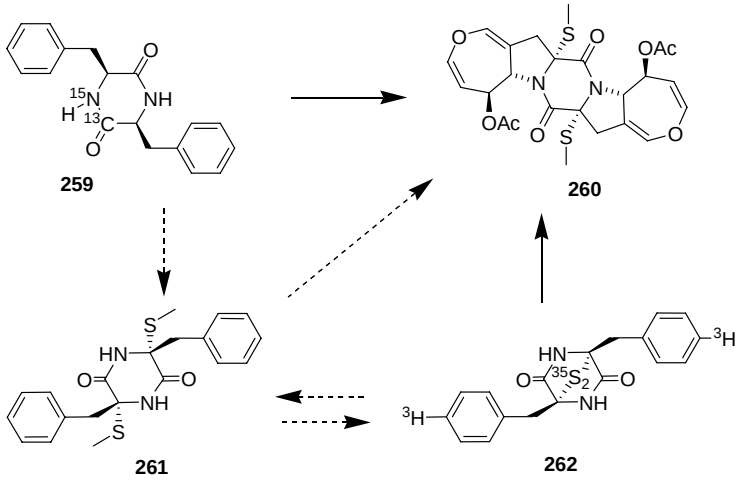
Scheme 1.14. Proposed biosynthetic pathway of sirodesmin PL (**165**) by *Leptosphaeria maculans.* (postulated intermediates in brackets) (Pedras et al., 1989; Gardiner et al., 2004).

1.4.3 Biosynthesis of other EPTs

Biosynthesis of sporidesmin (**258**), produced by *Pithomycas chartarum*, was conducted with ^{3}H and ^{14}C doubly labeled tryptophan **234b**-**234g**. When tryptophan with 3*R*-tritium label, i.e. (3*R*)-[3-^{3}H, 2-^{14}C]tryptophan (**234b**) or (3*R*)-[3-^{3}H, 3-^{14}C]tryptophan (**234c**), was fed to a culture, the tritium in the sporidesmin (**258**) obtained was almost lost (>10% retention compared with ^{14}C). Whereas when tritium was labeled at the 3*S* or 2' position of tryptophan, the tritium incorporated into sporidesmin had above 90% retention (Scheme 1.15). These results indicated that the configuration of C-3 in tryptophan remained the same when it was incorporated into sporidesmin, which suggested that the hydroxylation reaction at C-3 in sporidesmin is stereospecific (Kirby and Varley, 1974).



Scheme 1.15. Biosynthesis of **258** from **234** by cultures of *Pithomycas chartarum*.

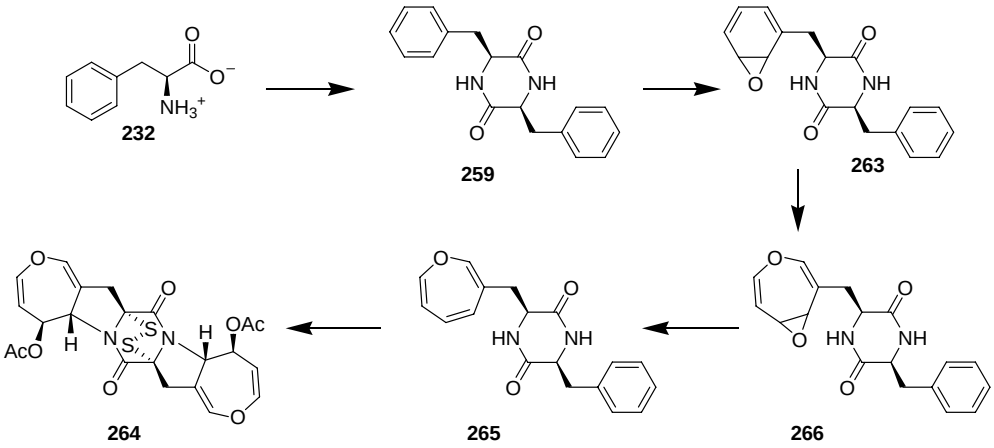


Scheme 1.16. Proposed biosynthetic pathway of **260**. (Full line denoted the incorporation; dash line denoted the possible biotransformation).

Neuss, Boente and their co-workers demonstrated that phenylalanine (Neuss et al., 1968), and *cyclo*-(L-phe-L-phe) were incorporated into bisdethiobis(methylthio)acetylaranotin (**260**) efficiently (Boente et al., 1981), using radioactive labeled materials. But *cyclo*-(L-phe-D-phe) and *cyclo*-(D-phe-D-phe) did not

incorporate into bisdethiobis(methylthio)acetylaranotin (**260**) efficiently (Boente et al., 1981). The intact incorporation of *cyclo*-(L-[^{15}N]phenyla1anyl-L-[1-^{13}C]phenylalanyl) (**259**) suggested that *cyclo*-(L-phe-L-phe) is the possible intermediate in the biosynthesis of bisdethiobis(methylthio)acetylaranotin (**260**) (Scheme 1.16, Boente et al., 1981).

Later on, metabolite **261** was isolated from cultures of *A. terreus*, suggesting the possibility to introduce sulfur into bisdethiobis(methy1thio)acetylaranotin (**260**) immediately after the formation of dioxopiperazine ring (Kirby et al., 1983). Further, the authors demonstrated that **262** (^{3}H: ^{35}S ratio, 4.86) was incorporated into **260** with low dilution of ^{3}H, which suggested **262** was a biosynthetic precursor of **260**. However, the ^{3}H: ^{35}S ratio (3.82) was somewhat altered. Therefore, this conclusion has to be taken cautiously (Kirby et al., 1983).



Scheme 1. 17. Proposed biosynthetic pathway of aranotin (**264**).

The biosynthesis of aranotin (**264**) was also studied by feeding radioactive phenylalanine to the culture of *Avachniofus Aureus*. No further incorporation experiments have been done yet. Scheme 1.17 was the proposed biosynthetic pathway of aranotin (**264**) (Nagaraja.R et al., 1968; Neuss et al., 1968).

1.5 SUMMARY

The chemical interactions between fungi and plants are dynamic and involve metabolites such as phytotoxins and elicitors produced by fungi as well as phytoanticipins and phytoalexins produced by plants. Phytotoxins can damage plant tissues and help the colonization of fungi on plants. Elicitors, which are detrimental to the fungal colonization of plants, are evidence of the fungus-plant co-evolution. Some of the indisputable cell components or metabolites produced by fungi are signals that can be recognized by potential host plants through the selection of co-evolution. The recognition of elicitors will induce the plant defense responses which include for example, the production of phytoalexins and hypersensitive response. Induced phytoalexins and preformed phytoanticipins are two layers of a plant's chemical defenses, which most of the time are important to inhibit further growth of fungi.

More intriguing, another group of fungal pathogens are hemibiotrophic. They normally start biotrophic life style and become necrotrophic at a later stage of growth. Therefore, they possess the ability to produce HSTs and elicitor/virulence factors. The hemibiotrophic fungus *L. maculans* is an economically important phytopathogen from which HSTs phomalide (**179**) and depsilairdin (**183**) were isolated. But are these the only HSTs produced by *L. maculans*? Moreover, the elicitor/virulence factors produced by *L. maculans* are still unknown. A practical way to find elicitor/virulence factors and phytotoxins is to manipulate the medium components and incubation conditions (discussed in section 1.2.3.2). Therefore, elicitors and more of the potential phytotoxins produced by *L. maculans* will be discovered using a bioassay guided isolation from various cultures of *L. maculans*.

CHAPTER 2: RESULTS AND DISCUSSION

2.1 POTENTIAL ELICITORS FROM *LEPTOSPHAERIA MACULANS*

To search for elicitors and phytotoxins produced by *L. maculans*, metabolites produced in different culture conditions were analyzed by HPLC and isolated using an elicitor-toxin activity guided assay. Two basic media, minimal medium (MM) and potato dextrose broth (PDB) medium as well as several modified MM and PDB media were employed for the preparation of the cultures of *L. maculans*. The various culture conditions are summarized in Table 2.1. The elicitor-toxin activity assays were carried out employing *B. juncea* cv. Cutlass (brown mustard, resistant to *L. maculans*), *B. napus* cv. Westar (canola, susceptible to *L. maculans*) and *Sinapis alba* cv. Ochre (white mustard, resistant to *L. maculans*). Sixteen metabolites were isolated from *L. maculans* for the first time, among them, eleven metabolites are new. A detailed isolation procedure of these metabolites is described in the experimental section. In the following sections HPLC analysis of the metabolites produced under various culture conditions is described first, followed by a brief description of the isolation of the metabolites. Lastly, the structure determination of the new metabolites and the metabolites isolated for the first time from *L. maculans* is described and discussed.

Table 2.1. Culture conditions used to grow *Leptosphaeria maculans*.

#	Media	Amendments[a] (concentration)	***Days of incubation before addition of amendments***
1	MM	-	-
2	MM	NaCl (0.17 M)	-
3	MM	NaCl (0.17 M)	3
4	MM	NaCl (0.34 M)	3
5	MM	NaCl (0.34 M)	4
6	MM	NaCl (0.68 M)	3
7	MM	NaCl (0.68 M)	4
8	MM	NaCl (0.68 M)	5
9	PDB	-	-
10	PDB	25 °C	-
11	PDB	27 °C	-
12	PDB	29 °C	-
13	MM	29 °C	-
14	MM	$MgSO_4 \cdot 7H_2O$ (2.0×10^{-4} M)	-
15	MM	$MgSO_4 \cdot 7H_2O$ (1.0×10^{-3} M)	-
16	MM	$MgSO_4 \cdot 7H_2O$ (4.0×10^{-3} M)	-
17	MM	- thiamine	-
18	MM	- thiamine + leaves of brown mustard	-
19	MM	Spirobrassinin (1.0×10^{-4} M)	3
20	MM	Camalexin (1.0×10^{-4} M)	3
21	PDB	Spirobrassinin (1.0×10^{-4} M)	3
22	PDB	Camalexin (1.0×10^{-4} M)	3
23	V_8	Glucose (5.5×10^{-2} M)	-

[a]All the cultures were incubated at room temperature (23°C) unless stated otherwise.

2.1.1 Metabolites produced in minimal media

2.1.1.1 Standard conditions

Minimal medium (MM) is a chemically defined medium. Similar to the previous observation (Férézou et al., 1977 and 1980a), when *L. maculans* was incubated in MM under standard conditions (Table 2.1, entry 1), sirodesmin PL (**165**), deacetylsirodesmin PL (**166**) and phomamide (**171**) were detected as the major metabolites in culture broth (Figure 2.1). However, these metabolites have not been tested for their elicitor activities. Also, most of the minor metabolites from the culture broth and metabolites from mycelia have not been isolated and tested for their elicitor and phytotoxin activities. Therefore, an elicitor-toxin activity assay guided isolation was carried out to find elicitors and potentially new phytotoxins produced by *L. maculans* in MM under standard conditions.

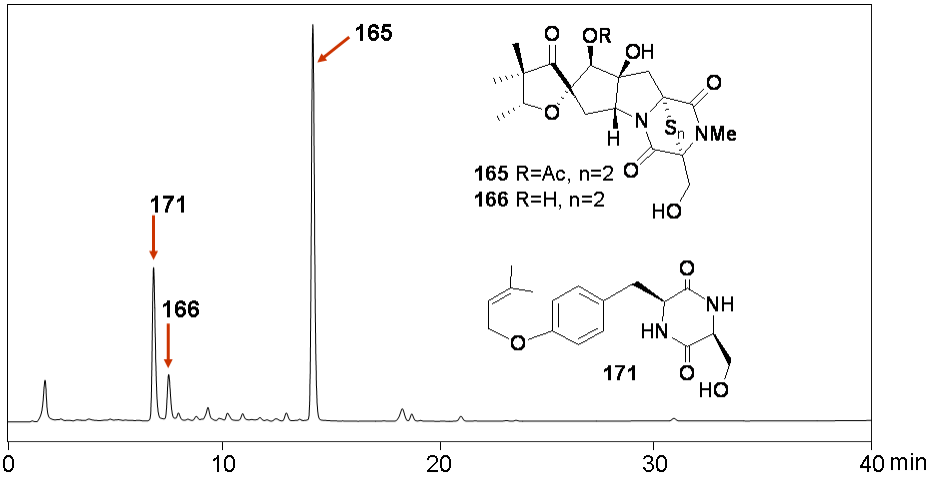


Figure 2.1. HPLC chromatogram of EtOAc extract of broth of *Leptosphaeria maculans* incubated in minimal medium (MM).

A total of 20 liters of the cultures of *L. maculans* IBCN 57 (BJ 125) was prepared in standard MM. After 7 days of incubation, mycelia were separated from the broth by vacuum filtration and the broth was extracted with EtOAc. The EtOAc layer was concentrated to give 7.2 g residue, the residue (EtOAc extract) was assayed, showing

elicitor-toxin activity. Therefore the EtOAc extract was fractioned using various chromatographic methods to give metabolites **267** to **270** and **272** to **275** as well as **288** (Section 4.2.1.1). The aqueous layer did not show elicitor-toxin activity. Therefore it was autoclaved and discarded. The mycelia were then extracted with MeOH. The MeOH layer was concentrated and the residue was resuspended in MeOH/H_2O (1:1 v/v) then extracted with hexane. The hexane layer was concentrated to give 3.4 g residue and the residue (hexane extract) showed elicitor activity. Therefore, the hexane extract was fractioned to give metabolites **14** and **31**, as described in Section 4.2.1.1. The MeOH/H_2O (1:1 v/v) layer was concentrated to give 12.6 g residue. This residue did not show elicitor activity, and D-mannitol (**301**) was isolated as the major component.

Leptomaculin A (**267**) and leptomaculin B (**268**)

The molecular formula of compound **267 (**$C_{19}H_{27}N_3O_6S$) obtained by HRMS-EI indicated eight degrees of unsaturation. The ^{1}H NMR ($CDCl_3$) of compound **267** (Table 2.2) displayed a pattern similar to that of sirodesmin PL (**165**), i.e. two singlets (δ_H 1.07, 3H, and 1.00, 3H), a doublet (δ_H 1.26, 3H) and a quartet (δ_H 3.87, 1H) accounting for all protons of ring A, two doublets of doublets (δ_H 2.69, 1H, and 1.84 1H) coupled to another doublet of doublets (δ_H 4.59, 1H) accounting for H_2-12 and H-13 of ring B. Two doublets (δ_H 2.93, 1H and δ_H 2.24, 1H) accounted for H_2-5 in ring C. A singlet (δ_H 3.44, 3H) accounting for the N-methyl group of ring D. After D_2O exchange, a doublet (δ_H 3.07, 1H) disappeared and another doublet (δ_H 3.91, 1H) became a singlet, accounting for coupling due to hydroxyl group at C-7 and H-7, respectively. Moreover a singlet at δ_H 4.20, 1H disappeared accounting for hydroxyl group at C-6. According to this analysis, compound **267** has the same skeleton in rings A, B and C as those of sirodesmin PL (**165**). A doublet (δ_H 2.84, 3H) became a singlet and a quartet (δ_H 6.55, 1H) disappeared after D_2O exchange, which indicated a secondary methyl amide. The methylene hydroxyl group corresponding to the OH group at C-14 in sirodesmin skeleton was not observed. A methylene AB spin system (δ_H 4.23, dd, 1H, and 3.87, dd, 1H) indicated that the methylene hydroxyl group was not present. Therefore, the structure of compound **267** was different from that of sirodesmin PL (**165**) at ring D.

Four carbonyl carbons were displayed in the ^{13}C NMR spectrum. The carbonyl carbons at δ_C 223.4 and 157.4 were assigned to be C-9 and C-1, respectively, as in sirodesmin PL (**165**). δ_C 171.3 was assigned to be the carbonyl C-15 of an amide moiety. The δ_C 185.1 was assigned as a thio carbonyl. The position of the amide carbonyl was assigned based on HMBC 2D NMR correlations (Figure 2.2). C-15 (δ_C 171.3) showed correlations with H_2-5 (δ_H 2.93 and 2.24), H_2-3 (δ_H 4.23 and 3.87) and H_3-16 (δ_H 2.84). The nitrogen methyl H_3-14 (δ_H 3.44) showed correlations with C-3 (δ_C 59.5) and C-2 (δ_C 185.1), H_2-3 (δ_H 4.23 and 3.87) showed correlation with C-2 (δ_C 185.1). Therefore structure **267** was proposed. Crystallization of **267** yielded single crystals by slow evaporation of a solution of metabolite **267** in MeOH-CH_2Cl_2-hexane (1:5:4). The X-ray diffraction data (CCDC 684017) confirmed the proposed structure of compound **267** (Figure 2.3).

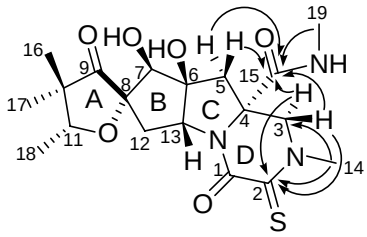


Figure 2.2. Selected HMBC correlations of leptomaculin A (**267**).

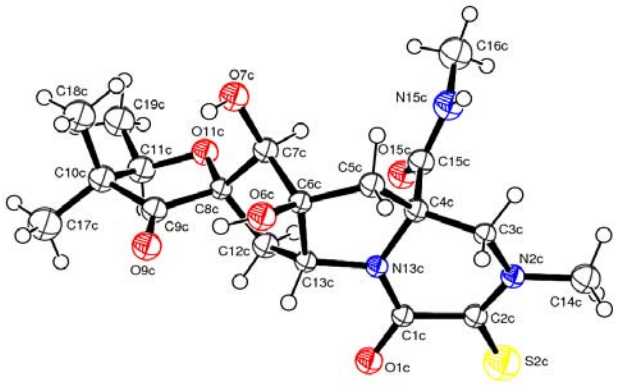


Figure 2.3. X-ray ORTEP diagram of leptomaculin A (**267**).

The ^{1}H and ^{13}C NMR ($CDCl_3$) spectra of compound **268** (Table 2.2) were similar to those of compound **267**. The characteristic resonances of ring A, B and C in the sirodesmin skeleton were observed, although three proton resonances were somehow overlapped at δ_H 3.84-3.91 which accounted for H-3b, H-7 and H-11. The ^{1}H NMR (DMSO-d_6) of compound **268** showed well separated spin systems, and the D_2O exchange results were consistent with those of compound **267**. The major difference between compound **268** and **267** was that the thiocarbonyl at δ_C 185.1 in compound **267** was shifted upfield to δ_C 155.5 in compound **268**. This change is in agreement with the molecular formula of compound **268** ($C_{19}H_{27}N_3O_7$, HRMS-EI), i.e., the thiocarbonyl was replaced by an oxygen carbonyl. The stereochemistry of compound **268** was assigned based on the NOESY 2D NMR (Figure 2.4). H-7 (δ_H 3.61) showed correlations with H-12b (δ_H 1.71) and H-5a (δ_H 2.62). These two correlations were consistent with the stereochemistry of sirodesmin PL (**165**), which suggested that H-7 (δ_H 3.61) and H-5a (δ_H 2.62) were on the lower face of the B/C rings. Therefore, H-5b (δ_H 2.09) was on the upper face of the B/C rings. H-5b (δ_H 2.09) showed a correlation with H-3a (δ_H 2.84), indicating that H-3a (δ_H 2.84) and H-5b (δ_H 2.09) were close in space and both were on the upper face of the C/D rings. Based on the above analysis, carbonyl carbon C-15 of compound **268** was on the lower face of C/D rings, which was consistent with compound **267** as shown in Figures 2.2 and 2.4.

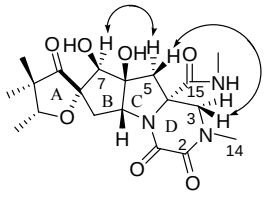


Figure 2.4. Selected NOESY correlations of leptomaculin B (**268**).

Table 2.2. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) chemical shifts (ppm) and multiplicities (*J* in Hz) of leptomaculins A (**267**) and B (**268**).

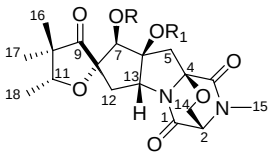
	Leptomaculin A (**267**) (CD_3OD)		Leptomaculin B (**268**) ($(CD_3)_2SO$)	
C /H #	δ_C	δ_H	δ_C	δ_H
1	158.2	---	155.5	---
2	186.7	---	157.4	---
3	60.4	4.03, 1H, d, (13.4)	56.1	3.75, 1H, d, (12.9)
		4.14, 1H, d, (13.4)		3.94, 1H, d, (12.9)
4	69.8	---	67.8	---
5	46.0	2.83, 1H, d, (14.3)	45.2	2.62, 1H, d, (14.1)
		2.25, 1H, d, (14.3)		2.09, 1H, d, (14.0)
6	85.8	---	83.2	---
7	80.4	3.80, 1H, s	78.4	3.61, 1H, d, (5.8)
8	92.7	---	91.3	---
9	223.1	---	221.7	---
10	48.6	---	46.9	---
11	80.9	3.87, 1H, q, (6.2)	79.2	3.83, 1H, q, (6.2)
12	37.5	1.93, 1H, dd, (14.9, 5.7)	35.0	1.71, 1H, dd, (14.4, 7.1)
		2.57, 1H, dd, (14.9, 9.4)		2.44, 1H, dd, (14.4, 9.1)
13	69.3	4.50, 1H, dd, (9.3, 5.6)	66.0	4.15, 1H, dd, (8.9, 7.1)
14	43.9	3.41, 3H, s	34.3	2.82, 3H, s
15	173.8	---	171.5	---
16	27.3	2.76, 3H, s	26.5	2.65, 3H, d, (4.8)
17	20.4	1.03, 3H, s	19.6	0.97, 3H, s
18	17.3	0.98, 3H, s	16.3	0.87, 3H, s
19	14.7	1.22, 3H, d, (6.3)	14.1	1.14, 3H, d, (6.3)
Others				C-6 -OH, 4.60, s
				C-7 -OH, 5.57, d, (5.6)
				-NH, 8.32, q, (5.0)

Leptomaculin C (**269**) and deacetylleptomaculin C (**270**)

The molecular formula of compound **269** ($C_{20}H_{27}N_2O_8$) obtained by HRMS-ESI indicated nine degrees of unsaturation. The absence of sulfur atoms indicated that compound **269** did not contain a sulfur bridge as that in sirodesmin PL. The ^{1}H NMR ($CDCl_3$) of compound **269** (Table 2.3) displayed a similar pattern as that of sirodesmin PL (**165**), i.e. two singlets (δ_H 1.06, 3H, and 1.01, 3H), a doublet (δ_H 1.26, 3H) and a quartet (δ_H 3.88, 1H) accounting for all protons of ring A. Two doublets of doublets (δ_H 2.54, 1H, and 1.59 1H) coupled to another doublet of doublets (δ_H 4. 35, 1H) accounting for H_2-12 and H-13 of ring B. Two doublets at δ_H 3.50 (1H) and δ_H 2.54 (1H) accounted for H_2-5 in ring C. The hydroxyl group at C-6 was displayed at δ_H 3.27, but no signal for hydroxyl group at C-14 was observed. Upon acetylation of compound **269** with pyridine and Ac_2O, only one acetyl group was introduced. In the ^{13}C NMR of compound **269**, two carbonyl carbons (δ_C 167.2 and 166.6) accounting for C-1 and C-3, and δ_C 169.6 and δ_C 221.2 accounting for acetyl carbonyl C-19 and ketone carbonyl C-9, respectively. All four carbonyl groups as well as rings A, B, C and D accounted for eight degrees of unsaturation. Compound **269** had the same nine degrees of unsaturation as sirodesmin PL (**165**) ($C_{20}H_{26}N_2O_8S_2$). One of the methylene protons at C-5 was substantially shielded (δ_H 3.50 and 2.54, H_2-5), which is an ABq (δ_H 3.27, H_2-5) in sirodesmin PL (**165**). Therefore, the methylene hydroxyl group was attached to C-4 to form an ether bridge. The configurations of C-2 and -4 were assigned to be 2*S* and 4*S*, i.e. the ether bridge was above the plane of rings C and D, as shown below. Assuming that compound **269** has the same biosynthetic origin as sirodesmin PL (**165**), the absolute configurations at C-6, 7, 8, 10, 11 and 13 were assigned similar to sirodesmin PL (**165**) and compound **269**. Since H_2-14 and H-13 of compound **269** showed correlation in NOE experiments, therefore, protons H_2-14 were on the upper face of the C/D rings, i.e. *cis* relative to H-13. From a more polar fraction, Compound **270**, a deacetyl derivative of compound **269**, was isolated and confirmed the structure assignment of compound **270**.

Table 2.3. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) chemical shifts (ppm) and multiplicities (*J* in Hz) of leptomaculin C (**269**) and ^{1}H NMR ($CDCl_3$, 500 MHz) of deacetylleptomaculin C (**270**) and deacetylleptomaculin C (**271**).

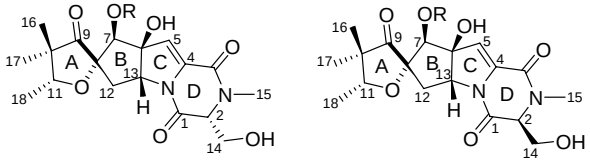
	269		**270**	**271**
C#	δ_C	δ_H	δ_H	δ_H
1	167.2	---	---	---
2	61.8	4.00, 1H, bs	4.00, 1H, d, (2.3)	4.01, 1H, bs
3	166.6	---	---	---
4	95.3	---	---	---
5	42.9	3.50, 1H, d, (16.2)	3.46, 1H, d, (15.4)	3.92, 1H, d, (16.7)
		2.54, 1H, d, (16.3)	2.39, 1H, d, (15.4)	2.68, 1H, d, (16.7)
6	84.6	---	---	---
7	80.4	4.95, 1H, s	3.99, 1H, bs	5.64, 1H, s
8	89.4	---	---	---
9	221.2	---	---	---
10	47.6	---	---	---
11	80.3	3.88, 1H, q, (6.3)	3.85, 1H, q, (6.4)	3.70, 1H, q, (6.3)
12	35.9	2.54, 1H, dd, (13.9, 8.0)	2.45, 1H, dd, (14.5, 8.3)	2.32, 1H, dd, (14.8, 8.5)
		1.59, 1H, dd, (14.0, 8.2)	1.85, 1H, dd, (14.9, 3.8)	1.88, 1H, bd, (14.6)
13	67.0	4.35, 1H, dd, (8.1, 8.1)	4.29, 1H, dd, (8.3, 3.7)	4.72, 1H, bd, (8.3)
14	66.1	4.07, 1H, dd, (9.1, 2.4)	4.05, 1H, dd, (8.9, 2.2)	4.04, 1H, bd, (9.7)
		4.01, 1H, bd, (8.4)	4.00, 1H, bd, (9.1)	3.96, 1H, bd, (8.8)
15	31.7	3.10, 3H, s	3.10, 3H, s	3.09, 3H, s
16	20.5	1.06, 3H, s	1.04, 3H, s	1.02, 3H, s
17	17.6	1.01, 3H, s	1.01, 3H, s	0.93, 3H, s
18	14.4	1.25, 3H, d, (6.3)	1.24, 3H, d, (6.4)	1.24, 3H, d, (6.3)
19	169.6	---	---	---
20	20.7	2.10, 3H, s	---	2.09, 3H, s
22		---	---	2.04, 3H, s



269 R=Ac, R_1=H
270 R=R_1=H
271 R=R_1=Ac

Leptomaculin D (**272**) and E (**274**) and deacetylleptomaculin D (**273**) and E (**275**)

Metabolite **272** had structure moieties similar to sirodesmin PL (**165**) in rings A and B according to ^{1}H NMR ($CDCl_3$) (Table 2.4); two singlets (δ_H 1.11, 3H, and 1.03, 3H), a doublet (δ_H 1.25, 3H) and a quartet (δ_H 3.95, 1H) accounting for all protons of ring A. Two doublets of doublets (δ_H 2.92, 1H, and 1.63 1H) coupled to another doublet of doublets (δ_H 4. 48, 1H) accounted for H_2-12 and H-13 of ring B. As well, a much lower field singlet (H-7, δ_H 4. 86, 1H) typical of acetylated OH group at C-7 (singlet acetyl methyl group at δ_H 2.10, 3H). Two D_2O exchangeable resonances at δ_H 3.56 and 2.44 are OH groups at C-6 and -14 respectively. A singlet at δ_H 6.39 (1H) indicated the presence of a double bond in compound **272**. Two sp^2 carbons at δ_C 134.3 and 119.4 in ^{13}C NMR spectrum of compound **272** (Table 2.5) showed correlation with this low field singlet in HMQC and HMBC spectral data. The replacement of the ABq resonance as H_2-5 in sirodesmin PL (**165**) with a singlet (δ_H 6.39, 1H) in the spectrum of compound **272** indicated that the position of double bond was between C-4 and C-5. A broad doublet at δ_H 4.08 (1H) and a broad doublet of doublets at δ_H 4.00 (1H) coupled to a doublet of doublets at δ_H 4.03 (1H) were assigned to H_2-14 and H-2, respectively. Based on the above analysis, the structure of metabolite **272** was proposed.



272 R=Ac
273 R=H

274 R=Ac
275 R=H

Table 2.4. ^{1}H NMR ($CDCl_3$, 500 MHz) chemical shifts (ppm) and multiplicities (*J* in Hz) of leptomaculins D (**272**) and E (**274**) and deacetylleptomaculins D (**273**) and E (**275**).

H #	Leptomaculin D (**272**) δ_H	Leptomaculin E (**274**) δ_H	Deacetylleptomaculin D (**273**) δ_H	Deacetylleptomaculin E (**275**) δ_H
2	4.03, 1H, dd, (3.1, 2.0)	4.01, 1H, dd, (3.6, 1.7)	4.02, 1H, s	4.00, 1H, s
5	6.39, 1H, s	6.39, 1H, s	6.08, 1H, s	6.15, 1H, s
7	4.86, 1H, s	4.85, 1H, s	3.94, 1H, s	3.93, 1H, s
11	3.95, 1H, q, (6.4)	3.94, 1H, q, (6.3)	3.88, 1H, q, (6.3)	3.95, 1H, q, (6.3)
12	1.63, 1H, dd, (14.0, 9.0) 2.92, 1H, dd, (14.0, 8.5)	1.55, 1H, dd, (13.9, 8.9) 2.85, 1H, dd, (13.9, 8.7)	1.60, 1H, dd, (14.3, 7.1) 2.72, 1H, dd, (14.3, 8.5)	1.72, 1H, dd, (14.1, 7.3) 2.80, 1H, dd, (14.1, 8.7)
13	4.48, 1H, dd, (8.8, 8.7)	4.50, 1H, dd, (8.7, 8.7)	4.55, 1H, dd, (8.3, 7.3)	4.48, 1H, dd, (7.9, 7.9)
14	4.00, 1H, dd, (11.2, 3.4) 4.08, 1H, dd, (11.2, 2.0)	3.99, 1H, dd, (11.2, 3.6) 4.09, 1H, dd, (11.2, 3.6)	4.05, 1H, dd, (11.9, 2.8) 4.07, 1H, dd, (11.8, 3.1)	4.01, 1H, dd, (11.8, 12.1) 4.05, 1H, dd, (11.8, 11.6)
15	3.07, 3H, s	3.08, 3H, s	3.09, 3H, s	3.08, 3H, s
16	1.11, 3H, s	1.11, 3H, s	1.04, 3H, s	1.09, 3H, s
17	1.03, 3H, s	1.03, 3H, s	1.00, 3H, s	1.01, 3H, s
18	1.25, 3H, d, (6.4)	1.25, 3H, d, (6.3)	1.22, 3H, d, (6.3)	1.24, 3H, d, (6.3)
20	2.10, 3H, s	2.09, 3H, s	---	---

Compound **274**, an epimer of compound **272**, was isolated from the fraction containing compound **272** by PTLC using multiple developments. These two metabolites were very similar in all the aspects of ^{1}H NMR and ^{13}C NMR data (Tables 2.4 and 2.5). In order to confirm the skeleton and stereochemistry of compound **272** and **274**, attempts

to obtain crystals or the Mosher ester derivative (**282**) of compound **272** (Scheme 2.1, Ward and Rhee, 1991) failed due to the limited amount of compound. Later, the structures of compounds **272** and **274** were confirmed by the preparation of the diacetyl derivatives of compounds **281a** and **281b** from sirodesmin PL (**165**) (Scheme 2.1). The disulfur bridge of sirodesmin PL was readily reduced using PPh_3 and the resulting sirodesmin H (**167**) was derivatized (Ac_2O) to give compound **276**. After hydrolysis to remove the acetyl group from the primary hydroxyl at C-14 of compound **276**, a pair of epimers, compounds **280a** and **280b,** were obtained after reaction of the hydrolysis product of compound **276** (Raney nickel in MeOH at room temperature for one hour). Compounds **280a** and **280b** were separated and acetylated to give compounds **281a** and **281b**, respectively. Treatment of naturally occurring metabolites **272** and **274** with pyridine/acetic anhydride gave two products which are identical to compounds **281a** and **281b**, respectively (Scheme 2.1). Therefore, the structures of compounds **272** and **274** were confirmed. Another pair of epimers compounds **273** and **275** were isolated from a more polar fraction. Their structures were determined to be the deacetyl derivatives of compounds **272** and **274** from analysis of their spectroscopic data and preparation of their acetyl derivatives **281a** and **281b**.

Table 2.5. ^{13}C NMR ($CDCl_3$, 125 MHz) chemical shifts (ppm) of leptomaculins D (**272**) and E (**274**) and deacetylleptomaculins D (**273**) and E (**275**).

C #	Leptomaculin D (**272**) δ_C	Leptomaculin E (**274**) δ_C	Deacetylleptomaculin D (**273**) δ_C	Deacetylleptomaculin E (**275**) δ_C
1	161.6	162.2	162.3	161.5
2	65.9	66.1	66.4	66.4
3	157.1	157.3	157.9	157.7
4	134.3	134.2	134.4	134.2
5	119.4	119.4	118.2	118.6
6	83.8	83.8	85.1	85.1
7	79.0	78.9	79.2	79.1
8	88.5	88.3	89.9	90.1
9	222.4	222.3	222.4	222.6
10	47.7	47.7	47.9	47.9
11	80.6	80.6	80.4	80.8
12	34.6	35.1	35.6	34.9
13	67.0	66.4	66.8	66.9
14	62.0	61.9	61.6	61.8
15	32.3	32.4	32.5	32.4
16	20.7	20.7	20.5	20.4
17	17.6	17.7	17.2	17.2
18	14.6	14.6	14.7	14.6
19	169.7	169.6	---	---
20	20.7	20.7	---	---

However, the configuration of C-2 in compounds **272** to **275** could not be assigned directly from NOESY spectra, because of the overlap of signals of H-14 and H-2 (ranging from δ_H 3.9 to 4.1) in all four epimers. Acetylated derivatives of sirodesmin

PL (**165**) have been reported (Pedras et al, 1990) and displayed well separated resonances of H-14, H-13 and H-2 in ^{1}H NMR. Therefore diacetyl- (**281b** and **281a**) and 6-mono acetyl- (**280b** and **280a**) derivatives of new compounds **272** to **275** were prepared (Scheme 2.1). Both derivative pairs gave the desired spectra in which H-14, H-13 and H-2 were separated. Compound **280b** showed clear signals in the NOESY spectrum, displaying a correlation between H-13 and H-14a (Figure 2.5). By contrast, **280a** did not show correlation between H-13 and H-14 (Figure 2.5). Assuming that compounds **272** to **275** have the same configurations in rings A, B, C and D as sirodesmin PL (**165**), e. g. H-13 of compounds **272** to **275** were on the upper face of C/D rings. Therefore H_2-14 is on the upper face of C/D rings in compounds **274** and **275**. Further support came from comparison of the NOESY data of compounds **280a**, **280b** and **276**. X-ray diffraction data of compound **276**, which crystallized readily from a solution of CH_2Cl_2-hexane (1: 9) to give a single crystal, indicated that compound **276** kept the same configurations as sirodesmin PL (**165**). As shown in Figure 2.5, compounds **276** and **280b** have the same correlation between H-13 and H-14a, but no correlation between H-13 and H-14a was observed in compound **280a**. Therefore, the configuration of C-2 in compound **280b** is *S*, the same as compound **276**. Consequently, the configuration of C-2 in compounds **273** and **275** was determined to be *S*, and for the same reasons compounds **272** and **273** have *R* configuration at C-2 (Scheme 2.1).

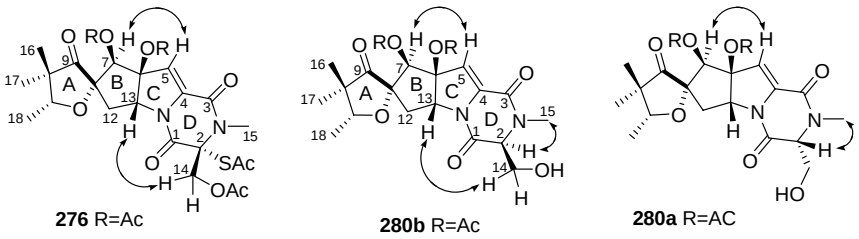
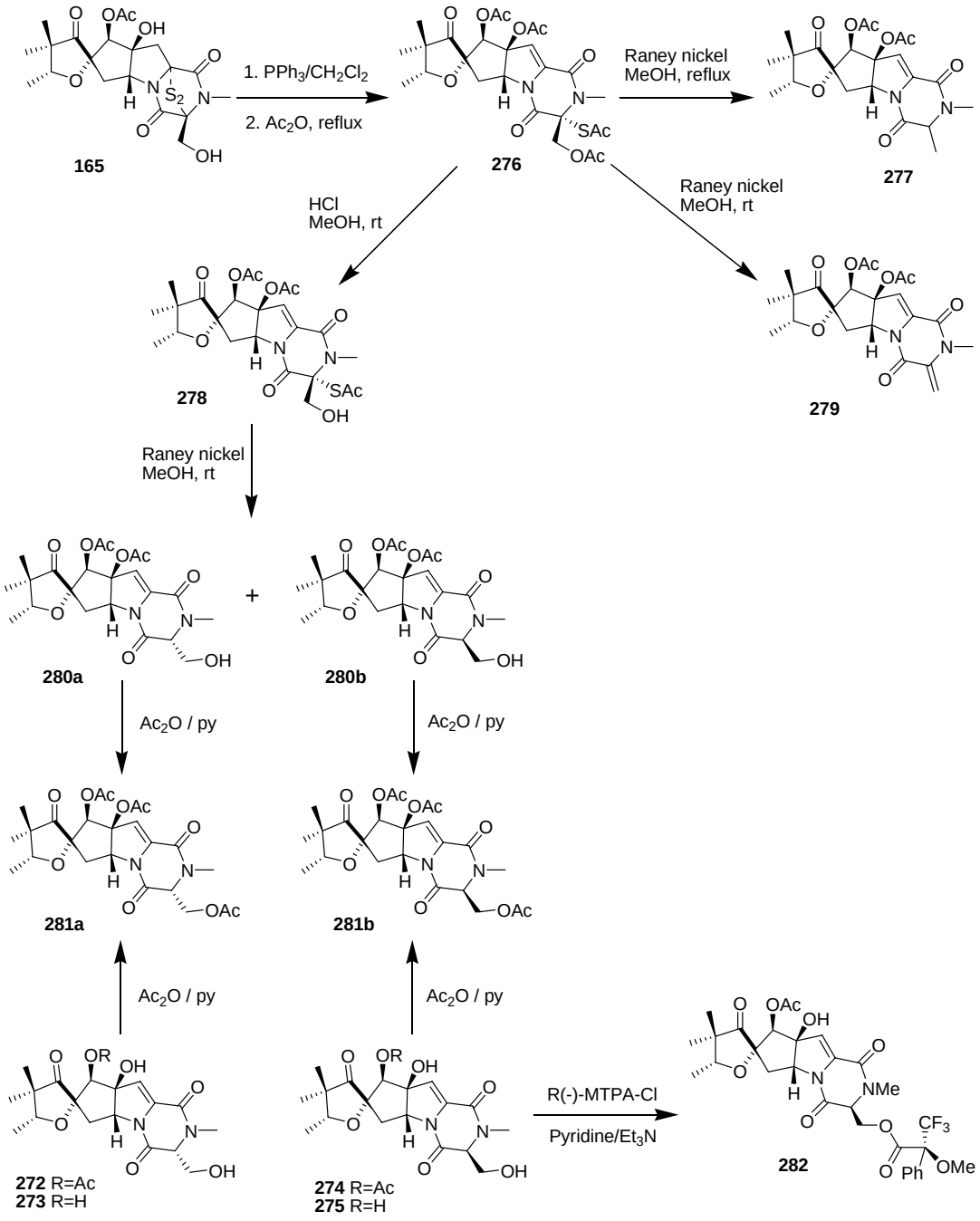


Figure 2.5. Selected NOESY correlations of compounds **276**, **280b** and **280a**.



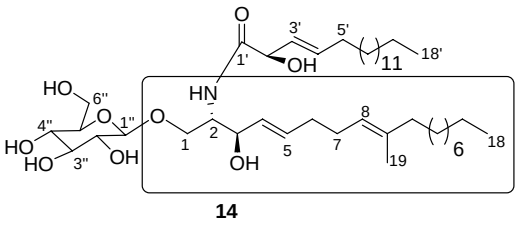
Scheme 2.1. Chemical derivatization of sirodesmin PL (**165**) and leptomaculins D (**272**) and E (**274**).

It is worth noting that when compound **276** reacted with Raney nickel (Scheme 2.1), compound **279** with an e*xo*-double bond was produced at room temperature

whereas compound **277** was obtained under reflux. Only after the acetyl group was removed by hydrolysis did addition of Raney nickel yield the pair of desired epimers **280a** and **280b.** However, deacetyl intermediate compound **278** was not stable (concentration or FCC purification), as intermolecular acetyl-transfer was observed. Therefore the reaction mixture of methanolysis of compound **276** was immediately used for the next step without further purification. The structure of compound **278** was proposed based on the ^{1}H NMR and LC-MS data of the methanolysis reaction mixture of compound **276**.

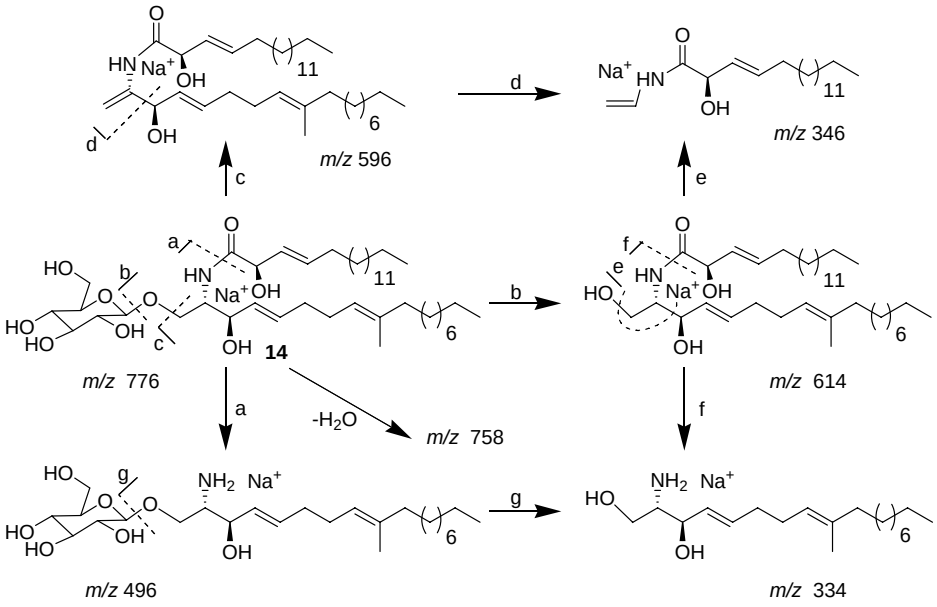
Cerebroside C (**14**) and D (**31**)

The molecular formula of compound **14** $[M+1]^{+}$ **(**$C_{43}H_{80}NO_9$) obtained by HRMS-ESI, indicated four degrees of unsaturation. The ^{1}H NMR spectrum of compound **14** showed most of the signals as broad peaks with no coupling and overlapping. When the ^{1}H NMR spectrum was recorded at 47 ºC the signals from a likely sugar moiety and double bond protons became sharper. Therefore 1D and 2D NMR spectra were recorded at 47 ºC in $CDCl_3$. The ^{1}H NMR spectrum characteristic of sphingolipids was observed and elucidated from COSY 2D NMR data. Two broad singlets at δ_H 4.01 and 3.87 (H_2-1) coupled to the broad singlet at δ_H 4.09 (H-2), which in turn coupled with an exchangeable doublet at δ_H 7.35 (NH) indicated the amino alcohol part of the sphingosine moiety (Figure 2.6) and the amide moiety corresponding to the carbonyl at δ_C 174.6. A broad singlet at δ_H 4.58 (H'-2) coupled to a doublet of doublets at δ_H 5.53 (H'-3) and a doublet of triplets at δ_H 5.86 (H'-4), which in turn coupled to a broad singlet at δ_H 2.07 (H'-5), accounted for the unsaturated moiety of the fatty acid residue. A broad doublet at δ_H 5.77 (H-5) coupled with a broad singlet at δ_H 2.07 (H-6) and a doublet of doublets at δ_H 5.47 (H-4), which in turn coupled with a broad singlet at δ_H 2.07 (H-3), accounted for the unsaturated moiety of sphingosine. The sugar moiety signals were also observed in the range at δ_H 4.38 to 3.38, although the signals did not show a clear spin system. Therefore, compound **14** was proposed to be a sphingolipid.



14

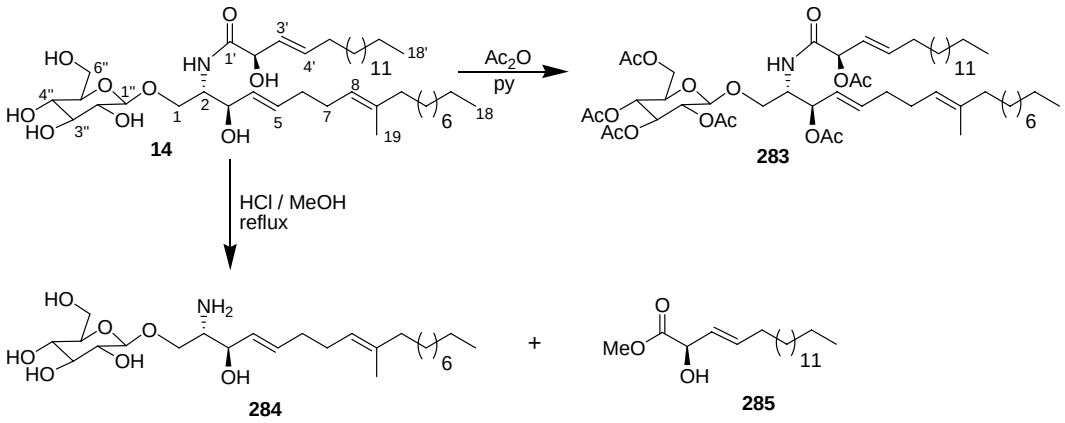
Figure 2.6. Structure of cerebroside C (**14**). The cycled part is a sphingoid base moiety.



Scheme 2.2. Fragmentation pattern of cerebroside C (**14**) in ESI-MS/MS.

The lengths of the fatty acid and sphingoid base were established by ESI-MS/MS analysis and chemical degradation. The ESI-MS/MS spectrum of compound **14** was obtained by using the $[M + Na]^+$ pseudomolecular ion at *m/z* 776 as the parent ion. The fragmentation pattern is summarized in Scheme 2.2. The spectrum displayed two intense peaks at *m/z* 496 $[M + Na – 280]^+$, and 346 $[M + Na – 430]^+$, and several other peaks such as *m/z* 758 $[M + Na – 18]^+$, 614 $[M + Na – 162]^+$, 596 $[M + Na – 180]^+$ and 334 $[M + Na – 442]^+$. The peak at *m/z* 496 originated from the loss of C_{18} 2-hydroxyl fatty acid group (Scheme 2.2., a). The peak at *m/z* 346 originated from, first, the loss of the sugar moiety to give fragment *m/z* 596 (Scheme 2.2., c) followed by the loss of the C_{17} chain of sphingoid base fragment (*m/z* 596) by breaking the C-2 –N bond (Scheme 2.2.,

d, Costantino et al. 2007). The peak at *m/z* 614 was accounted for by loss of the sugar moiety (Scheme 2.2., b), which can also give the peak at *m/z* 346 (Scheme 2.2., e). The peak at *m/z* 334 can derive from either peaks of *m/z* 614 or *m/z* 496 (Scheme 2.2., f and g, respectively). Based on the information from the ESI-MS/MS fragmentation and molecular formula, it was concluded that this sphingolipid **14** contained three parts: a C-18 fatty acid component, a C-19 sphingoid base and a glucopyranose residue.



Scheme 2.3. Methanolysis and acetylation of cerebroside C (**14**).

The chemical degradation of compound **14** was carried out using methanol in 1N HCl (reflux, Scheme 2.3). After workup a methyl fatty acid ester **285** was isolated together with another minor saturated fatty acid ester **286**. HRMS-EI suggested the molecular formula of **285** to be $C_{19}H_{36}O_3$ and its optical rotation was $[\alpha]_D$= - 43 (c, 0.1) (lit. $[\alpha]_D$=-42, (c, 0.50, $CHCl_3$), Takanami et al., 2005). Therefore the fatty acid chain was determined to be (2*R*,3*E*)-2-hydroxy-3-octadecenoic acyl moiety. Because of the limited amount of material, the sphingoid residue **284** was not recovered from the aqueous layer during the workup. However, the structural detail of sphingoid base was further determined from the analysis of the 1D and 2D NMR of compound **14** when the spectra were recorded in MeOD-d_4 at 47 ºC. After searching the literature it was found that a mixture of $CDCl_3$ and MeOD-d_4 is the proper solvent for recording NMR spectra of glycosphingolipids (Sitrin et al., 1988). This is probably because of the formation of micelles in less polar solvent, i.e. $CDCl_3$, the polar head of glucose is aggregated and

consequently the resolution of the NMR signals is decreased and the broadened signals overlap with the signals from the fatty chains (Sitrin et al., 1988). As a result the signals in the range δ_H 4.38 to 3.38 were much less informative. However, in the ^{1}H NMR (MeOD-d_4) of compound **14,** a broad triplet (δ_H 5.53, H-8) couple with a broad singlet (δ_H 2.04, H-7) and a singlet (δ_H 1.60, H_3-19) showed correlation with this sp^2 carbon (δ_C 125.0, C-8) indicating a methyl branched vinyl sphingoid chain. The position of the double bonds was located to be at C-4 and C-8 positions through HMBC and COSY 2D NMR spectroscopic data (Figure 2.7). The ^{13}C NMR spectroscopic data (Table 2.6) of compound **14** matches the reported data of cerebroside C (**14**) (Koga et al., 1998). Based on all the data, the sphingolipid **14** was determined to be cerebroside C (**14**) and the minor component was cerebroside D (**31**). These two compounds, cerebroside C (**14**) and cerebroside D (**31**) were isolated before from the rice blast fungus (*Magnaporthe grisea*) and proven to be general elicitors (Koga et al., 1998).

Table 2.6. ^{1}H NMR (MeOD-d_4, 500 MHz, 47 ºC) and ^{13}C NMR (MeOD-d_4, 125 MHz, 47 ºC) chemical shifts (ppm) and multiplicities (*J* in Hz) of cerebroside C (**14**).

C#.	Sugar		Fatty acid		Sphingoid base	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	104.9	4.28, 1H, d, (7.7)	175.6	-	69.8	4.09, 1H, m; 3.75, 1H, dd, (10.4, 3.6)
2	75.3	3.21, 1H, m	74.3	4.44, 1H, d, (6.0)	55.0	3.99, 1H, m
3	78.2	3.36, 1H, m	129.3	5.53, 1H, m	73.2	4.15, 1H, m
4	71.9	3.29, 1H, m	135.0	5.84, 1H, m	131.2	5.49, 1H, m
5	78.3	3.29, 1H, m	33.5	2.06, 2H, m	134.6	5.74, 1H, m
6	63.0	3.87, 1H, m; 3.68, 1H, m	30.3-30.9	1.39, 2H, m	33.9	2.06, 2H, m
7	-	-	30.3-30.9	1.29, 2H, m	28.9	2.06, 2H, m
8	-	-	30.3-30.9	1.29, 2H, m	125.0	5.15, 1H, m
9	-	-	30.3-30.9	1.29, 2H, m	137.0	-
10	-	-	30.3-30.9	1.29, 2H, m	40.9	1.98, 2H, t, (7.2)
11	-	-	30.3-30.9	1.29, 2H, m	29.3	1.39, 2H, m
12	-	-	30.3-30.9	1.29, 2H, m	30.3-30.9	1.29, 2H, m
13	-	-	30.3-30.9	1.29, 2H, m	30.3-30.9	1.29, 2H, m
14	-	-	30.3-30.9	1.29, 2H, m	30.3-30.9	1.29, 2H, m
15	-	-	30.3-30.9	1.29, 2H, m	30.3-30.9	1.29, 2H, m
16	-	-	33.1	1.29, 2H, m	30.3-30.9	1.29, 2H, m
17	-	-	23.8	1.29, 2H, m	30.3-30.9	1.29, 2H, m
18	-	-	14.4	0.90, 3H, t, (6.5)	33.1	1.29, 2H, m
19	-	-	-	-	23.8	1.29, 2H, m
20	-	-	-	-	14.4	0.90, 3H, t, (6.5)
21	-	-	-	-	16.3	1.60, 3H, s

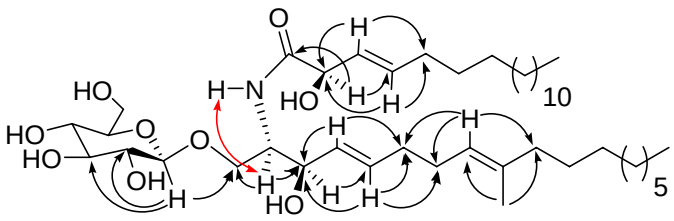
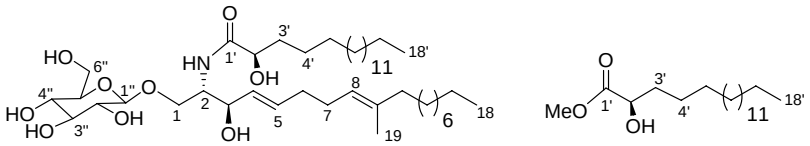


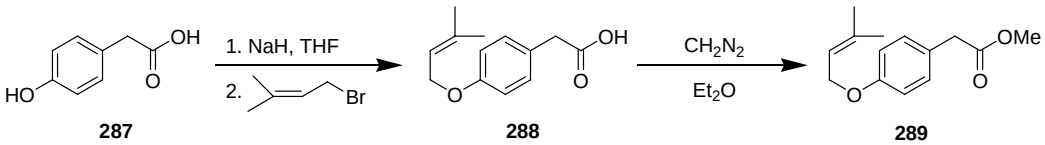
Figure 2.7. Selected HMBC (one arrow curves) and COSY (double arrow curves, N-H↔H-2) correlations of cerebroside C (**14**).



31 **286**

4-Prenyl-benzeneacetic acid (**288**)

The molecular formula of compound **288** ($C_{13}H_{16}O_3$) obtained by HRMS-EI indicated six degrees of unsaturation. A triplet (δ_H 5.49, 1H) coupled with a doublet (δ_H 4.47, 2H) plus two singlets (δ_H 1.79, 3H and 1.73, 3H) accounted for an O-prenyl group. Two doublets (δ_H 7.15, 2H and 6.84, 2H) accounted for a 1,4-substituted benzene ring, and a broad singlet (δ_H 3.54, 2H) for the methylene protons. Compound **288** and its methyl ester **289** were prepared from 4-hydroxyl-benzeneacetic acid **287** to confirm the proposed structure (Scheme 2.4). Compound **288** was isolated before from a mutant of *Aspergillus ochraceus* (Awad et al., 2005).

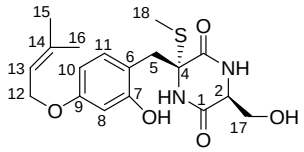


287 **288** **289**

Scheme 2.4. Preparation of methyl ester of compound **289** from 4-hydroxyl-benzeneacetic acid (**287**)

Metabolite (**290**)

The molecular formula of metabolite **290 (**$C_{18}H_{24}N_2O_5S$) obtained from HRMS-ESI indicated eight degrees of unsaturation and that it contained a sulfur atom. The structure of the metabolite was elucidated based on the ^1H, ^{13}C NMR, HMQC and HMBC spectra. A multiple (δ_H 5.38, 1H) coupled with a doublet (δ_H 4.40, 2H, J = 6.5 Hz) plus two singlets (δ_H 1.72, 3H and 1.67, 3H) accounted for an *O*-prenyl group. A doublet (δ_H 6.98, 1H, J = 8.4 Hz) coupled with a doublet of doublets (δ_H 6.31, 1H, J = 8.4, 2.4 Hz) which inturn coupled with a doublet (δ_H 6.34, 1H, J = 2.4 Hz) suggested a 1,2,4-trisubstituted benzene ring. Two doublets of doublets (δ_H 3.69, 1H, J = 10.9, 5.8 Hz, and δ_H 3.50, 1H, J = 10.8, 3.3 Hz) coupled to another doublet of doublets (δ_H 3.39, 1H, J = 5.8, 3.3 Hz) accounted for H_2-17 and H-2 of the dioxopiperazine ring. Two doublets of the methine protons (δ_H 3.27, 1H, J = 13.9 Hz and δ_H 2.97, 1H, J = 13.8 Hz) indicated C-4 of the dioxopiperazine ring was substitured. This substitution group was assigned to be a methylthio of which the methyl group was a singlet (δ_H 2.17, 3H) in ^1H NMR spectrum and displayed correlation with C-4 in HMBC spectrum. Two D_2O exchangible broad singlets (δ_H 8.28, 1H, 8.19, 1H) were assigned to the N-H of the dioxopiperazine ring. A D_2O exchangible broad singlet (δ_H 9.37, 1H) was assingned to the phenol proton, which is the extra substitution group compared with the phomamide phenyl ring. Based on the above analysis the structure of metabolite **290** was proposed. No further data were collected due to the limited amount of sample, therefore, the structure of metabolite **290** needs to be further confirmed.



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2.1.1.2 High NaCl

High NaCl concentration (0.17 M to 0.68 M) was used to induce stress in *L. maculans* in culture. In a preliminary experiment (Table 2.1, entries 2 and 3), a new peak (metabolite **293)** was observed at 2.4 min in the HPLC chromatogram of EtOAc extracts of broth and the production of sirodesmin PL was decreased about 30 % when NaCl (0.17 M) was added into the MM culture. When NaCl (0.17 M) was added into the 3-day-old MM culture, the production of sirodesmin PL was similar to that of control cultures. Therefore, a series of experiments with different concentrations of NaCl and different ages of cultures was conducted to maximize the production of the metabolite **293** and to inhibit the production of sirodesmin PL (Table 2.1, entries 4 to 8). The results are presented in Figures 2.8, 2.9 and 2.10. As the concentration of NaCl increased, the production of sirodesmin PL decreased (Figure 2.9). The earlier the NaCl was added, the lower was the production of sirodesmin PL (Figure 2.9). Therefore, the concentration of NaCl and the age of culture to which NaCl was added affected the production of sirodesmin PL. When NaCl (0.68 M) was added to 4-day-old cultures, the production of sirodesmin PL was inhibited completely (Figure 2.9), while the production of new metabolite **293** reached a maximum (Figure 2.10). Another interesting observation was that the mycelia turned green on the 7^{th} day under these conditions (Table 2.1, entry 7) and became black, eventually, on the 9^{th} day (Figure I. 1, page xxxii). A large scale culture (12 L) was prepared using these conditions (Table 2.1 entry 7) to isolate the new metabolite **293** from EtOAc extract of culture broth. The known blue pigment bulgarein (**294**) was isolated from mycelia extract (see experimental, section 4.2.1.2).

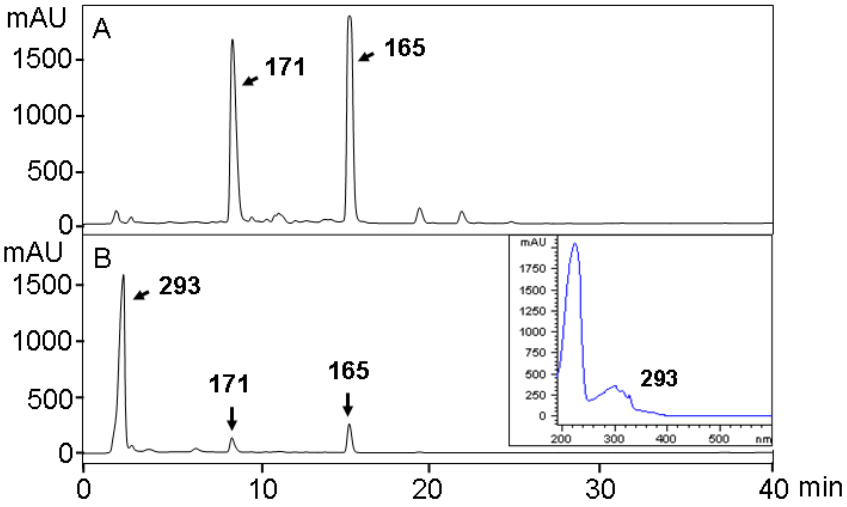


Figure 2.8. HPLC chromatograms of extracts of cultures of *Leptosphaeria maculans* incubated in MM for 7 days. A: Control; B: NaCl (0.68 M) added to 4-day-old cultures.

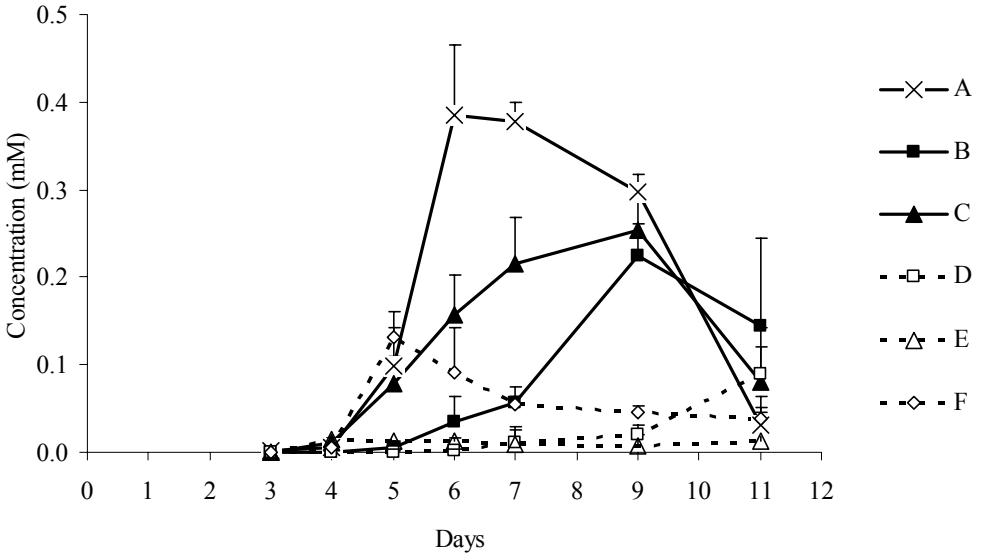


Figure 2.9. Production of sirodesmin PL (**165**) in MM containing high NaCl (0.34M and 0.68M). NaCl added to cultures with different incubation times (day). (A control; B, 0.34 M, 3 day; C, 0.34 M, 4 day; D, 0.68 M, 3 day; E, 0.68 M, 4 day ; F, 0.68 M, 5 day)

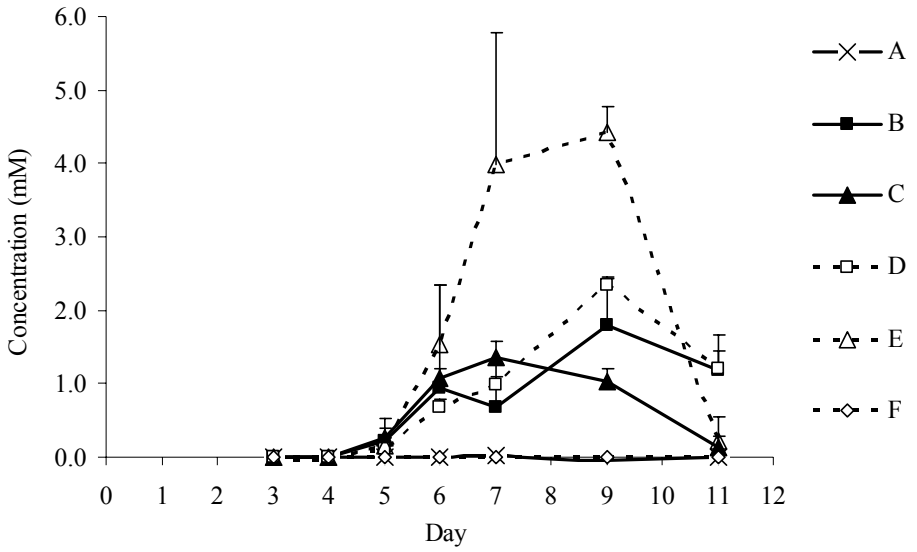
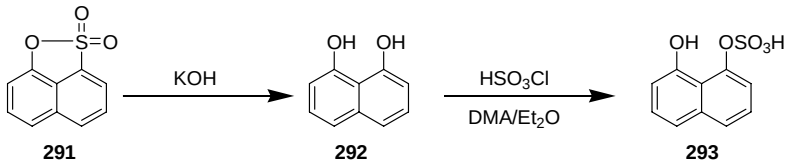


Figure 2.10. Production of metabolite **293** in MM containing high NaCl (0.34M and 0.68M). NaCl added to cultures with different incubation times (day). (A, control (no metabolite **293** was detected); B, 0.34 M, 3 day; C, 0.34 M, 4 day; D, 0.68 M, 3 day; E, 0.68 M, 4 day ; F, 0.68 M, 5 day)

8-Hydroxynaphthalene-1-sulfate (**293**)

HRMS-ESI ($[M-1]^-$) of metabolite **293** indicated the molecular formula $C_{10}H_7O_5S$. ^1H NMR spectra data (D_2O) showed all signals at low field range (δ_H 6.9 - 7.8) indicating a substituted aromatic system, e.g. naphthalene moiety. The spin systems were elucidated from ^1H COSY spectral data. Namely, two protons at δ_H 6.99 (d, J = 7.6 Hz, 1H) and δ_H 7.51 (d, J = 8.3 Hz, 1H) together with a proton at δ_H 7.42 (dd, J = 8.2, 7.6 Hz, 1H) accounted for one spin system. The other spin system showed overlapping resonances for two protons at δ_H 7.47 (d, J = 5.1 Hz, 2H) and another signal at δ_H 7.79 (dd, J = 4.9, 4.4 Hz, 1H), suggesting an unsymmetrical naphthyl nucleus. Altogether the spectroscopic data suggested that the new metabolite was 1,8-dihydroxylnaphthalene sulfate **293** , which was confirmed by synthesis using commercially available 1,8-naphthosultone (**291**) (Scheme 2.5) (Burkhardt and Lapworth, 1926; Ragot et al., 1999). Commercially available 1,8-naphthosultone was heated with KOH at 300 °C to yield compound **292** which was reacted with HSO_3Cl to yield metabolite **293** (Experimental).

Although **293** appeared to slowly hydrolyze to 1,8-dihydroxynaphthalene **292** on standing in aqueous solution, only a very small amount of **292** was detected in the EtOAc broth extracts (ca. 5% of **292** determined by HPLC).



Scheme 2.5. Synthesis of metabolite **293**

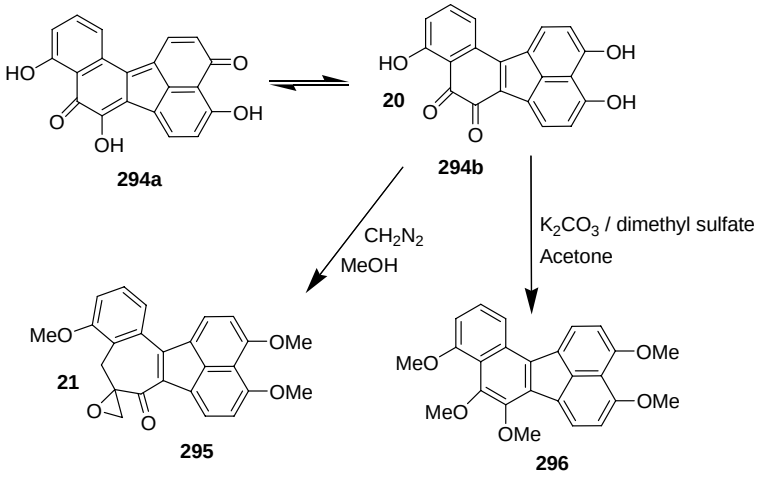
Bulgarein (**294**)

HRMS-ESI ($[M-1]^-$) of metabolite **294** indicated a molecular formulae $C_{20}H_9O_5$ i.e., an unsaturation number of sixteen. The ^1H NMR (DMSO-d_6) spectrum displayed ten protons, three of which were exchangeable (δ_H 13.48, bs, 2H and 12.66, bs, 1H). Of the remaining seven signals, three broad singlets at δ_H 7.57, 7.38 and 6.67 changed multiplicity when the ^1H NMR spectrum was recorded at ca. 58 °C; the broad singlets at δ_H 7.57 and 6.67 became broad doublets and the broad singlet at δ_H 7.38 became a broad triplet. The remaining signals at δ_H 8.27 (d, J = 9.0 Hz, 1H), 7.96 (d, J = 8.0 Hz, 1H), 6.59 (d, J = 8.0 Hz, 1H), and 6.40 (d, J = 9.0 Hz, 1H) indicated the structure to be aromatic. Furthermore, the ^{13}C NMR spectral data suggested that this aromatic system contained two carbonyls (δ_C 183.1, 2C), and three C-O bonds (δ_C 160.6, 3C).

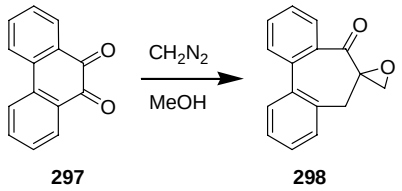
Treatment of this blue metabolite (**294a**/**294b**) with diazomethane (Scheme 2.6) gave a product that appeared to contain an oxymethylene, a methylene and three methoxyl groups, according to its ^1H NMR spectrum (δ 8.37, d, J = 7.9 Hz; 8.11, d, J = 8.1 Hz; 7.44, m; 7.03, m; 4.14, s; 4.11, s; 3.91, s; 3.49, bd J = 13.9 Hz; 3.44, bd J = 13.9 Hz; 3.05, bd J = 6.6 Hz; 2.90, bd J = 6.8 Hz). The HRMS spectrum confirmed the presence of ten additional protons and five carbons ($C_{25}H_{20}O_5$, i.e. unsaturation number of sixteen). Thus, it appeared that diazomethane treatment methylated three hydroxyl groups, and provided two additional methylenes. However, methylation with K_2CO_3 and dimethyl sulfate in acetone gave a reduced pentamethoxy derivative ($C_{25}H_{22}O_5$, i.e.

unsaturation number of fifteen) (Scheme 2.6). These spectroscopic data and chemical transformations are consistent with the structure of bulgarein (**294a**/**294b**), a fungal metabolite produced by *Bulgaria inquinans* whose structure was elucidated by Edwards and Lockett in 1976 for which ^{1}H and ^{13}C NMR spectroscopic data were not provided (Edwards and Lockett, 1976; Fujii et al., 1993).

A reaction similar to that observed between bulgarein (**294a**/**294b**) and diazomethane was reported for 9,10-phenanthrenedione (**297**), which also yielded a product resulting from double insertion of methylene (**298**, Scheme 2.7) (Eistert et al., 1968).



Scheme 2.6. Preparation of the derivatives **295** and **296** from metabolite **294**



Scheme 2.7. Preparation of the derivative **298** from compound **297** (Eistert et al., 1968).

2.1.2 Metabolites produced in potato dextrose media

PDB medium is not a chemically defined medium, it contains substantial amounts of unidentified components. Therefore, only major new metabolites produced after incubating spores of *L. maculans* were isolated and identified. No sirodesmins (**165-169**) were isolated from PDB media culture of *L. maculans* incubated at room temperature (Table 2.1, entry 9). Known metabolite **212** was produced as the major component (Pedras et al, 2007). However, no obvious new peaks were identified in this culture medium (Table 2.1, entry 9).

The temperature is an important factor that influences the production of metabolites by fungi. Time course experiments with variation of temperature were planned before the start of this research work. However, an accidental power failure resulting in temperature increase of the incubation room (ca. 27 ºC), led to the observation of several new peaks (maculansins) in the HPLC chromatograms of cultural extracts (Figure 2.11). The crude extracts of culture broths showed high toxicity to both brown mustard and canola. Therefore time course experiments with higher temperatures (Table 2.1, entries 10 to 12) were carried out to optimize the production of these new peaks. When PDB medium was incubated at higher temperature (27 ºC) for 4 days, maculansin A (**299**) was produced at higher concentration (Table 2.1, entry 11; Figure 2.12), while metabolite **212** was produced at maximum concentration seven days after incubation at 25 ºC (Table 2.1, entry 10; Figure 2.13). Therefore, five liter cultures of *L. maculans* were prepared by incubation of spores of *L. maculans* in PDB (27 ºC for 4 days; Table 2.1, entry 11). The broth was separated from mycelia by vacuum filtration and extracted with EtOAc. The EtOAc extract was submitted to various chromatographic methods to give maculansin A (**299**), B (**300**) and a mixture of analogues of maculansin A (**299**) (Section 4.2.2.).

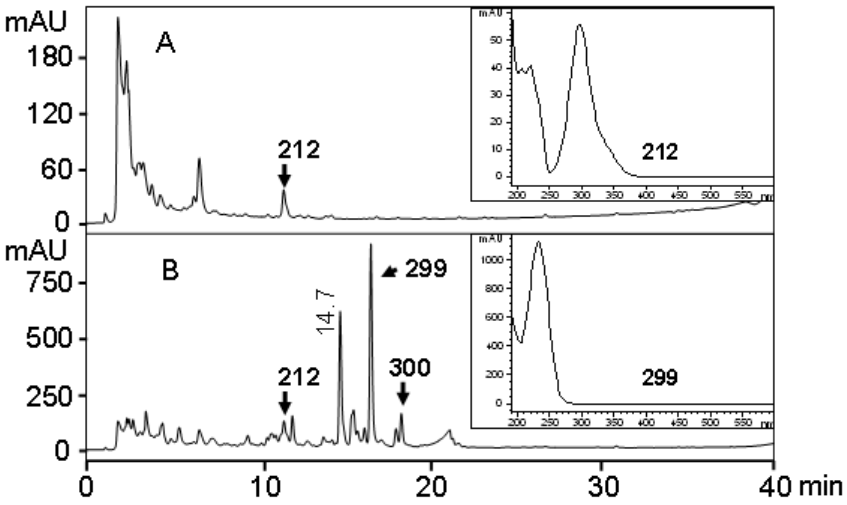
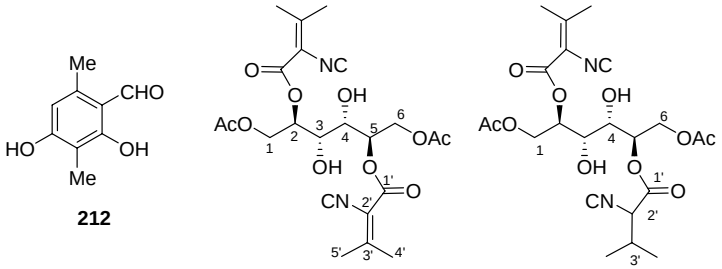


Figure 2.11. HPLC chromatograms of extracts of cultures of *Leptosphaeria maculans* grown in potato dextrose medium (PDB); A: EtOAc extract of culture incubated at 23 °C for four days; B: EtOAc extract of culture incubated at 27 °C for four days; numbered peaks identified as: 2,4-dihydroxy-3,6-dimethylbenzaldehyde (**212**), maculansin A (**299**), maculansin B (**300**), peak at 14.7 min identified as mixture of metabolites related to **299**. Inserts: UV/Vis spectra of metabolites **212** and **299**.



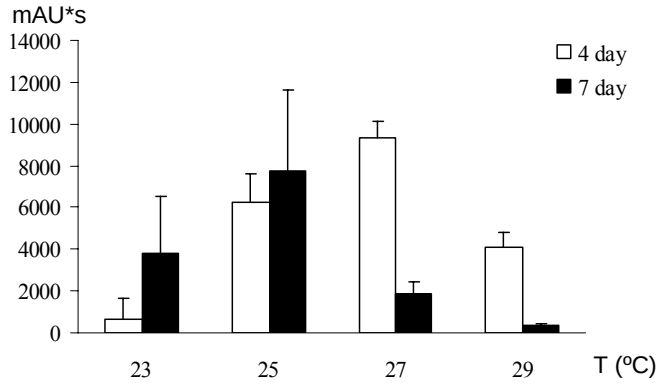


Figure 2.12. Production of maculansin A (**299**) at different temperatures.

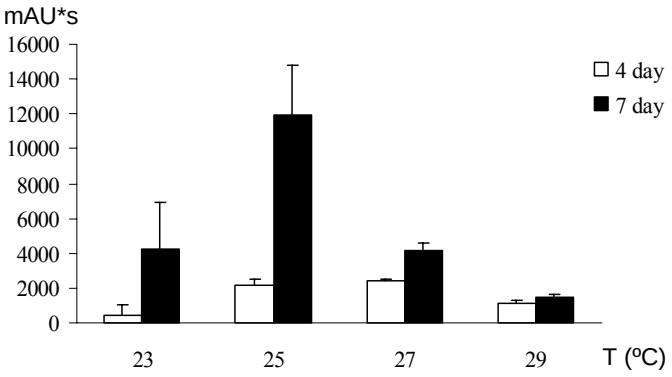
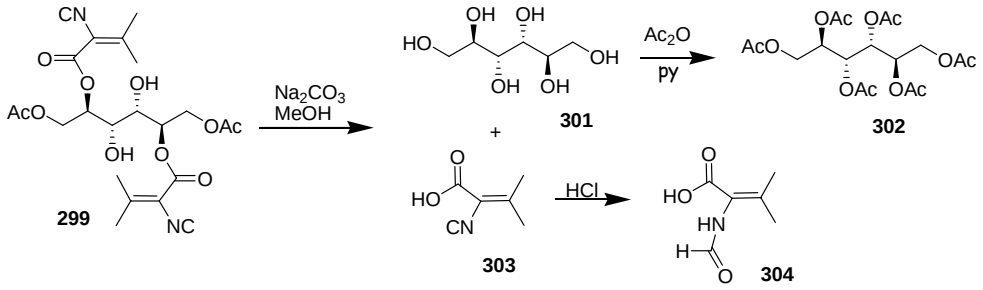


Figure 2.13. Production of metabolite **212** at different temperatures.

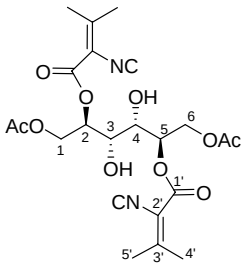
Maculansin A (**299**)

HRMS-EI of metabolite **299** suggested $C_{22}H_{28}N_2O_{10}$ (*m/z* 380.1749) as the molecular formula. In the ^{1}H NMR of metabolite **299** (Table 2.7) a methylene moiety (H-1) at δ_H 4.64 and 4.42 was attached to an acetyl group and coupled to a methine proton (H-2) at δ_H 5.13 (^{1}H COSY). A methine proton (H-3) at δ_H 3.89 (t, J = 8.2 Hz) was coupled to H-2 (δ_H 5.13, m) and an exchangeable proton (δ_H 3.00, d, J = 7.7 Hz) (COSY and D_2O exchange experiments). Three methyl groups at δ_H 2.11, 2.18 and 2.31 were attached to sp^2 carbons. These signals accounted for all 14 proton resonances in ^{1}H NMR. A total of 11 carbon signals were displayed in the ^{13}C NMR. Considering the molecular formula of this metabolite ($C_{22}H_{28}N_2O_{10}$, HRMS-EI), this data suggested that

299 possessed a symmetry element. The backbone of metabolite **299** was determined to be D-mannitol after hydrolysis and by comparison of the spectral data and $[\alpha]_D$ of an authentic sample with acetylation product **302** (Scheme 2.8). Two types of substituents were attached to the D-mannitol backbone. An acetyl group was assigned to -O-C-1 from an HMBC correlation between the carbonyl carbon at δ_C 171.6 and H_2-1 and the methyl group at δ_H 2.11. The second substituent was elucidated as follows. Both methyl groups at δ_H 2.31 and 2.18 showed similar correlation with an sp^2 carbon at δ_C 158.7 and 115.0 which were assigned to dimethyl allyl moiety. H-3 showed a correlation with one sp^2 carbon at δ_C 160.5. This carbon was assigned to the conjugated carbonyl carbon. The IR spectrum contained a characteristic absorption at 2116 cm^{-1}, which indicated the presence of isonitrile functional group. The isonitrile carbon which has no correlation with any protons was assigned to the peak at δ_C 168.0 (Karuso et al., 1989). Therefore a 2-isonitriledehydrovaline moiety was attached at position 3 of the D-mannitol **301** backbone. Therefore, the structure of new phytotoxin **299** was elucidated as 1,6-diacetyl-2,5-bis(2-isocyano-3-methyl-2-butenoate)-D-mannitol. Compound **303** was isolated from the basic hydrolysis (^{1}H NMR), however, it seemed to polymerize to yield a complex mixture.



Scheme 2.8. Hydrolysis and preparation of acetylated derivative of the sugar backbone of metabolite **299**.



299

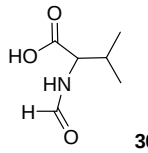
Table 2.7. ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) chemical shifts (ppm) and multiplicities (*J* in Hz) of maculansin A (**299**) (in $CDCl_3$).

	Maculansin A (**299**)	
C/H #	δ_C	δ_H
1, 6	62.3	4.64, 2H, dd, (12.5, 2.5)
		4.41, 2H, dd, (12.5, 4.2)
2, 5	72.8	5.13, 2H, m
3, 4	67.4	3.89, 2H, t, (8.2)
1′	160.5	---
2′	115.0	---
3′	158.3	---
4′/5′	21.4	2.31, 6H, s
5′/4′	25.1	2.18, 6H, s
6′	168.0	---
1″	171.6	---
2″	21.1	2.11, 6H, s
(O)H	---	3.00, 2H, d, (7.7)

Maculansin B (**300**) and related metabolites

From a less polar fraction, a mixture of several compounds having structural characteristics similar to those of **299** was obtained and further separated by preparative TLC, as described in the experimental, to afford components with R_t = 18.3 min (**300**)

and 14.7 min (Figure 2.11). Although HPLC analysis suggested a chromatographically homogeneous material, preliminary inspection of the ^{1}H NMR spectral data of the component with R_t = 18.3 min (**300**) indicated that this sample contained a mixture of at least two compounds (complex spectrum showing a polyol backbone with two sets of signals for some protons). In addition, comparison of this spectrum with that of **299** indicated that two doublets at δ_H 1.14 and 1.04 coupled to a methine proton at δ_H 2.38 in addition to the two methyl groups at δ_H 2.3 and 2.2 observed in **299**. That is, one of the 2-isocyano-3-methyl-2-butenoyl fragments attached to the polyol was replaced with a 2-isocyano-3-methylbutanoyl moiety, whereas the acetyl groups and the backbone remained intact. This conclusion was consistent with the $[M+1]^+$ *m/z* 483 obtained for this sample (LC-MS-ESI). As well, the polyol backbone of **300** was determined to be D-mannitol after hydrolysis and acetylation of a mixture of **299** and **300**, as reported for maculansin A (**299**). Therefore, the inseparable mixture **300** contained two epimers due to the acidity of H-2' and is proposed to be 1,6-diacetyl-2-(2-isocyano-3-methyl-2-butenoyl)-5-(2-isocyano-3-methylbutanoyl)-D-mannitol and named maculansin B (**300**).



305

From the same fraction containing maculansin A (**299**), an inseparable mixture was isolated from a more polar fraction. The presence of doublet methyl groups at δ_H 1.1 and 1.0, a multiplet at δ_H 2.4 (1H), and absence of two methyl groups at δ_H 2.3 and 2.2 as in maculansin A (**299**) indicated that the two 2-isocyano-3-methyl-2-butenoyl moieties of maculansin A (**299**) were reduced to 2-isocyano-3-methylbutanoyl moieties. Upon basic hydrolysis of this mixture, N-formylvaline (**305)** was obtained from organic extracts, and D-mannitol hexaacetate (**302**) was obtained by acetylation of the residue of the aqueous layer of basic hydrolysis. Compound **305**, which was obtained from basic hydrolysis of this mixture of analogues of maculansin A (**299**), was stable enough to be isolated and identified from spectroscopic data. (Gloer et al., 1988). Due to the acidic

nature of the proton at the β position of the 2-isocyano-3-methylbutanoyl moiety, four diastereomers are possible and inseparable, which results in a complex ^{1}H NMR spectrum (Gloer et al., 1988). Therefore, this sample contained a mixture of structural isomers likely due to the presence of two 2-isocyano-3-methylbutanoyl substituents and absence of an acetyl substituent. Because of the complex ^{1}H NMR spectrum, the position of these moieties could not be assigned, therefore structures are not proposed.

2.1.3 Production of metabolites under other stress conditions

Additional stress conditions were used to test the production of new metabolites that may be potential elicitors or phytotoxins. First, a preliminary experiment in using culture incubation at higher temperature was conducted at 29°C (Table 2.1, entry 13). Under this condition the production of sirodesmin PL (**165**) reached a maximum at around the fourth day, three days earlier than standard conditions, and became barely detectable after seven days. However, no new peaks were detected. Therefore, no further experiments were carried out by varying incubation temperatures when *L. maculans* was grown in MM.

Second, the amount of $MgSO_4 \cdot 7H_2O$ was adjusted to 1/10 (2.0×10^{-4} M), half (1.0×10^{-3} M) and two times (4.0×10^{-3} M) that in standard MM (Table 2.1, entries 14, 15 and 16 respectively). As shown in Figure 2.14, the maximum production of sirodesmin PL (**165**) is related to the amount of sulfur. When $MgSO_4 \cdot 7H_2O$ was 1/10 and 1/2 of the standard amount the production of sirodesmin PL (**165**) decreased close to 29 fold and two fold, respectively, compared to the control; about 1.4 fold increase of the production of sirodesmin PL was observed when twice the amount of $MgSO_4 \cdot 7H_2O$ was supplied to the MM culture. However, no new peaks were detected.

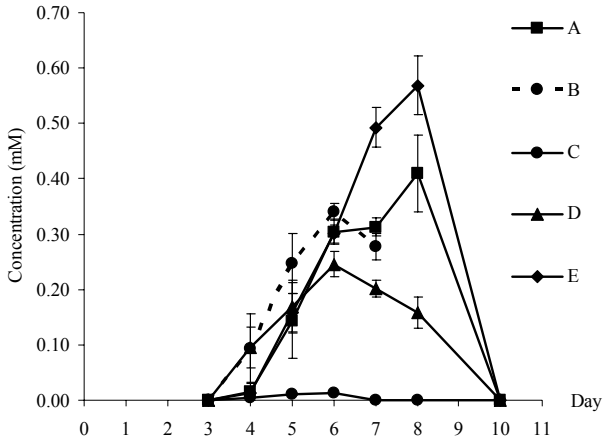


Figure 2.14. Production of sirodesmin PL (**165**) in different MM. A, control; B, removal of thiamine but with adding leaves of brown mustard; C, $MgSO_4 \cdot 7H_2O$ (2.0×10^{-4} M); D, $MgSO_4 \cdot 7H_2O$ (1.0×10^{-3} M); E, $MgSO_4 \cdot 7H_2O$ (4.0×10^{-3} M).

Third, the role of thiamine in the production of sirodesmin PL by *L. maculans* in MM was tested. When thiamine was removed from the medium, no sirodesmin PL was detected (Table 2.1, entry 17). When thiamine was removed from the medium but with adding leaves of brown mustard (Table 2.1, entry 18), the production of sirodesmin PL (**165**) was close to that in control samples (Figure 2.14). This is most likely because the leaves contained thiamine. Metabolite **212**, which cannot be detected in MM in standard culture conditions, was detected at the 5th, 6th and 7th day of incubation when leaves were added into the MM culture. This may indicate that some components from leaves can induce the production of compound **212** by *L. maculans*.

Fourth, phytoalexins were added into the culture of *L. maculans* in MM to mimic the stress conditions caused by plants. Spirobrassinin (**122**) or camalexin (**306**) (5×10^{-4} M) were added to three-day-old MM cultures (Table 2.1, entries 19 and 20). A slight decrease in the production of sirodesmin PL (**165**) was observed (Figure 2.15), but no new peaks were detected by HPLC. Based on previous results no higher concentrations of phytoalexins were used because the growth of the fungus was inhibited at higher concentration of phytoalexins (Pedras and Taylor, 1993).

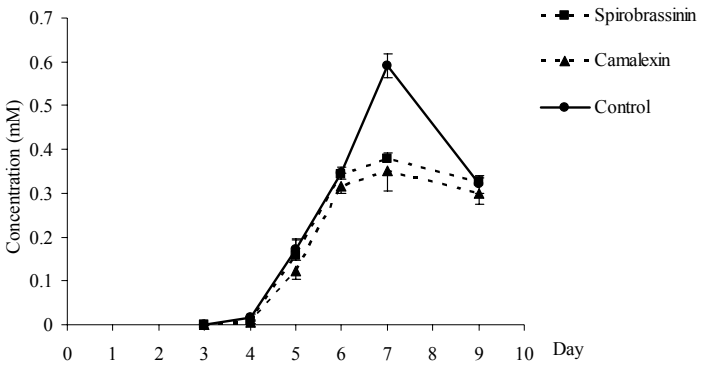


Figure 2.15. Production of sirodesmin PL (**165**) in MM in presence of the phytoalexins camalexin (**306**) and spirobrassinin (**122**).

Fifth, phytoalexins were also added into the culture of *L. maculans* in PDB media (Table 2.1, entries 21 and 22). The production of metabolite **212** was slightly increased when spirobrassinin (**122**) was present, while in the presence of camalexin (**306**) the production of metabolite **212** was increased about two-fold after seven days of incubation (Figure 2. 16). However, no new peaks were detected.

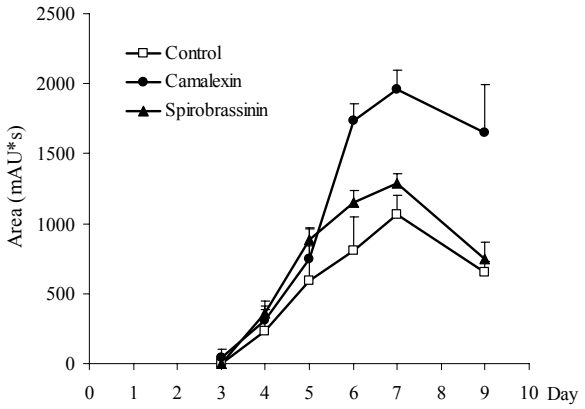
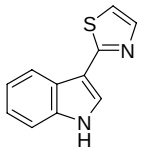
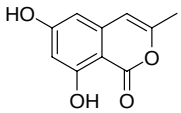


Figure 2.16. Production of metabolite **212** in PDB media in presence of phytoalexins camalexin (**306**) and spirobrassinin (**122**).



306



307

At last, V_8 juice was modified and used as a medium to culture *L. maculans* (Table 2.1, entry 23; experimental 4.2.3). Trace amount of sirodesmin PL was detected. A peak at 10.8 minutes was detected to be 6,8-dihydroxy-3-methylisocoumarin (**307**) based on UV and retention time of HPLC-DAD data. The production of total metabolites by *L. maculans* in this medium was very low (ca. 30 mg/ L). Therefore, no further study was conducted with this stress condition.

2.2 BIOLOGICAL ACTIVITY

2.2.1 Elicitor activity

One of the standard methods to determine the elicitor activity of a metabolite is to test if the metabolite is able to induce the production of phytoalexins in plants (Hahn, 1996; Keen, 1975; Montesano et al., 2003). Specific elicitors produced by *L. maculans* are expected to induce the production of phytoalexins in brown mustard (*B. juncea* cv. Cutlass, resistant to *L. maculans*) but not in canola (*B. napus* cv. Westar, susceptible to *L. maculans*). General elicitors produced by *L. maculans* are expected to induce the production of phytoalexins in both resistant and susceptible plants. Elicitor activity assays were carried out as described in the experimental section. Leaves of whole plants were treated with a fraction or a purified metabolite in a solution of MeOH/H_2O (1:1 v/v). After incubating plants for two days, the leaves were excised, extracted and the less-polar fractions of the extracts were analyzed by HPLC-DAD. The results are summarized in Table 2.8. Sirodesmin PL (**165**) and deacetylsirodesmin PL (**166**) (at concentrations of 1.0 or 0.5 mM) induced the production of phytoalexins such as brassilexin (**118**), cyclobrassinin (**119**), rutalexin (**127**) and spirobrassinin (**122**) in

brown mustard and spirobrassinin (**122**) in canola **(**Figures 2.17 and 2.18**)**. Therefore, sirodesmin PL (**165**) and deacetylsirodesmin PL (**166**) are general elicitors. A mixture of cerebrosides C (**14**) and D (**31**) (at concentrations of 1.0 or 0.8 mM) induced the production of the phytoalexin rutalexin (**127**) only in brown mustard but not in canola **(**Figure 2.19**)**. Therefore cerebrosides C (**14**) and D (**31**) displayed selective elicitor activity.

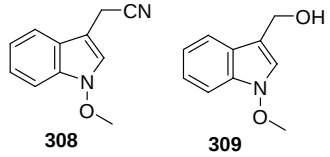
Table 2.8. Bioassay results of metabolites isolated from cultures of *Leptosphaeria maculans*

Compounds (mM)	Phytotoxin activity (damage index)			Elicitor activity[a]	
	Brown mustard	White mustard	Canola	Brown mustard	Canola
299 (1.0)	4.8±0.5	4.3±1.5	4.6±1.1	N. E.[c]	N. E.
299 (0.2)	2.8±0.8	1.2±0.6	0.3±0.4	N. E.	N. E.
299 (0.1)	2.4±0.7	0	0	N. E.	N. E.
299 (0.02)	1.0±0.5	-	0	N. E.	N. E.
300 (1.0)	Necrosis	-	Necrosis	N. E.	N. E.
14/**31** (1.0)	0	-	0	**127**	N. E.
165 (1.0)	4.7±0.5	3.7±0.8	2.1±0.6	**127**, **122**	**122**
165 (0.4)	Necrosis	-	-	**118**, **119, 127**, **122**	-
165 (0.2)	4.1±0.3[b]	4.3±0.7[b]	2.2±0.5[b]	**122**	**122**
166 (0.1)	Necrosis	-	Necrosis	**122**	**122**
166 (0.01)	0	-	0	N. E.	N. E.
167 (0.5)	Necrosis	-	Necrosis	N. E.	N. E.
4 & **169** (1.0)	Necrosis	-	Necrosis	N. E.	N. E.
171(1.1)	0	-	0	N. E.	N. E.
171(0.9)	0	-	-	10.2 min	-
272 (1.0)	0	-	0	N. E.	11.0 min
274 (1.0)	0	-	0	N. E.	N/E
268 (1.0)	0	-	0	N. E.	11.0 min
267 (1.1)	0	-	0	N. E.	N. E.
293 (0.1)	0	-	0	N. E.	N. E.
294 (0.6)	0	-	0	N. E.	N. E.
212 (6.0)	0	0	0	-	-
212 (0.1)	0	-	0	N. E.	N. E.
288 (0.6)	0	0	0	-	-

[a]Elicitor activity was determined by induction of the production of phytoalexins. [b]Data were collected when plants were incubated for two days. [c] N. E. = no effect.

Cerebrosides C (**14**) and D (**31**) were found previously to be produced by a wide range of phytopathogens and appeared to induce the production of phytoalexins in rice plants as well as disease resistance to the rice blast fungus (Umemura et al., 2004). The only phytoalexin induced by cerebrosides C (**14**) and D (**31**) was rutalexin (**127**) (Figure 2.17). The phytoalexin induced by sirodesmin PL and deacetylsirodesmin PL at all the concentrations and replicates is spirobrassinin (**122**) (Figures 2.18 and 2.19) although a few other phytoalexins were detected occasionally when plants were treated with sirodesmin PL or deacetylsirodesmin PL.

Metabolites **169**, **168**, **167**, **267**, **268**, **272**, **274**, **293**, **294**, **212**, **299**, **300**, and **171** did not show elicitor activity at the concentrations tested (Table 2.8).



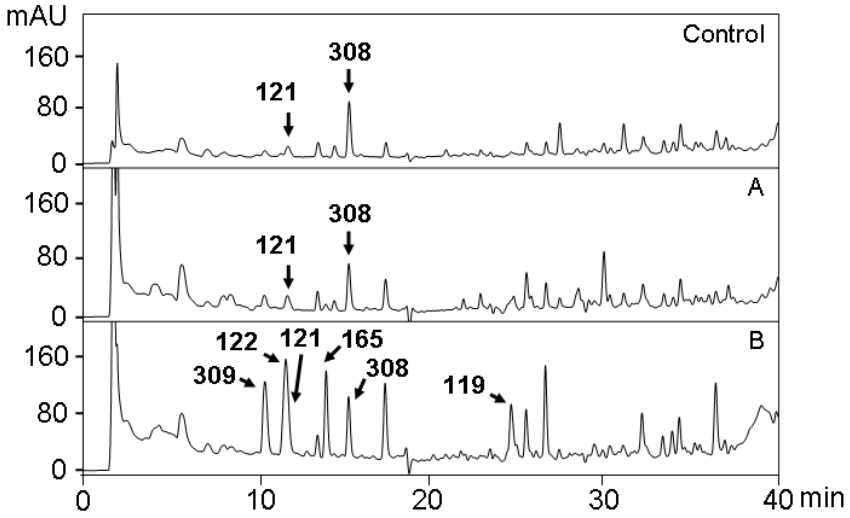


Figure 2.17. HPLC chromatograms of extracts of leaves of brown mustard (*B. juncea* cv. Cutlass resistant to *L. maculans*) treated with toxins. A, maculansin A (**299**) and B, sirodesmin PL (**165**). Numbered peaks identified as: indole-3-acetonitrile (**122**), 1-methoxy-indole-3-acetonitrile (**308**), 1-methoxy-indole-3-methanol (**309**), spirobrassinin (**122**), and cyclobrassinin (**119**).

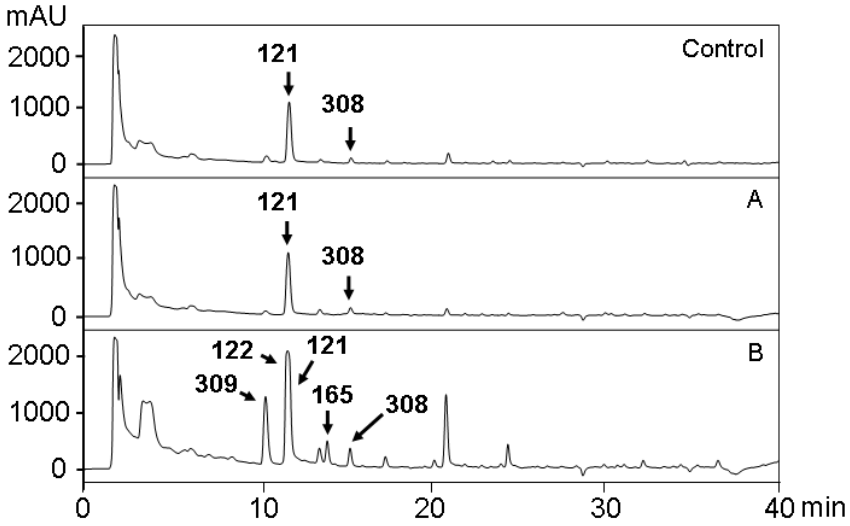


Figure 2.18. HPLC chromatograms of extracts of leaves of canola (*B. napus* cv. Westar, susceptible to *L. maculans*) treated with toxins. A, maculansin A (**299**); B, sirodesmin PL (**165**). Numbered peaks identified as: indole-3-acetonitrile (**121**), 1-methoxy-indole-3-acetonitrile (**308**), 1-methoxy-indole-3-methanol (**309**), and spirobrassinin (**122**).

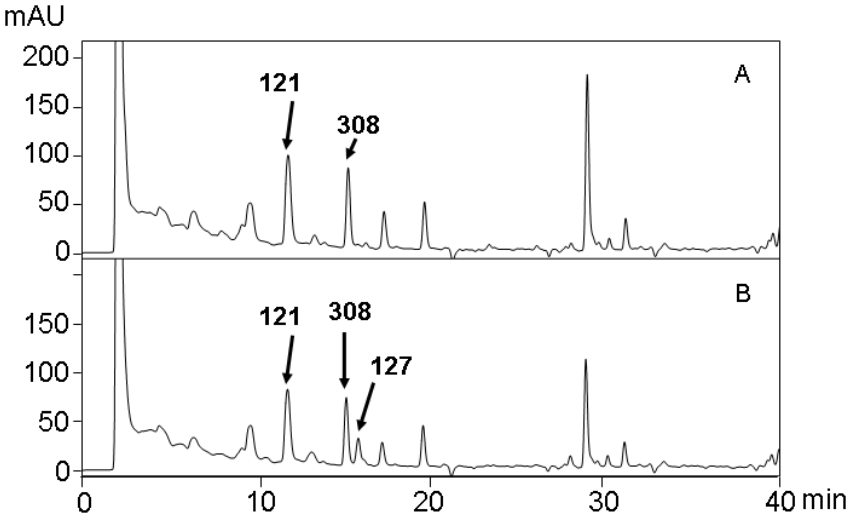


Figure 2.19. HPLC chromatograms of extracts of leaves of brown mustard (*B. juncea* cv. Cutlass, resistant to *Leptosphaeria maculans*) treated with a mixture of cerebrosides C (**14**) and D (**31**). A control; B, treated with a mixture of cerebroside C (**14**) and D (**31**). Numbered peaks identified as: indole-3-acetonitrile (**121**), 1-methoxy-indole-3-acetonitrile (**308**), and rutalexin (**127**).

2.2.2 Phytotoxicity and growth inhibition

Phytotoxicity assays were carried out on resistant (*B. juncea* cv. Cutlass and *S. alba* cv. Ochre) and susceptible (*B. napus* cv. Westar) plants. The phytotoxicity of metabolites isolated from *L. maculans* to each plant species is summarized in Table 2.8. Sirodesmins (**165** to **169**) caused necrosis and chlorosis on leaves of both resistant (*B. juncea* cv. Cutlass and *S. alba* cv. Ochre) and susceptible (*B. napus* cv. Westar) plants, as previously reported (Pedras et al., 1990).

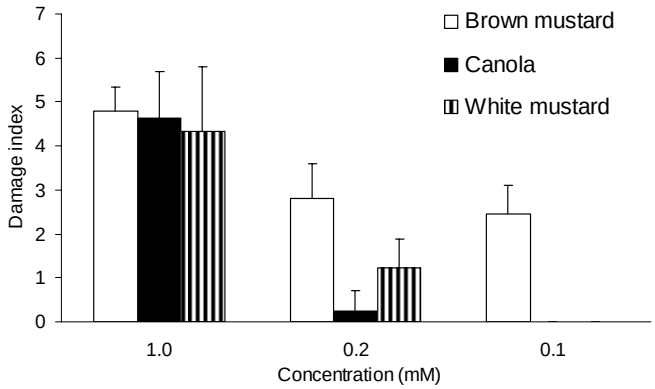


Figure 2.20. Phytotoxicity of maculansin A (**299**) to brown mustard (*B. juncea* cv. Cutlass), canola (*B. napus* cv. Westar) and white mustard (*S. alba* cv. Ochre).

The damage on the leaves was rated using a damage index number (the number is proportional to the size of the lesion, see experimental section). Although maculansin A (**299**) did not elicit the production of phytoalexins in *B. juncea* cv. Cutlass or *B. napus* cv. Westar, it causes leaf damage even at 2×10^{-5} M concentration on *B. juncea* cv. Cutlass (damage index 1, Figures I.2, I.3, and I.4, page xxxii and xxxiii). At high concentration (1×10^{-3} M) maculansin A **(299**) caused similar damage to *B. juncea* cv. Cutlass (resistant to *L. maculans*) and *B. napus* cv. Westar (susceptible to *L. maculans*) and *S. alba* cv. Ochre (resistant to *L. maculans*), about 4.5 damage index (Figure 2.20). At lower concentration maculansin A (**299**) (2×10^{-4} M) caused smaller lesions on *B. napus* cv. Westar than on *B. juncea* cv. Cutlass (damage index close to 3). At a concentration of 1×10^{-4} M, maculansin A (**299**) still caused lesions (damage index 2.2)

on *B. juncea* cv. Cutlass but no lesions on *B. napus* cv. Westar or *S. alba* cv. Ochre (Figure 2.20). That is, maculansin A (**299**) is more toxic to resistant plants, the reverse of the effect caused by *L. maculans*.

The lesions caused by maculansin A (**299**) and sirodesmin PL (**165**) on *B. juncea* cv. Cutlass and *S. alba* cv. Ochre were similar at 1×10^{-3} M, but the lesions caused by sirodesmin PL (**165**) on *B. napus* cv. Westar was about half the size of those caused by maculansin A (**299**) at the same concentration (Figure 2.21). Maculansin A (**299**) causes larger lesions on the susceptible plants than sirodesmin PL (**165**). Moreover, the irregular lesions caused by maculansin A (**299**) are similar to those caused by the pathogen and the toxin depsilairdin (**183**) (Pedras et al., 2004), suggesting that it is able to diffuse in the leaf tissue. On the other hand, the lesion caused by sirodesmin PL (**165**) is a circle, the size of each drop indicating immediate lysis of leaf cells.

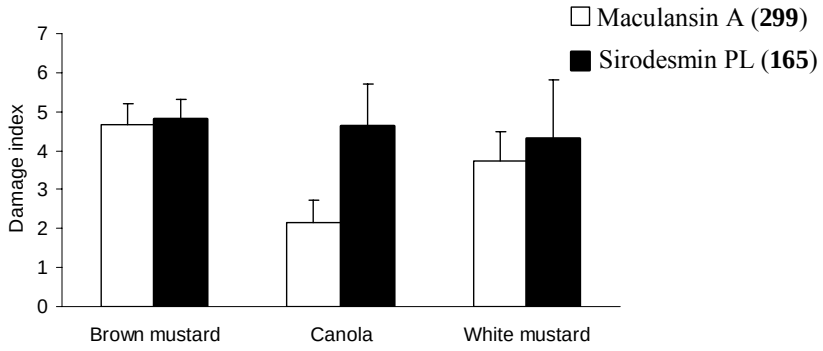


Figure 2.21. Damage caused by phytotoxins maculansin A (**299**) and sirodesmin PL (**165**) (1.0 mM) on brown mustard (*B. juncea* cv. Cutlass), canola (*B. napus* cv. Westar) and white mustard (*S. alba* cv. Ochre).

Metabolite **212** has been isolated from *Valsa ambiens* and several other fungi (Ayer et al., 1993; Mitova et al., 2006) and showed root and hypocotyl growth inhibition effect on lettuce (Jiao et al., 1995). Therefore, the root growth inhibition effect of metabolite **212** was tested on host plants. The seeds of *B. juncea* cv. Cutlass and *B. napus* cv. Westar were incubated in PDB media in the presence of metabolite **212** (5×10^{-4} M). The inhibition effect is shown in Figure 2.22. The root length of canola treated

with metabolite **212** measured 0.3 ± 0.1 cm, while control roots measured 1.9 ± 0.7 cm. Similarly, the root length of brown mustard treated with metabolite **212** measured 0.2 ± 0.1 cm and 4.9 ± 1.2 cm in control plants. Metabolite **212** showed about 6 fold and 25 fold inhibition effects on canola and brown mustard roots, respectively. This is the first time that the root growth inhibition effect of a metabolite (**212)** of *L. maculans* has been reported. Metabolite **212** was not detected in the infected plants *in vivo*; however, metabolite **212** was produced as the major component in PDB media and in lower amounts in MM.

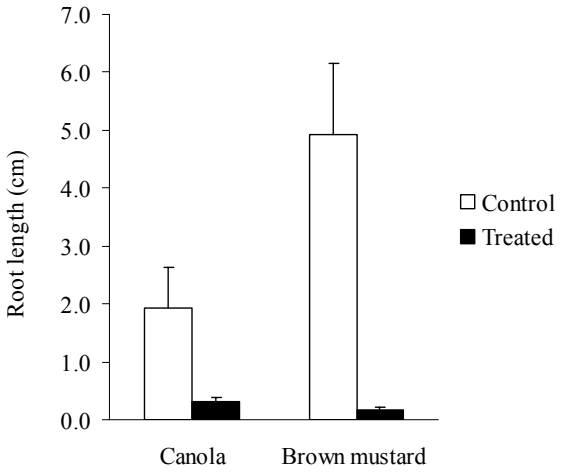


Figure 2.22. Root length of seedlings of canola and brown mustard treated with metabolite **212** (5×10^{-4} M).

2.3 BIOSYNTHESIS OF SIRODESMIN PL (**165**)

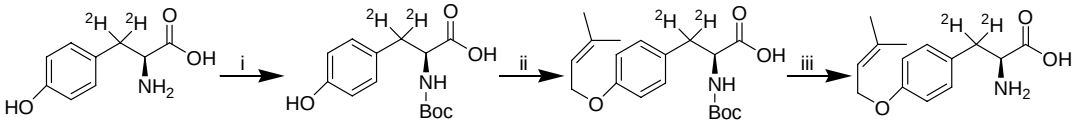
As mentioned in the Introduction, phomamide was the only intermediate identified in the biosynthetic pathway of sirodesmins (**165**) (Férézou et al., 1980b), while phomalirazine was a proposed intermediate (Pedras et al., 1989). The isolation of leptomaculins (**272** and **274**) triggered an interest to find out if they were substrates used by *L. maculans* enzymes for the introduction of sulfur to give sirodesmin PL (**165**). If the dideuterated precursor **251a** was incorporated intact into sirodesmin PL (**165**), then leptomaculins (**272** and **274**) could not be the intermediates used for the introduction of

sulfur into sirodesmin PL (**165**). Therefore, [3,3-$^2H_2$]-(L)-tyrosine and [5,5-$^2H_2$]phomamide were chosen as useful substrates to clarify this question. Another question to answer is at what stage does the prenylation happen, after or before the formation of the dioxopiperazine **252**. If (*E*)-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-(L)-tyrosine was incorporated into sirodesmin PL (**165**) intact, prenylation happens before the formation of the dioxopiperazine. Alternatively, both could be alternative biosynthetic pathways.

Therefore, the study of sirodesmin PL (**165**) biosynthesis was carried out by incubation of deuterated potential precursors with a wild type isolate (BJ125) of *L. maculans*. The potential precursors included commercially available amino acids [3,3-$^2H_2$]-(L)-tyrosine and [2,3,3-$^2H_3$]-(L)-serine as well as [3,3-$^2H_2$]*O*-prenyl-(L)-tyrosine, (*E*)-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-(L)-tyrosine, [5,5-$^2H_2$]cyclo-(L)-tyr-(L)-ser, and [5,5-$^2H_2$]phomamide, which were synthesized as described below (also see experimental section). The incorporation or nonincorporation of deuterium was detected by using ^1H NMR, ^{13}C NMR, LC-HRMS-ESI and HRMS-EI.

2.3.1 Synthesis of potential biosynthetic precursors

Deuterium labeled potential precursors [3,3-$^2H_2$]*O*-prenyl-(L)-tyrosine (**312a**), (*E*)-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-(L)-tyrosine (**312b**), [5,5-$^2H_2$]cyclo-(L)-tyr-(L)-ser (**319**) and [5,5-$^2H_2$]phomamide (**171a**) were synthesized using modifications of reported methods.

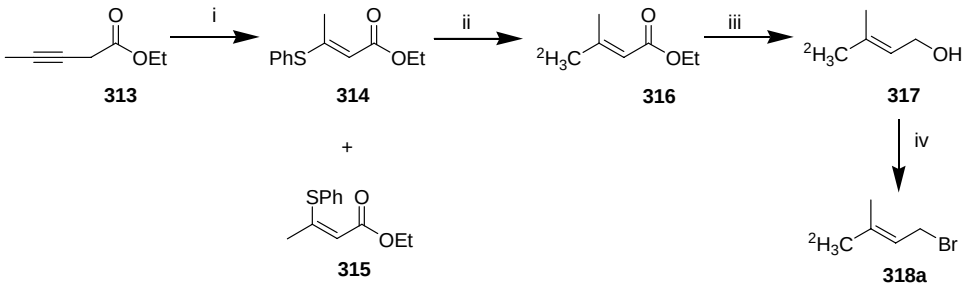


251a **310a** **311a** **312a**

Scheme 2.9. Synthesis of [3,3-$^2H_2$]*O*-prenyl-(L)-tyrosine (**312a**). Reagents and conditions: (i) t-Boc-anhydride/NaOH, dioxane, quantitative; (ii) a) NaH/THF, b) **318**, rt, 70%; (iii) 155 ºC, 60%.

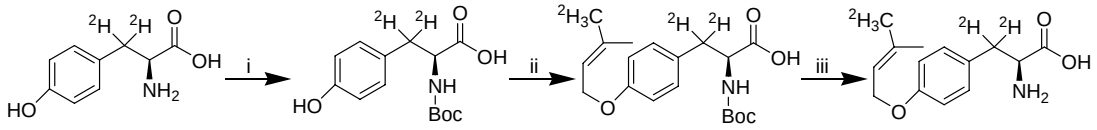
Compound **311a** was prepared by prenylation of *t*-Boc protected tyrosine **310a** with prenyl bromide (Fraile et al., 1996); **311a** was heated at 155 ºC for one hour to give **312a** 42% total yield (Scheme 2.9).

Compound **318a** was not commercially available, therefore its preparation followed reported methods with minor modification (Scheme 2.10, Thulasiram et al., 2006). Benzenethiol was treated with NaOH in EtOH followed by addition of ethyl 2-butynoate to give a mixture of E/Z isomers **314** (68%) and **315 (17%)**. The E isomer **314** was readily separated from Z isomer by FCC. **314** was treated with CuI in THF first, then deuterated methyl Grignard reagent was added to the cuprate of **314** at -15 ºC to afford **316** in 80% yield (Christie et al., 1981). Although both reported methods gave similar yield, the method used in this study (Christie et al., 1981) had two advantages: first, the deuterated Grignard reagent was used in lower amount than the method reported by Thulasiram et al. (2006) and second, the reaction temperature was carried out at -15 ºC instead of -70 ºC. After reduction of **316** with LAH, then bromination with PBr_3, (*E*)-[4,4,4-$^2H_3$]3-methyl-2-butenyl bromide (**318**) was yielded as an oil (yield 35 % in four steps, Scheme 2.10).



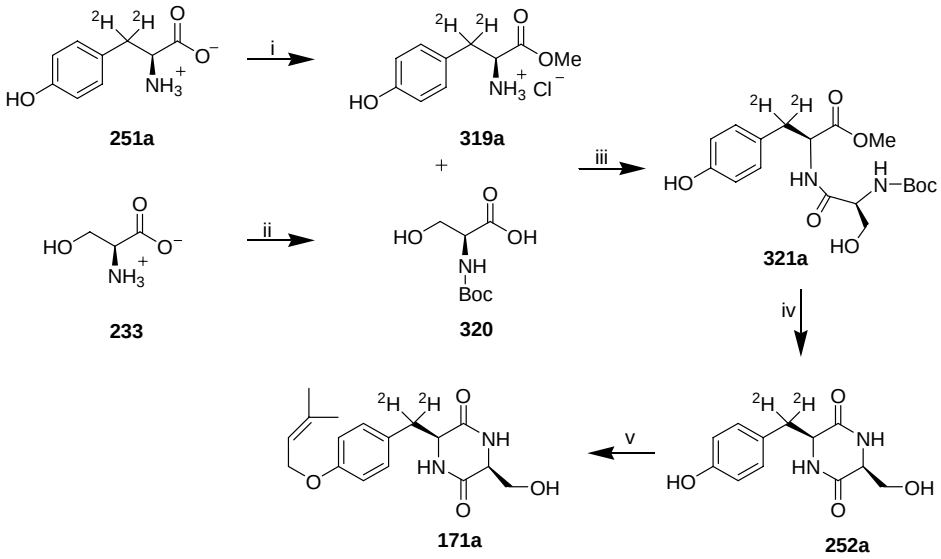
Scheme 2.10. Synthesis of (*E*)-[4,4,4-$^2H_3$]-prenylbromide (**318a**). Reagents and conditions: (i) PhSH/NaOH, EtOH, rt, **314**, 68%; **315**, 17%; (ii) CuI/THF, CD_3MgI/Et_2O, -15 ºC, 80%; (iii) LAH, Et_2O, 80%; (iv) PBr_3, Et_2O, 80%.

Compound **312b** was prepared similarly to **312a** by coupling of (*E*)-[4,4,4-$^2H_3$]3-methyl-2-butenol (**318a**) with [3,3-$^2H_2$]-(L)-tyrosine (**310a**) (Scheme 2.11).



251a **310a** **311b** **312b**

Scheme 2.11. Synthesis of (*E*)-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-(L)-tyrosine (**312b**). Reagents and conditions: (i) t-Boc-anhydride/NaOH, dioxane, quantitative; (ii) a) NaH/THF b) **318a**, rt, 70%; (iii) 155 ºC, 60%.



251a **319a** **233** **320** **321a** **171a** **252a**

Scheme 2.12. Synthesis of [5,5-$^2H_2$]cyclo-(L)-tyr-(L)-ser (**252a**) and [5,5-$^2H_2$]phomamide (**171a**). Reagents and conditions: (i) $SOCl_2$, MeOH, reflux, quantitative; (ii) t-Boc-anhydride/NaOH, dioxane, rt, quantitative; (iii) TEA / EDCI / CH_2Cl_2 -15°C, 96%; (iv) a) formic acid, rt. 60 min, b) *sec*-butyl-alcohol / toluene, 65°C, 90%; (v) a) NaOH, b) prenyl bromide, 67%.

Compounds **252a** and **171a** were prepared following a reported procedure (Scheme 2.12, Férézou et al., 1980a). Compound **321a** was obtained in 96% yield by coupling *t*-Boc-(L)-serine with methyl ester tyrosine using EDCI. Compound **321a** readily cyclized under heating in *sec*-butyl-alcohol/toluene after removal of the protecting group using formic acid to yield a white precipitate (**252a**). After lyophilization of NaOH treated **252a**, the residue was prenylated to afford **171a** in 67%

yield. Racemization during the synthesis was ruled out by comparison of the specific optical rotations of the synthetic and naturally occurring phomamide (Férézou et al., 1980a).

2.3.2 Incorporation of potential precursors

Deuterium labeled potential precursors were added to three-day-old cultures of *L. maculans* and cultures were further incubated for two days. Experiments were carried out in triplicates, control experiments were carried out using the corresponding natural abundance (non-deuterium containing) potential precursors. The broth was filtered off and extracted with EtOAc. The EtOAc extracts were separated by PTLC to give sirodesmin PL (**165**) and phomamide (**171**). Incorporation of deuterium into sirodesmin PL (**165**) and phomamide (**171**) was detected using ^{1}H NMR, ^{13}C NMR, HRMS-EI and LC-HRMS-ESI. Results are summarized in Table 2.9.

Table 2.9. Incorporation[a,b,c] of deuterated compounds into phomamide (**171**) and sirodesmin PL (**165**) in cultures of *Leptosphaeria maculans*.

Entry	Deuterated compound	Phomamide (**171**) % of ^{2}H (analytical method)	***Sirodesmin PL (165)* % of ^{2}H (analytical method)**
1	**251a**	32±3 (HRMS-EI: monodeuterated = 20; dideuterated = 12)[a] 30±5 (^{1}H NMR: monodeuterated = 19; dideuterated = 11, H_2-5a,5b)	32±2 (HRMS-EI monodeuterated = 19; dideuterated = 13) [a] 30±2 (^{1}H NMR, H-7)[b]
2	**312a**	41±7 (HRMS-EI: monodeuterated = 24; dideuterated = 17) [a] 39±1 (^{1}H NMR: monodeuterated = 21; dideuterated = 18, H_2-5a,5b)	35±7 (HRMS-EI monodeuterated = 21; dideuterated = 15) [a] 32±3 (^{1}H NMR, H-7)[b]
3	**233d**	3±1 (HRMS-EI)[a]	4±2 (HRMS-EI) [a]
4	**171a**	-	30±10 (HRMS-EI monodeuterated = 4; dideuterated = 26) [a] 27 (^{1}H NMR, H-7)[b]
5	**251a**	3±1 (HRMS-EI: monodeuterated = 1; dideuterated = 2) [a]	2±0 (HRMS-EI: monodeuterated = 0; dideuterated = 2) [a]
6	**312b**	23 (HRMS-ESI: tetradeuterated = 16; pentadeuterated = 7) [c] 26 (^{1}H NMR, ^{2}H-5 = 19; $^2H_2$-5 = 7)	13 (HRMS-ESI tetradeuterated = 9; pentadeuterated = 4) [c] 23 (^{1}H NMR, H-7)[b]

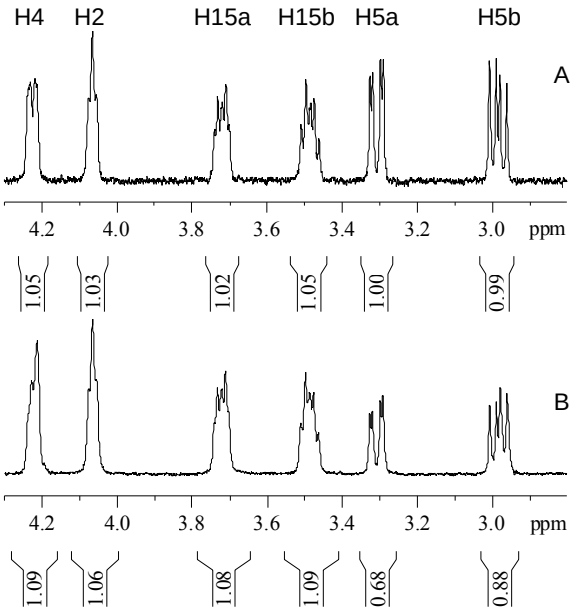
[a.] The percentage of incorporation (I) was determined by HRMS-EI and was calculated using the formula: $I = \{([M+n]^+ - [M+n]^+_{Ctl}) / ([M]^+ + [M+1]^+ + [M+2]^+)\} \times 100$, (n = 1, 2);

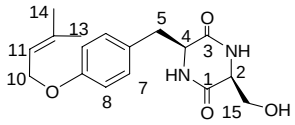
$[M+n]^+$ = intensity of deuterated molecular ion; $[M+n]^+_{Ctl}$ = intensity of molecular ion of control samples (natural abundance).

b. Resolution of signals was enhanced through a Lorentz-Gaussian line-shape transformation (gb = 0.3, lb = -1).

c. The percentage of incorporation (I) was determined by HRMS-ESI (positive mode) and was calculated using the formula: $I = \{([M+1+n]^+ - [M+1+n]^+_{Ctl}) / ([M+1]^+ + [M+2]^+ + [M+5]^+ + [M+6]^+)\} \times 100$, (n = 4, 5); $[M+n]^+$ = intensity of deuterated molecular ion; $[M+n]^+_{Ctl}$ = intensity of molecular ion of control samples (natural abundance).

Initial analysis of HRMS-EI data established that (i) [3,3-$^2H_2$]-(L)-tyrosine (**251a**), [3,3-$^2H_2$]*O*-prenyl-(L)-tyrosine (**312a**), (*E*)-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-(L)-tyrosine (**312b**), and [5,5-$^2H_2$]phomamide (**171a**) were incorporated efficiently (> 10%) into sirodesmin PL (**165**); (ii) [3,3-$^2H_2$]-(L)-tyrosine (**251a**), [3,3-$^2H_2$]*O*-prenyl-(L)-tyrosine (**312a**), and (*E*)-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-(L)-tyrosine (**312b**) were incorporated efficiently (>20%) into phomamide (**171**); (iii) [2,3,3-$^2H_3$]-(L)-serine (**233d**) and [5,5-$^2H_2$]cyclo-(L)-tyr-(L)-ser (**252a**) were incorporated poorly (<5%) into phomamide (**171**) and sirodesmin PL (**165**).





171

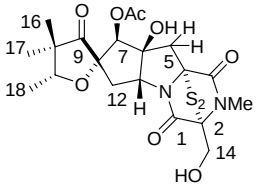
Figure 2.23. Sections of the ^{1}H NMR spectrum of phomamide (**171**). A, control, B, fed with [3,3-$^{2}H_2$]-(*L*)-tyrosine (**251a**).

In the ^{1}H NMR spectrum of phomamide (Table 2.10) two methylene protons (H_2-5) were well separated at δ_H 3.30 (H-5a, dd, J = 14.0, 3.5 Hz) and δ_H 3.00 (H-5b, dd, J = 14.0, 8.9 Hz), so the integration of H-5a and H-5b could be obtained. The resonances of H-5a and H-5b of phomamide were integrated as ca. 0.7 and 0.9, respectively, when [3,3-$^{2}H_2$]-(*L*)-tyrosine (**251a**) was used as the feeding material, whereas control samples showed the integration of ca. 1 as expected (Figure 2.23). Therefore, there was incorporation of deuterium and a higher deuterium percentage at H-5a (30%) than at H-5b (10%). The observation of unequal distribution of deuterium indicated the partial exchange of deuterium during the incorporation of [3,3-$^{2}H_2$]-(*L*)-tyrosine (**251a**) into phomamide. This result was consistent with the result obtained from the HRMS-EI data of phomamide isolated from cultures fed with [3,3-$^{2}H_2$]-(*L*)-tyrosine (**251a**). The $[M+1]^+$ and $[M+2]^+$ ions were ca 20% and 12% higher than those of control samples, respectively, indicating that ca 20% of phomamide was monodeuterated and ca 12% was dideuterated (calculation see note in Table 2.9). That is the result calculated from HRMS-EI suggested that 32% of phomamide was biosynthesized from incorporation of [3,3-$^{2}H_2$]-(*L*)-tyrosine (**251a**), which was very close to the result calculated from ^{1}H

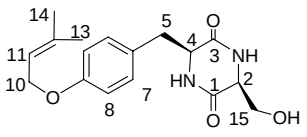
NMR using deuterium enrichment at H-5a (30%). Therefore, the deuterium enrichment at H-5a represents both mono- and di- deuterated phomamide. Furthermore, the percentage of deuterium enrichment at H-5b (10%) was almost equal to the percentage of dideuterated phomamide calculated from the HRMS-EI (12%), which indicates that the deuterium enrichment at H-5b represents the dideuterated phomamide. Since both HRMS-EI and ^{1}H NMR gave consistent results, it is clear that the partial deuterium exchange happened selectively at H-5b position. Similar results were obtained in the experiments when [3,3-$^{2}H_2$]*O*-prenyl-(L)-tyrosine (**312a**) was fed to the cultures of *L. maculans* (Table 2.9).

Table 2.10. ^{13}C NMR ($CDCl_3$, 125MHz) and ^{1}H NMR ($CDCl_3$, 500MHz) spectral data of phomamide (**171**). Chemical shifts (ppm) and multiplicities (*J* in Hz)

H #	^{13}C NMR	^{1}H NMR
1	166.3	
2	56.3	4.06, 1H, m
3	167.5	
4	56.3	4.22, 1H, m
5	39.5	a. 3.30, 1H, dd, (14.0, 3.5)
		b. 2.99, 1H, dd, (14.0, 8.9)
6	127.0	
7	130.9 (2C)	7.13, 2H, d, (8.4)
8	115.5 (2C)	6.90, 2H, d, (8.4)
9	158.6	
10	65.1	4.51, 2H, d, (6.6)
11	119.7	5.49, 1H, t, (6.6)
12	138.7	
13	18.5	1.76, 3H, s
14	26.1	1.81, 3H, s
15	64.1	a. 3.72, 1H, m
		b. 3.48, 1H, m
NH		6.14, 1H, s; 5.83, 1H, s
OH		2.30, 1H, bs



165



171

Table 2.11. ^{13}C NMR ($CDCl_3$, 125MHz) and ^{1}H NMR ($CDCl_3$, 500MHz) spectral data of sirodesmin PL (**165**). Chemical shifts (ppm) and multiplicities (*J* in Hz)

H #	^{13}C NMR	^{1}H NMR
1	163.1	
2	77.3	
3	165.3	
4	75.5	
5	43.8	3.27, 2H, brABq (17.1)
6	82.4	
7	79.2	5.56, 1H, s
8	89.7	
9	223.5	
10	47.8	
11	80.3	3.95, 1H, q, (6.2)
12	33.9	1.75, 1H, dd, (14.2, 8.2)
		2.77, 1H, dd, (14.2, 8.8)
13	67.3	4.34, 1H, m,
14	61.1	4.35, 1H, m,
		4.31, 1H, m,
15	27.6	3.16, 3H, s
16	20.6	1.04, 3H, s
17	17.5	1.12, 3H, s
18	14.4	1.27, 3H, d, (6.2)
19	170.1	
20	20.7	2.11, 3H, s
OH-6		4.20, 1H, s
OH-14		3.39, 1H, dd, (9.0, 6.4)

The deuterium enrichment in sirodesmin PL (**165**) from feeding experiments was also analyzed using HRMS-EI, ^{1}H NMR and ^{13}C NMR spectral data. In the ^{1}H NMR spectrum (Table 2.11) of sirodesmin PL (**165**) the resonance at δ_H 3.27 is H_2-5, where the deuterium is expected to be incorporated. This resonance integrated for ca. 2 in the natural abundance samples of sirodesmin PL (**165**) and for < 2 (1.4-1.6) in samples resulting from incorporation of [3,3-$^{2}H_2$]-(L)-tyrosine. However, the quantification of mono and di deuterated sirodesmin PL cannot be obtained from the integration of H_2-5 because these two methylene protons appeared as a broad AB quartet. Unexpectedly, a shoulder peak of proton H-7 (δ_H 5.56), that was not present in control samples (Figure 2.24), was displayed in the samples of purified sirodesmin PL (**165**) from feeding experiments using [3,3-$^{2}H_2$]-(L)-tyrosine (**312a**). Integration of these two signals using resolution enhancement (Lorentz-Gaussian line-shape transformations and baseline corrections) resulted in two well separated singlets at δ_H 5.556 and δ_H 5.550 in a 2:1 ratio (Figure 2.24B). The upfielded singlet (H-7, δ_H 5.560) was caused by the replacement of protons H-5 with deuterium (this statement is discussed in next paragraph). Therefore, about 33% of sirodesmin PL (**165**) had deuterium at C-5 position, which was consistent with the HRMS-EI results of sirodesmin PL purified from incorporation of [3,3-$^{2}H_2$]-(L)-tyrosine (**251a**). The intensity of $[M]^+$ of sirodesmin PL was very weak, so the base peak $[M\text{-}S_2]^+$ was used to calculate the percentage of deuterium incorporation. The intensity of the $[M\text{-}S_2\text{+}1]^+$ and $[M\text{-}S_2\text{+}2]^+$ ions were about 19% and 13% higher than those of control samples respectively, suggesting both monodeuterated (19%) and dideuterated (13%) sirodesmin PL were present in the sirodesmin PL isolated from the cultures incubated with [3,3-$^{2}H_2$]-(L)-tyrosine (**251a**). Therefore, the total amount of deuterated sirodesmin PL was about 32% calculated from HRMS-EI.

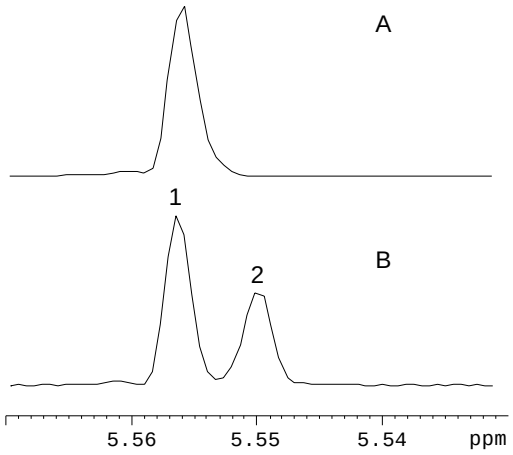


Figure 2.24. Sections of the ^{1}H NMR spectrum of sirodesmin PL (**165**). A, H-7 signal of natural abundance; B, ^{1}H NMR, H-7 signal (peaks 1 at δ 5.556 and 2 at δ 5.550) of **165** due to partial deuteration at C-5.

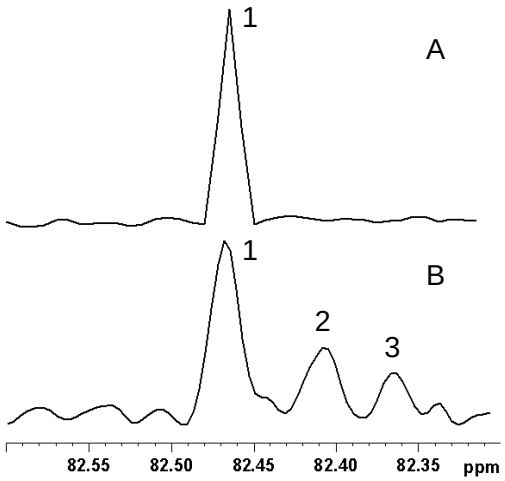


Figure 2.25. Sections of the ^{13}C NMR spectrum of sirodesmin PL (**165**). A, control, peak 1: C-6 signal of natural abundance; B, peak 2: C-6 signal of **165b**, and peak 3: C-6 signal of **165b** due to partial deuteration at C-5.

When considering the isotope effect of ^{2}H-5 on H-7, one would doubt that it is possible to observe the isotope effect when two nuclei are four bonds away in a nonconjugated system. But it is still possible to observe the isotope effect where two nuclei are five bonds away but close enough in space, termed "intrinsic steric isotope

effect (ISIE)" (Anet and Dekmezian, 1979). When the proton decoupled ^{13}C NMR of sirodesmin PL isolated from feeding [3,3-$^{2}H_2$]-(L)-tyrosine was inspected, the isotope effect of two nuclei through two bonds (β-isotope effect) was observed in both C-4 (δ_C 75.46, 75.40, 75.32) and C-6 (δ_C 82.44, 82.38, 82.34, Figure 2.25), which again indicated two types of deuterium containing sirodesmin PL (mono- and di- deuterated) were present in the sirodesmin PL samples because of the presence of the additional two upfield peaks (δ_C 75.40, 75.32 of C-4 and δ_C 82.38, 82.34 of C-6). The α-isotope effect (two nuclei through one bond) was not observed at C-5 because the splitting of C-5 is expected due to spin-spin coupling between ^{13}C-5 and ^{2}H-5, i.e. $^{13}\underline{C}(^{2}HH)$: triplet, 1:1:1 intensity, and $^{13}\underline{C}(^{2}H_2)$ quintet, 1:2:3:2:1 intensity. This splitting reduced the intensity of each peak and consequently the peaks were buried in the background noise. No isotope effect on C-7 was observed from ^{2}H-5, because the isotope effect decreases as the number of bonds increases. The above results and analysis fit in the theory of isotope effect through bonds (Schneider, 2007). Now considering the isotope effect through space, the direct distance between deuterium $^{2}H_2$-5 to H-7 was calculated to be 2.47 Å (H-5a - H-7) and 3.59 Å (H-5b - H-7) based on Molecular Mechanics Calculations (Spartan 06 software package, method MMFF94). It would be expected that only the deuterated molecules having the deuterium close enough (< 3 Å) can show intrinsic steric deuterium isotope effects (Anet and Dekmezian, 1979). Therefore the peak at δ_H 5.550 was the result of the deuterium isotope effect caused by the deuterium located at the H-5a position (*pro* S hydrogen) through space but not H-5b (*pro* R hydrogen) (Figure 2.26). This conclusion gives a basis to analyze the deuterium incorporation pattern from [3,3-$^{2}H_2$]-(L)-tyrosine (**251a**) into sirodesmin PL (**165**).

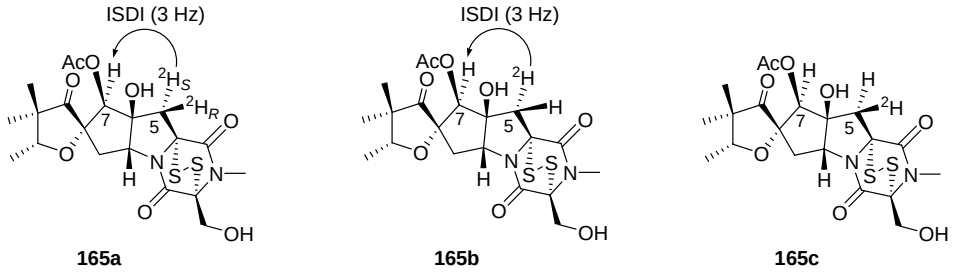
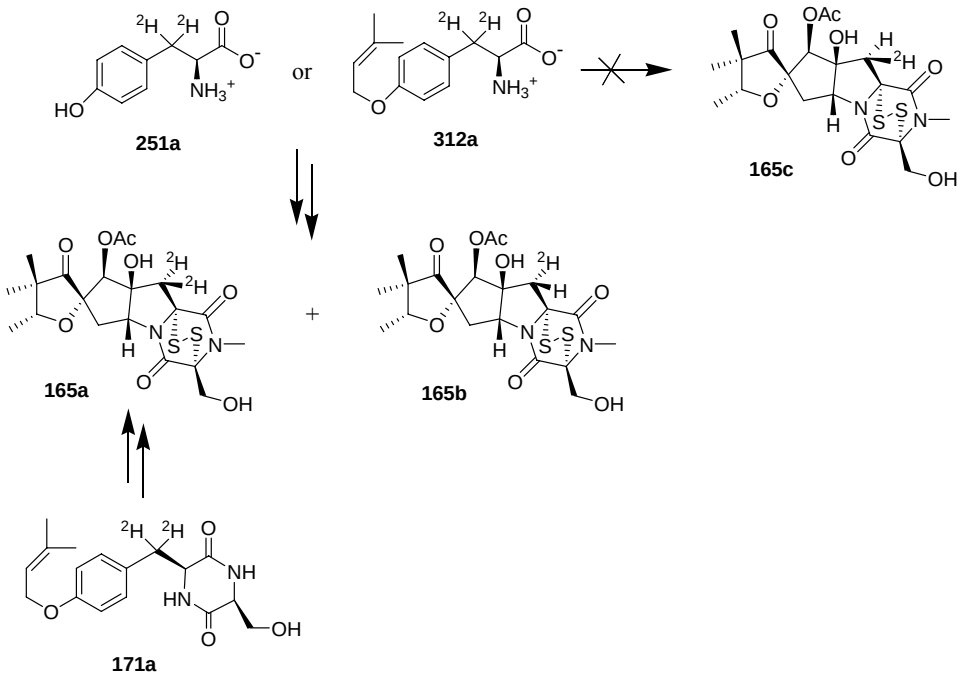


Figure 2.26. Intrinsic steric deuterium isotope (ISDI) effect detected in sirodesmin PL (**165**). ISDI of $^{2}H_{S}$-5 on the ^{1}H NMR chemical shift of H-7 detected in **165a** and **165b** was ca. 3 Hz. (No ISDI was detected in **165c**).

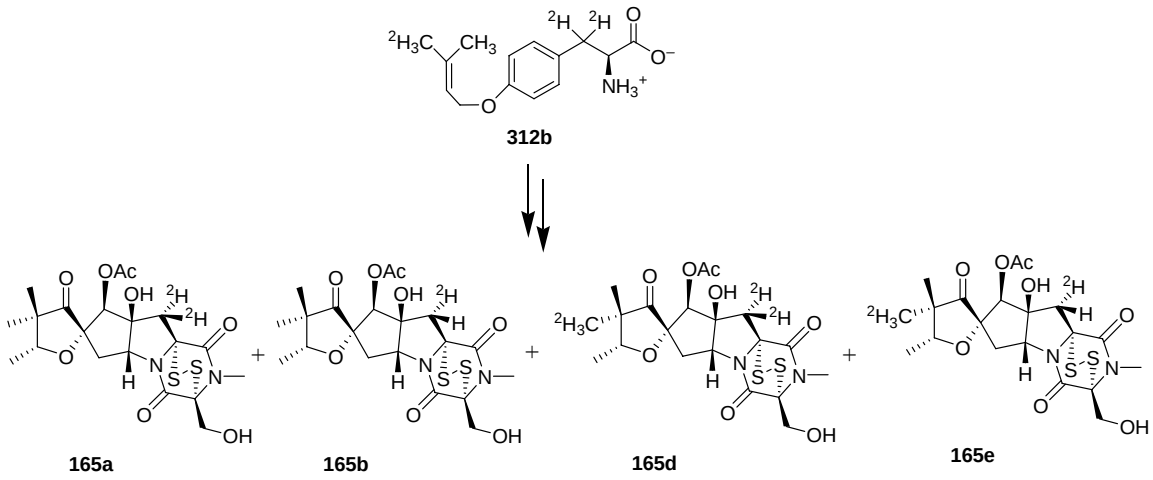
It was concluded from HRMS-EI results of sirodesmin PL resulting from incorporation of [3,3-$^{2}H_{2}$]-(L)-tyrosine (**251a**) that 13% was dideuterated and 19% was monodeuterated. However, it is impossible to distinguish where the deuterium is located in the monodeuterated sirodesmin PL, i.e., only at *pro* R, only at *pro* S or randomly distributed at both positions from HRMS-EI data. The total enrichment of deuterium is about the same whether calculated from the HRMS-EI data (ca. 32%) or calculated from the isotope effect at H-7 shown in the ^{1}H NMR spectrum (ca. 33%.). Dideuterated sirodesmin PL contributed only 13% to the isotope effect seen on H-7. Therefore, the remaining 20% (33%-13%) isotope effect on H-7 has to come from the deuterium located at the *pro* S position. This number is about the same as monodeuterated sirodesmin PL determined from HRMS-EI (19%), which means all the monodeuterated sirodesmin PL has the deuterium located at *pro* S position. Therefore, a stereo-specific β-deuterium exchange happened during the incorporation of [3,3-$^{2}H_{2}$]-(L)-tyrosine (**251a**) into sirodesmin PL (**165**, Scheme 2.13). The *pro* R deuterium was preferentially exchanged. Similar results were obtained from feeding experiments with [3,3-$^{2}H_{2}$]*O*-prenyl-(L)-tyrosine (**312a**, Table 2.9). Actually, as discussed before for the incorporation of [3,3-$^{2}H_{2}$]-(L)-tyrosine (**251a**) or [3,3-$^{2}H_{2}$]*O*-prenyl-(L)-tyrosine (**312a**) into phomamide (**171**), the different deuterium enrichment at H-5a and H-5b was also noticed, which indicated selective exchange of the β-deuterium. But the resonances of the *pro* S and *pro* R protons cannot be assigned in the ^{1}H NMR spectrum of phomamide (**171**) due to rotation of the bond between C-4 and C-5.



Scheme 2.13. Incorporation of [3,3-$^2H_2$]L-tyrosine (**251a**), [3,3-$^2H_2$]*O*-prenyl-L-tyrosine (**312a**) and [5,5-$^2H_2$]phomamide (**171a**) into sirodesmin PL(**165**). Dideuterated (**165a**) and monodeuterated (**165b**) sirodesmin PL were formed when **251a** or **312a** was fed to the cultures of *Leptoshphaeria maculans* but **165b** is not formed; only **165a** not **165b** and **165c** formed from intact incorporation of [5,5-$^2H_2$]phomamide (**171a**) by *L. maculans*.

Further analysis of the feeding experiment results from incorporation of [5,5-$^2H_2$]phomamide into sirodesmin PL indicated that this β-deuterium exchange did not happen after the formation of phomamide (**171**, Scheme 2.13). When dideuterated [5,5-$^2H_2$]phomamide was fed to the culture, about 26% sirodesmin PL was dideuterated, only a small amount, 4%, of sirodesmin PL was monodeuterated. Considering the likely re-incorporation of hydrolyzed fragment of [5,5-$^2H_2$]phomamide, the detection of 4% monodeuterated sirodesmin PL is not surprising. Therefore, the stereospecific deuterium exchange happened before the formation of phomamide (after formation of phomamide no β-deuterium exchange happened). Consequently, the proposed intermediates leptomaculins (**272** and **274**) and deacetylleptomaculins (**273** and **275**) were ruled out as intermediates in the biosynthesis of sirodesmin PL for introduction of the sulfur bridge.

If the double bond precursor (**272** or **274**) was formed to introduce sulfur, when dideuterated phomamide was fed to the culture, only monodeuterated sirodesmin PL would be detected. Similar β-deuterium exchange had been noticed in the study of the biosynthesis of gliotoxin (Bűlock et al., 1972). In that study the *pro* S proton was selectively exchanged.



Scheme 2.14. Incorporation of [3,3,5',5',5'-$^2H_5$]*O*-prenyl-L-tyrosine (**312b**) into sirodesmin PL (**165**). Pentadeuterated (**165d**) and tetradeuterated (**165e**) as well as dideuterated (**165a**) and monodeuterated (**165b**) sirodesmin PL were formed.

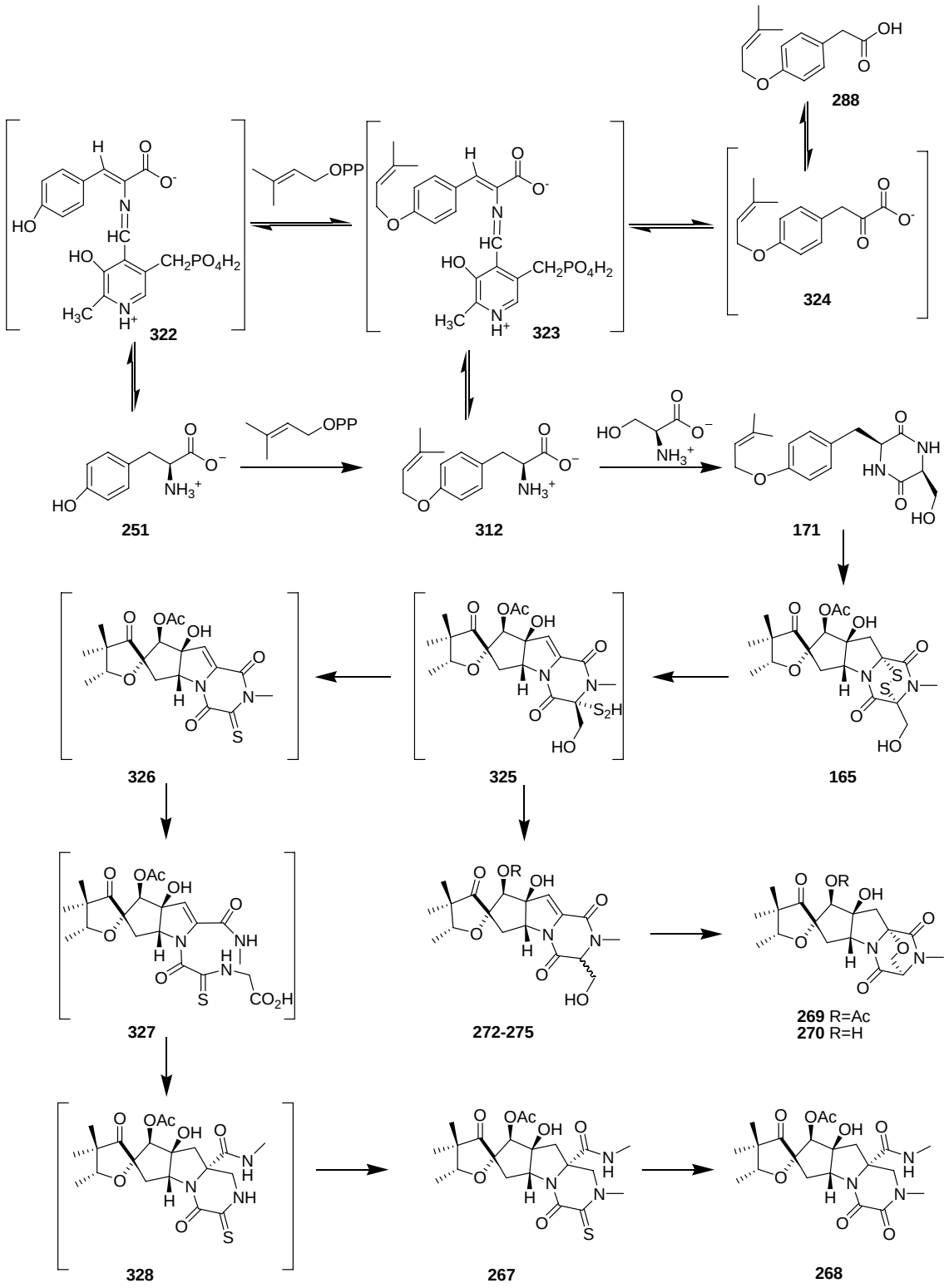
Another question to be answered in this study was at what stage does prenylation happen in the biosynthetic pathway of sirodesmin PL (**165**). The deuterium enrichment obtained from incubation of [5,5-$^2H_2$]cyclo-(L)-tyr-(L)-ser (**251a**) was very low, i.e., 3% and 2% into phomamide and sirodesmin PL, respectively (Table 2.9). On the other hand, [5,5-$^2H_2$]phomamide gave 30% deuterium enrichment. It seems that the dioxopiperazine (**252**) is not the likely intermediate in the biosynthesis of sirodesmin PL. Considering the high incorporation of [3,3-$^2H_2$]*O*-prenyl-(L)-tyrosine (**312a**) into phomamide (**171**) and sirodesmin PL (**165**), it is more likely that prenylated tyrosine will first couple to serine to form phomamide (**171**) instead of coupling of tyrosine and serine, then prenylation to form phomamide (**171**). Doubly labeled pentadeuterated (*E*)-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-

(L)-tyrosine (**312b**) were synthesized and incubated with cultures. Both pentadeuterated and tetradeuterated phomamide (7% and 16%) and sirodesmin PL (4% and 9%) were detected using LC-MS-ESI from samples produced in cultures of *L. maculans*, which indicated the intact incorporation of prenylated tyrosine (Scheme 2.14). The results obtained from the incubation of pentadeuterated (*E*)-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-(L)-tyrosine (**312b**) strongly supported the proposal that prenylation happens before the coupling of two amino acids in the biosynthetic pathway of sirodesmin PL (**165**). In addition, ^1H NMR spectrum of sirodesmin PL from the incorporation of (*E*)-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-(L)-tyrosine (**312b**) clearly showed that CH_3-17 of sirodesmin PL was derived from C^2H_3-5'of (*E*)-[3,3,5',5',5'-$^2H_5$]*O*-prenyl-(L)-tyrosine (**312b**), which agrees with previous data (Férézou et al., 1980a).

The deuterium incorporation into phomamide (**171**) and sirodesmin PL (**165**) obtained from the incubation with [2,3,3-$^2H_3$]-(L)-serine (**233d**) was very low, i.e., 3% and 4% respectively (Table 2.9). The substantially lower incorporation of serine than tyrosine could be due to the faster incorporation of serine into other primary pathways such as the synthesis of proteins and tryptophan and/or degradation to pyruvate and glycine. A rather low incorporation of both amino acids tyrosine and serine into phomamide and sirodesmin PL was reported previously (Férézou et al., 1980b).

Scheme 2.15 is proposed to summarize the results obtained from both feeding experiments with deuterated potential precursors (this section, section 2.3.2) and isolation of new metabolites from cultures of *L. maculans* in MM under standard conditions (section 2.1.1.1). Considering the β proton exchange when tyrosine or prenyl tyrosine was fed to cultures of *L. maculans* and the isolation of metabolite **288**, although alternative biosynthetic pathways are possible, it is proposed that tyrosine maybe conjugated with pyridoxal phosphate through a reversible α,β-elimination process (Vederas and Floss, 1980) to form an intermediate **322** which is prenylated to intermediate **323**. Hydrolysis of intermediate **323** to remove pyridoxal phosphate yields either compound **312** or α-keto-acid **324** and vise versa. α-Keto-acid **324** then is converted to compound **288** by decarboxylation and oxidation. Because of the reversible α,β-elimination process in the formation of intermediates **322** and **323**, one of the β deuterium is exchanged with medium proton when tyrosine or prenyl tyrosine is

incorporated into phomamide (**171**) and sirodesmin PL (**165**). However, if tyrosine or prenyl tyrosine is directly incorporated into phomamide and sirodesmin, then no β deuterium exchange happens, which yields the intact incorporation of tyrosine and prenyl tyrosine into phomamide and sirodesmin PL. Because both of these two biosynthetic pathways are present, mono- and di-deuterated phomamide and sirodesmin PL are detected in the feeding experiments. After the formation of phomamide, no proton exchange was observed suggesting that new metabolites leptomaculins **272** to **275** were, possibly, the further metabolites of sirodesmin PL, as proposed in Scheme 2.15. The formation of the double bond at C-5,6 position of sirodesmin PL is likely due to the break of the sulfur bridge, which gives intermediate **325**. Completely removal of sulfur atoms from intermediate **325** yields leptomaculins **272** to **275**, which in turn leads to the formation of leptomaculins **269** and **270**. Retention of the sulfur atom and removal of the hydroxyl methylene group of intermediate **325** leads to the formation of leptomaculins A (**267**) and B (**268**) through plausible intermediate **326**, **327** and **328**.



Scheme 2.15. Proposed biosynthesis and catabolism of sirodesmin PL (**165**). (postulated intermediates in brackets, adapted from Pedras and Yu, 2008).

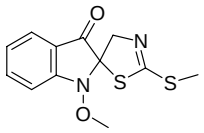
2.4 MACROMOLECULAR ELICITORS AND PHYTOTOXINS PRODUCED BY *LEPTOSPHAERIA MACULANS*

An increasing number of elicitors and phytotoxins identified, recently, are macromolecules. For example, Nep1-like proteins (NLPs) are protein elicitors isolated from oomycetes, fungi and bacteria (Gijzen and Nurnberger, 2006). The protein ***Avr1***, which was identified as a race specific elicitor from race 1 of fungus *F. oxysporum,* elicited the defense response of tomato carrying resistance gene ***I-1*** and resulted in the incompatible interaction between race 1 and tomato carrying resistance gene ***I-1*** (Houterman et al., 2008). Only a few HSTs were isolated and identified in recent years, for example ABR-toxin, a 27.5 kDa protein, was purified from germinating spores of *Alternaria brassicae* on leaves of cabbage, the host plant species (Parada et al., 2008). Therefore, isolation and identification of potential macromolecular elicitors and phytotoxins produced by *L. maculans* is important. The production of elicitor and/or phytotoxin active metabolites by fungi can be very sensitive to fungal growth conditions (Discussed in section 1.2.3.2). For example, the host-selective AB-toxin is produced by germinating spores of *Alternaria brassicicola* only on leaves of host plant species, but not on nonhost plant species or in culture media (Otani et al., 1998). The specific production of AB-toxin was found to be elicited by an oligosaccharide derived from host plants species (Oka et al., 2005). Therefore, fungal spore germination fluids produced by *L. maculans* inoculated on leaves of canola are also likely to produce potential elicitors and/or phytotoxins.

Preliminary experiments were carried out to find and optimize conditions from which potential elicitors and/or phytotoxins can be isolated and identified. The age of the plants, the concentration of fungal spores used for inoculation and the time of incubation on excised leaves were studied. The first two leaves of three-week-old plants of *B. napus* cv. Westar were excised and incubated in a Petri dish. Spore suspensions at a concentration of 2.5×10^{7}/mL had higher germination rate, while almost no spore germination was observed at ten times higher concentration (2.5×10^{8}/mL). After two days of incubation about 70% spores germinated while in another independent experiment 90% spores germinated. Although the time required to observe spore germination varies, two days was adequate as determined by inspection of the spore

suspension droplets on leaves under the microscope (40 μL spore suspension droplets were used for each inoculation site). The spores were removed from suspensions to yield SGF by centrifugation (3500 rpm, for 15 minutes, twice). Control leaves were treated similarly with sterile water. From the above optimized conditions SGF (ca. 1.5 mg/mL, 18 leaves) and control (ca. 1.0 mg/ mL, 15 leaves) were collected and analyzed by HPLC and ^{1}H NMR.

The HPLC chromatograms showed that both SGF and control solution had a peak at R_t = 4 min. SGF contained more peaks around R_t 2.1 to 3.5 min. Less polar materials were not detected maybe because the concentration was too low. ^{1}H NMR of SGF showed some typical amino acid signals, such as the multiple resonances at δ_H 2.0-3.0 also several doublets at δ_H 1.5-0.9 could be isopropyl moieties. A few signals were also displayed within the aromatic proton range (δ_H 6.6-7.5). The resonances of ^{1}H NMR of control showed polyol protons at δ_H 3.5-4.5 range similar to those observed in spectra of SGF but other resonances were too broad to be assigned. The elicitor-toxin bioassays of SGF were conducted using both brown mustard and canola. The phytoalexin erucalexin (**329**) was detected in the extracts of brown mustard leaves treated with SGF but not in the control. No phytoalexins were detected in the extracts of leaves of canola treated with SGF or controls. The leaves treated with SGF showed yellowing and necrosis on both brown mustard (Figure I.5, page xxxiv) and canola. The cotyledons of brown mustard also showed large yellowish areas when treated with SGF. No damage was observed in the controls, therefore, SGF appeared to contain elicitors and/or phytotoxins.



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CHAPTER 3: CONCLUSION AND FUTURE WORK

The elicitor-toxin activity bioassay guided isolation of elicitors and phytotoxins produced by *L. maculans* lead to the isolation of general elicitors, sirodesmin PL (**165**) and deacetylsirodesmin PL (**166**) and specific elicitors, cerebrosides C (**14**) and D (**31**) from MM culture under standard conditions (Table 2.1, entry 1). As well, root growth inhibitor **212** and phytotoxins maculansins A (**299**) and B (**300**) were isolated from PDB cultures (Table 2.1, entry 11).

In this work, brown mustard (resistant to *L. maculans*) produced the phytoalexin rutalexin (**127**) in response to a mixture of cerebrosides C (**14**) and D (**31**); canola (susceptible to *L. maculans*) did not appear to respond to the mixture of cerebrosides C (**14**) and D (**31**), as no phytoalexins were detected. Cerebroside C (**14**) was shown to be a sphingolipid elicitor in several plant species (Umemura et al., 2004). Sphingolipids are fungal cell wall components. It was reported that conversion of sphingolipids to glycosphingolipids by glucosyltransferase is essential for spore germination, hyphal growth and cell cycle in *Aspergillus* species (Levery et al., 2002). Therefore, sphingolipids act as general elicitor/microbe associated molecular patterns (MAMPs) in the interaction of pathogens and host plants. However, brown mustard and canola responded to cerebrosides C (**14**) and D (**31**) differently, which is consistent with the susceptibility of canola to the pathogen *L. maculans*. Therefore, cerebrosides could be used as potential biologically derived control agents for the screening and selection of disease-resistant plants (Bautista-Banos et al., 2006; Graniti, 1991).

Maculansins A (**299**) and B (**300**) caused significant damage on leaves of both susceptible and resistant plants but did not elicit the production of phytoalexins. Compared to cerebrosides and maculansins, sirodesmins seemed to act as dual agents. They damaged plant leaves and elicited the production of phytoalexins in both resistant and susceptible plants. Which, if any, do these bioactive metabolites play a role in the interaction between *L. maculans* and its host plants? The attempt to identify these

metabolites in leaves of *B. napus* (Westar) infected by *L. maculans* gave results that were not consistent and convincing. Only recently, sirodesmin PL was detected in leaves of canola infected by *L. maculans* (Elliott et al., 2007). Nonetheless, further work related with these bioactive metabolites needs to be carried out to understand their role in the interaction between *L. maculans* and its host plants.

Although eight new metabolites, leptomaculins A–E (**267**–**270** and **272**–**274**), were isolated from elicitor active fractions, purified individual metabolites did not show elicitor or toxin activities when tested on brown mustard (*B. juncea* cv. Cutlass, resistant) and canola (*B. napus* cv. Westar, susceptible). This was likely caused by the presence of sirodesmins, which are present in most fractions due to their large range of polarity and interconversion (Pedras et al., 1990). Nonetheless, the biosynthesis of these new metabolites is intriguing. Leptomaculins D (**272**) and E (**274**) as well as acetyl leptomaculins D (**273**) and E (**275**) were ruled out as intermediates involving the introduction of sulfur into sirodesmins because of the intact incorporation of [5,5-$^2H_2$]phomamide (**171a**) into sirodesmin PL (**165**). Instead, they are likely the further catabolism products of sirodesmin PL (**165**), as proposed in Scheme 2.15. Similarly, leptomaculin C (**269**) and acetylleptomaculin C (**270**) were also proposed as the catabolic products of sirodesmin PL (**165**) (Scheme 2.15). However, leptomaculin A (**267**) and leptomaculin B (**268**), the first naturally occurring examples of a 2,3-oxopiperazinethione/dioxopiperazine, were biosynthesized through multiple steps with significant structural modification (Scheme 2.15), suggesting a special role of these two metabolites in the metabolism of *L. maculans* (Pedras and Yu, 2008).

Recently, a large number of orphan pathways were detected in microorganisms, which suggested that it is possible to discover a large number of secondary metabolites from one microorganism species (Gross, 2007). The isolation of bulgarein (**294**) and 8-hydroxynaphthalene-1-sulfate (**293**) from high NaCl MM media conditions and maculans A (**299)** and B (**300**) from PDB media demonstrated that diverse secondary metabolites can be obtained from one fungal species by varying culture conditions (Bode et al., 2002). The composition of culture media and amendments are literally unlimited. Therefore, there is a great potential to discover additional biologically active metabolites from *L. maculans*, which needs to be further explored.

Suggested future work

1. Isolation of macromolecular elicitors produced by *L. maculans* from SGF.
2. Identification of intermediates in the biosynthetic pathway of sirodesmin PL using genomics-guided strategies.
3. Study of melanin biosynthetic pathway using high concentrations of NaCl (Table 2.1, entry 7).

CHAPTER 4: EXPERIMENTAL

4.1 GENERAL METHODS

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON; solvents were HPLC grade and used as such. Organic extracts were dried with Na_2SO_4 and solvents removed under reduced pressure in a rotary evaporator. Flash column chromatography (FCC) was carried out using silica gel grade 60, mesh size 230 – 400 Å. Preparative thin layer chromatography (prep TLC) was carried out on silica gel plates, Kieselgel 60 F_{254} (20 × 20 cm × 0.25 mm), compounds were visualized under UV light. Specific rotations $[\alpha]_D$ were determined at ambient temperature on a polarimeter using a 1 mL, 10 cm path length cell; the units are 10^{-1} deg cm^2 g^{-1} and the concentrations are reported in g/100 mL.

Nuclear magnetic resonance (NMR) spectra (^1H, ^{13}C, HMQC – heteronuclear multiple quantum coherence, HMBC – heteronuclear multiple bond coherence) were recorded on Bruker Avance 500 spectrometers. High resolution (HR) electron impact (EI) mass spectra (MS), were obtained on a VG 70 SE mass spectrometer, employing a solids probe.

HPLC analysis was carried out with Agilent high performance liquid chromatographs equipped with quaternary pump, automatic injector, and diode array detector (DAD, wavelength range 190 - 600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), having an in-line filter. Mobile phase: 75% H_2O - 25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient, and at a flow rate 1.0 mL/min.

Minimal medium (MM) contains the following chemicals:

Solution 1: KNO_3 (3.12 g/L, 30.8 mM), K_2HPO_4 (0.75 g/L, 4.31 mM), KH_2PO_4 (0.75 g/L, 5.51 mM), NaCl (0.10 g/L, 1.71 mM), asparagine (0.28 g/L, 2.12 mM);

Solution 2: $CaCl_2 \cdot 2H_2O$ (0.10 g/L, 0.68 mM), $MgSO_4 \cdot 7H_2O$ (0.50 g/L, 2.03 mM);
Solution 3: $ZnSO_4 \cdot 7H_2O$ (0.395 mg/L, 1.37 μM), $CuSO_4 \cdot 5H_2O$ (0.079 mg/L, 0.32 μM), $MnSO_4 \cdot 4H_2O$ (0.0405 mg/L, 0.18 μM), MoO_3 (85%, 0.0175 mg/L, 0.12 μM), ferric citrate (0.535 mg/L, 2.16 μM), $Na_2B_4O_7 \cdot 10H_2O$ (0.0375 mg/L, 0.10 μM);
Solution 4: thiamine (0.1 mg/L, 0.38 μM);
Glucose: (15 g/L, 83.3 mM).

Solutions 1 and 3 and glucose were mixed and the pH of the mixed solution was adjusted to 6.55 and autoclaved. The autoclaved solution was combined with solutions 2 and 4 (solutions 2 and 4 are sterilized, separately). Canadian virulent isolate of *L. maculans* IBCN 57 (BJ 125) was grown in Erlenmeyer flasks (250 mL) containing MM (100 mL) inoculated with fungal spores (10^8) and incubated on a shaker at 120 rpm, at 23 ± 2 °C for 7 days. Cultures were grown in triplicates.

4.2 POTENTIAL ELICITORS FROM *LEPTOSPHAERIA MACULANS*

4.2.1 Metabolites produced in minimal media

4.2.1.1 Standard condition

A total of 20 liters of cultures of *L. maculans* IBCN 57 (BJ 125) was prepared in standard MM (Table 2.1 entry 1, see 4.1 General Methods). Mycelia were separated from broth by vacuum filtration; the broth was extracted with EtOAc. The aqueous layer did not show elicitor activity (direct application of aliquot of aqueous layer on plant leaves), therefore it was autoclaved and discarded. The EtOAc layer was concentrated to yield 7.2 g of residue, which showed elicitor activity. Therefore the EtOAc extracts were fractioned as described in the following section. Mycelia were extracted with MeOH, the MeOH layer was concentrated, and the residue was resuspended in a solution of MeOH/H_2O (1:1 v/v) and then extracted with hexane. The MeOH/H_2O (1:1 v/v) layer was concentrated to give 12.6 g of residue. This residue did not show elicitor activity, and D-mannitol (**301**) was isolated as the major component. The hexane layer was

concentrated to give 3.4 g of residue and the residue (hexane extract) showed elicitor activity. Therefore, the hexane extracts were fractioned as described in experimental.

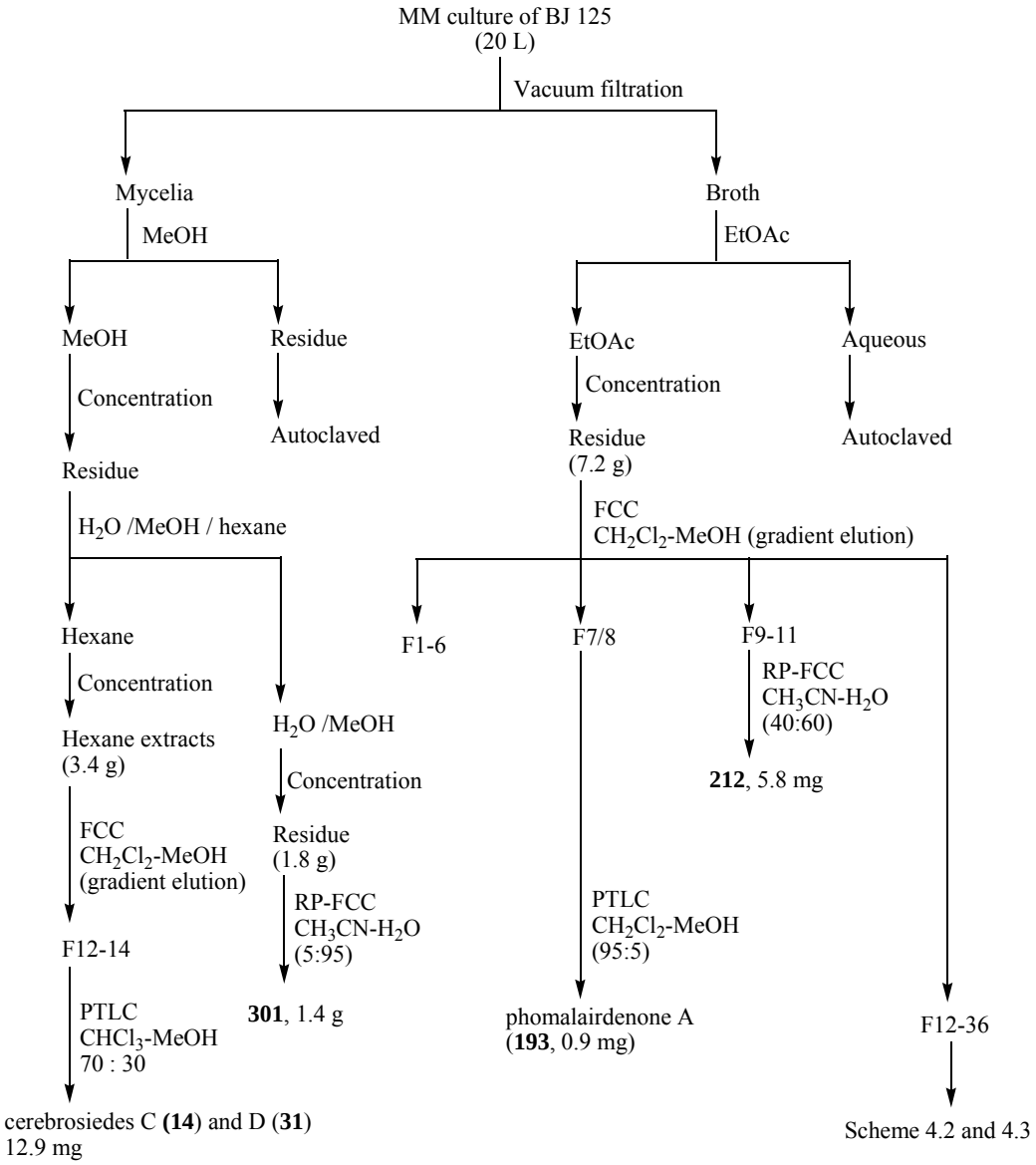
The EtOAc extract (7.2 g) was subjected to FCC on silica gel and eluted with a gradient of CH_2Cl_2–MeOH to yield 36 fractions (each 50 mL). Fractions F_{1-6} and F_{31-36} did not show elicitor activity, and F_{9-11} contained mainly sirodesmins (**165-169**), whereas $F_{24/25}$ contained mainly phomamide (**171**). Therefore, these fractions were not fractionated further. The remaining fractions showed either elicitor activity or contained new metabolites and were further fractionated as summarized in Schemes 4.1 to 4.3.

$F_{7/8}$ was subjected to prep. TLC (CH_2Cl_2–MeOH, 95:5) to give phomalairdenone A **193** (0.9 mg). F_{9-11} was applied to reversed phase chromatography, CH_3CN–H_2O, (40:60) to give benzoaldehyde **212** (5.8 mg). F_{12-14} was applied to reversed phase chromatography, CH_3CN–H_2O, (25:75). Leptomaculin A (**267**, 1.5 mg) was obtained by prep. TLC (toluene–EtOAc–HOAc, 50:40:10, multiple developments) of fraction 2. Leptomaculin A (**269**, 1.5 mg) and deacetylleptomaculin C (**270**, 0.3 mg) were obtained by reverse phase column (CH_3CN–H_2O, 15:85) from a nonhomogenous PTLC fraction (toluene–EtOAc–HOAc, 50:40:10, multiple developments) of fraction seven.

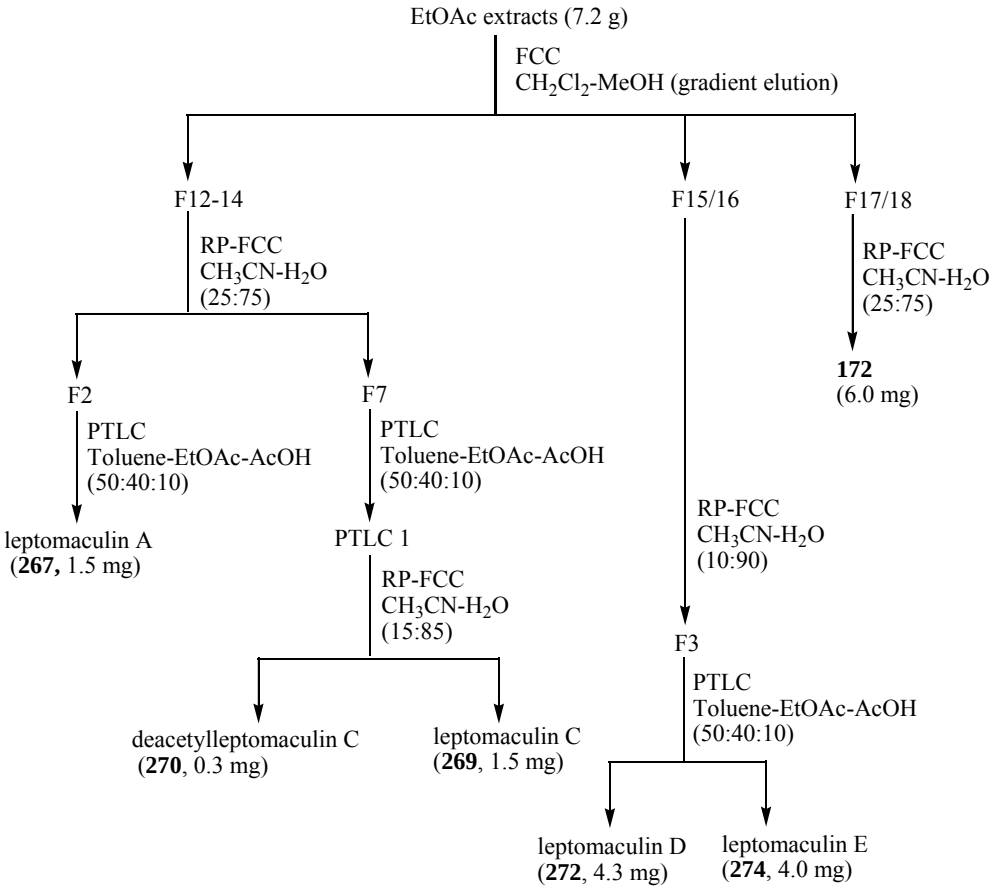
$F_{15/16}$ was applied to reversed phase chromatography, CH_3CN–H_2O, (10:90) 28 fractions were collected. Fractions 11 to 16 were combined and further subjected to prep. TLC (toluene–EtOAc–HOAc, 50:40:10, multiple developments) to yield leptomaculin D (**272**, 4.3 mg) and leptomaculin E (**274**, 4.0 mg). $F_{17/18}$ was applied to reversed phase chromatography, CH_3CN–H_2O, (25:75) to give **172** (6.0 mg). F_{19} to F_{22} were combined and applied to reversed phase chromatography, CH_3CN–H_2O, (20:80) and metabolite **290** was obtained by prep. TLC (MeOH–EtOAc, 10:90, multiple developments) of fraction nine.

$F_{23/24}$ was applied to reversed phase chromatography, CH_3CN–H_2O, (10:90). Deacetylleptomaculins D (**273**, 3.7 mg) and E (**275**, 2.5 mg) were obtained from prep. TLC (toluene–EtOAc–HOAc, 50:40:10, multiple developments) of fraction three. $F_{25/26}$ was applied to reversed phase chromatography, CH_3CN–H_2O, (10:90). Leptomaculin B (**268**, 4.1 mg) was obtained from prep. TLC (CH_2Cl_2–MeOH, 70:30) of fraction five. F_{27-30} was applied to prep. TLC (CH_2Cl_2–MeOH–AcOH, 80:20:1) and followed by prep. TLC (toluene–EtOAc–HOAc, 80:10:10) to give **288** (3.6 mg).

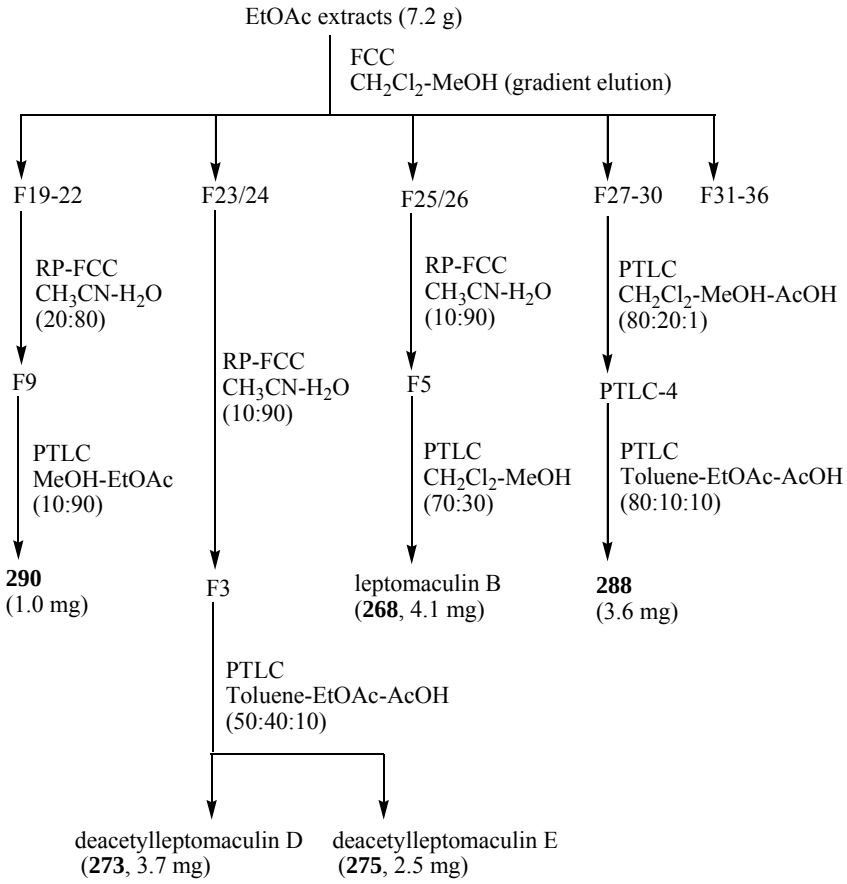
The hexane extract (3.4 g) was subjected to FCC on silica gel and eluted with a gradient of CH_2Cl_2–MeOH to yield 20 fractions (each 50 mL). Fractions F_{13} to F_{16} showed elicitor activity. Therefore, F_{13} to F_{16} were combined and subjected to prep. TLC ($CHCl_3$–MeOH, 70:30) to give 12.9 mg inseparable mixture of cerebroside C (**14**) and D (**31**).



Scheme 4.1. Flow chart for separation of metabolites from standard MM culture of BJ125.



Scheme 4.2. Flow chart for separation of metabolites from standard MM culture of BJ125.



Scheme 4.3. Flow chart for separation of metabolites from standard MM cultures of BJ125.

Leptomaculin A (**267**)

HPLC: R_t= 4.7 min.

^{1}H NMR and ^{13}C NMR see Table 2.2.

m.p. 252-256 °C decomposed.

HRMS-EI: *m/z* 425.1614, $C_{19}H_{27}N_3O_6S$, calcd. 425.1621. MS-EI *m/z* 425 (61%), 367 (100%).

FTIR (KBr): 3325, 3090, 2970, 2929, 2871, 1738, 1683, 1652 cm^{-1}.

UV (MeOH): λ_{max} (log ε) 202 (4.07), 274 (3.70), 312 (3.86) nm.

$[\alpha]_D$ = + 56 (c 0.10, MeOH).

Leptomaculin B (**268**)

HPLC: R_t = 3.3 min.

^{1}H NMR and ^{13}C NMR see Table 2.2.

HRMS-EI: *m/z* 409.1852, $C_{19}H_{27}N_3O_7$, calcd. 409.1849. MS-EI: *m/z* 409 (5%), 351(100%).

FTIR (KBr): 3438, 3290, 2927, 2856, 1740, 1674 cm^{-1}.

UV (MeOH): λ_{max} (log ε) 224 (3.81) nm.

$[\alpha]_D$ = - 45 (c 0.10, MeOH).

Leptomaculin C (**269**)

HPLC: R_t = 7.4 min.

^{1}H NMR and ^{13}C NMR see Table 2.3.

HRMS-EI: *m/z* 423.1767, $C_{20}H_{27}N_2O_8$, calcd. 423.1767. MS-EI: *m/z* 423(10%), 422(8%), 365(100%), 323(57%).

FTIR (KBr): 3379, 2974, 1751, 1710, 1395, 1236 cm^{-1}.

Deacetylleptomaculin C (**270**)

HPLC: R_t = 4.7 min.

^{1}H NMR see Table 2.3.

HRMS-ESI: *m/z* $[M+1]^+$ 381.1660, $C_{18}H_{25}N_2O_7$, calcd. 381.1656.

FTIR (KBr): 3368, 2927, 2841, 1743, 1674, 1017 cm^{-1}.

Leptomaculin D (**272**)

HPLC: R_t = 4.8 min.

^{1}H NMR and ^{13}C NMR see Tables 2.4 and 2.5, respectively.

HRMS-EI: *m/z* 422.1678, $C_{20}H_{26}N_2O_8$, calcd. 422.1676. MS-EI: *m/z* 422 (4%), 380 (10%), 344 (18%), 223 (74%), 200 (86%), 140 (100%).

FTIR (KBr): 3425, 2972, 2934, 2872, 1751, 1677, 1644, 1446, 1231, 1081, 738 cm^{-1}.

UV (MeOH): λ_{max} (log ε) 220 (3.92), 254 (3.87) nm.

$[\alpha]_D$ = - 47 (c 0.22, $CHCl_3$).

Deacetylleptomaculin D (**273**).

HPLC: R_t = 3.8 min.

^1H NMR and ^{13}C NMR see Tables 2.4 and 2.5, respectively.

HRMS-EI: *m/z* 380.1591, $C_{18}H_{24}N_2O_7$, calcd. 380.1584. MS-EI: *m/z* 380(66%), 344(22%), 223(90%), 211(73%), 180(100%), 158(84%), 70 (46%).

FTIR (KBr): 3375, 2927, 1750, 1675, 1640, 1464, 1405, 1085 cm^{-1}.

UV (MeOH): λ_{max} (log ε) 221 (3.96), 255 (3.94) nm.

$[\alpha]_D$ = - 82 (c 0.14, $CHCl_3$).

Leptomaculin E (**274**).

HPLC: R_t = 6.2 min.

^1H NMR and ^{13}C NMR see Tables 2.4 and 2.5, respectively.

HRMS-EI: *m/z* 422.1675, $C_{20}H_{26}N_2O_8$, calcd. 422.1689. MS-EI: *m/z* 422 (21%), 223 (57%), 200 (67%), 140 (100%).

FTIR (KBr): 3416, 2974, 2935, 1752, 1681, 1645 cm^{-1}.

UV (MeOH): λ_{max} (log ε) 219 (4.01), 254 (3.97) nm.

$[\alpha]_D$ = - 66 (c 0.21, $CHCl_3$).

Deacetylleptomaculin E (**275**).

HPLC: R_t = 3.1 min.

^1H NMR and ^{13}C NMR see Tables 2.4 and 2.5, respectively.

HRMS-EI: *m/z* 380.1585, $C_{18}H_{24}N_2O_7$, calcd. 380.1584. MS-EI: *m/z* 380 (10%), 362 (12%), 223 (50%), 180 (43%), 158 (100%), 140 (43%), 70 (21%).

FTIR (KBr): 3414, 2968, 2930, 1750, 1676, 1641, 1466, 1083 cm^{-1}.

UV (MeOH): λ_{max} (log ε) 221 (4.04), 256 (3.99) nm.

$[\alpha]_D$ = - 93 (c 0.13, $CHCl_3$).

Cerebroside C (**14**)

^{1}H NMR and ^{13}C NMR see Table 2.6.

HRMS-ESI: $[M+1]^+$ *m/z* 754.5827, $C_{43}H_{80}NO_9$, calcd. 754.5827;

HRMS-ESI-MS^2: $[M+Na]^+$ *m/z* 776.5686, *m/z* 758, 614, 596, 496, 346, 334.

Metabolite (**290**)

HPLC: GRADSCR R_t= 9.2 min.

^{1}H NMR (500 MHz, DMSO-d_6): δ 9.37 (bs, 1H), 8.28(bs, 1H), 8.19 (s, 1H), 6.98 (d, *J* = 8.4 Hz, 1H), 6.34 (d, *J* = 2.4 Hz, 1H), 6.31 (d, *J* = 8.4, 2.4 Hz, 1H,), 5.38 (m, 1H), 5.02 (t, *J* = 5.7 Hz, 1H), 4.40 (d, *J* = 6.5 Hz, 2H), 3.69 (dd, *J* = 10.9, 5.8 Hz, 1H), 3.50 (dd, *J* = 10.8, 3.3 Hz, 1H), 3.39 (dd, *J* = 5.8, 3.3 Hz, 1H), 3.27 (d, *J* = 13.9 Hz, 1H), 2.97 (d, *J* = 13.8 Hz, 1H), 2.17 (s, 3H), 1.72 (s, 3H), 1.67 (s, 3H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 165.6 (2C), 158.6, 156.3, 136.8, 132.1, 120.0, 113.8, 105.5, 102.0, 67.0, 64.1, 62.0, 57.2, 39.0, 25.4, 18.0, 12.9.

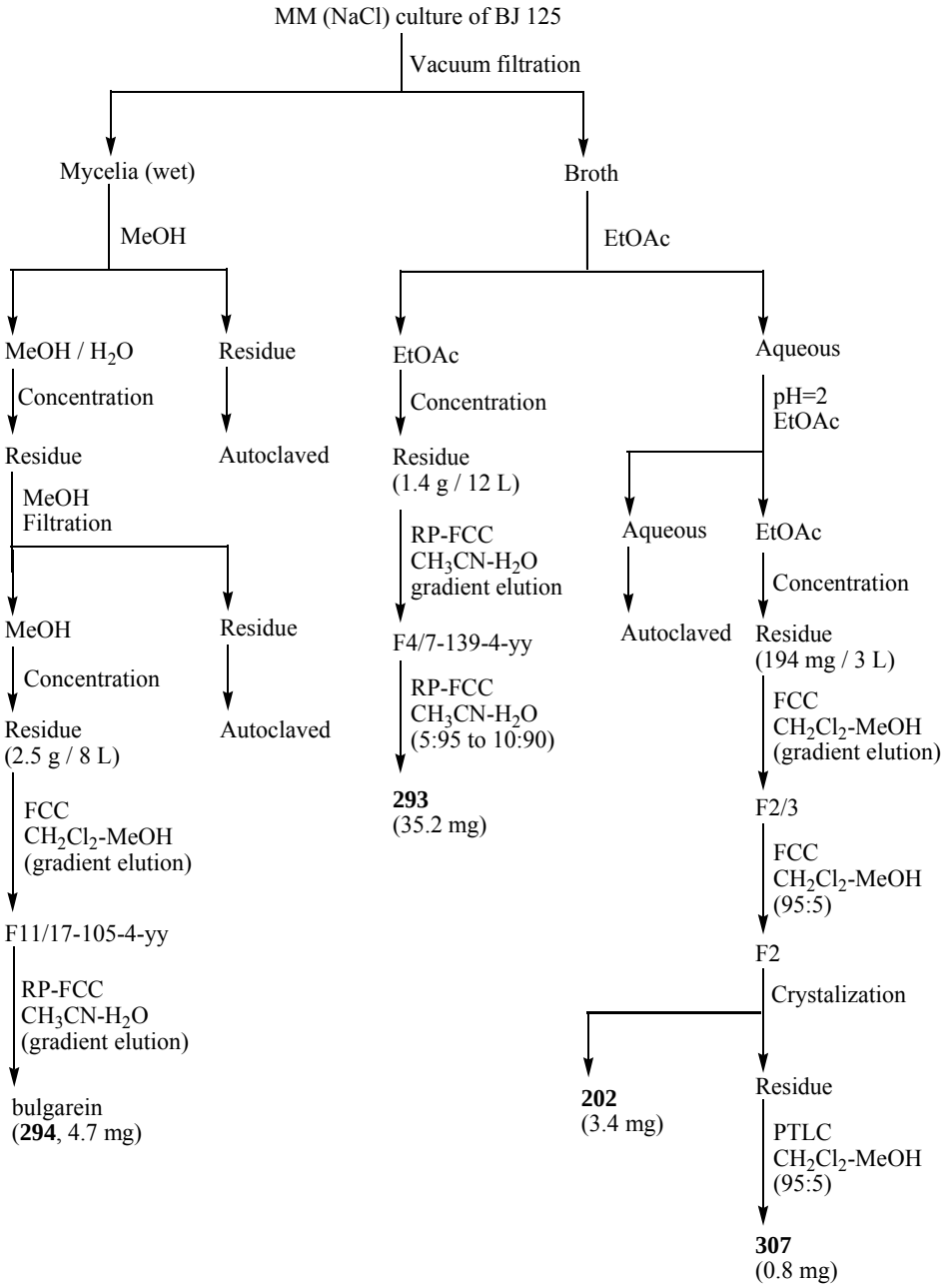
HRMS-ESI *m/z* $[M+Na]^+$ 403.1298, $C_{18}H_{24}N_2O_5NaS$, calcd. 403.1298;

4.2.1.2 High NaCl

Canadian virulent isolate of *L. maculans* IBCN 57 (BJ 125) was grown in 250 mL Erlenmeyer flasks (100 mL minimal medium) inoculated with fungal spores (1×10^8). The cultures were incubated on a shaker at 120 rpm and 23 ± 2 °C. Different amounts of NaCl (1.0 g / 100 mL, 2.0 g / 100 mL or 4.0 g / 100 mL) were added at different incubation times (0, 3, 4, or 5 day) (Table 2.1, entries 2 to 8). Cultures (10 mL) were collected on the 4th, 5th, 6th, 7th, 9th and 11th days, extracted with EtOAc (20 mL × 3) and extracts were analyzed by HPLC (triplicate cultures).

Isolation of metabolites (Table 2.1, entry 7): Isolate BJ 125 culture was grown in 250 mL Erlenmeyer flasks (100 mL minimal medium) inoculated with BJ 125 spores

(1×10^8). The cultures were incubated on a shaker (150 rpm) at 23 ± 2 °C for 4 days, and then NaCl (4 g / 100 mL) was added. On the 7th day the cultures were harvested. The broth was separated from mycelia by vacuum filtration. A total of 12 liters of broth was collected and extracted with EtOAc. The EtOAc extracts (1.4 g) was applied to Rp-18 reverse phase FCC (CH_3CN-H_2O, gradient elution). F_{4-7} (66 mg) was further purified with Rp-18 reverse phase FCC (CH_3CN-H_2O, 5:95) to give 35.2 mg of compound **293** (Scheme 4.4). Mycelia were washed with distilled water twice and extracted with MeOH (200 mL× 3). The MeOH extract was concentrated to 50 mL and filtered. The filtrate was concentrated to dryness to give 2.5g residue. The residue was subjected to FCC and eluted with MeOH-CH_2Cl_2 (5 % MeOH to 25 % MeOH gradient). The elute from 20 % MeOH/CH_2Cl_2 (50 mg) was subjected to Rp-18 reverse phase chromatography, 10% MeOH-H_2O to 50% MeOH-H_2O gradient elution, F_{12} to F_{18} gave 4.7 mg of blue color pigment bulgarein **294** (Scheme 4.4).



Scheme 4.4. Flow chart for separation of metabolites from high NaCl MM cultures of BJ125.

8-hydroxyl-naphthalenylsulfate (**293**)

m. p. 223-225 °C decomposed.

HPLC: GRADSCR R_t= 2.3 min.

^{1}H NMR (500 MHz, D_2O): δ 7.79 (dd, *J* = 4.9, 4.4 Hz, 1H), 7.51 (d, *J* = 8.3 Hz, 1H), 7.47 (bd, *J* = 5.1 Hz, 2H), 7.42 (dd, *J* = 8.0, 7.8 Hz, 1H); 6.99 (d, *J* = 7.7 Hz, 1H);

^{13}C NMR (125 MHz, D_2O): δ 151.3, 146.7, 136.8, 127.4, 126.5, 126.0, 120.3, 118.0, 117.4, 111.5.

HRMS-ESI: *m/z* $[M-1]^-$ 239.0024, $C_{10}H_7O_5S$, Calcd. 239.0019; MS-EI: *m/z* 160 (100%);

FTIR (KBr): 3329, 1628, 1602, 1583, 1235 cm^{-1};

UV (MeOH): λ_{max} (log ε): 225 (4.55), 302 (3.77), 316 (3.69), 330 (3.61) nm.

Bulgarein (**294**)

HPLC: GRADSCR R_t= 4.1 (broad) min.

^{1}H NMR (500 MHz, DMSO-d_6): δ 13.48 (bs, 2H), 12.66 (bs, 1H), 8.27 (d, *J* = 9.0 Hz, 1H), 7.96 (d, *J* = 8.0 Hz, 1H), 7.57 (bs, 1H), 7.38 (bs, 1H), 6.67 (bs, 1H), 6.59 (d, *J* = 8.0 Hz, 1H), 6.40 (d, *J* = 9.0 Hz, 1H);

^{13}C NMR (125 MHz, DMSO-d_6): δ 183.1 (2C), 160.6 (3C), 137.2 (C-6), 137.1, 134.5 (C-12), 134.1, 127.9 (C-18), 123.6, 122.3, 119.9, 119.7, 116.7 (C-7), 115.4, 114.7, 112.3, 109.3, 109.1;

HRMS-ESI *m/z* $[M-1]^-$ 329.0441, $C_{20}H_9O_5$, calcd. 329.0455;

FTIR (KBr): 3397, 2938, 1619, 1585, 1465, 1213 cm^{-1};

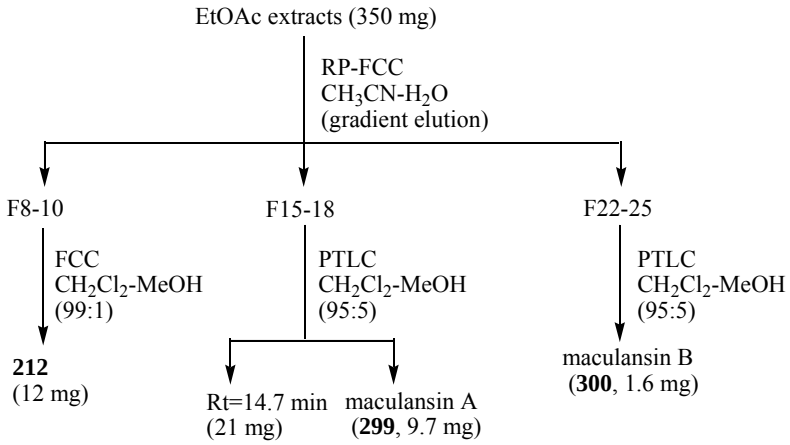
UV (MeOH): λ_{max} (log ε) 631 (4.04), 376 (3.79), 252 (4.32) nm.

4.2.2 Metabolites produced in potato dextrose medium

HPLC analysis of metabolites in PDB cultures (Table 2.1, entries 9 to 12): Canadian virulent isolate of *L. maculans* IBCN 57 (BJ 125) was grown in 250 mL Erlenmeyer flasks containing PDB medium (100 mL) inoculated with fungal spores

(10^8) and incubated on a shaker at 120 rpm, at 23, 25, 27, and 29 °C. Cultures (20 mL), 4-day-old or 7-day-old, were filtered to separate the broth from mycelia. The broth was extracted with EtOAc (20 mL × 3) and the EtOAc layer was dried with Na_2SO_4, and then concentrated. The residue was dissolved in CH_3CN (1 mL) and analyzed by HPLC.

Isolation of metabolites (Table 2.1, entry 11): A total of 5 L PDB media culture of *L. maculans* was prepared as reported above. After 4 days the culture was filtered, and the broth was extracted with EtOAc. The EtOAc extract was concentrated to give 360 mg of residue. The residue was separated by FCC (RP-18, CH_3CN–H_2O, 10:90 to 40:60, gradient elution) to yield 30 fractions (15 ml per fraction). $F_{8\text{-}10}$ was further purified by FCC (MeOH-CH_2Cl_2 0:100 to 5:95, gradient elution) to give compound **212** (10.5 mg). $F_{15\text{-}18}$ (45.1 mg) was further purified by prep TLC (MeOH-CH_2Cl_2, 5:95) to yield maculansin A (**299**, 9.7 mg) and an inseparable mixture (21 mg) with R_t = 14.7 min. $F_{22\text{-}25}$ contained a mixture of maculansin type metabolites that were further separated by prep TLC to yield an epimeric mixture of maculansin B (**300**, 1.6 mg) (Scheme 4.5).



Scheme 4.5. Flow chart for separation of metabolites from PDB cultures of BJ125.

Maculansin A (**299**)

HPLC: R_t = 16.4 min.

^{1}H NMR (500 MHz, $CDCl_3$): δ 5.13 (m, 2H), 4.64 (dd, *J* = 12.5, 2.5 Hz, 2H), 4.41 (dd, *J* = 12.5, 4.2 Hz, 2H), 3.89 (t, *J* = 8.2 Hz, 2H), 3.00 (d, *J* = 7.7 Hz, 2H, D_2O exchangeable), 2.31 (s, 6H), 2.18 (s, 6H), 2.11 (s, 6H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 171.6, 168.0, 160.5, 158.3, 115.0, 72.8, 67.4, 62.3, 25.1, 21.5, 21.1.

HRMS-EI: *m/z* 480.1749, $C_{22}H_{28}N_2O_{10}$, calcd. 480.1744. MS-EI: *m/z* 480 (3%), 465 (22%), 115 (100%).

FTIR (KBr): 3460, 2960, 2923, 2116, 1740, 1732, 1222 cm^{-1}.

UV (MeOH): λ_{max} (log ε) 231 (4.2) nm;

$[\alpha]_D$ = - 71 (c 0.10, $CHCl_3$)

Maculansin B (**300**)

HPLC R_t = 18.3 min.

^{1}H NMR (500 MHz, $CDCl_3$): δ 5.2 (m, 1H), 5.1 (m, 1H), 4.6 (m, 2H), 4.4 (m, 2H), 4.28 (d, *J* = 4.2 Hz, 1H) , 3.8 (m, 2H), 3.07 (d, *J* = 7.4 Hz, 1H, OH), 2.93 (d, *J* = 8.3 Hz, 1H, OH), 2.4 (m, 1H), 2.32 (s, 3H), 2.19 (s, 3H), 2.10 (s, 3H), 2.09 (s, 3H), 1.14 (d, *J* = 6.8 Hz, 3H), 1.04 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 171.5 (2C), 158.8, 116.6, 115.0, 74.0, 72.5, 67.6 (2C), 62.8, 61.9 (2C), 24.8, 21.3 (2C), 20.8 (2C), 19.5, 16.6, (C-1′, -6′, and -1′′ were not detected). ^{13}C NMR spectral data were obtained from analysis of HMQC and HMBC correlations.

HRMS-ESI: $[M+Na]^+$ *m/z* 505.1807, $C_{22}H_{28}N_2O_{10}Na$, calcd. 505.1792.

HRMS-ESI-MS/MS: *m/z* 505 (100%), 380 (40%), 378 (70%), 253 (27%).

4.2.3 Production of metabolites in other stress conditions

Minimal medium cultures incubated at 29°C (Table 2.1, entry 13)

The minimal medium culture was prepared the same as in the general method (Table 2.1, entry 1), except the incubation temperature was at 29°C. Culture broth (10 mL) was extracted with EtOAc (25 mL × 2) on the 4^{th} and 7^{th} days after inoculation of spores and the extracts were analyzed by HPLC (triplicates).

Minimal medium cultures amended with different amount of $MgSO_4 \cdot 7H_2O$ (Table 2.1, entries 14, 15 and 16)

The condition in Table 2.1 entry 14 was prepared as follows: $MgSO_4 \cdot 7H_2O$ (15.0 mg, 0.060 mmol) and $CaCl_2 \cdot 2H_2O$ (30.0 mg, 0.20 mmol) were added to distilled water (30 mL) and the solution was autoclaved. Ten mL of the latter solution was added to an autoclaved solution (90 mL) of a mixture of solution 1 (10 mL), solution 3 (0.1 mL), distilled water (80 mL) and glucose (1.5 g). The spores (10^8) of Canadian virulent isolate of *L. maculans* IBCN 57 (BJ 125) was grown in 250 mL Erlenmeyer flasks containing this prepared minimal medium (100 mL). The remaining conditions were the same as that in the general method (triplicates). The conditions in Table 2.1 entries 15 and 16 were prepared similarly with $MgSO_4 \cdot 7H_2O$ at 75.0 mg (0.30 mmol) and 300.0 mg (1.20 mmol), respectively. Culture broth (10 mL) was collected on the 4^{th}, 5^{th}, 6^{th}, 7^{th}, 8^{th} and 10^{th} days, extracted with EtOAc (20 mL × 3) and the extracts were analyzed by HPLC.

Minimal medium cultures amended without thiamine (Table 2.1, entry 17)

Canadian virulent isolate of *L. maculans* IBCN 57 (BJ 125) was grown in 250 mL Erlenmeyer flasks containing MM (100 mL) without addition of thiamine, inoculated with fungal spores (10^8). The cultures were incubated on a shaker at 120 rpm, 23 ± 2 °C. Culture broth (10 mL) was collected on the 4^{th}, 5^{th}, 6^{th}, and 7^{th} days, extracted with EtOAc (20 mL × 3) and the extracts were analyzed by HPLC (duplicates).

Minimal medium cultures amended with leaves of brown mustard without thiamine (Table 2.1, entry 18)

Fresh leaves (four leaves, ca 0.40 g wt) of brown mustard were added into a 250 mL Erlenmeyer flask containing minimal medium (100 mL) without addition of thiamine. Then spores (10^8) of Canadian virulent isolate of *L. maculans* IBCN 57 (BJ 125) was inoculated in thus made media. Cultures were incubated on a shaker at 120 rpm, 23 ± 2 °C. Culture broth (10 mL) was collected on the 4th, 5th, 6th, and 7th days, extracted with EtOAc (20 mL × 3) and the extracts were analyzed by HPLC (triplicates).

Minimal medium cultures amended with phytoalexins (Table 2.1, entries 19and 20)

Canadian virulent isolate of *L. maculans* IBCN 57 (BJ 125) was grown in 250 mL Erlenmeyer flasks containing minimal medium (100 mL) inoculated with fungal spores (1×10^8). Cultures were incubated on a shaker at 120 rpm, at 23 ± 2 °C for 3 days and solutions of camalexin (final concentration 1×10^{-4} M in MM) or spirobrassinin (final concentration 1×10^{-4} M in MM) was added to separate cultures. Cultures (10 mL) were collected on the 4th, 5th, 6th, 7th and 9th days, extracted with EtOAc (20 mL × 3) and the extracts were analyzed by HPLC. Controls (0.2 mL DMSO) were treated similarly.

PDB medium cultures amended with phytoalexins (Table 2.1, entry 21 and 22)

Isolate BJ 125 was grown in 250 mL Erlenmeyer flasks containing PDB media (100 mL) inoculated with fungal spores (1×10^8). The cultures were incubated on a shaker at 120 rpm, at 23 ± 2 °C for 3 days and the 0.2 mL DMSO solution of camalexin (1×10^{-4} M) or spirobrassinin (1×10^{-4} M) were added into the cultures. From the 4th to 7th day, cultures (10 ml) were extracted with EtOAc (20 mL × 3) every 24 hours and the extracts were analyzed by HPLC. Controls with 0.2 mL DMSO were treated similarly.

V_8 juice medium culture (Table 2.1, entry 23)

Glucose (6.0 g) was added into a mixture of V_8 juice (60 mL) diluted with H_2O (540 mL). The mixture was stirred for 5 minutes, transferred into falcon tubes (40 mL/ tube) and centrifuged for 10 minutes at 3500 rpm. The supernatant was filtered through a nylon cloth. The filtrate was collected and autoclaved. After cooling to room temperature, the medium was transferred to Erlenmeyer flasks (100 mL / flask). The spores (10^8) of Canadian virulent isolate of *L. maculans* IBCN 57 (BJ 125) were inoculated into the above prepared medium and incubated on a shaker at 120 rpm. Culture broth (10 mL) was collected on the 4^{th}, 5^{th}, 6^{th}, 7^{th} and 9^{th} days, extracted with EtOAc (20 mL × 3) and the extracts were analyzed by HPLC (triplicates).

4.2.4 Synthesis and chemical derivatization of metabolites

Acetylleptomaculin C (**271**)

Pyridine (0.1 mL) and acetic anhydride (0.1 mL) were added to compound **10** (1.0 mg) at room temperature and stirred overnight. The solvents were removed to yield compound **12** (1.1 mg). HPLC: R_t = 12.4 min. ^{1}H NMR see Table 2.3. HRMS-EI: *m/z* 464.1799, $C_{22}H_{28}N_2O_9$, calcd. 464.1795. MS-EI: *m/z* 464 (22%), 407 (100%), 365 (54%). FTIR (KBr): 3401, 2959, 1755, 1712, 1232 cm^{-1}.

Compound **276**

Acetic anhydride (5 mL) was added to sirodesmin H (**169**, 589 mg, 1.30 mmol) and the reaction solution was heated at 110 °C, overnight. The reaction mixture was concentrated and the residue was fractionated using FCC, EtOAc-CH_2Cl_2 (1:4) to give compound **276** (581 mg, yield 77%). HPLC: t_R = 20.1 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.49 (s, 1H), 5.40 (s, 1H), 5.03 (dd, *J* = 8.9, 4.9 Hz, 1H), 4.61 (d, *J* = 11.2 Hz, 1H), 4.37 (d, *J* = 11.2 Hz, 1H), 3.74 (q, *J* = 6.3 Hz, 1H), 3.07 (s, 3H), 2.54 (dd, *J* = 14.5, 8.9 Hz, 1H), 2.31 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H), 1.94 (dd, *J* = 14.5, 4.8 Hz,

1H), 1.21 (d, *J* = 6.3 Hz, 3H), 1.00 (s, 3H), 0.97 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 218.0, 191.8, 169.9, 169.3, 168.8, 159.3, 156.6, 135.6, 115.9, 90.8, 86.9, 79.9, 79.6, 77.0, 65.9, 63.4, 47.5, 34.8, 31.0, 30.1, 21.2, 20.7, 20.5, 20.1, 17.5, 14.2. HRMS-EI: *m/z* 580.1717, $C_{26}H_{32}N_2O_{11}S$, calcd. 580.1727. MS-EI: *m/z* 580 (6%), 505 (24%), 385 (49%), 287 (60%), 245 (100%), 205 (67%); FTIR (KBr): 2971, 2928, 1752, 1691, 1443, 1363, 1236, 1044 cm^{-1}; UV (MeOH): λ_{max} (log ε) 223 (4.09), 267 (4.15) nm; $[\alpha]_D$= + 43 (*c* 0.11, MeOH), $[\alpha]_D$= +26 (c 0.20, $CHCl_3$).

Compounds **280a** and **280b**

Compound **276** (12.8 mg, 0.022 mmol) was dissolved in 1% HCl in MeOH (10 mL) at room temperature and kept overnight. After concentration, Raney nickel (1.5 mL) in MeOH (5 mL) was added to the residue and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was filtered and the solids washed with MeOH (5 mL × 4), the combined methanolic phase was filtered through a celite pad, and the filtrate was concentrated and applied to a RP-C_{18} FCC, CH_3CN-H_2O (20:80). Further chromatography of polar fraction (prep TLC, MeOH-CH_2Cl_2, 5:95) yielded compound **280b** (0.9 mg, 9% yield). HPLC: t_R = 8.9 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.48 (s, 1H), 5.49 (d, *J* = 1.0 Hz, 1H), 4.92 (dd, *J* = 8.0, 1.0 Hz, 1H), 4.08 (brd, *J* = 11.5 Hz, 1H), 4.02 (dd, *J* = 3.0, 1.5 Hz, 1H), 3.94 (dd, *J* = 11.5, 3.0 Hz, 1H), 3.77 (q, *J* = 6.0 Hz, 1H), 3.08 (s, 3H), 2.47 (dd, *J* = 15.0, 8.0 Hz, 1H), 2.35 (bs, 1H, OH), 2.24 (bd, *J* = 15.0 Hz, 1H), 2.05 (bs, 6H), 1.20 (d, *J* = 6.0 Hz, 3H), 0.98 (s, 3H), 0.97 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 216.9, 169.2, 168.8, 161.6, 156.7, 137.0, 114.6, 93.3, 87.5, 80.9, 79.8, 67.1, 66.2, 62.3, 47.5, 35.6, 32.3, 21.3, 20.8 (2C), 17.8, 15.3. HRMS-EI: *m/z* 464.1785, $C_{22}H_{28}N_2O_9$, calcd. 464.1795. MS-EI: *m/z* 464 (8%), 422 (38%), 404 (44%), 380 (54%), 362 (100%), 314 (52%), 223 (78%); FTIR (KBr): 3471, 2971, 2939, 1755, 1674, 1642, 1448, 1378, 1233, 1034 cm^{-1}. UV (MeOH): λ_{max} (log ε) 220 (4.05), 259 (4.06) nm; $[\alpha]_D$ = + 77 (*c* 0.10, MeOH). Further chromatography of less polar fraction (prep TLC MeOH-CH_2Cl_2, 5:95) yielded compound **280a** (2.9 mg, 28% yield). HPLC: t_R = 9.5 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.29 (s, 1H), 5.18 (s, 1H), 5.00 (dd, *J* = 8.5, 6.5 Hz, 1H), 4.14 (brd, *J* = 12.0 Hz, 1H), 4.03 (brd, *J* = 3.5 Hz, 1H), 3.93 (dd, *J* = 12.0,

4.0 Hz, 1H), 3.80 (q, *J* = 6.0 Hz, 1H), 3.07 (s, 3H), 2.76 (bs, 1H, D_2O exchangeable), 2.62 (dd, *J* = 14.0, 8.5 Hz, 1H), 2.09 (s, 3H), 2.03 (s, 3H), 1.74 (dd, *J* = 14.0, 6.0 Hz, 1H), 1.22 (d, *J* = 6.0 Hz, 3H), 1.02 (s, 3H), 1.01 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 217.6, 169.9, 169.5, 162.9, 157.5, 137.4, 114.2, 90.5, 86.7, 80.4, 79.6, 66.4, 63.8, 61.9, 47.5, 36.1, 32.2, 21.5, 20.6 (2C), 17.4, 14.8. HRMS-EI: m/z 464.1807, $C_{22}H_{28}N_2O_9$, calcd. 464.1795. MS-EI: *m/z* 464 (5%), 422 (26%), 380 (29%), 362 (42%), 314 (100%), 223 (58%); FTIR (KBr): 3428, 2971, 2928, 1752, 1682, 1647, 1438, 1373, 1233, 1039 cm^{-1}; UV (MeOH): λ_{max} (log ε) 221 (3.91), 257 (3.94) nm; $[\alpha]_D$ = + 4.6 (c 0.10, MeOH).

Compound **281b**

Pyridine (100 mL) and acetic anhydride (200 mL) were added to compound **280b** (2.1 mg, 0.005 mmol) at room temperature. After standing overnight, the solution was concentrated to yield compound **281b** (2.3 mg, quantitative). HPLC: R_t = 14.6 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.48 (s, 1H), 5.46 (s, 1H), 4.97 (dd, *J* = 9.0, 3.5 Hz, 1H), 4.73 (dd, *J* = 11.5, 3.5 Hz, 1H), 4.33 (dd, *J* = 11.5, 3.0 Hz, 1H), 4.20 (dd, *J* = 3.5, 3.0 Hz, 1H), 3.79 (q, *J* = 6.4 Hz, 1H), 3.07 (s, 3H), 2.64 (dd, *J* = 14.9, 9.0 Hz, 1H), 2.06 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.92 (dd, *J* = 14.9, 3.4 Hz, 1H), 1.23 (d, *J* = 6.4 Hz, 3H), 1.01 (s, 3H), 1.00 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 217.3, 170.1, 169.1 (2C), 160.1, 156.1, 136.1, 115.3, 92.2, 87.2, 80.8, 79.6, 66.2, 63.3, 62.7, 47.5, 36.7, 32.7, 21.5, 21.1, 20.7 (2C), 17.7, 15.6. HRMS-EI: *m/z* 506.1882, $C_{24}H_{30}N_2O_{10}$, calcd. 506.1900. MS-EI: *m/z* 506 (2%), 464 (10%), 422 (21%), 404 (100%), 344 (38%), 205 (45%); FTIR (KBr): 2970, 2928, 2874, 1750, 1688, 1647, 1375, 1236, 1044 cm^{-1}; UV (MeOH): λ_{max} (log ε) 220 (3.99), 260 (4.00) nm; $[\alpha]_D$ = + 104 (*c* 0.10, MeOH); $[\alpha]_D$ = + 136 (*c* 0.19, $CHCl_3$).

Compound **281a**

Compound **281a** was obtained similarly to compound **281b** from acetylation of compound **280a**. HPLC: R_t = 14.2 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.44 (s, 1H), 5.44 (s, 1H), 4.95 (dd, *J* = 9.0, 2.5 Hz, 1H), 4.69 (dd, *J* = 11.5, 3.0 Hz, 1H), 4.37 (dd, *J* = 11.5, 2.0 Hz, 1H), 4.18 (dd, *J* = 3.0, 2.0 Hz, 1H), 3.75 (q, *J* = 6.5 Hz, 1H), 3.06 (s, 3H),

2.57 (dd, *J* = 14.5, 9.0 Hz, 1H), 2.07 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.92 (dd, *J* = 14.5, 2.5 Hz, 1H), 1.20 (d, *J* = 6.5 Hz, 3H), 1.00 (s, 3H), 0.98 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 217.3, 170.2, 169.0, 168.9, 161.0, 156.6, 136.4, 115.0, 92.2, 87.1, 80.3, 79.6, 65.7, 63.7, 62.0, 47.5, 36.7, 32.4, 21.0, 20.8, 20.7, 20.6, 17.7, 15.0. HRMS-EI: m/z 506.1899, $C_{24}H_{30}N_2O_{10}$, calcd. 506.1900. MS-EI: *m/z* 506 (13%), 464 (70%), 422 (43%), 404 (100%), 344 (39%), 265 (47%); FTIR (KBr): 2960, 2925, 2854, 1746, 1690, 1649, 1377, 1242, 1048 cm^{-1}; UV (MeOH): λ_{max} (log ε) 221 (4.02), 257 (4.04) nm; $[\alpha]_D$ = + 10 (c 0.10, MeOH); $[\alpha]_D$ = + 25 (c 0.15, $CHCl_3$).

Compound **277**

Raney nickel (2 mL) in MeOH (5 mL) was added to compound **276** (34.2 mg, 0.059 mmol) in MeOH (5 mL) and heated at 55 °C for 6 h. After cooling to room temperature, the reaction mixture was filtered and the solids washed with MeOH (5 mL × 4), the combined methanolic phase was filtered through a celite pad, the filtrate was concentrated and the residue was separated using prep TLC (EtOAc-CH_2Cl_2, 2:3) to yield **277** (10.5 mg, 40% yield). HPLC: R_t = 13.7 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.46 (s, 1H), 5.52 (d, *J* = 1.0 Hz, 1H), 4.90 (d, *J* = 7.0 Hz, 1H), 4.02 (q, *J* = 7.0 Hz, 1H), 3.67 (q, *J* = 6.5 Hz, 1H), 3.05 (s, 3H), 2.40 (dd, *J* = 15.0, 8.0 Hz, 1H), 2.18 (brd, *J* = 15.0, 1H), 2.05 (brs, 6H), 1.53 (d, *J* = 7.0 Hz, 3H), 1.17 (d, *J* = 6.5 Hz, 3H), 0.97 (s, 3H), 0.95 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 217.3, 169.2, 168.9, 154.0 (2C), 137.8, 136.2, 116.2, 103.7, 92.6, 87.2, 80.3, 79.6, 66.7, 47.5, 36.3, 30.0, 21.0, 20.7 (2C), 17.7, 15.0. HRMS-EI: *m/z* 448.1848, $C_{22}H_{28}N_2O_8$, calcd. 448.1846. MS-EI: *m/z* 448 (21%), 404 (64%), 344 (76%), 205 (100); FTIR (KBr): 2978, 1757, 1687, 1648, 1454, 1431, 1372, 1237 cm^{-1}; UV (MeOH): λ_{max} (log ε) 258 (3.97), 220 (4.00) nm; $[\alpha]_D$ = + 57 (c 0.11, MeOH).

Compound **279**

Raney nickel (1 mL) in MeOH (5 mL) was added to compound **276** (28 mg, 0.048 mmol) in MeOH (5 mL) at room temperature and stirred for 1 h. The reaction

mixture was filtered and the solids washed with MeOH (5 mL × 4), the combined methanolic layer was filtered through a celite pad, the filtrate was concentrated and separated using prep TLC (EtOAc-CH_2Cl_2, 2:3) to yield **279** (12 mg, 56% yield): HPLC: R_t = 17.5 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.54 (s, 1H), 5.88 (d, *J* = 1.5 Hz, 1H), 5.53 (d, *J* = 1.0 Hz, 1H), 5.02 (d, *J* = 1.5 Hz, 1H), 5.00 (dd, *J* = 9.0, 2.5 Hz, 1H), 3.72 (q, *J* = 6.5 Hz, 1H), 3.30 (s, 3H), 2.55 (dd, *J* = 15.0, 9.0 Hz, 1H), 2.08 (dd, *J* = 15.0, 2.5 Hz, 1H), 2.06 (s, 3H), 2.04 (s, 3H), 1.17 (d, *J* = 6.5 Hz, 3H), 0.99 (s, 3H), 0.96 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 217.3, 169.4, 168.9, 163.5, 155.7, 137.0, 114.5, 93.6, 87.8, 80.3, 80.0, 67.3, 60.3, 47.7, 35.3, 32.4, 21.1, 20.9, 20.5, 19.9, 17.9, 15.1. HRMS-EI: *m/z* 446.1694, $C_{22}H_{26}N_2O_8$, calcd. 446.1689. MS-EI: *m/z* 446 (21%), 404 (64%), 344 (76%), 205 (100); FTIR (KBr): 2970, 2928, 1755, 1689, 1609, 1432, 1233 cm^{-1}; UV (MeOH): λ_{max} (log ε) 286 (4.27) nm; $[\alpha]_D$= + 48 (*c* 0.11, MeOH).

Methanolysis of sphingolipids (**14** and **31**)

Sphingolipids (**14** and **31,** 5.0 mg) were refluxed with a solution of MeOH (2 mL) and HCl (1N, 0.5 mL) for overnight. The reaction mixture was cooled and extracted with hexane (4 mL × 3). The hexane layer was concentrated and the residue was applied to a pipet column FCC (CH_2Cl_2 / MeOH, 98: 2) to give a mixture of fatty acid methyl esters **285** (77%) and **286** (23%). Compound **285**: HRMS-EI: *m/z* 312.2664, $C_{19}H_{36}NO_3$, calcd. 312.2664. MS-EI: *m/z* 312 (1.2%), 253 (100%), 149 (16%), 109 (27%), 95 (45%), 81 (35%). ^{1}H NMR (500MHz, $CDCl_3$): δ 5.89(dt, *J* = 15.3, 6.9 Hz, 1H), 5.50(dd, *J* = 15.3, 6.2 Hz, 1H), 4.62 (bs, 1H), 3.81 (s, 3H), 2.84 (d, *J* = 5.3, Hz, 1H, D_2O exchangeable), 2.06 (dt, *J* = 7.2, 7.1 Hz, 2H) 1.39 (m, 2H), 1.27 (bs, 24H), 0.89 (t, *J* = 6.8 Hz, 3H). Compound **286**: HRMS-EI: *m/z* 314.2815, $C_{19}H_{38}NO_3$, calcd. 314.2821. MS-EI: *m/z* 314 (17%), 253 (100%), 111 (17%), 97 (36%), 83 (50%).^{1}H NMR (500MHz, $CDCl_3$): δ 4.19 (m, 1H), 3.80 (s, 3H), 2.67 (d, *J* = 4.9, Hz, 1H, D_2O exchangeable), 1.78 (m, 1H), 1.63 (m, 1H), 1.26 (bs, 28H), 0.89 (t, *J* = 6.8 Hz, 3H) .

4-Prenyl-benzeneacetic acid (**288**)

NaH (60% in mineral oil, 28 mg, 0.39 mmol) was washed with hexane (2 mL × 2) then added 4-hydroxyphenylacetic acid (**287**) (20 mg, 0.13 mmol) in anhydrous THF (2 mL) at 30 °C. After 60 minutes dimethylallyl bromide (33 μL, 0.39 mmol) was added and stirred for 36 hours. The reaction mixture was concentrated, diluted with aq NaOH (0.1 M, 10 mL) and extracted with diethyl ether (15 mL × 2). The aqueous solution was then acidified to pH 2 and re-extracted with EtOAc (20 mL × 3), the EtOAc layer was dried over Na_2SO_4 and concentrated to yield the product **288** (27 mg, 94% yield) HPLC: t_R = 7.0 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.15 (d, *J* = 7.7 Hz, 1H), 6.84 (d, *J* = 7.7 Hz, 1H), 5.49 (bt, *J* = 6.8 Hz, 1H), 4.47 (d, *J* = 6.7 Hz, 2H), 3.54 (bs, 2H), 1.79 (s, 3H), 1.73 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 158.3, 138.3, 130.6, 126.0, 119.9, 115.0, 65.0, 26.0, 18.4.

Methyl-4-Prenyl-benzeneacetate (**289**)

Excess CH_2N_2 in Et_2O was added to an Et_2O (15 mL) solution of **288** (13 mg, 0.059 mmol), after 30 minutes 0.1M acetic acid was added until the solution became colorless. The reaction solution was concentrated to give **289** (13.7 mg, 99% yield) HPLC: t_R = 26.0 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.19 (d, *J* = 7.7 Hz, 1H), 6.87 (d, *J* = 7.7 Hz, 1H), 5.50 (m, 1H), 4.50 (d, *J* = 6.7 Hz, 2H), 3.69 (s, 3H), 3.57 (bs, 2H), 1.80 (s, 3H), 1.74 (s, 3H). FTIR (KBr): 2923, 1738, 1511, 1236 cm^{-1}.

1,8-Dihydroxynaphthalene (**292**)

1,8-Naphthosultone (**291**, 240.0 mg, 1.20 mmol) and KOH (1.2 g, 21.4 mmol) were heated together in a reaction vial at 300 °C in a sand bath for about 20 minutes until the mixture became a homogeneous black liquid. The reaction mixture was cooled to room temperature and hydrochloric acid (concentrated HCl-H_2O, 1:2) was added with stirring until neutral pH was obtained. H_2O (10 mL) was added into the mixture and the mixture was extracted with EtOAc (25 mL×3). EtOAc layers were dried with Na_2SO_4 and concentrated. The concentrated residue was applied to FCC (EtOAc/hexane, 20: 80)

to give compound **292** as a white solid (120 mg, yield 63%). All spectroscopic data are identical to the reported data (Ragot et al., 1999).

8-hydroxyl-naphthalenylsulfate (**293**)

HSO_3Cl (25μL, 0.38 mmol) was added slowly into a solution of diethyl ether (156 μL) and N,N-dimethylaniline (156 μL, 1.25 mmol) at -10 °C. After 1,8-dihydroxynaphthalene **292** (20.0 mg, 0.125 mmol) was added, the reaction mixture was warmed to 0 °C and stirred for 1 hour. 60% NaOH (1 mL) was added into the reaction mixture with vigorous stirring then the reaction mixture was adjusted to pH = 8 with 1 M H_2SO_4 and extracted with EtOAc (20 mL × 4). EtOAc layer was concentrated to gave a white residue, which was purified by reverse phase FCC (CH_3CN/H_2O, 10 : 90) to give compound **293** as a gray solid (14.1 mg, yield 47%).

D-mannitol-hexaacetate (**302**)

Maculansin A (**299**, 8.0 mg, 0.017 mmol) was dissolved in MeOH (4.0 mL) then excess Na_2CO_3 (10.0 mg) was added. The reaction mixture was stirred for 4 hours at room temperature and concentrated. The residue was dissolved in 5 mL water. The pH of the solution was adjusted to 2 and extracted with CH_2Cl_2 (10 mL×3). The aqueous layer was concentrated to dryness. Pyridine (500 μL) and acetic anhydride (500 μL) were added to the residue and stirred for 5 hours at room temperature. After removal of reagents the residue was applied to PTLC (MeOH-CH_2Cl_2, 4:96) to give compound **302** (6.7 mg, 93 %). The ^{1}H NMR spectrum of compound **302** was identical to that of authentic D-mannitol hexaacetate, and optical rotation ($[\alpha]_D$ = 13 (c 0.51, $CHCl_3$)) was close to authentic D-mannitol hexaacetate ($[\alpha]_D$ = 12 (c 0.26, $CHCl_3$)). CH_2Cl_2 layer was concentrated to give a mixture of components which could not be identified.

The mixture of metabolites with R_t = 14.7 min (9.5 mg) was dissolved in MeOH (5 mL) then Na_2CO_3 (2 mg) was added. The reaction mixture was stirred overnight then concentrated. The residue was dissolved in 10 mL water. The pH of the solution was adjusted to 2 with 1.0 M HCl and extracted with EtOAc. The EtOAc layer was

concentrated to give 2.4 mg N-formylvaline (**305)**. ^{1}H NMR (500 MHz, $CDCl_3$) 8.30 (s, 1H), 6.14 (bd, *J* = 8.0 Hz, 1H), 4.71 (dd, *J*= 8.7, 4.5 Hz, 1H), 2.31 (m, 1H), 1.02 (d, *J* = 6.9 Hz, 3H), 0.98 (d, *J* = 6.9 Hz, 3H). HRMS-ESI: *m/z* $[M-1]^-$ 144.0650, $C_6H_{10}NO_3$, , calcd. 144.0655. MS-EI: *m/z* 128 (6%), 100 (100%), 85 (87%), 72 (25%). The aqueous layer was concentrated to dryness then pyridine (1 mL) and acetic anhydride (2 mL) were added to the residue and stirred overnight. After removal of reagents the residue was applied to PTLC (MeOH-CH_2Cl_2 4: 96) to give compound **302** (4.4 mg). The ^{1}H NMR spectra and optical rotation data ($[\alpha]_D$ = 5 (c 0.2, $CHCl_3$)) of compound **302** were similar to commercial D-mannitol hexaacetate.

Compound **295**

An excess amount of diazomethane was added to a suspension of methanol containing 4.6 mg compound **294**, and kept overnight. After concentration, the residue was applied to PTLC (EtOAc/hexane 1:1, two developments) to give 1.3 mg of compound **295**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.37 (d, *J* = 7.9 Hz, 1H), 8.11 (d, *J* = 8.1 Hz, 1H), 7.44 (m, 2H), 7.03 (m, 3H), 4.14, (s, 3H), 4.11 (s, 3H), 3.91 (s, 3H), 3.49 (bd *J* = 13.9 Hz, 1H), 3.44 (bd *J* = 13.9 Hz, 1H), 3.05 (bd *J* = 6.6 Hz, 1H), 2.90 (bd *J* = 6.8 Hz, 1H), HRMS-EI: *m/z* 400.1312, $C_{25}H_{20}O_5$, calcd. 400.1311. MS-EI: *m/z* 400 (100%), 371 (72%).

Compound **296**

K_2CO_3 (482 mg, 3.50 mmol) and dimethyl sulfate (120 µL, 0.88 mmol) were added into a mixture of acetone (10 mL) and bulgarein **294** (40 mg of a fraction containing bulgarein **294**). The reaction mixture was refluxed for 3 hours and the solution was concentrated to give an oily residue. The residue was applied to PTLC (EtOAc/hexane, 1:1) to yield compound **296 (**3.8 mg), R_f = 0.4; HPLC: GRADSCR M. R_t = 34.7 min; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.36 (d, *J* = 8.0 Hz, 1H), 8.26 (bd, *J* = 8.0 Hz, 2H), 7.44 (t, *J* = 8.0, 8.0 Hz, 1H), 7.00 (bd, *J* = 8.0 Hz, 1H), 6.99 (bd, *J* = 8.0 Hz, 1H), 6.86 (d, *J* = 8.0 Hz, 1H), 4.20 (s, 3H), 4.11 (s, 6H), 4.07 (s, 3H), 4.01 (s, 3H); ^{13}C

NMR (500 MHz, $CDCl_3$): δ 158.7, 157.8, 157.1, 148.0, 147.8, 135.5, 131.8, 130.4, 130.1, 130.0, 127.7, 126.3, 126.0, 125.6, 120.7, 117.1, 114.4, 107.2, 106.9, 105.2, 62.3, 60.9, 56.7 (2C), 56.3. HRMS-EI: *m/z* 402.1464, $C_{25}H_{22}O_5$, calcd. 402.1467. MS-EI: *m/z* 402(100%), 388(20%), 387(71%); FTIR (KBr): 2958, 2927, 2854, 1602, 1576, 1459, 1425, 1263 cm^{-1}; UV (MeOH): λ_{max} (log ε) 406 (4.34), 345 (4.63), 331 (4.52), 251 (4.84), 226 (4.66) nm.

4.3 BIOASSAY OF METABOLITES FROM *LEPTOSPHAERIA MACULANS*

4.3.1 Elicitor activity bioassay

Plants (*B. napus* cv. Westar and *B. juncea* cv. Cutlass) were grown in a growth chamber with 16 h illumination (fluorescent and incandescent, 450-530 μmol s^{-1} m^{-2})/8 h dark, at 24 ±2° C. After two weeks, purified compounds or factions of extracts dissolved in MeOH-H_2O (1:1 v/v) solutions were sprayed to the leaves. After two days, the leaf tissues were frozen in liquid N_2, crushed with a glass rod and extracted with EtOAc (50 mL). EtOAc extract was dried over Na_2SO_4 and concentrated in a rotary evaporator. The residue was dissolved in 1% MeOH/CH_2Cl_2, applied to a mini silica gel column (Pasteur pipet) and eluted with 1% MeOH/CH_2Cl_2 (5 mL), this 5 ml solution was concentrated, the residue was dissolved in CH_3CN (80 μL) and analyzed by HPLC (20 μL injection volume). Control leaves were treated similarly employing a MeOH-H_2O 1:1 solution.

4.3.2 Phytotoxicity bioassay

Plants were grown in the same conditions as described above. After the third leaf was half developed (about two weeks), purified compounds or factions of extracts dissolved in MeOH-H_2O (1:1, v/v) solution were applied to leaves by pipetting droplets (5 μL) to puncture sites of leaves (6 sites each leaf). After two days, the damaged areas were measured using a stencil having cut out circles with different diameters, and the

measured diameters were converted to the damage index as Table 4.1. All bioassays were carried out in triplicates.

Table 4.1. Scale for conversion of damaged area to damage index.

Lesion diameter (mm)	<1.5	1.6-2.3	2.4-3.1	3.2-3.9	4.0-4.7	4.8-5.5	5.6-6.3	6.4-7.0
Damage index	0	1	2	3	4	5	6	7

4.3.3 Growth inhibition bioassay

Seeds of brown mustard (20) and canola (20) were sterilized by soaking in Javex (10 %/ v/v) for 10 minutes, washed with water, air dried and incubated in PDB medium (0.9 g in 100 ml) amended with compound **212** (0.5 mM). The dishes were sealed with Parafilm and kept in darkness at room temperature. After 7 days the root length of seedlings was measured with a ruler.

4.4 BIOSYNTHESIS OF SIRODESMIN PL (**165**)

4.4.1 Synthesis of potential biosynthetic precursors

*4.4.1.1 Synthesis of O-prenyl-L-tyrosine (**312**)*

t-Boc-*O*-prenyl-L-tyrosine (**311**)

NaH (60% in mineral oil, 69.6 mg, 2.90 mmol) was washed with hexane (2 mL × 2) and added to a stirred solution of *t*-Boc-L-tyrosine (**310**) (281 mg, 1.00 mmol) in dry

THF (8 mL) at room temperature. After 10 minutes dimethylallyl bromide (162 μL, 1.20 mmol) was added and the reaction mixture was stirred overnight. The reaction mixture was then concentrated, diluted with aq NaOH (0.5 M, 15 mL) and extracted with diethyl ether (15 mL × 2). The remaining aqueous solution was acidified to pH 2 and was extracted with EtOAc (20 mL × 4). The combined EtOAc extract was dried over Na_2SO_4 and concentrated to yield the product **311** (285 mg, 82% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.09 (d, *J* = 8.5 Hz, 2H), 6.86 (d, *J* = 8.5 Hz, 2H), 5.49 (t, *J* = 7.0 Hz, 1H), 4.91 (d, *J* = 7.0 Hz, 1H), 4.55 (m, 1H), 4.49 (d, *J* = 7.0 Hz, 2H) 3.13 (dd, *J* = 5.0, 14.0 Hz, 1H) 3.05 (dd, *J* = 5.5, 14 Hz, 1H), 1.80 (s, 3H), 1.74 (s, 3H), 1.43 (bs, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 176.7, 158.1, 156.0, 138.3, 130.5 (2C), 128.4, 119.9, 114.9 (2C), 80.4, 64.9, 55.4, 37.1, 28.5, 26.0, 18.4. HRMS-EI: *m/z* 349.1895, $C_{19}H_{27}NO_5$, calcd. 349.1889. MS-EI: *m/z* 349 (1%), 225 (19%), 207 (15%), 164 (31%), 107 (100%).

<u>*O*-prenyl-L-tyrosine (**312**)</u>

t-Boc-*O*-prenyl-L-tyrosine (**311**) (52 mg, 0.15 mmol) was heated at 155 ± 2°C under atmosphere of argon for 60 min, and the resulting yellowish solid was washed with MeOH to yield *O*-prenyl-L-tyrosine (**312**) as a white solid (20 mg, 54% yield). HPLC t_R 7.2 min. M.p. 204-206 °C. ^{1}H NMR (500 MHz, D_2O, very low solubility) δ: 7.28 (d, *J* = 8.5 Hz, 2H), 7.03 (d, *J* = 8.5 Hz, 2H), 5.54 (t, *J* = 7.0 Hz, 1H), 4.64 (d, *J* = 7.0 Hz, 2H), 3.97 (bt, *J* = 7 Hz, 1H) 3.24 (dd, *J* = 5.0, 14.5 Hz, 1H), 3.09 (dd, *J* = 8.0, 14.5 Hz, 1H), 1.82 (s, 3H), 1.78 (s, 3H). ^{1}H NMR (500 MHz, 0.5% NaOH in D_2O, good solubility) δ: 7.22 (d, *J* = 8.5 Hz, 2H), 6.98 (d, *J* = 8.5 Hz, 2H), 5.52 (t, *J* = 7.0 Hz, 1H), 4.61 (d, *J* = 7.0 Hz, 2H), 3.47 (bt, *J* = 6.0 Hz, 1H), 2.94 (dd, *J* = 13.5, 5.5, 1H), 2.81 (dd, *J* = 13.5, 7.0 Hz, 1H), 1.80 (s, 3H), 1.76 (s, 3H). ^{13}C NMR (125 MHz, 0.5% NaOH D_2O) δ: 183.2, 157.1, 142.0, 131.7, 131.2 (2C), 118.9, 115.7 (2C), 65.8, 58.1, 40.5, 25.6, 17.9. HRMS-EI: *m/z* 249.1367, $C_{14}H_{19}NO_3$, calcd. 249.1366. MS-EI: *m/z* 249 (4%), 175 (24%), 107 (100%).

FTIR (KBr): 2958, 2928, 2868, 1611, 1561, 1512, 1246, 1016 cm^{-1}.

*4.4.1.2 Synthesis of [3,3-$^2H_2$]O-prenyl-L-tyrosine (**312a**)*

[3,3-$^2H_2$]-*t*-Boc-*O*-preny-L-tyrosine (**311a**) (170 mg, 0.48 mmol) was heated as reported above for **311** and the product obtained was crystallized from MeOH-H_2O to yield **312a** (58.2 mg, 48% yield) as white crystals. ^{1}H NMR (500 MHz, D_2O) δ: 7.28 (d, *J* = 8.5 Hz, 2H), 7.03 (d, *J* = 8.5 Hz, 2H), 5.54 (t, *J* = 7.0 Hz, 1H), 4.64 (d, *J* = 7.0 Hz, 2H), 3.94 (s, 1H), 1.82 (s, 3H), 1.78 (s, 3H). HRMS-EI: *m/z* 251.1492, $C_{14}H_{17}D_2NO_3$, calcd. 251.1490. MS-EI: *m/z* 251 (2%), 183 (10%), 177 (10%), 109 (100%). FTIR (KBr): 2960, 2928, 2868, 1614, 1560, 1512, 1248, 1012 cm^{-1}.

*4.4.1.3 Synthesis of E-[4,4,4-$^2H_3$]-3-methyl-2-butenyl bromide (**318a**)*

(*E*)-ethyl-3-phenylsulfanyl-2-butenoate (**314**) and (*Z*) isomer (**315**):

NaOH (189 mg, 4.7 mmol) was added to a stirred solution of phenylthiol (436 µL, 4.3 mmol) in EtOH (3.6 mL). After stirring for 30 min at room temperature, a solution of ethyl 2-butynoate (**313**) (500 µL, 4.3 mmol) was added. After 2 h the reaction mixture was quenched with aq. acetic acid (295 µL in 20.0 mL of water), the mixture was extracted with ethyl ether, the combined organic extract was washed with 4% aq. NaOH and water, dried over $MgSO_4$, filtered, and concentrated on a rotary evaporator. The residue was chromatographed over silica gel (10% ether/hexane) to give *E* isomer (**314**) (649 mg, 68% yield) and *Z* isomer (**315**) (162 mg, 17% yield). The spectroscopic data were identical to that reported in literature (Thulasiram et al., 2006).

Ethyl-[5,5,5-$^2H_3$]-3-methyl-2-butenoate (**316**)

C^2H_3I (836 µL, 13.1 mmol) was added to Mg (319 mg, 13.1 mmol) in dry diethyl ether (10 mL) at room temperature under argon and the reaction mixture was stirred for 40 min (most Mg dissolved). CuI (1.37 g, 7.21 mmol) was added to a solution of *E*-ethyl-3-phenylsulfanyl-2-butenoate (**314**) (1.46 g, 6.56 mmol) in dry THF (10 mL) at -15°C and stirred for 10 minutes. C^2H_3MgI in Et_2O was cooled to 0 °C and added drop-wise to the cuprate mixture over 30 min. The mixture was stirred for 2 h after which

saturated NH_4Cl (20 mL) was poured into the reaction mixture, the resulting mixture was stirred at rt for 60 min and extracted with diethyl ether (20 mL × 4). The organic layer was dried over $MgSO_4$ and concentrated to yield ethyl [4,4,4-$^2H_3$]3-methyl-2-butenoate (**316**) as an oil (805 mg, crude yield 94%), which was used in the next step without further purification. The spectral data were identical to that reported for this compound (Thulasiram et al., 2006).

E-[5,5,5-$^2H_3$]-3-methyl-2-butenol (**317**)

LAH (358 mg, 9.43 mmol) was added to a solution of ethyl [4,4,4-$^2H_3$]3-methyl-2-butenoate (**316**) (805 mg, 6.15 mmol) in diethyl ether (20 mL) at -5 °C under argon. The reaction mixture was stirred at 0 °C for 30 min, was cooled to -5 °C and 15% aq. NaOH (0.96 mL) and H_2O (1 mL) were added sequentially. After excess $MgSO_4$ was added to the mixture the white slurry was stirred at 0 °C for 1 hour, filtered, and washed with diethyl ether. The combined organic extract was concentrated to yield *E*-[4,4,4-$^2H_3$]3-methyl-2-butenol (**317**) as an oil (521 mg, crude yield 95%), which was used in the next step without further purification.

E-[4,4,4-$^2H_3$]-3-methyl-2-butenyl bromide (**318a**)

PBr_3 (278 µL, 2.93 mmol) was added to a solution of *(E)*-[4,4,4-$^2H_3$]3-methyl-2-butenol (**317**) (521 mg, 5.85 mmol) in dry diethyl ether (20 mL) at -5 °C under argon. After allowing the reaction mixture to stir for 30 minutes, H_2O (1 mL) and 10% $NaHCO_3$ (10 mL) were added to quench the reaction and the organic layer was separated. The organic layer was washed with 10% $NaHCO_3$, dried over $MgSO_4$, and concentrated to 2 mL (if concentrated long enough, it gave 706 mg oily residue with crude yield of 80%). The crude product (2 mL) was passed through a basic Al_2O_3 mini-column. After concentration of the fraction, *E*-[4,4,4-$^2H_3$]3-methyl-2-butenyl bromide (**318a**) was obtained as an oil (406 mg, yield 46%), which was used in the preparation of [3,3,5',5',5'-$^2H_5$]*O*-prenyl-L-tyrosine (**312b**) without further purification.

*4.4.1.4 Synthesis of Z-[3,3,5',5',5'-$^2H_5$]O-prenyl-L-tyrosine (**312b**)*

[3,3,5',5',5'-^{2}H$_5$]-*t*-Boc-*O*-prenyl-L-tyrosine (**311b**)

[3,3,5',5',5'-^{2}H$_5$]-*t*-Boc-*O*-prenyl-L-tyrosine (**311b**) was prepared as reported above for *t*-Boc-*O*-prenyl-L-tyrosine (**311a**). ^{1}H NMR (500 MHz, $CDCl_3$): δ7.09 (d, *J* = 8.5 Hz, 2H), 6.86 (d, *J* = 8.5 Hz, 2H), 5.49 (t, *J* = 7.0 Hz, 1H), 4.92 (d, *J* = 7.0 Hz, 1H), 4.55 (d, *J* = 7.0 Hz, 1H), 4.49 (d, *J* = 7.0 Hz, 2H), 1.74 (s, 3H), 1.43 (bs, 9H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 175.9, 158.3, 155.8, 138.4, 130.5 (2C), 127.7, 119.8, 115.0 (2C), 80.6, 64.9, 54.5, 28.5, 18.4; HRMS-EI: *m/z* 354.2199, $C_{19}H_{22}D_5NO_5$, calcd.354.2203. MS-EI: *m/z* 354 (1%), 227 (7%), 209 (9%), 109 (100%).72 (76%).

[3,3,5',5',5'-^{2}H$_5$]*O*-prenyl-L-tyrosine (**312b**)

The preparation of [3,3,5',5',5'-^{2}H$_5$]*O*-prenyl-L-tyrosine (**312b**) is similar to that of *O*-prenyl-L-tyrosine (**312a**). ^{1}H NMR (500 MHz, D_2O) δ: 7.28 (d, *J* = 8.5 Hz, 2H), 7.04 (d, *J* = 8.5 Hz, 2H), 5.54 (t, *J* = 7.0 Hz, 1H), 4.64 (d, *J* = 7.0 Hz, 2H), 3.97 (s, 1H), 1.78 (s, 3H). ^{1}H NMR (500 MHz, CD_3OD) δ: 7.20 (d, *J* = 7.0 Hz, 2H), 6.88 (d, *J* = 7.0 Hz, 2H), 5.44 (t, *J* = 5.5 Hz, 1H), 4.52 (d, *J* = 5.5 Hz, 1H), 3.70 (s, 1H), 1.74 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ: 174.2 (from HMBC), 159.8, 138.6, 131.6 (2C), 129.1 (from HMBC), 121.4, 116.3 (2C), 65.9, 57.7, 36.9 (from HMBC), 25.0 (from HMBC, HMQC), 18.3. HRMS-EI: *m/z* 254.1674, $C_{14}H_{14}D_5NO_3$, calcd. 254.1679. MS-EI: *m/z* 254 (2%), 110 (57%), 109 (100%), 72 (32%).

*4.4.1.5 Synthesis of methyl-L-Tyr-t-Boc-L-Ser (**321**) and methyl [5,5-$^2H_2$]-L-Tyr-t-Boc-L-Ser (**321a**)*

L-Tyrosine methyl ester (**319**) (117 mg, 0.45 mmol) was added to a solution of *t*-Boc-L-serine (**320**) (93 mg, 0.45 mmol) in CH_2Cl_2 (1.5 ml). The reaction mixture was cooled to -15 °C and triethylamine (69µl, 0.5 mmol) and EDCI (87 mg, 0.45 mmol) were added -15°C. After 20 hours the reaction mixture was transferred into a separatory funnel with ethyl acetate (10 mL) and $NaHCO_3$ (1M, 10 mL). After removal of the ethyl

acetate layer the aqueous layer was adjusted to pH 4 with 1M H_2SO_4, and was extracted with ethyl acetate (10 mL × 5). The combined ethyl acetate extract was dried over Na_2SO_4 and was concentrated with a rotary evaporator to give compound **321** (159 mg, yield 92%). ^{1}H NMR (500MHz, $CDCl_3$): δ 7.07 (d, *J* = 6.1 Hz, 1H), 6.96 (d, *J* = 8.3 Hz, 2H), 6.73 (d, *J* = 8.3 Hz, 2H), 6.56 (bs, 1H), 5.59 (m, 1H), 4.83 (m, 1H), 4.16 (s, 1H), 3.95 (m, 1H), 3.77 (s, 3H), 3.60 (s, 1H), 3.24 (s, 1H), 3.13 (dd, *J* = 4.9 Hz, 4.1 Hz, 1H), 2.99 (dd, *J* = 14.1 Hz, 6.6 Hz, 1H), 1.46 (s, 9H). Methyl [5,5-$^2H_2$]*t*-Boc-L-Tyr-L-Ser (**321a**) was synthesized similarly.

*4.4.1.6 Synthesis of [5,5-$^2H_2$]cyclo-L-tyr-L-ser (**251a**)*

Methyl [5,5-$^2H_2$]*t*-Boc-L-Tyr-L-Ser (**321a**) (53 mg, 0.14 mmol) in formic acid (5 mL) was allowed to stand at room temperature for 60 min, the solvent was removed under vacuum (water bath temp. < 30 °C) and *sec*-butyl-alcohol (5 mL) in toluene (1.4 mL) was added. After heating at 65 °C for 6 h, the solvent was evaporated under vacuum to give a white solid **251a** (32 mg, 90% yield). HPLC R_t = 2.7 min. ^{1}H NMR (500 MHz, D_2O): δ 7.13 (d, *J* = 8.5 Hz, 2H), 6.91 (d, *J* = 8.5 Hz, 2H), 4.40 (s, 1H), 4.01 (dd, *J* = 3.5, 6.5, 1H), 3.42 (dd, *J* = 3.5, 11.5 Hz, 1H), 2.65 (dd, *J* = 6.5, 11.5 Hz, 1H). HRMS-EI: *m/z* 252.1074, $C_{12}H_{12}D_2N_2O_4$, calcd. 252.1079. MS-EI: *m/z* 252 (5%), 234 (9%), 109 (100%); FTIR (KBr): 3188, 3047, 2920, 1685, 1664, 1519, 1462 cm^{-1}.

*4.4.1.7 Synthesis of [5,5-$^2H_2$]phomamide (**171a**)*

[5,5-$^2H_2$]Cyclo-L-Tyr-L-Ser (**251a**, 20 mg, 0.079 mmol) in aqueous KOH (8 × 10^{-3} M, 15 mL) at 0 °C, was allowed to stir for 2 h and then freeze-dried. Dry DMF (1 mL) was added to the freeze-dried residue followed by addition of prenyl bromide (**318a**) (30 μL, 0.24 mmol) and stirring at 35 °C. After 6 h the solvent was removed under vacuum (water-bath temperature was < 40 °C), the residue was diluted with distilled water (15 mL) and was sequentially extracted with hexane (5 mL × 2) and ethyl acetate (20 mL × 3); the ethyl acetate extract was dried over Na_2SO_4 and concentrated under vacuum to yield [5,5-$^2H_2$]phomamide (**171a**, 17 mg, 67% yield). HPLC R_t = 8.2

min. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.13 (d, *J* = 8.5 Hz, 2H), 6.90 (d, *J* = 8.5 Hz, 2H), 6.16 (s, 1H, NH), 5.84 (s, 1H, NH), 5.49 (t, *J* = 6.5 Hz, 1H), 4.51 (d, *J* = 6.5 Hz, 2H), 4.21 (s, 1H), 4.06 (t, *J* = 5.0 Hz, 1H), 3.72 (m, 1H), 3.49 (m, 1H), 2.33 (bs, 1H, OH), 1.81 (s, 3H), 1.76 (s, 3H).

HRMS-EI: *m/z* 320.1708, $C_{17}H_{20}D_2N_2O_4$, calcd. 320.1705. MS-EI: *m/z* 320 (3%), 252 (31%), 144 (72%), 109 (100%), 69 (44%); FTIR (KBr): 3196, 3038, 2962, 1674, 1666, 1511, 1463 cm^{-1}. $[\alpha]_D$ = - 137 (c 0.11, MeOH).

4.4.2 Incorporation of potential precursors

Minimal medium (MM) was prepared as described in the experimental section (General methods). Canadian virulent isolate of *L. maculans* IBCN 57 (BJ 125) was grown in 250 mL Erlenmeyer flasks containing MM (100 mL) inoculated with fungal spores (10^9) and incubated on a shaker at 130 rpm, at 23 ± 2 °C for 3 days. Deuterium labeled compounds [3,3-$^{2}H_2$]-L-tyrosine (**251a**), [3,3-$^{2}H_2$]*O*-prenyl-L-tyrosine (**312a**), (Z)-[3,3,5',5',5'-$^{2}H_5$]*O*-prenyl-L-tyrosine (**312b**), [5,5-$^{2}H_2$]cyclo-L-tyr-L-ser (**251a**), [5,5-$^{2}H_2$]phomamide (**171a**) or [2,3,3-$^{2}H_3$]-L-serine (**233d**) were dissolved in sterile distilled water and added to the cultures of *L. maculans* in three flasks (0.50 mmoles/ 100 mL). Non-labeled compounds were added to the culture in another three flasks and the cultures thus prepared were used as controls. On the 5th day the broth of each flask was extracted with EtOAc (100 mL × 3) and combined extracts were concentrated to dryness. The residue was separated by prep. TLC (MeOH-CH_2Cl_2, 10: 90) to give sirodesmin PL (**165**) (R_f = 0.75, ca. 90 mg/L) and phomamide (**171**) (R_f = 0.25, ca. 10 mg/L).

4.5 MACROMOLECULAR ELICITORS PRODUCED BY *LEPTOSPHAERIA MACULANS*

4.5.1 Preparation of spore germination fluids (SGF)

A total of 20 leaves (the first two leaves) of 21-day-old *B. napus* cv. Westar were cut from the base of petioles. The lower (or back) sides of leaves were scratched with a surgical blade (about 20 sites) and spore suspension (30 μL, 2.5×10^7 spores / mL) was applied on each scratched site. The leaves were kept upside down in moisturized Petri dishes. The Petri dishes were sealed by using Parafilm and kept in darkness at 23 ± 1 ºC for two days. The control was treated similarly using sterile distilled water (15 mL). After two days about 67% spores germinated (from the inspection of the germinating spore suspension under the microscope). The spore germination suspension droplets from treated sites were collected into a falcon tube and centrifuged at 3500 rpm for 15 minutes. The supernatant was decanted into a falcon tube and centrifuged at 3500 rpm for 15 minutes. The supernatant was inspected under a microscope to ensure that no spores remained (normally centrifuged two times to remove all the spores and germinating spores). The total supernatant (SGF) was 10 mL. The control solution was 8 mL. The SGF and control solution were kept at 4 ºC.

4.5.2 Bioassay of spore germination fluids (SGF)

The upper side of leaves of two-week-old *B. napus* cv. Westar and *B. juncea* cv. Cutlass were scratched with a surgical blade (about 15 sites). SGF (40 μL) was applied on each scratched site. After two days the leaves were collected and HPLC samples of leaf extracts were prepared similarly as described in Section 4.3.1. The control was treated similarly to the solution obtained from control leaves during the preparation of SGF.

The cotyledons and leaves of two-week-old *B. napus* cv. Westar and *B. juncea* cv. Cutlass were cut from the base of petioles. The upper side of cotyledons and lower (or back) sides of leaves were scratched with a surgical blade, 2 and 6 sites,

respectively. SGF (40 μL) was applied on each scratched site. The cotyledons and leaves were kept in moisturized Petri dishes. The Petri dishes were sealed with Parafilm and kept at 23 ± 1 ºC for two days under constant light. Toxicity was compared with the control by visual inspection of damage on the leaves.

CHAPTER 5: REFERENCES

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